EPIDEMIOLOGICAL TYPING

A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus*-*A. baumannii* complex

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Different PCR-based DNA fingerprinting techniques were evaluated for typing 26 clinical isolates belonging to the *Acinetobacter calcoaceticus*-*A. baumannii* complex. Seven isolates belonged to a previously defined outbreak while 19 isolates were unrelated epidemiologically. The PCR-based DNA fingerprinting techniques used were: (i) repetitive extragenic palindromic (REP) PCR; (ii) enterobacterial repetitive intergenic consensus (ERIC) PCR; (iii) randomly amplified polymorphic DNA with M13 forward primer; (iv) restriction analysis of the amplified 16S rRNA gene (ARDRA-16S); and (v) restriction analysis of an amplified region containing the 16S–23S rRNA spacer region and part of the 23S rRNA gene (ARDRA 23S + spacer). The discrimination index for the PCR-based DNA fingerprinting techniques was: 0.99 for REP; 0.94 for ERIC; 0.87 for M13; 0.60 for ARDRA-16S digested with *Hpa* II and <0.50 for ARDRA 23S + spacer. It was concluded that REP-PCR possessed high discriminatory power and reproducibility in comparison with the other PCR-based DNA fingerprinting techniques, and is a simple and rapid typing method for use in epidemiological studies of isolates belonging to the *A. calcoaceticus*-*A. baumannii* complex.

Introduction

Nosocomial outbreaks of infection involving members of the *Acinetobacter calcoaceticus*-*A. baumannii* complex have been reported frequently [1–6]. An ability to distinguish isolates of the *A. calcoaceticus*-*A. baumannii* complex is useful for investigating the source of outbreaks of infection, the relationship between isolates from different patients and the environment, and the identities of multiple isolates from the same patient. Methods that have been used include antibiotic resistance patterns [7], phage typing [8], total plasmid content [9], serological typing [10], whole-cell [11, 12] and cell-envelope [13, 14] protein electrophoresis, ribotyping [15], and low-frequency restriction analysis of chromosomal DNA [16, 17]. Some of these techniques have a low discriminatory power, whereas others require a considerable amount of time, equipment and expertise to perform. In addition, with some of these techniques, various in-vivo or in-vitro conditions can change the phenotypic characteristics of certain organisms [18] and can generate variable results. Although ribotyping and low-frequency restriction analysis of chromosomal DNA have a good discrimination index, they are complex to perform.

Numerous reports have also demonstrated that PCR is useful for typing many micro-organisms [19–21]. Several PCR-based DNA fingerprinting techniques have been used to study nosocomial outbreaks involving members of the *A. calcoaceticus*-*A. baumannii* complex, including arbitrarily primed (AP) PCR with primer M13 [22, 23], enterobacterial repetitive intergenic consensus (ERIC) PCR [24] and repetitive extragenic palindromic (REP) PCR [25]. However, these techniques have not been compared thus far for their reproducibility and discriminatory power by fingerprinting isolates of a single collection. The purpose of this study was to evaluate several PCR-based DNA-fingerprinting techniques — AP-PCR with primer M13, ERIC-PCR, REP-PCR and amplified rDNA restriction analysis (ARDRA) — for typing strains belonging to the *A. calcoaceticus*-*A. baumannii* complex.

Materials and methods

Bacterial strains

In total, 26 clinical isolates belonging to the *A. calcoaceticus*-*A. baumannii* complex were analysed, with initial identification based on the biochemical criteria established by Bouvet and Grimont [26]. The epidemiology of the isolates had been examined previously by low-frequency restriction analysis of

chromosomal DNA [17]. Of the 26 strains, seven belonged to the same outbreak and 19 were unrelated epidemiologically.

DNA preparation and PCR reaction conditions

Bacteria were grown on MacConkey's agar overnight. Half a colony of each isolate was suspended in 25 µl of distilled sterile H₂O, 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 KCl, 3.0 mM MgCl₂, gelatin 0.1% w/v, 400 µM dNTPs, and 1 µM of the appropriate primers, except as noted below were added, together with 2.5 U of *Taq* polymerase (Gibco-BRL). The mixture was overlaid with oil and amplified by PCR (see below). To amplify rDNA, the extracted DNA was purified to a higher level with the InstaGene DNA Purification Matrix (BioRad), used according to the manufacturer's instructions. The extracted DNA was diluted 1 in 10, 25 µl of the diluted DNA was boiled for 10 min, and 25 µl of the reaction mixture described above was added together with *Taq* polymerase.

AP-PCR

AP-PCR fingerprinting was performed as described previously [24], with a primer concentration of 0.25 µM and the following PCR program: 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. Amplified DNA products were resolved by electrophoresis on agarose 2% w/v gels.

REP-PCR and ERIC-PCR

The primers used had the following sequences: 5'-IIIGCGCCGICATCAGGC-3' and 5'-ACGTCTTATCAGGCCTAC-3' (REP); and 5'-ATGTAAGCTCCTGGG-GATTCAC-3' and 5'-AAGTAAGTGACTGGGGGT-3' (ERIC). PCR amplification was performed with 30 cycles of 94°C for 1 min, 40°C (REP) or 52°C (ERIC) for 1 min and 65°C for 8 min, with a single final extension of 65°C for 16 min. Samples (20 µl) of each PCR end-product were analysed on agarose 2% w/v gels.

Restriction analysis of the amplified 16S rRNA gene and an amplified region containing the 16S–23S rRNA spacer region and part of the 23S rRNA gene

The primers used to amplify the 16S rRNA gene were 5'-TACCTTGTTACGACTT-3' and 5'-AGAGTTTGATCITGGA-3'. For amplification of the spacer region between the genes coding for 16S and 23S rRNA and a portion of the gene coding for 23S rRNA gene, the primers used were 5'-AAGTCGTAACAAGGTA-3' and 5'-TACTGGTTCATCATCGGTCA-3'. In both cases,

PCR was performed with 30 cycles of 94°C for 70 s, 55°C for 150 s, and 72°C for 180 s. A 15-µl portion of each PCR end-product was digested, according to the manufacturer's instructions, with 1 U of *Hinf*I, *Hpa*II, *Taq*I, *Alu*I, (Promega), *Rsa*I, *Hph*I, *Hha*I, *Nla*IV (New England Biolabs) or *Dde*I (Boehringer Mannheim) Restriction fragment length polymorphisms (RFLPs) were determined after electrophoresis of the restricted DNA on agarose 1% w/v plus NuSieve agarose 1.5% w/v.

Determination of reproducibility

Reproducibility was defined as the ability of a technique to yield the same result when the same strain was tested three times on different days with different DNA preparations, and was expressed as the percentage of strains that gave the same profile in three separate experiments.

Results

AP-PCR

DNA fingerprints generated with M13 forward primer are shown in Fig. 1A. Ten different banding patterns were distinguished (Table 1), composed of 2–4 DNA fragments of 0.5–2.1 kb. PCR profiles of the seven strains involved in the outbreak showed the same patterns (strain nos. 41 and 661 in Figs 1 and 2). The small differences in fingerprints caused by minor DNA fragments were demonstrated repeatedly.

REP- and ERIC-PCR

The profiles generated with REP primers contained several bands, ranging in size from 0.1 to 5 kb. REP-PCR analysis revealed 18 different banding patterns (Table 1 and Fig. 1B). The DNA banding pattern obtained with primer combination ERIC1R + ERIC2 was not as heterogeneous as that with REP-PCR. The profiles comprised 4–8 bands of <2 kb and 13 different banding patterns were obtained. A 1.5-kb fragment was present in 95% of the ERIC patterns (Fig. 1C). Strains belonging to the outbreak had the same profile by either REP-PCR or ERIC-PCR.

ARDRA

Primers complementary to rRNA regions that are well-conserved throughout the eubacterial kingdom were used to amplify two regions: (i) almost the entire 16S rRNA gene; and (ii) a region between the genes coding for 16S and 23S rRNA plus a portion of the gene coding for 23S rRNA. The amplified products comprised a DNA fragment of c. 1.5 kb for 16S rDNA (Fig. 2) and a fragment of 1.1 kb for 23S rDNA + spacer (data not shown). Initially, only one PCR product was found in all the strains following either amplification strategy. Therefore, restriction digestions

Fig. 1. Patterns obtained by PCR with: **A**, M13 forward primer; **B**, REP; **C**, ERIC. Lanes: **M**, DNA mol. wt marker VI (pBR328 DNA-*Bgl*I + pBR328-*Hinf*I); 1, strain 60; 2, strain 74; 3, strain 86; 4, strain 167; 5, strain 195; 6, strain 661; 7, strain 37; 8, strain 72; 9, strain 61; 10, strain 65; 11, strain 31; 12, strain 77; 13, strain 183; 14, strain 92; 15, strain 41; 16, strain 78; 17, strain 58; 18, strain 83; 19, strain 188; 20, strain 36; 21, strain 68.

were performed on the PCR amplicons and the resulting fragments were separated by electrophoresis: neither *Dde*I nor *Hae*III could differentiate between the isolates; *Hinf*I, *Taq*I, *Rsa*I and *Hha*I generated

two patterns, as did *Nla*IV and *Hph*I but with a different distribution among the strains; *Alu*I produced two patterns of different distribution; *Hpa*II was the only enzyme that generated three different patterns

Table 1. Subdivision of isolates belonging to the *A. calcoaceticus*-*A. baumannii* complex by different PCR-based DNA fingerprinting techniques

Strain no.	Outbreak	Number of patterns obtained with				
		PFGE*	AP-PCR	ERIC	REP	16S rDNA-RFLP
60	No	12	1	1	1	1
74	No	14	2	2	2	2
86	No	9	3	3	3	3
167	No	17	4	4	4	1
195	No	21	2	5	5	2
1, 6, 21, 23, 39, 41, 661	Yes	7	5	6	6	4
37	No	18	6	7	7	2
72	No	11	2	6	8	3
61	No	4	2	8	9	2
65	No	10	7	9	10	3
31	No	16	7	9	11	3
77	No	1	2	10	12	2
183	No	19	7	5	13	2
92	No	20	8	11	14	1
78	No	2	2	10	12	2
58	No	8	2	6	8	3
83	No	15	9	9	15	2
188	No	5	8	6	16	2
36	No	3	9	12	17	1
68	No	13	10	13	18	5

*Results from reference 17.

(Table 2). As an example, the *Hinf*I and *Hpa*II digests of PCR products from 16S rRNA genes of all types are shown in Fig. 2A and 2B. No polymorphisms were detected in 23S + spacer regions with *Hpa*II, *Rsa*I and *Alu*I, while *Hinf*I generated two different digestion patterns (data not shown). The other restriction enzymes were not used with 23S + spacer PCR products.

Discriminatory power and reproducibility

The discrimination index and reproducibility of each typing method are shown in Table 3. ARDRA, with either 16S rRNA or 23S + spacer PCR products, was the least discriminatory method with indices of 0.74 or <0.50, respectively. A combination of *Hpa*II and *Hinf*I increased the discrimination index from 0.6 (*Hpa*II alone) to 0.72, and with a combination of *Hinf*I, *Hpa*II, *Alu*I and *Nla*IV to 0.74. AP-PCR with M13 primer had a moderate discrimination index (0.87), while REP-PCR and ERIC-PCR both had a high discrimination index, although REP-PCR showed better reproducibility than ERIC-PCR.

Discussion

Nosocomial infections caused by organisms belonging to the *A. calcoaceticus*-*A. baumannii* complex are an increasing problem, especially in immunosuppressed patients, and have become a major cause of hospital-acquired infections worldwide. Rapid and accurate identification of strains involved in an outbreak is important, both for limiting the severity of the outbreak and for tracing the source of the infecting organism. Despite intensive investigations, no simple, sensitive,

rapid and highly discriminatory system for typing members of the *A. calcoaceticus*-*A. baumannii* complex has so far emerged. Current typing methods are based on either phenotyping (e.g., antimicrobial susceptibility testing, biotyping, phage typing, protein electrophoresis) or genotypic analysis (e.g., plasmid analysis, restriction analysis of chromosomal DNA with or without pulsed-field gel electrophoresis). The relative advantages and disadvantages of these methods have been reviewed previously [27]. In general, genotypic methods have improved the ability to demonstrate differences between strains of nosocomial origin. Plasmid analysis has proved to be a useful means of demonstrating clonality among organisms involved in hospital epidemics [28], but is not ideal as 20–30% of *A. calcoaceticus*-*A. baumannii* strains do not contain plasmids [5, 17], rendering them non-typable by this method. Ribotyping and low-frequency restriction analysis of chromosomal DNA are labour-intensive processes and require several days for completion.

The present study investigated several PCR-based DNA-fingerprinting techniques. Such techniques have been used previously to delineate outbreaks [22–25], and seem to possess a high degree of discrimination, ease and speed. In AP-PCR, arbitrarily selected primers are annealed to the template DNA under low-stringency conditions for the initial cycles of DNA amplification, which allows interactions between the primers and target DNA in regions containing base mismatches. As these interactions may occur at multiple sites on both strands of a complex genome, only a single primer sequence is required for a given reaction. Graser *et al.* [22] amplified DNA from *A. calcoaceticus*-*A. baumannii* strains with a primer

Fig. 2. Patterns obtained after restriction of the amplified 16S rDNA PCR products with: A, *Hinf*I; B, *Hpa*II. Lanes: M, DNA mol. wt marker VI (pBR328 DNA-*Bgl*I + pBR328 DNA-*Hinf*I); A, PCR product of 16S rDNA with no cleavage by restriction endonucleases; 1, strain 41; 2, strain 661; 3, strain 60; 4, strain 77; 5, strain 183; 6, strain 188; 7, strain 188; 8, strain 78; 9, strain 74; 10, strain 78; 11, strain 74; 12, strain 86; 13, strain 86; 14, strain 72; 15, strain 92; 16, strain 167; 17, strain 58; 18, strain 68; 19, strain 65; 20, strain 195; 21, strain 31.

Table 2. Overview of ARDRA-16S rDNA patterns obtained for isolates belonging to the *A. calcoaceticus*-*A. baumannii* complex

Strain no.	16S rDNA-RFLP type					Pattern no.
	<i>Hinf</i> I	<i>Hpa</i> II	<i>Alu</i> I	<i>Nla</i> IV	Combined	
60	B	B	B	B	BBBB	1
74	A	C	B	A	ACBA	2
86	A	B	B	A	ABBA	3
167	B	B	B	B	BBBB	1
195	A	C	B	A	ACBA	2
1, 3, 21, 23, 39, 41, 661	A	A	A	A	AAAA	4
37	A	C	B	A	ACBA	2
72	A	B	B	A	ABBA	3
61	A	C	B	A	ACBA	2
65	A	B	B	A	ABBA	3
31	A	B	B	A	ABBA	3
77	A	C	B	A	ACBA	2
183	A	C	B	A	ACBA	2
92	B	B	B	B	BBBB	1
78	A	C	B	A	ACBA	2
58	A	B	B	A	ABBA	3
83	A	C	B	A	ACBA	2
188	A	C	B	A	ACBA	2
36	B	B	B	B	BBBB	1
68	A	B	B	B	ABBB	5

Table 3. Reproducibility and discriminatory index of several PCR-based DNA fingerprinting techniques for 26 isolates belonging to the *A. calcoaceticus*-*A. baumannii* complex

Typing method	Number of types	Discrimination index	Reproducibility (%)
AP-PCR	10	0.87	86
REP-PCR	18	0.99	90
ERIC-PCR	13	0.94	66
ARDRA-16S*	5	0.74	100
ARDRA-23S + spacer [†]	2	<0.50	100

*Combination of the following restriction enzymes: *Hinf*I, *Hpa* II, *Alu* I and *Nla* IV.

[†]23S rDNA + spacer digested with *Hinf*I.

consisting of the core sequence of the M13 phage, and found a discriminatory power similar to that of low-frequency restriction analysis of chromosomal DNA. In contrast, the present study found a discrimination index of 0.87 for this technique, in comparison with an index of 1.0 for low-frequency restriction analysis of chromosomal DNA, which was considered to be the reference technique. In a previous study the discriminatory power of ribotyping and AP-PCR was very similar [23].

Another PCR-based fingerprinting technique was described by Versalovic *et al.* [29], who used consensus primers to amplify sequences located between successive repetitive elements — repetitive extragenic palindromic elements (REP) and enterobacterial repetitive intergenic consensus sequences (ERIC) — in gram-negative bacteria. REP and ERIC-based primers have also been used to investigate gram-positive bacteria, including *Staphylococcus aureus* [30] and *Streptococcus pneumoniae* [31]. In the present study, ERIC-PCR had a higher discrimination index than AP-PCR, but a lower discrimination index than REP-PCR and low-frequency restriction analysis

of chromosomal DNA. Reboli *et al.* [25] applied REP and ERIC-PCR to characterise *A. baumannii* isolates from adult intensive care units, and also found that REP-PCR was more effective in discriminating strains than ERIC-PCR. In contrast, Struelens *et al.* [24] found agreement between ERIC-PCR and low-frequency restriction analysis of chromosomal DNA when investigating an outbreak of infections caused by *A. baumannii*.

Ribosomal operons have also been targeted for PCR-RFLP typing. Results from 16S rDNA analysis are useful for identification to the species level [32], including identification of *Acinetobacter* spp. [33], but are not useful for typing members of the *A. calcoaceticus*-*A. baumannii* complex, as shown in this study and others [33]. In the present study, the difference between ARDRA-16S profiles A (five DNA fragments) and B (four DNA fragments) generated by digestion with *Hinf*I (Fig. 2A) could result from loss of a *Hinf*I site, since the size (*c.* 1.1 kb) of the large DNA fragment in profile B is the sum of the sizes of the 789-bp and 300-bp DNA fragments of profile A. The difference observed

between profiles A (five DNA fragments) and B (four DNA fragments) generated by digestion with *Hpa*II (Fig. 2B) could be explained if the genomic DNA from *Acinetobacter* contained two or more copies of 16S rDNA. Loss of an *Hpa*II site from one or more of the copies could generate profile A from profile B as profile A showed an extra 200-bp DNA fragment (i.e., the sum of the 130-bp and 70-bp fragments from profile B). The heterogeneity in one or more copies of 16S rDNA is also suggested by a reproducible decrease in the intensity of the 130-bp and 70-bp fragments, indicating that there is less DNA present. Thus, although slight differences are observed between the ARDRA-16S patterns, the strains studied probably belong to the same species of the *A. calcoaceticus*-*A. baumannii* complex. The intergenic spacer region between the genes coding for 16S rRNA and 23S rRNA is useful for identification of eubacteria [34] and for typing *Rochalima henselae* [35], but in the present study with ARDRA of 23S rDNA + spacer, despite the high reproducibility demonstrated, only limited variation was found among the *A. calcoaceticus*-*A. baumannii* strains examined, thereby showing a low discrimination index.

In conclusion, REP-PCR was found to be a rapid method, with a high degree of reproducibility and discriminatory power, which seems to be well-suited for epidemiological studies of strains belonging to the *A. calcoaceticus*-*A. baumannii* complex.

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