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Validation of Use of Whole-Cell Repetitive Extragenic Palindromic Sequence-Based PCR (REP-PCR) for Typing Strains Belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* Complex and Application of the Method to the Investigation of a Hospital Outbreak

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***Acinetobacter* spp. are being reported with increasing frequency as causes of nosocomial infection. In order to identify reservoirs of infection as quickly as possible, a rapid typing method that can differentiate epidemic strains from environmental and nonepidemic strains is needed. In 1993, a cluster of *Acinetobacter baumannii* isolates from five patients in the adult intensive therapy unit of our tertiary-care teaching hospital led us to develop and optimize a rapid repetitive extragenic palindromic sequence-based PCR (REP-PCR) typing protocol for members of the *Acinetobacter calcoaceticus-A. baumannii* complex that uses boiled colonies and consensus primers aimed at repetitive extragenic palindromic sequences. Four of the five patient isolates gave the same REP-PCR typing pattern as isolates of *A. baumannii* obtained from the temperature probe of a Bennett humidifier; the fifth isolate had a unique profile. Disinfection of the probe with 70% ethanol, as recommended by the manufacturer, proved ineffective, as *A. baumannii* with the same REP-PCR pattern was isolated from it 10 days after cleaning, necessitating a change in our decontamination procedure. Results obtained with REP-PCR were subsequently confirmed by ribotyping. To evaluate the discriminatory power (*D*) of REP-PCR for typing members of the *A. calcoaceticus-A. baumannii* complex, compared with that of ribotyping, we have applied both methods to a collection of 85 strains that included representatives of six DNA groups within the complex. Ribotyping using *Eco*RI digests yielded 53 patterns ($D = 0.98$), whereas 68 different REP-PCR patterns were observed ($D = 0.99$). By computer-assisted analysis of gel images, 74 patterns were observed with REP-PCR ($D = 1.0$). Overall, REP-PCR typing proved to be slightly more discriminatory than ribotyping. Our results indicate that REP-PCR typing using boiled colonies is a simple, rapid, and effective means of typing members of the *A. calcoaceticus-A. baumannii* complex.**

Acinetobacter spp. are opportunistic pathogens that are being implicated in sporadic epidemics of hospital infection with increasing frequency (2, 4, 26, 35), and cases of community-acquired *Acinetobacter* infection have also been recorded (21, 25). *Acinetobacter* spp. can cause a wide range of clinical conditions, including pneumonia, septicemia, urinary tract infections, wound infections, endocarditis, and meningitis (5, 9, 13, 35). Immunocompromised patients are at particular risk of being infected by bacteria of this genus, as are the elderly and those requiring mechanical ventilation. Treatment can be complicated by the multiple-drug resistance of many isolates (2, 32, 37). Contaminated hospital equipment or colonized hands of hospital staff have previously been identified as reservoirs for *Acinetobacter* spp. in epidemics (5, 9, 19, 29). In many cases, the true source of infection cannot be traced, because members of the genus *Acinetobacter* are widespread in the hospital environment and can be isolated from sinks, tap water, the air, and dust or can be present as commensal organisms of human skin and the respiratory tract (1, 5, 14). Typing schemes that allow rapid differentiation of epidemic strains from the numerous incidental strains likely to be encountered in a ward are urgently needed.

DNA-DNA hybridization studies have shown that there are at least 19 DNA groups (genomic species) within the genus *Acinetobacter* (7, 8, 17, 38). Strains belonging to some of these groups are so similar that phenotypic differentiation between them is not always possible (12, 18). These phenotypically similar but genotypically distinct DNA hybridization groups have been designated the *Acinetobacter calcoaceticus-A. baumannii* complex (18). This complex currently comprises DNA group 1, *A. calcoaceticus* sensu stricto Bouvet and Grimont (7); DNA group 2, *A. baumannii*; unnamed DNA group 3 (7); unnamed DNA group 13 (38); and two more as yet unnamed DNA groups (17). With the exception of DNA group 1, which is regarded as an environmental species, each genomic species within the complex has been linked to nosocomial infections (12, 17, 26). It is thus essential that typing schemes used for epidemiological studies be able to distinguish between isolates in the *A. calcoaceticus-A. baumannii* complex.

A variety of methods, including biotyping, antibiotic resistance typing (28), comparison of outer membrane protein profiles (12), plasmid profiling (15, 33), ribotyping (16), and analysis of restriction fragment length polymorphisms using

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TABLE 1. Strains isolated at the Leeds General Infirmary used in this study

Strain	Date of isolation (mo/day/yr)	Ward	Source	API 20NE profile	Species[a]	Growth at 44°C	Glucose oxidation	REP-PCR profile[b]	Ribotype
16\6	1/5/93	ITU	Sputum	4041471	*A. baumannii*	+	+	1	2M
16\7	1/7/93	ITU	Tracheal secretion	4041471	*A. baumannii*	+	+	1	2M
16\8	1/8/93	ITU	Sputum	4041053	*A. baumannii*	+	+	2	2N
16\9	1/9/93	ITU	Sputum	4041470	*A. baumannii*	+	+	1	2M
16\10	12/31/92	ITU	Leg wound	4041470	*A. baumannii*	+	+	1	2M
16\11[c]	12/?[d]/92	?	Blood culture	4010050	*Acinetobacter haemolyticus*	−	+	3	A[e]
16\12	1/12/93	ITU	Bennett temp probe	4041070	*A. baumannii*	+	+	1	2M
16\13	1/12/93	ITU	Sluice sink	4000051	*A. haemolyticus*	−	−	4	B[e]
16\14[c]	12/?/92	?		4001473	*A. baumannii*	+	+	5	2L
16\15[c]	12/?/92	?		4201073	*A. baumannii*	+	+	6	13L
16\16[c]	12/?/92	?	Blood culture	0001032	*Acinetobacter lwoffii*	−	−	7	C[e]
16\17	1/12/93	ITU	Bennett temp probe	4041070	*A. baumannii*	+	+	1	2M
16\22	1/22/93	ITU	Bennett temp probe (10 days after cleaning)	4041071	*A. baumannii*	+	+	1	2M

[a] As determined from API 20NE profile.
[b] Refers to profiles shown in Fig. 1.
[c] Random isolates of *Acinetobacter* spp. from services other than ITU made in December 1992.
[d] ?, unknown.
[e] Denotes a ribotype that indicates that the strain does not belong to the *A. calcoaceticus-A. baumannii* complex.

pulsed-field gel electrophoresis (22, 23), have been used successfully for the typing of *Acinetobacter* spp. These methods can be complex and time-consuming to perform or can rely on unstable phenotypic characteristics. A PCR-based fingerprinting system that uses consensus primers for the repetitive extragenic palindromic (REP) sequences found in many bacterial chromosomes has been shown to be applicable to a wide range of bacterial species (11, 40–42) and is known as REP-PCR. The highly conserved REP sequence is approximately 35 nucleotides long, includes an inverted repeat, and can occur in the genome singly or as multiple adjacent copies (36). Recently, Reboli et al. (30) described the use of REP-PCR to characterize isolates of *A. baumannii* from hospitalized patients. The method effectively discriminated between epidemic and sporadic isolates but required time-consuming genomic DNA isolation, purification, and quantitation. In 1993, a cluster of *A. baumannii* isolates from patients in the intensive therapy unit (ITU) of the Leeds General Infirmary prompted us to develop a rapid REP-PCR-based typing method for *Acinetobacter* spp. that utilizes boiled cultures. Here, we describe the use of this method to identify the reservoir of infection in the outbreak and a systematic evaluation of the discriminatory power of REP-PCR typing versus ribotyping, based on analysis of a comprehensive collection of strains belonging to the *A. calcoaceticus-A. baumannii* complex.

MATERIALS AND METHODS

Description of the outbreak. During the 10-day period between 31 December 1992 and 9 January 1993, *A. baumannii* was isolated from five patients in the ITU of the Leeds General Infirmary, a 1,000-bed tertiary-care teaching hospital. These isolates were designated 16\6 to 16\10, and details are given in Table 1. Isolates 16/6 and 16/9 were isolated from two patients with *A. baumannii* pneumonia. The other three patients did not exhibit symptoms of infection and were considered to be only colonized. Isolates were identified by using the API 20NE system (Biomerieux, Marcy l'Etoile, France). An environmental screen of the ward was performed in an attempt to trace the source of the contamination. *Acinetobacter* spp. were cultured from a sluice sink on the ward (isolate 16\13) and the temperature probe of a Bennett humidifier (Puritan Bennett, London, United Kingdom). Upon primary isolation, two different colony morphologies were observed among the growth from the probe, and an example of each (isolates 16\12 and 16\17; Table 1) was retained for study.

Bacterial strains. To test the discriminatory power of REP-PCR typing versus ribotyping, 85 strains belonging to the *A. calcoaceticus-A. baumannii* complex were examined, and they are listed below (see Tables 2 to 6). Most of the strains originated from clinical sources in Sweden, Denmark, and The Netherlands and have been used in previous studies (16, 17). None of the strains were thought to be linked epidemiologically. DNA groups 1, 2, 3, and 13 were represented by 10, 28, 27, and 16 strains, respectively. Two strains from each of the two unnamed DNA groups within the complex were also included (see Table 6). Representatives of all of the ribotypes that have been encountered to date were included in the collection.

Strains were maintained on Iso-Sensitest agar (code CM471; Unipath, Basingstoke, United Kingdom) for routine purposes. For long-term storage, strains were suspended in glycerol broth at −70°C.

Ribotyping. DNA was extracted and digested with *Eco*RI, and ribotyping was performed as described previously (16). If two isolates produced the same banding pattern when the DNA was digested with *Eco*RI, a further level of discrimination could sometimes be obtained by repeating the test with restriction enzymes *Cla*I and/or *Sal*I. The ribotype designation of each strain consists of a number corresponding to the DNA group of the strain, an uppercase letter indicating differences within each DNA group determined by use of *Eco*RI, and, where necessary, a lowercase letter that indicates further differentiation determined by use of *Cla*I and/or *Sal*I. It has been shown previously that the ribotype pattern of a strain contains bands that identify the DNA group to which the strain belongs (16).

Oligonucleotide primers. The primer pair REP1R-I (5′-IIIICGICGICATCIG GC-3′) and REP2-I (5′-ICGICTTATCIGGCCTAC-3′) (40) was used to amplify putative REP-like elements in the bacterial DNA. These primers have the nucleotide inosine at ambiguous positions in the REP consensus sequence. Inosine contains the purine base hypoxanthine and is able to base pair with A, C, G, or T. Oligonucleotides were prepared by the Department of Pathology, University of Leeds, Leeds, United Kingdom.

Rapid template preparation for PCR. Strains were grown overnight on Iso-Sensitest agar at 30°C. After incubation, four or five discrete colonies of each strain were removed with a sterile wooden toothpick and emulsified in 50 μl of sterile distilled water in a 0.5-ml Eppendorf tube. To lyse the cells, the tubes were heated for 5 min at 95°C with an Omnigene thermal cycler (Hybaid Ltd., Middlesex, United Kingdom) and then frozen at −20°C while the rest of the PCR reagents were prepared. Frozen crude template was always used on the day it was prepared.

Phenol-chloroform extraction of genomic DNA. A more rigorous method was used to prepare template DNA from a minority of strains that did not give results with the method outlined above. Briefly, strains were grown overnight on Iso-Sensitest agar and the growth from approximately one-quarter of a plate was resuspended in 500 μl of extraction buffer (0.1 M NaOH, 1 M NaCl, 0.5% sodium dodecyl sulfate) and boiled for 15 min. Three extractions with phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma) were then performed. Genomic DNA was precipitated by adding 1 ml of ethanol and placing the solution at −70°C for 30 min. DNA was harvested by centrifugation (12,000 × *g*) for 15 min. The pellet was washed with diethyl ether, dried under a vacuum at room temperature, and then redissolved in 100 μl of sterile ultrapure water. For PCR, 4 μl of the solution was used as a template.

PCR amplification. Amplification reactions were performed in a final volume of 50 μl. Mg^{2+}-free PCR buffer was purchased as a 10× concentrate consisting of 500 mM KCl–100 mM Tris-HCl (pH 9.0)–1% Triton X-100 (Promega Cor-

poration, Madison, Wis.) and used at a final concentration of 1×. Each reaction mixture contained 5 μl of 10× PCR buffer; 2 U of Super-*Taq* DNA polymerase (HT Biotechnology, Cambridge, United Kingdom); 1 mM $MgCl_2$; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.); 5 μl of dimethyl sulfoxide; 50 pmol of each primer; and 5 μl of thawed crude template DNA or 4 μl of phenol-chloroform-extracted DNA. A negative control (template DNA omitted) to detect reagent contamination was included in each run. The reaction mixtures were vortexed briefly and overlaid with 4 drops of mineral oil. Amplification reactions were carried out in an air-cooled Omnigene thermal cycler (Hybaid Ltd.) with an initial denaturation (3 min at 95°C) followed by 30 cycles of denaturation (30 s at 90°C), annealing (1 min at 45°C), and DNA molecule extension (8 min at 65°C), with a single final extension (16 min at 65°C). Aliquots (13 μl) of each sample were subjected to electrophoresis in 1.5% agarose gels. Amplified products were detected by being stained with ethidium bromide and photographed with Polaroid type 665 film.

All 85 strains were coded and then subjected to REP-PCR analysis, and the resulting fingerprints were compared without knowledge of the ribotype or DNA group of each strain.

Visual inspection of fingerprints. Prior to the computer-assisted analysis outlined below, the REP-PCR fingerprints of strains were compared by visual inspection. The fingerprint of each strain was compared with that of every other strain in the study by one observer (A. M. Snelling). The molecular sizes of amplimers were judged by comparison with concurrently run molecular weight standards. Profiles were considered to be highly similar when all visible bands from two isolates had the same apparent migration distance. Variations in the intensity or shape of bands were not taken into account. The absence of up to two bands from a fingerprint was allowed, when all other visible bands in the fingerprints matched, before isolates were considered different by visual inspection (30, 41).

Computer-assisted analysis of fingerprints. Dendron software (Solltech, Inc., Oakdale, Calif.) (31) run on an Apple Macintosh Quadra 800 computer was used to compare the REP-PCR fingerprints. Photographs of each gel were scanned into the Dendron data file with a Microtek ScanMaker IIxe scanner. The software was used to correct the lanes of each gel for angle distortion, and the lanes were then scanned for pixel density. The patterns were normalized by equating the *Bgl*I-cut λ DNA molecular weight markers between gels. The Dendron package calculated the similarity value (S_{AB}) for each pair of fingerprints on the basis of band positions alone, using the following formula:

$$S_{AB} = \frac{\sum_{i=1}^{k} (a_i + b_i - [a_i - b_i])}{\sum_{i=1}^{k} (a_i + b_i)}$$

where a_i and b_i are the intensities of band i in patterns A and B, respectively, and k is the number of bands. Since band position alone was used, the values of a_i and b_i were either 0 (not present) or 1 (present). S_{AB} ranges from 0 to 1.0, where 0 indicates that the fingerprints have no bands in common and 1.0 indicates that the fingerprints are identical.

The unwarping of gels requires some subjective input from the investigator, so pairs of strains with S_{AB} of ≥0.75 were regarded as highly similar and not distinguished by REP-PCR. The cutoff of 0.75 was arrived at by our experience gained using the software to compare profiles of the same strain run on different gels with various degrees of gel warping.

Discriminatory power. The discriminatory power of REP-PCR as a typing method for members of the *A. calcoaceticus-A. baumannii* complex was assessed by using the discriminatory index (D) of Hunter and Gaston (27). This index represents the probability that two unrelated strains will be characterized as being of different types by a given typing system. D is calculated by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types observed, and n_j is the number of strains belonging to the jth type.

RESULTS

Optimization and reproducibility of the REP-PCR protocol. Strains 16\6 (ribotype 2M), 10095 (ribotype Z), and 12398 (ribotype 3P) were used in preliminary tests to optimize the PCR protocol. The Mg^{2+} concentration was observed to have a profound effect on the complexity of the banding pattern, with relatively high concentrations leading to the amplification of more fragments but with increased smearing and loss of resolution. A final concentration of 1 mM Mg^{2+} in combination with Promega PCR buffer was found to give profiles with a resolution and number of amplimers suitable for strain typing. Raising the PCR extension temperature from 65 to 68 or 72°C was found to inhibit the amplification of fragments larger than approximately 1.5 kb. The addition of dimethyl sulfoxide at a final concentration of 10% was found to promote the amplification of bands larger than 2 kb and also to improve the reproducibility and clarity of the patterns. It has been shown previously that dimethyl sulfoxide improves PCR amplification when a template with a complex secondary structure is used (34). Annealing temperatures between 39 and 49°C were assessed for their affect on the reproducibility of the profiles. Few amplimers were formed when the annealing temperature was as high as 49°C, and the yield of each fragment was very poor. An annealing temperature of 45°C was found to give reproducible patterns with a sufficient number of amplimers for typing purposes. To check reproducibility, strain 16\6 was tested on six separate occasions and found to give the same banding pattern.

The average number of amplimers in each REP-PCR profile was 10, with sizes ranging from approximately 0.1 to 5 kb. High-molecular-weight fragments can be PCR artifacts (3) and are close to the upper limit of *Taq* polymerase extension. We found that amplimers larger than 5 kb were not always reproducible for a given strain, and therefore such amplimers were not included in the profile comparisons; such a protocol has also been recommended by previous workers (41, 42).

The simple template preparation protocol was found to work without the need for additional steps such as washing the cells, provided that only a small amount (just sufficient to cover the end of a toothpick) of culture was used and that strains were grown on Iso-Sensitest agar. The use of blood-containing media sometimes resulted in smeared patterns, possibly as a result of more cations and substances inhibitory to PCR, in the template (data not shown). Only four of the strains used in this study gave poor results with this rapid method. Strain R466 (ribotype 2Be) did not produce any amplimers when lysed cells were used as a template, while strains 133 (2I), 353 (13Ap), and 89 (13G) consistently gave a smear down the length of the lane with one or two weak bands visible. When phenol-chloroform-extracted DNAs from these strains were tested, reproducible, usable profiles containing 3 to 10 amplimers were obtained.

Investigation of the hospital outbreak. Isolates 16\6 to 16\17 were subjected to REP-PCR according to the protocol given in Materials and Methods, with lysed cells as templates. The results are shown in Fig. 1. Four of the ITU patient isolates and the two from the Bennett humidifier temperature probe all gave the same PCR profile (lanes 1, 2, 4, 5, 7, and 12). The isolates from the fifth ITU patient (16\8) and the sluice sink (16\13) each gave a unique profile, as did four other clinical isolates originating from other wards in the preceding month that were included as controls. Chart review revealed that each of the ITU patients whose *Acinetobacter* isolate gave the same profile as the isolates from the temperature probe had recently used a Bennett humidifier. The patient from whom sputum isolate 16\8 was obtained had used a Conchapak humidifier. The latter has a disposable, single-use cartridge and does not require a temperature probe inserted in the Y-piece connector adjacent to the endotracheal tube. The epidemiological data provided by REP-PCR typing were subsequently confirmed by ribotyping (Fig. 2). The isolates with the same REP-PCR profile were all ribotype 2M and confirmed to be *A. baumannii*

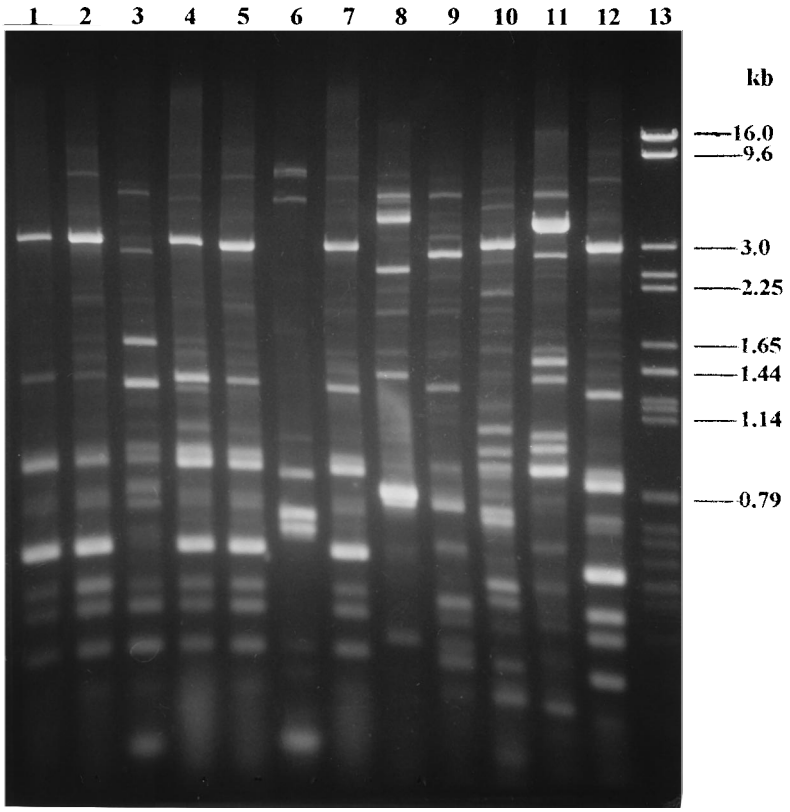

FIG. 1. REP-PCR profiles of 12 *Acinetobacter* spp. isolates from the Leeds General Infirmary (see Table 1 for details). Lanes 1 to 12, isolates 16\6, 16\7, 16\8, 16\9, 16\10, 16\11, 16\12, 16\13, 16\14, 16\15, 16\16, and 16\17, respectively; lane 13, *Bgl*I-cut λ DNA size marker.

(Table 1). Biotypes, as determined with API 20NE strips, did not yield epidemiologically useful data, as there were minor variations in the profiles of isolates that were otherwise indistinguishable by REP-PCR and ribotyping (Table 1). Despite this, the API 20NE profiles correctly determined the genomic species of isolates, as they were also deduced and confirmed from the ribotype patterns.

The temperature probe was disinfected with a 70% ethanol disposable wipe and left to dry, as recommended by the manufacturer. Ten days later, *A. baumannii* with the same REP-PCR profile and ribotype as the epidemic strain was again isolated from it (isolate 16\22).

Comparison of REP-PCR typing and ribotyping. The ribotypes of the 85 strains were determined (Tables 2 to 6), and the strains were then coded before being subjected to REP-PCR according to the protocol given in Materials and Methods with templates derived from lysed cells. The resulting PCR profiles were analyzed, and decisions about which profiles were highly similar were made without knowledge of the DNA group or ribotype of each strain.

Examples of some of the profiles are shown in Fig. 3 and 4. Among the 85 strains, 68 unique REP-PCR profiles were noted by visual inspection. It was discovered that when strains were grouped on the basis of highly similar profiles (up to two bands different) or identical profiles, they also belonged to the same DNA group. There were 11 cases in which two or more strains with the same ribotype were discriminated by REP-PCR. Alternatively, there were nine examples in which pairs of strains suggested by visual inspection to have identical REP-PCR profiles were discriminated by ribotype. The computer-assisted analysis confirmed the finding that strains with the same REP-PCR profiles belonged to the same DNA group.

When compared visually, several of the DNA group 1 profiles were very similar in terms of the number and relative positions of many of the bands, but upon closer inspection small differences in the sizes of some of the bands were noticed. Consequently, a computer-generated dendrogram showing the S_{AB}s of DNA group 1 strains depicted the profiles as being heterogeneous (data not shown). Strains ATCC 23055 and R54 were both ribotype 1D, but their REP-PCR profiles differed by a total of four bands. The profile of each of the DNA group 1 strains contained a motif that consisted of a bright amplimer of approximately 650 bp and a weaker amplimer of approximately 870 bp. This motif can be seen in Fig. 3 for strains ATCC 17902-2, ATCC 23055, and R 582 (lanes 5 through 7). Overall, 10 REP-PCR profiles and nine *Eco*RI ribotypes were represented in this selection of DNA group 1 strains.

Details for the 28 strains belonging to DNA group 2 that were studied are given in Table 3, and the dendrogram based on the computer-assisted comparison of the REP-PCR profiles of these strains is shown in Fig. 5. Strains 189 and 50853-82 have the same ribotype and were not distinguished by REP-PCR (S_{AB} = 0.89), as shown in Fig. 5. Likewise, the ribotype 2J strains 65 and 147 were not distinguished, but this pair had a profile different from that of ribotype 2J strain R1019. The latter gave the same profile as R872, which is ribotype 2L. The results of the computer-assisted analysis were in good agreement with the visual analysis, apart from the profiles of some of the ribotype 2A strains. Upon visual inspection, the REP-PCR

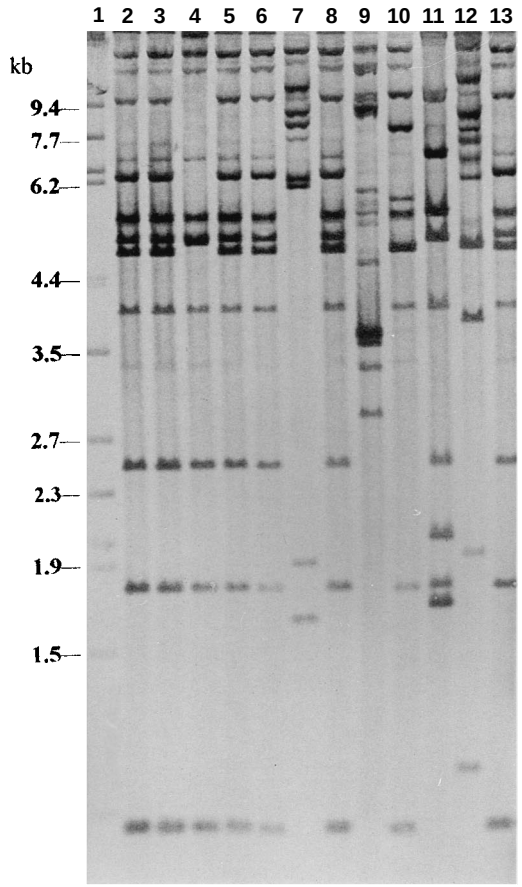

FIG. 2. *Eco*RI ribotype patterns of 12 *Acinetobacter* spp. isolates from the Leeds General Infirmary (see Fig. 1 for corresponding REP-PCR profiles). Lane 1, mixture of *Hin*dIII- and *Sty*I-cut λ DNA size markers; lanes 2 to 13, isolates 16\6, 16\7, 16\8, 16\9, 16\10, 16\11, 16\12, 16\13, 16\14, 16\15, 16\16, and 16\17, respectively.

profiles of strains 189 (2Aa), 50853-82 (2Aa), AB 2444 (2Ab), R112 (2Ac), R1141 (2Ad), and R210 (2Ad) appeared to be highly similar. The more stringent analysis afforded by the computer software split this group so that the 2Aa strains were indistinguishable from each other but different from the other ribotype 2A strains (Fig. 5). Strains AB 2444 and R1141 also gave identical profiles (Fig. 3, lanes 8 and 9). Overall, 21 REP-PCR profiles delineated by the computer-assisted analysis, 13 *Eco*RI ribotypes, and 18 ribotypes based on digestion with *Eco*RI and *Cla*I and/or *Sal*I were represented among these DNA group 2 strains.

TABLE 2. DNA group 1 strains (*A. calcoaceticus* sensu stricto Bouvet and Grimont [7]) analyzed by REP-PCR in this study

Strain[a]	Isolation site	Ribotype
ATCC 17902-2	Soil	1A
42	Wound	1B
R944	Unknown (clinical)	1C
ATCC 23055^T	Soil	1D
R54 (74) (= LMD 22.17)	Soil	1D
R582 (66)	Soil	1E
R584 (68)	Soil	1F
R583 (67)	Soil	1G
59	Sputum	1H
132	Wound	1I

[a] Previous strain designations as reported by Gerner-Smidt (16) are given in parentheses.

TABLE 3. DNA group 2 strains (*A. baumannii*) analyzed by REP-PCR in this study

Strain[a]	Isolation site	Ribotype
189	Ulcer	2Aa
50853-82	CSF[b]	2Aa
AB 2444	Unknown (clinical)	2Ab
AB 2445	Unknown (clinical)	2Ab
R112 (13)	Wound	2Ac
R210 (1)	Sputum	2Ad
R1141	Unknown	2Ad
R466 (28)	Eye	2Be
ATCC 17904	Urine	2Bf
91	Sputum	2Bg
ATCC 19606^T	Urine	2C
R1063 (78) (= NCTC 7844)	Unknown	2C
107	Wound	2D
R1052 (77) (= LMD 82.52)	Unknown	2E
ATCC 9955	CSF	2F
ATCC 17978	CSF	2G
R413 (29)	Pus	2H
133	Wound	2I
144	Wound	2I
R872 (18)	Urine	2L
10086	Urine	2I
R1019 (27)	Wound	2J
147	Wound	2J
65	Wound	2J
R1093 (60)	Sputum	2K
R447 (24)	Catheter tip	2L
10073	Blood	2L
16\6	Sputum	2M

[a] Previous strain designations as reported by Gerner-Smidt (16) are given in parentheses.
[b] CSF, cerebrospinal fluid.

Twenty-seven DNA group 3 strains were examined (Table 4). Among this collection, 25 REP-PCR profiles, 17 *Eco*RI ribotypes, and 22 ribotypes based on digestion with *Eco*RI and *Cla*I and/or *Sal*I were represented.

Table 5 gives the ribotypes of the 16 strains belonging to DNA group 13 that were studied. Four strains, namely, R2284 (13Ao), R2285 (13Ao), 165 (13Ao), and 53893-82 (13Ap), were thought to have the same REP-PCR profile as determined by visual inspection, but each of these strains produced a high background down the length of the lane, making interpretation difficult. The computer analysis grouped R2284 and R2285 together with an S_{AB} of 0.77, which is just within the cutoff point (0.75) for strains not distinguished from each other. Strains 53893-82 and 165 were found to cluster with the previous two strains at S_{AB}s of 0.67 and 0.54, respectively. Fourteen REP-PCR profiles, as determined by the computer-assisted analysis, 10 *Eco*RI ribotypes, and 12 ribotypes based on digestion with *Eco*RI and *Cla*I and/or *Sal*I were represented among the DNA group 13 strains.

Strains 5804 and 10090 (Table 6) belong to an as yet unnamed DNA group in the *A. calcoaceticus-A. baumannii* complex that is most closely related to DNA group 13 (17). Each of these strains produced a unique REP-PCR profile (data not shown). Strains 10095 and 10169 (Table 6) belong to another unnamed DNA group that is related to groups 1 and 3 (17). These two strains each gave rise to a unique REP-PCR profile (data not shown).

Determination of the discriminatory index of REP-PCR and ribotyping. The discriminatory indices of both REP-PCR and

TABLE 4. DNA group 3 (unnamed) strains analyzed by REP-PCR in this study

Strain[a]	Isolation site	Ribotype
128	Sputum	3Ah
ATCC 17922	Unknown	3Ai
55	Surgical gown	3B
R1163 (31)	Toe web	3Ck
62	Wound	3Ck
204	Wound	3Ci
ATCC 19004	CSF[b]	3Cj
R411 (61)	Sputum	3D
R532 (59)	Urine	3E
10790	Dialysate	3Fm
41	Wound	3Fm
79	Wound	3Fm
102	Wound	3Fm
R502 (37)	Drain	3Fm
162	Wound	3Fn
212	Wound	3G
143	Urine	3H
R1020 (36)	Bronchus	3H
40	Wound	3I
R509 (63)	Bronchus	3J
176	Conjunctiva	3K
9907	Urine	3L
10078	Vagina	3M
12174a	Urine	3N
10088	Ulcer	3O
12398	Sputum	3P
10084	Umbilicus	3Q

[a] Previous strain designations as reported by Gerner-Smidt (16) are given in parentheses.
[b] CSF, cerebrospinal fluid.

ribotyping were high. The discriminatory indices of ribotyping using *Eco*RI digests were 0.98 (s = 53) for all strains considered together and in the range of 0.87 (DNA group 13) to 0.98 (DNA group 1) for each DNA group considered separately. When strains were ribotyped on the basis of *Eco*RI digests and *Cla*I and/or *Sal*I digests, 65 different types were identified and D was 0.99 for the 85 strains considered together. For individual DNA groups, the value ranged from 0.94 (DNA group 13)

TABLE 5. DNA group 13 (Tjernberg and Ursing Gp13 [38]) strains analyzed by REP-PCR in this study

Strain[a]	Isolation site	Ribotype
165	Urine	13Ao
R503 (65)	Unknown	13Ao
R2284	Respiratory tract	13Ao
R2285	Respiratory tract	13Ao
353	Sputum	13Ap
53893-82	CSF[b]	13Ap
R2376	Sputum	13Bq
ATCC 17903	Unknown	13Br
100	Gastric fistula	13C
R2624	Forehead	13D
53937bb	Unknown (clinical)	13E
R2627	Rectum	13F
89	Wound	13G
R412 (62)	Blood	13H
R2041	Unknown (clinical)	13I
12112	Blood	13J

[a] Previous strain designations as reported by Gerner-Smidt (16) are given in parentheses.
[b] CSF, cerebrospinal fluid.

TABLE 6. Strains belonging to unnamed DNA groups within the *A. calcoaceticus-A. baumannii* complex analyzed by REP-PCR in this study

Strain	Isolation site	Ribotype
5804[a]	Blood	V
10169[b]	Sputum	X
10090[a]	Ulcer	Y
10095[b]	Abscess	Z

[a] Strain belongs to a DNA group most closely related to DNA group 13.
[b] Strain belongs to a DNA group most closely related to DNA groups 1 and 3.

to 0.98 (DNA group 1). With REP-PCR and visual analysis of the profiles, 68 different types were observed and D was 0.99 for the 85 strains. With the computer-assisted analysis, 74 different REP-PCR profiles were observed and D was 1.0, which is the theoretical maximum. When the DNA groups were analyzed separately, the value was in the range of 0.98 (DNA groups 2 and 13) to 1.0 (DNA group 1).

DISCUSSION

The information provided by REP-PCR typing allowed us to rapidly identify the reservoir of *A. baumannii* contamination that had caused pneumonia in two seriously ill ITU patients in our hospital as being the temperature probe of a Bennett humidifier. The same strain had colonized the respiratory tract of a third patient and the leg wound of a fourth. The isolate

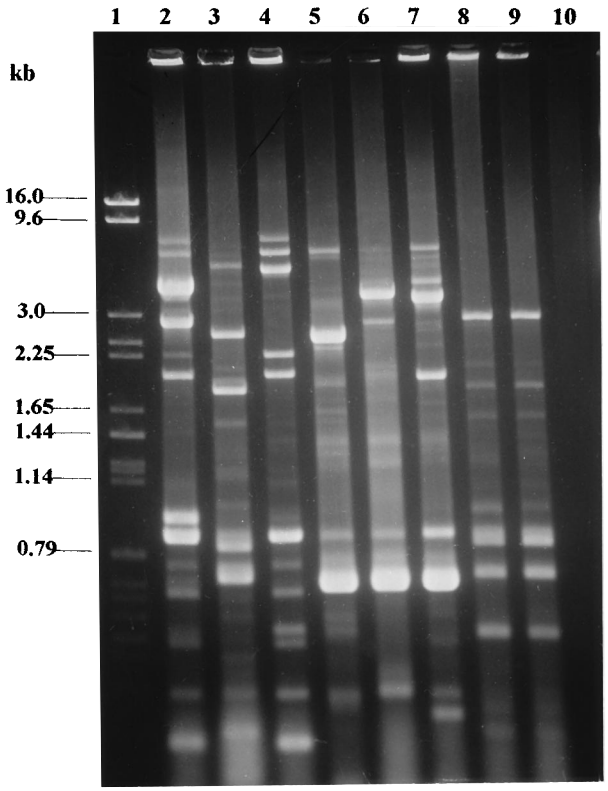

FIG. 3. Examples of REP-PCR profiles of strains belonging to the *A. calcoaceticus-A. baumannii* complex, generated by using lysed cells as templates. Lane 1, *Bgl*I-cut λ DNA size marker; lanes 2 to 9, strains (and ribotypes) 10088 (3O), ATCC 9955 (2F), R502 (3Fm), ATCC 17902-2 (1A), ATCC 23055 (1D), R582 (1E), AB 2444 (2Ab), and R1141 (2Ad), respectively; lane 10, negative control.

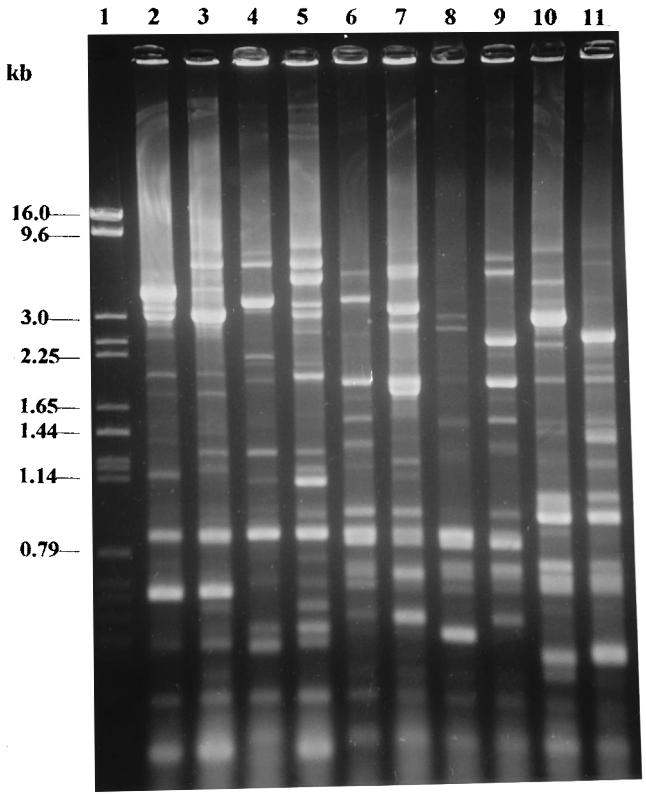

FIG. 4. Examples of REP-PCR profiles of strains belonging to the *A. calcoaceticus-A. baumannii* complex, generated by using lysed cells as templates. Lane 1, *Bgl*I-cut λ DNA size marker; lanes 2 to 11, strains (and ribotypes) 10078 (3M), 128 (3Ah), 10084 (3Q), R1020 (3H), 10086 (2I), ATCC 17904 (2Bf), 65 (2J), 144 (2I), 12112 (13J), and R2624 (13D), respectively.

obtained from the sputum of a fifth patient was shown to be a separate, unrelated strain for which no environmental source was identified. This strain may have originated from the patient's own bowel flora rather than the environment of the ward. Routine disinfection of the temperature probe with ethanol proved to be ineffective at removing the contamination, and the remarkable ability of *A. baumannii* to survive on inanimate dry surfaces was illustrated by the fact that the same strain was again isolated from the probe 10 days after the probe was cleaned. Additional tests in our laboratory have confirmed that ethanol does not adequately remove *Acinetobacter* contamination from the lumen of this type of device (data not shown). The capacity of certain *Acinetobacter* spp. to survive on dry surfaces for long periods is an additional factor to be considered when attempting to prevent nosocomial infections and is a cause for serious concern (20). We now sterilize the probes with the Sterrad low-temperature plasma sterilizing system (Johnson and Johnson Medical, Slough, United Kingdom), which uses free radicals generated from hydrogen peroxide in a plasma cloud to destroy bacteria. Previous workers have highlighted in-line temperature and oxygen monitor probes as potential reservoirs for *Acinetobacter* infection and recommended rigorous decontamination of such devices between uses for each patient (10). Berthelot et al. (6) reported a similar problem with ventilator temperature probes involving *Pseudomonas cepacia* contamination. Previous outbreaks of *Acinetobacter* infection linked to ventilatory equipment have also highlighted the need for careful decontamination of reusable tubing and pressure monitors (9, 24, 39).

REP-PCR typing provided a degree of discrimination among the 85 strains belonging to the *A. calcoaceticus-A. baumannii* complex studied that was equal to that afforded by ribotyping. The discriminatory index, which gives an indication of the probability that two unrelated strains will be typed differently, was extremely high with both methods. When ribotypes were assigned on the basis of *Eco*RI digests alone, *D* was 0.98, and this value increased to 0.99 when strains were ribotyped on the basis of *Eco*RI digests and *Cla*I and/or *Sal*I digests. Visual inspection of the REP-PCR profiles of the 85 strains revealed 68 different profiles and a *D* of 0.99; this value rose to the theoretical maximum of 1.0 when profiles were compared on the basis of S_{AB}s by computer. Since it does not involve DNA extraction, restriction endonuclease digestion, or hybridization, REP-PCR using boiled cells is significantly faster and simpler to perform than ribotyping. The method is ideally suited to investigations that require high-throughput strain typing.

We encountered just three strains that produced profiles consisting of smears with only one or two visible bands and only one strain that did not give a profile at all when boiled cells were used as a template. It is not yet known whether these strains have nucleases that rapidly degrade the DNA released from the boiled cells or a higher content of substances that can inhibit PCR, such as lipopolysaccharide. These strains did give discriminatory profiles when phenol-chloroform-extracted DNA was used. Although differences in the REP-PCR profiles generated with boiled lysates versus phenol-chloroform-extracted DNA can be obtained, this does not affect the ability of investigators to discriminate between isolates tested in parallel with the same template preparation method. When typing strains, investigators should adhere to just one of the protocols.

There were no cases in which one REP-PCR profile was shared by strains from different DNA groups. Although some DNA group 1-specific amplimers were identified, they accounted for a small proportion of the amplimers that made up the REP-PCR profiles, and some strains belonging to other DNA groups were not precluded from having amplimers of a similar size in their profiles. Consequently, strains from each DNA group did not cluster together when a dendrogram showing the relationship between the REP-PCR profiles of all 85 strains was constructed (data not shown). Ribotype profiles contain a greater proportion of DNA group-specific bands, so strains belonging to the *A. calcoaceticus-A. baumannii* complex do cluster into DNA groups when the ribotype profiles are subjected to analysis by the unweighted pair-group method with arithmetic averages (16).

REP elements are located between genes within an operon or at the end of an operon and can be in either orientation. Clusters of REP elements can be found in intergenic regions (36). Some of the amplimers formed by REP-PCR represent amplification of intergenic regions, while others include genes. It should be noted that the relationships shown in the dendrograms represent statistical relationships between banding patterns. There are currently insufficient data concerning the function and distribution of REP-like sequences within *Acinetobacter* chromosomes to determine how S_{AB}s correlate with genetic distances between strains. Until this question is resolved, visual comparison of REP-PCR profiles, without statistical analysis, will be suitable for use in most laboratories. Visual comparison works well provided that gels are standardized with respect to how far samples are run and that there is comparatively little angular distortion of the lanes. However, computer-assisted analysis of profiles is invaluable if large numbers of strains are to be analyzed or if a database is to be

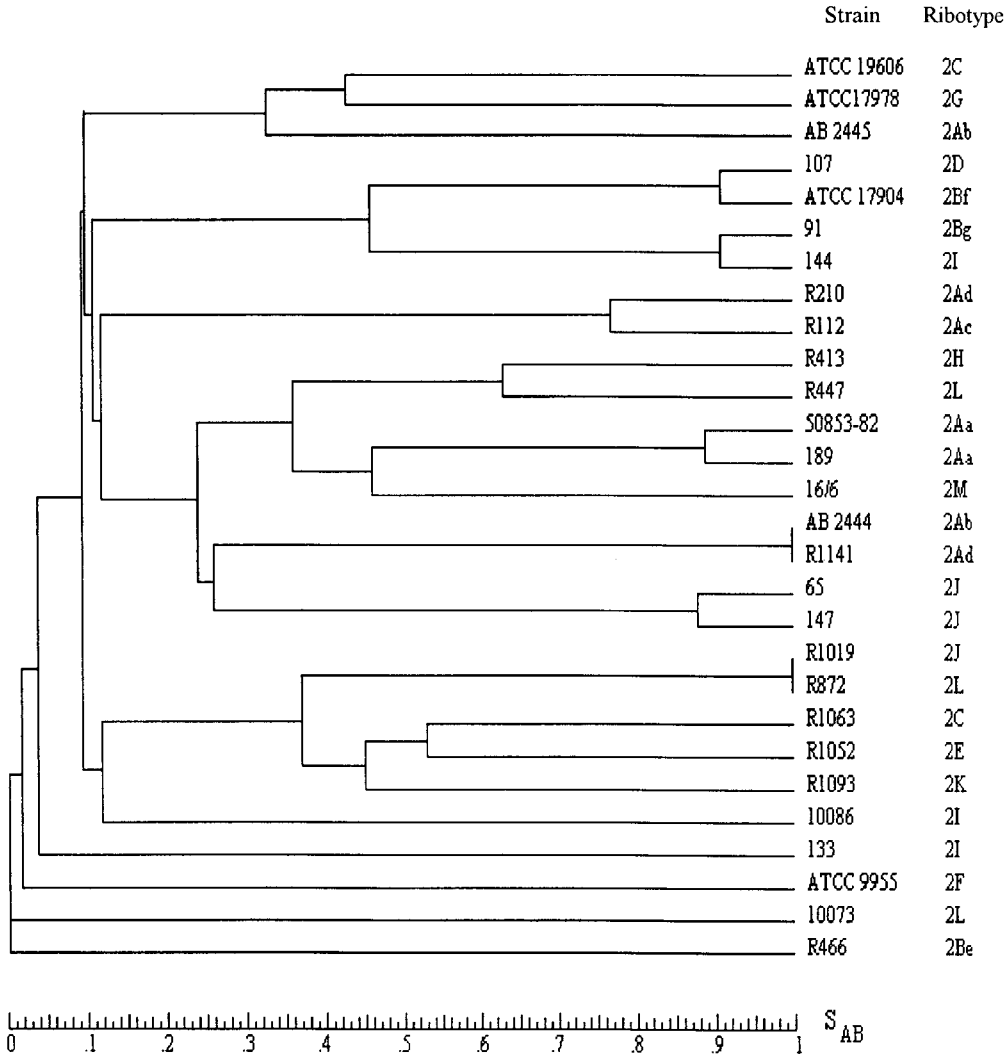

FIG. 5. Dendrogram based on computer-assisted comparison of REP-PCR profiles of 28 isolates belonging to DNA group 2 of the *A. calcoaceticus-A. baumannii* complex (see Table 3).

set up so that hospitals can determine rapidly if strains have been encountered previously.

When the REP-PCR profiles were compared, the intensities of individual bands were not taken into account. The yield of an amplimer is determined by such factors as the extent to which the sequence of the degenerate primers match chromosomal sequences at a particular location, the secondary structure of the chromosomal sequence being amplified, and the concentration of DNA in the reaction mixture. The last factor is difficult to standardize unless the template DNA is highly purified and quantified. When PCR fingerprints of this type are compared, the relative sizes of amplimers should be considered and not their relative intensities. However, since the presence or absence of faint bands could also reflect methodological variations (e.g., temperature variability in the thermocycler heating block), isolates with profiles that differ by just a few very weak bands should be retested together and the products should be run side by side for comparison.

When lysed colonies are used as templates, REP-PCR results can be obtained within 11 h of isolation of strains on agar plates. Contamination of PCR mixtures with airborne DNA molecules is a common problem, especially in laboratories in which space limitations mean that reactions have to be set up in the same room in which DNA extractions are performed. The lysed-colony method can help to reduce this type of contamination, since it requires minimal handling of the template and the DNA is released inside a closed tube when the cells are lysed by being boiled. The tube needs to be opened only once, when an aliquot is transferred to the PCR mix. Very small amounts of template are required for the test. Another advantage of REP-PCR is that the profiles for *Acinetobacter* strains are composed of amplimers that span a size range of approximately 100 bp to 4 kb. These amplimers can be resolved by using a 1.5% (wt/vol) gel made with a general-purpose agarose, whereas some PCR typing methods require expensive agaroses or polyacrylamide gels to resolve the very small amplimers that are produced.

Gräser et al. (23) reported the use of the core sequence of the M13 phage as a single primer in a PCR typing method for *A. baumannii* (DNA group 2). The method was used to fingerprint isolates from two *A. baumannii* outbreaks in a German intensive care unit. As in this study, DNA template for the PCR was prepared by boiling colonies, although a centrifugation step was also included. Unlike the REP-PCR method,

their RAPD protocol (23) produces very low yields of amplimers, and all of the 50-μl reaction mix had to be concentrated and run on a gel in order to visualize the profile. The reproducibility of the method may be poor if it relies on visualization from run to run of many amplimers of low yield. Primers specific for another class of repetitive sequence, the enterobacterial repetitive intergenic consensus (ERIC) sequence, have been used in a PCR typing protocol for *A. baumannii* (37). However, in that method, a very low annealing temperature (25°C) was used, and thus it is not clear if the primers annealed to genuine ERIC sequences or acted as arbitrary primers. Reboli et al. (30) reported that ERIC primers did not give discriminatory profiles with unrelated isolates of *A. baumannii.*

Compared with other DNA typing methods, REP-PCR typing offers a simple, rapid, and highly discriminatory means of identifying and comparing *Acinetobacter* strains. The method can also be applied to other species responsible for nosocomial infection (41, 42), making it a versatile and cost-effective tool for use in hospital diagnostic laboratories.

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