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RESEARCH ARTICLE

Combination of ARDRA and RAPD genotyping techniques in identification of *Acinetobacter* spp. genomic species

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Abstract A total of 10 non-repetitive multi-drug-resistant *Acinetobacter* strains were collected. With reference to *A. calcoaceticus* (ATCC23055), *A. baumannii* (ATCC19606), *A. lwoffii* (ATCC17986), and *A. junii* (NCTC5866), DNA fingerprint technique, amplified ribosomal DNA restriction analysis (ARDRA), and random amplified polymorphism DNA (RAPD) were carried out to identify the genomic species of *Acinetobacter* spp. The distances between them were calculated by the unweighted pair group method with arithmetic (UPGMA). Genotypes of *Acinetobacter* spp. were effectively classified and an *A. junii* together with nine *A. baumannii* isolates was genomically identified. The combination of ARDRA and RAPD DNA-fingerprint technique shows high complementarity, and could be a useful tool in *Acinetobacter* genomic species identification.

Keywords *Acinetobacter*, DNA fingerprinting, random amplified polymorphism DNA (RAPD), cluster analysis

1 Introduction

Members of the genus *Acinetobacter* are ubiquitous in soil and water and are an important cause of nosocomial infections, in which they can be recognized by using DNA-DNA hybridization. The genus *Acinetobacter* has recently been shown to comprise at least 20 genomic species. Numerous reports have commented on *Acinetobacter*-related hospital infections and outbreaks. Strains belonging to genomic species 5 (*A. junii*), 7 (*A. johnsonii*), 8, 10 and 13TU have been found more frequently to be associated with hospital infection and epidemic outbreaks, and were formerly regarded as of less clinical importance. In general, the ecology and epidemiology of *Acinetobacter* species are not well understood, mainly due to lack of practical and rapid methods to identify the isolates according to recent taxonomy. A rapid method is needed to genotype *Acinetobacter* isolates to determine their epidemiology and clonality during infectious outbreaks. By using amplified rDNA restriction analysis (ARDRA), together with random amplified polymorphic DNA (RAPD), we identified and collected genetic species of multi-drug resistant *Acinetobacter* isolators during the period of 2002–2004. Additionally, the effectiveness of the combination of the two methods was evaluated.

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2 Materials and methods

2.1 Bacterial strains

Sixteen bacterial strains, including *E. coli* ATCC25922 and *K. pneumonia* ATCC700603 were used for laboratory quality control. Ten clinical isolators were collected during 2002–2004. *A. calcoaceticus* (ATCC23055), *A. baumannii* (ATCC19606), *A. lwoffii* (ATCC17908), and *A. junii* (NCTC5866), which were generously donated by Dr. J M Coelhoare from the England Health Protection Agency, were used for typing and as references.

2.2 Reagents and software

Bacterial strains were cultured in Mueller-Hinton liquid medium (Oxoid, England). Bacterial genomic DNA was extracted using CTAB (Biotech Co. USA) protocol as described previously (Shahjahan et al., 1995). Reagents for PCR and restriction enzymes, such as *Alu*I, *Cfo*I, *Mbo*I, *Msp*I, and *Hin*fI were provided by TaKaRa Co., China. The amplicons and restricted 16S rDNA were quantified densitometrically using the Labworks 4.0 Software (UVP, CA). The genetic distances between the bacteria were calculated by unweighted pair group

method with arithmetic (UPGMA) using PC-ORD Cluster Analysis Software (USA).

2.3 Primary species identification

Initially, the clinical isolators were identified as *Acinetobacter baumannii* using API20NE (Biomerieux Co., France) with an identification rate of 87.0%–98.9%.

2.4 ARDRA genotyping

Genomic DNA was extracted using CTAB protocol (Shahjahan et al., 1995), and served as templates for the amplification of 16S rRNA and for RAPD. Universal primers for 16S rRNA amplification were 5′-TGG CTC AGA TTG AAC GCT GGC GGC-3′ (5′ end of 16S rRNA gene) and 5′-TAC CTT GTT ACG ACT TCA CCC CA-3′ (3′ end of 16S rRNA gene) (Rossau et al., 1991). PCR was carried out as described previously (Vaneechoutte et al., 1995). The length of the amplicon was about 1500 bp. The amplified DNA was used for restriction. Restriction was carried out for 1 h at 37°C in 20 μL of a commercially supplied incubation buffer containing 1 μL (about 5 U) of restriction enzymes *Alu*I (AGCT), *Cfo*I (GCGC), *Hin*fI (GANTC), *Mbo*I (GATC), and *Msp*I (CCGG), 10 to 15 μL of PCR product, reaction buffer 2 μL and bovine serum albumin or ddH_2O 2 μL. The volume of amplicon used in the restriction mixture was adjusted arbitrarily on the basis of visually observed fluorescence intensity of the amplified rDNA fragment in the control gel. Restriction was stopped by the addition of 5 μL of a loading buffer. Restriction fragment patterns were analyzed by gel electrophoresis of 10 mL of each restriction mixture at 7 V/cm for 3 h in 3% (w/v) agarose gel in Tris electrophoresis buffer. The bands on the gels were scanned with Labworks 4.0 and the digital information of the bands' molecular sizes was obtained. Numbers and densities were generated and calculated with UPGMA using PC-ORD Software. The Euclidean distances of these strains' congenic correlation were depicted with a dendrogram.

2.5 RAPD genotyping

RAPD genotyping was carried out as previously described with slight modifications (Carr et al., 2001; Gallego and Towner, 2001; Mathai et al., 2001; Koeleman et al., 1998). Random primers were AP2 (5′-GTTTCGCTCC-3′), AP4 (5′-AAGAGCCCGT-3′), AP5 (5′-AACGCGCAAC-3′), and AP6 (5′-CCCGTCAGC-3′). The PCR reaction mixture contained template DNA 2.0 μL, Taq polymerase 1.0 U, primer 1.0 μL (2 μmol/L) and dNTPs 200 μmol in a final volume of 25 μL. Initial denaturation (95°C for 6 min) was followed by 45 cycles of denaturation at 95°C for 45 s, annealing at 36°C for 45 s and at 72°C for 2 min, with a final extension step at 72°C for 10 min. A 0.4°C/s temperature changing rate was introduced in the PCR procedure. PCR products were resolved in 3% (w/v) agarose gels, followed by digital transformation, congenic distance calculation and dendrogram output as mentioned above.

3 Results

3.1 Bacterial genomic DNA and 16S rDNA

The molecular size of genomic DNA extracted by CTAB was about 60 kb with a λ260/λ280 ratio of 1.6–1.8. Bacterial 16S rDNA amplicons were about 1500 bp.

3.2 Electrophoresis of restricted 16S rDNA amplicons

Every strain had 2–5 electrophoresis bands which were clearly and easily recognized with conservative band in common (Fig. 1). Band patterns between reference strains diverged markedly, while clinical strains demonstrated fewer differences, indicating their genetic distance and phylogenetic relationship.

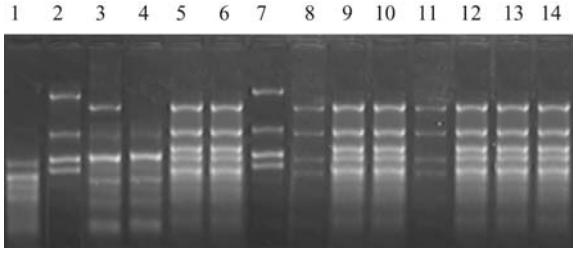

Fig. 1 Restriction patterns of *Hha*I of *Acinetobacter* spp. 16S rDNA. Lanes 1–14: ATCC 23056, ATCC 17908, ATCC 19606, NCTC 5866, A011, A018, ZN 3858, JA03, JB08, 0133, 2665, 2717, 4420 and 5181.

3.3 Outcomes of random amplified polymorphic DNA of *Acinetobacter*

Every strain had 5–15 electrophoresis bands with molecular sizes of 0.2–2.0 kb. Most of the bands were clearly and easily recognized with conservative band in common (Fig. 2). Band patterns between reference strains diverged markedly, demonstrating the genetic distance. The patterns of lane 2 (ATCC17908) and lane 7 (ZN3858) show high similarity, while the pattern of lane 3 (ATCC19606) was highly similar to that of other clinical strains.

3.4 Cluster analysis

3.4.1 UPGMA cluster analysis of ARDRA data

According to the level of 0.47 Dice coefficient, all the 14 strains fell into 4 cluster groups. Group A was

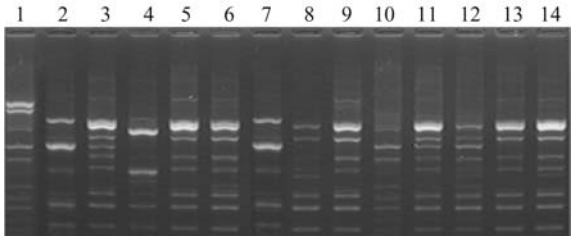

Fig. 2 AP5 random amplified polymorphic DNA patterns of *Acinetobacter* spp.. Lanes 1–14: ATCC23056, ATCC17908, ATCC19606, NCTC5866, A011, A018, ZN3858, JA03, JB08, 0113, 2665, 2717, 4420 and 5181.

ATCC23055. Group B contained *A. junii* strain ATCC17908 and ZN3858. Group C was NCTC5866. Group D consisted of 9 clinical strains and ATCC19606 (Fig. 3). Four reference strains fell into 4 groups as shown by the dendrogram, representing actual congenic correlation between these strains.

3.4.2 UPGMA cluster analysis of RAPD data

UPGMA cluster analysis of RAPD generates 4 groups as that in ARDRA but with a high resolution (Fig. 4).

The cluster analysis outcomes of these two genetic fingerprinting techniques demonstrated that *Acinetobacter junii* reference strains ATCC17908 and the clinical isolator ZN3858 in group B, *Acinetobacter baumannii* ATCC19606 and the rest 9 clinical isolators had a higher homology than that of standard reference strains. Therefore, each cluster group represented a genomic species in the genus *Acinetobacter*.

4 Discussion

Complexity in the taxonomy of the genus *Acinetobacter* necessitates the discovery of quick and accurate species identification methods (Van Looveren and Goossens, 2004). The overuse of antibiotics has led to antibiotic-resistant superbugs, which were conventionally regarded to be less virulent, less frequent and of less clinical importance, including *A. junii, A. Lwoffii* and others (van Dessel et al., 2002). The lack of practical, rapid identification methods, together with the need for epidemiological data, prompts *Acinetobacter* genus genotyping and species identification. To date, conventional biochemical identification systems (for example, Bouvet-Grimont systems) and commercial identification systems, including API system, have been shown to be problematic especially in discriminating among the genetically highly related glucose-acidifying genomic species 1, 2, 3, and 13. On the other hand, it has been shown that the DNA fingerprint technique can be very useful in *Acinetobacter* genomic species identification. Along with the pulse field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP), RAPD and ADRDA have been introduced in *Acinetobacter* species discrimination, which in turn has enhanced the accuracy and scientificity of genomic species identification. However, PFGE and AFLP techniques are associated with expensive laboratory equipments, more complex procedures and manipulation. Thus, it is not always practical for small labs (Biron et al., 2001).

16S rRNA is the chromosomal gene of 16S ribosomal DNA (rDNA) in prokaryotes and the most conserved gene in cell evolution. Each species has its special hot-spot of mutation in the nucleotide sequence of 16S rRNA, the

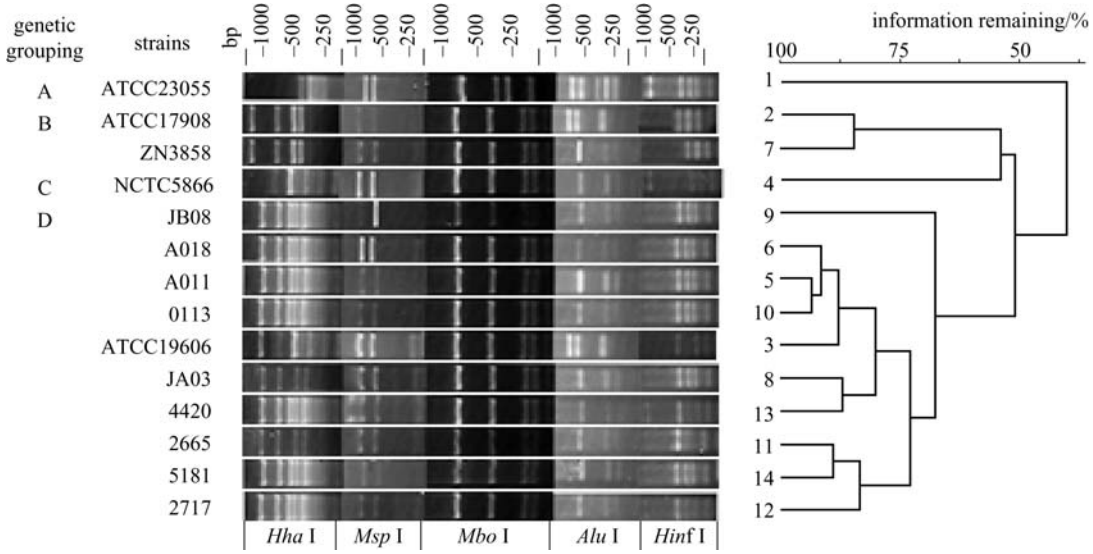

Fig. 3 Combination patterns of *Acinetobacter* 16S rDNA restricted by *Hha*I, *Msp*I, *Mbo*I, *Alu*I, *Hin*fI and the UPGMA cluster analysis. ZN3858 and ATCC17908 fall into group B, ATCC23055 and NCTC5866 represent groups A and C respectively, and all the others constitute group D.

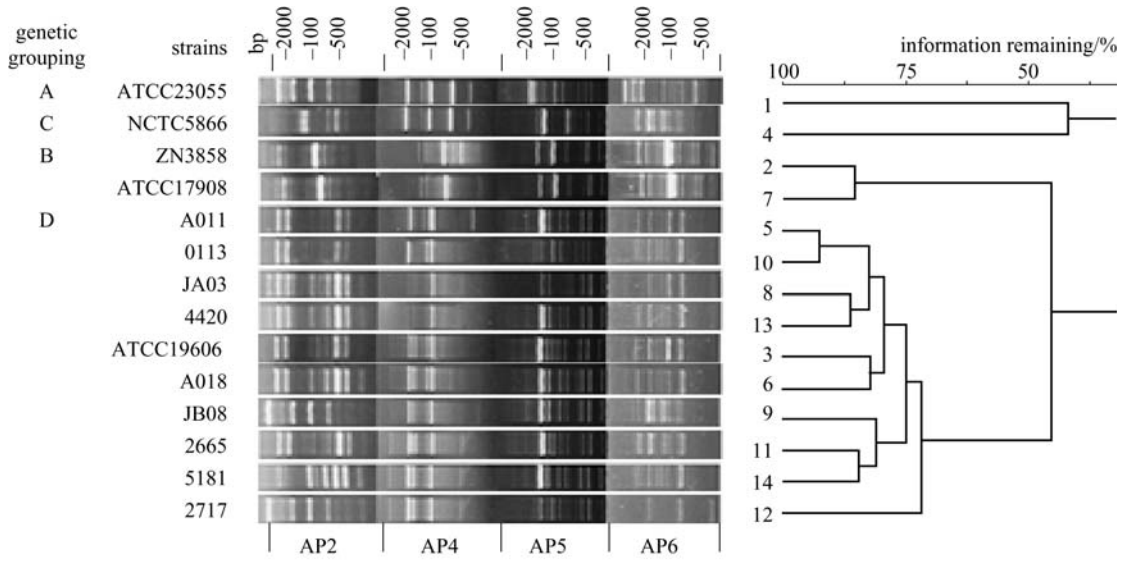

Fig. 4 Combination patterns of random primers amplified polymorphic DNA of *Acinetobacter* spp. and the UPGMA cluster analysis. ZN3858 and ATCC17908 fall into group B, ATCC23055 and NCTC5866 represent groups A and C, respectively, and all the others constitute group D.

restriction pattern of which can reveal the evolution divergence or congenic homology (Vaneechoutte et al., 1992). 16S rDNA can be amplified by PCR technique with conservative section based primers. ARDRA can be used to identify the species of different genera. The reproducibility of ARDRA is high (i.e., different cultures, cell suspensions, amplifications, and restrictions of the same strain yielded identical restriction patterns), but with a relatively lower resolution power (Rodas et al., 2003). To overcome this shortage, we have chosen 5 high-frequency restriction enzymes and jointly analyzed all the restriction patterns in combination with RAPD technique.

RAPD is an important DNA fingerprint detection method in biological genetics (Williams et al., 1990). PCR with arbitrary 10-mer primers will produce different molecular sizes and different richness amplicons, depending on the difference in DNA nucleotides of each genus or species, or the genetic divergence between them. Amplicon patterns can reflect the distance of evolution and thus, serve as genomic discrimination tools in biologic study. RAPD technique has a high sensitivity and resolution, but a low reproducibility. It can be influenced by the length or integrity of templates, the annealing temperature, PCR cycles, temperature changing rate, *etc*. In our study, we also jointly analyzed the RAPD amplicons of these strains, with combination of ARDRA data to reduce the influence of a lower reproducibility to the utmost.

Our study shows that cluster analysis of the combined data of ARDRA and RAPD could identify the genomic *Acinetobacter* species. ZN3858 was formerly identified as *A. baumannii* by API identification system, but is presently recognized as an *A. junii* strain. The multi-drug resistant *A. junii* isolator has rarely been reported (Zhang et al., 2005). In addition, we also successfully differentiated *A. calcoaceticus* strain from *A. baumannii* strain, which is hardly possible using bio-chemical characteristics and hence, the name ACB complex (*A. calcoaceticus-A. baumannii* complex) is used in clinical practice. Moreover, we found the inconsistency of ARDRA with RAPD in the dendrogram, which might be caused by the different discriminative powers.

In conclusion, ARDRA and RAPD techniques show apparent complementarity in sensitivity and reproducibility. We suppose that when the two techniques are combined, the disadvantages of each are minimized. As a result, it yields highly reproducible and informative DNA fingerprint data and thus, enhances the identification effectiveness. Additionally, these techniques are rapid and require fewer laboratory equipments. Therefore, the combination of ARDRA and RAPD DNA-fingerprint technique may be a useful tool in Acinetobacter genomic species identification and bacterial taxonomy.

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