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Multicenter Study Using Standardized Protocols and Reagents for Evaluation of Reproducibility of PCR-Based Fingerprinting of *Acinetobacter* spp.

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Seven laboratories in six European countries examined 40 isolates belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex to investigate whether standardized protocols and quality-controlled reagents could produce reliable, discriminatory, and reproducible PCR-based fingerprinting results. Four PCR protocols with different primers (primers DAF4, ERIC-2, M13, and REP1 + REP2) were used. The epidemiological conclusions reached by the participating laboratories were substantially correct, with 96.4% of the total isolate grouping allocations agreeing with the consensus view. All laboratories identified the main epidemiological clusters, and each laboratory also identified two non-outbreak-related isolates. There were no significant differences between the isolate grouping results obtained by the different protocols and with the different primers. Visual comparison indicated that the standardized protocols and reagents yielded reproducible fingerprint patterns, but with some variations in particular band intensities. Minor variations in fingerprint profiles were detected, but computer-assisted analysis of PCR fingerprints obtained on agarose gels demonstrated that 88.3 to 91.6% (depending on the source of DNA) of the patterns clustered correctly, while 96.4 to 98.9% of the patterns clustered correctly following automated high-resolution laser fluorescence analysis. Correlation of the patterns for isogenic isolates ranged from 83.3 to 86.6% but was slightly better (mean correlation, 87.1%) for centrally prepared DNA extracts than for DNA extracts prepared by individual laboratories (mean correlation, 84.7%). It was concluded that independently produced PCR fingerprint patterns can be obtained reproducibly for *Acinetobacter* spp. at the practical level if (i) quality-controlled reagents, (ii) standardized extraction of DNA, and (iii) standardized amplification conditions are used.

Identification and typing of microorganisms are vitally important in efforts to monitor the geographical spread of virulent or epidemic pathogens. Conventional phenotypic identification and typing methods have long been the mainstay of descriptive microbial epidemiology, but their use is normally confined to the group of organisms for which they were originally devised. More modern molecular biology-based methods of wider applicability are now available, and much interest has focused recently on rapid methods for DNA fingerprinting based on PCR (14). Such methods include the functionally interchangeable methods of arbitrarily primed amplification of chromosomal DNA (6, 19) and randomly amplified polymorphic DNA (RAPD) analysis (2, 6), involving PCR amplification of random fragments of genomic DNA with single primers with an arbitrary sequence, while an alternative approach, referred to as rep-PCR (21), amplifies intervening sequences located between highly repetitive DNA motifs. These methods are increasingly being used in many microbiology laboratories for the epidemiological typing of an ever expanding range of bacteria. However, difficulties arise when visually comparing the fingerprint banding patterns from a large number of isolates examined on different gels over extended time intervals. Other problems include a reported lack of reproducibility and difficulties with the standardization of equipment and reagents (7, 8, 12, 13). Some of these drawbacks have been overcome by combining PCR-based fingerprinting with automated laser fluorescence (ALF) analysis of DNA fragments in which computerized cluster analysis is used for recognition of related isolates within a single working day (5, 18). This technique achieves a high resolution of the PCR-based fingerprint patterns by using rapid denaturing sequencing gels that are capable of discriminating DNA products differing by only a single base (9). The resulting data are digitized and used for isolate comparisons and the generation of dendrograms without the loss of information that may occur during the scanning of gel images generated by other methods.

The present study was designed to address the problem of reproducibility by examining whether it would be possible to define appropriate PCR reagents, conditions, primers, and analysis methods that would produce reliable, discriminatory, and reproducible results at the practical level in different lab-

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oratories. For this purpose, seven laboratories in six European countries examined blindly (i.e., without prior knowledge of the epidemiological relationships) a panel of 40 isolates belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. This group of organisms is recognized as being of increasing nosocomial importance, particularly in intensive care units or hospital units with highly dependent patients (1). Each isolate included in the study was typed by four different PCR protocols with standardized reagents as well as standardized DNA extraction and amplification protocols. Ten of the isolates were also supplied as standard DNA samples in the form of crude lysates prepared centrally. Primers and PCR reagents were also prepared centrally for distribution to the participating laboratories. Reproducibility was assessed in three ways: first, by comparison of the isolate groupings (typing results) obtained independently by the participating laboratories; second, by central computer-assisted analysis of photographs of the PCR fingerprints on agarose gels generated independently by the participating laboratories; and third, by central ALF analysis of the actual PCR products generated by the individual laboratories.

MATERIALS AND METHODS

Bacterial isolates. Forty isolates belonging to the *A. calcoaceticus-A. baumannii* complex (Table 1) were included in the study. The identification of isolates to the genomic species level was confirmed by the technique of tRNA spacer fingerprinting (3). The isolates were derived from eight outbreaks of nosocomial infection in five different countries and were collected initially at the Leiden laboratory. Each isolate was then coded (labelled 1 to 40) by a nonparticipant in the study, prepared (see below), and distributed in a blind manner to all the participating laboratories by the Nottingham laboratory. All further work in each of the participating laboratories was performed in a blinded manner until the conclusion of the study. For initial distribution purposes, five to six small representative colonies from a pure culture grown on Iso-Sensitest agar (Oxoid, Basingstoke, United Kingdom) were emulsified in 1 ml of nutrient broth (Oxoid). Portions (25 μl) of this suspension were used to inoculate identical stabs of Iso-Sensitest agar for dispatch by airmail at ambient temperature (delivery time, 2 to 5 days) to each of the participating laboratories. On arrival, each coded isolate was stored at 4°C and was then restreaked onto Iso-Sensitest or blood agar plates and incubated overnight at 37°C to check for purity before proceeding further. Ten isolates were also supplied in the form of standard DNA extracts (labelled A to J). These were prepared centrally in the Nottingham laboratory by the method described below and were dispatched to participating laboratories by airmail at ambient temperature. Standard DNA extracts were stored at −20°C on receipt by the participating laboratories.

Preparation of DNA extracts. A small loopful of growth, representing three to four small representative colonies, was resuspended in 100 μl of sterile distilled water in a 0.5-ml microcentrifuge tube. These were heated for 15 min at 95°C, cooled on ice, and centrifuged at 12,000 × *g* for 20 s to remove the cell debris. These crude DNA extracts were either frozen at −20°C or were kept on ice for immediate use. Portions (2 μl) of these extracts were used in 25-μl PCR mixtures (see below) without further purification.

PCR primers. The following four primer sets (three single primers and one double primer) were used in the study: ERIC-2, 5′-AAGTAAGTGACTGGGG TGAGCG (17); M13, 5′-GAGGGTGGCGGTTCT (4); DAF4, 5′-CGGCAGC GCC (20); and the REP set (REP1 + REP2), 5′-IIIGCGCCGICATCAGGC + 5′-ACGTCTTATCAGGCCTAC (17).

The primers were chosen on the basis of previously published results (5, 10, 17–19) and included two primers (primers M13 and DAF4) that targeted conserved sequences at relatively high annealing temperatures and two primers (primers ERIC-2 and REP1 + REP2) that targeted enterobacterial repetitive sequences at lower annealing temperatures to allow for mismatches in *Acinetobacter* spp. All primers were prepared centrally as a single batch by Pharmacia Biotech (Freiburg, Germany) and were labelled with the carbocyanine dye Cy5 (Pharmacia Biotech) at the 5′ end during synthesis to permit subsequent ALF analysis of the PCR products. Each primer set was distributed to the participating laboratories already diluted to the precise concentration for immediate incorporation into the PCR mixtures (see below).

PCR mixtures. To ensure the maximum possible standardization, all reaction mixtures were based on Ready-To-Go RAPD Analysis Beads (Pharmacia Biotech). These beads are supplied in a quality-controlled, premixed, predispensed, room temperature-stable format that already contains Ampli*Taq* DNA polymerase and the Stoffel fragment, as well as all necessary buffer ingredients and nucleotides. The only additions required are sterile distilled H_2O, template DNA, and a suitable primer. Two different batches of RAPD Analysis Beads, manufactured at an interval of 3 months, were used during the study.

TABLE 1. Isolates of *Acinetobacter* included in the study

Isolate code no.[a]	Original isolate designation	Source[b]	Genomic species[c]
1	hg-d2041	Freiburg, GE	2
2	350211.96	Ghent, BE	2
3	V-11516	Cologne, GE	2
4	5177-88	Odense, DK	13TU
5	ruh3299	Nijmegen, NL	3
6	cw35	Nottingham, UK	2
7	hg-d2008	Freiburg, GE	2
8	350209.95	Ghent, BE	2
9	st-15961	Cologne, GE	2
10	pgs427	Odense, DK	13TU
11	ruh3294	Nijmegen, NL	3
12	cw33	Nottingham, UK	2
13	hg-d1986	Freiburg, GE	2
14	350213.98	Ghent, BE	2
15	st-14970	Cologne, GE	2
16	pgs387	Odense, DK	13TU
17	ruh3293	Nijmegen, NL	3
18	cw32	Nottingham, UK	2
19	hg-d1882	Freiburg, GE	2
20	350212.97	Ghent, BE	2
21	st-15598	Cologne, GE	2
22	pgs350	Odense, DK	13TU
23	ruh3296	Nijmegen, NL	3
24	cw31	Nottingham, UK	2
25	hg-d2006	Freiburg, GE	2
26	350208.94	Ghent, BE	2
27	pgs328	Odense, DK	13TU
28	ruh3292	Nijmegen, NL	3
29	cw5	Nottingham, UK	2
30	pgs14548	Copenhagen, DK	2
31	ruh2037	Venlo, NL	2
32	pgs14554	Copenhagen, DK	2
33	ruh2036	Venlo, NL	2
34	pgs14613	Copenhagen, DK	2
35	ruh2034	Venlo, NL	2
36	cw4	Nottingham, UK	2
37	pgs14545	Copenhagen, DK	2
38	pgs353	Odense, DK	13TU
39	ruh2033	Venlo, NL	2
40	ruh2032	Venlo, NL	2

[a] The isolate code numbers were allocated by a nonparticipant and were used in a blinded manner throughout this study.

[b] BE, Belgium; DK, Denmark; GE, Germany; NL, The Netherlands; UK, United Kingdom.

[c] As listed by Bergogne-Bérézin and Towner (1).

For the purposes of this study, 18 μl of sterile distilled H_2O, 5 μl of a primer set (5 pmol/μl), and 2 μl of a DNA extract were added to each Ready-To-Go tube held on ice. The tubes were then transferred to a preheated (95°C) thermal cycler (the precise equipment and whether or not a mineral oil overlay was used varied according to the individual laboratory), and the PCR was performed for each primer set as specified below. Each PCR was performed in duplicate, and each batch of PCRs included isolate 1 as a control reaction and a negative control containing 2 μl of sterile distilled H_2O instead of a DNA extract.

PCR conditions. PCR conditions were as follows for each individual primer set: (i) M13 primer, 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 50°C for 1 min, and 72°C for 20 s, followed by a final extension at 72°C for 5 min; (ii) DAF4 primer, 94°C for 2 min, followed by 45 cycles of 94°C for 40 s, 45°C for 40 s, and 72°C for 40 s, followed by a final extension at 72°C for 5 min; (iii) ERIC-2 primer, 94°C for 2 min, followed by 45 cycles of 94°C for 1 min, 25°C for 1 min, and 74°C for 2 min, with no final extension period; and (iv) REP primer set, 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min, followed by a final extension at 65°C for 16 min. All PCR products were stored at −20°C until required for analysis. Each participating laboratory performed a total of 400 PCRs: 40 coded isolates × 4 primer sets, in duplicate (which equals 320 reactions), plus 10 standard DNA samples × 4 primer sets, in duplicate (which equals 80 reactions).

Analysis of PCR products. For isolates 1 to 10 and DNA samples A to J, a 5-μl portion of the PCR end product was transferred to a sterile 100-μl tube which

was then sealed with parafilm and frozen at −20°C until all samples were ready. These samples were sent by overnight courier service to the Freiburg laboratory for analysis by ALF. Portions (up to 20 μl) of the PCR end products from all isolates and DNA samples were also analyzed locally in the participating laboratories by electrophoresis on 2% (wt/vol) agarose gels prepared in Tris-borate electrophoresis buffer (TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA). The precise electrophoresis apparatus varied according to the participating laboratory, but each gel contained size markers (100 Base-Pair Ladder; Pharmacia) in the first and last lanes plus one set of size markers after at least every six sample lanes on the gel. Electrophoresis (variable conditions according to the participating laboratory) was performed until the bromophenol blue tracking dye had migrated 10 cm. The gels were then stained with ethidium bromide (5 mg/liter in TBE) for 20 min, washed briefly with distilled water, and examined on a UV transilluminator. The PCR fingerprints were photographed, and the isolates were grouped by either a visual or an automated method according to the equipment available in each participating laboratory. Photographs of the PCR fingerprints obtained for isolates 1 to 10 and DNA samples A to J were sent to the Nottingham laboratory for central analysis of pattern reproducibility.

Thus, each participating laboratory supplied the following results: (i) an interpretation of clusters, based on visual or automated pattern analysis for the 40 coded isolates and the 10 standard DNA samples, (ii) photographs of the amplification patterns obtained on agarose gels with isolates 1 to 10 and DNA samples A to J for central computerized analysis of pattern reproducibility, and (iii) 5-μl portions of the fluorescence-labelled PCR products obtained with isolates 1 to 10 and DNA samples A to J for central computerized ALF analysis of pattern divergence and reproducibility.

Central analysis of pattern reproducibility. Photographs of PCR-based fingerprinting patterns visualized on gels were scanned at the Nottingham laboratory with a UMAX UC840 Scanner equipped with Adobe Photoshop Macintosh, version 2.5.1, software. Digital images were saved to a Macintosh Quadra 650 computer in the PICT format and were analyzed with the DENDRON program (version 2.0; Solltech Inc., Oakdale, Iowa). This program normalizes data from separate electrophoresis gels according to either internal size standards added to each track or (as in this study) sets of molecular size standards run at regular intervals in separate tracks. Alignment of the size standards by the computer allows inter- and intragel inconsistencies and variations in electrophoresis conditions to be corrected. The DENDRON program identifies the positions and intensities of the bands in each lane of a gel and then calculates a similarity coefficient (S_{AB}) for every pair of isolates. For the purposes of this study, the S_{AB} values were computed solely on the basis of band positions by using the Dice coefficient (11). The S_{AB} values are presented in a matrix and are then used to generate a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) in which the two or more isolates with the highest S_{AB} value are grouped into a cluster with a connection (or branch point) corresponding to that S_{AB} value along the horizontal axis of the dendrogram. The process continues in the direction of lower S_{AB} values until the dendrogram is complete. This central comparison of the isolate grouping positions obtained from the data generated independently by the participating laboratories allowed the reproducibility of the RAPD patterns to be assessed. A previous investigation (18) determined that good discrimination between genetically unrelated groups of *Acinetobacter* spp. was achieved with an S_{AB} value of 0.7. Making use of this threshold, PCR fingerprints were defined as concordant if PCR products from different participating laboratories yielded an S_{AB} value of ≥0.7 for the same isolate or DNA extract.

ALF analysis of PCR products. Amplification products from all laboratories were analyzed in parallel by a high-speed automated DNA fragment analysis device based upon an ALF analyzer (ALF Express; Pharmacia). Denaturing separating gels containing Hydrolink Long Ranger (5% [wt/vol]; A and T Biochemicals, Malvern, Pa.), 7 M urea (ALF grade; Pharmacia), and 0.6× TBE were prepared. A 1-μl portion of amplification product was denatured in 5 μl of stop solution (5 mg of dextran blue per ml of formamide) at 95°C for 5 min and was then applied to the gel. Electrophoresis was in 0.6× TBE at a constant voltage of 800 V (current limit, 50 mA) and a constant temperature of 45°C. Amplification patterns were identified by the fluorescence emitted by DNA fragments passing a fixed laser beam and were reconstructed as fluorescence density profiles. Data were stored and subsequently normalized by Fragment Manager (Pharmacia) software according to the internal size markers of 100 and 1,064 bp (5) added to each lane of the gel and the 100 Base-Pair Ladder run in every seventh lane. Similarity analysis of the digital data was carried out with Pearson's product moment correlation coefficient, and cluster analysis was carried out by UPGMA (GelCompar program; Applied Maths, Kortrijk, Belgium). A previous investigation (5) determined that good discrimination between genetically unrelated groups of *Acinetobacter* spp. was achieved by this method with a correlation coefficient of <70. Making use of this correlation threshold, PCR fingerprints were defined as concordant if PCR products from different participating laboratories yielded a pattern correlation coefficient of ≥70 for the same isolate or DNA extract.

TABLE 2. Isolate grouping conclusions reached by the participating laboratories

Isolate code no.	Isolate group allocated by individual laboratories[a]						
	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
1	a	a	a	a	a	a	a
2	b	b	b	b	b	b	b
3	c	c	c	c	c	c	c
4	d	d	d	d	d	d	d
5	e	e	e	e	e	e	e
6	c	c	c	c	c	c	c
7	a	a	a	a	a	a	a
8	b	b	b	b	b	b	b
9	c	c	c	c	c	c	c
10	d	d	d	d	d	d	d
11	e	e	e	e	e	e	e
12	c	c	**b**	c	c	c	c
13	a	a	a	a	a	a	a
14	b	b	b	b	b	**a**	b
15	c	c	c	c	c	c	c
16	d	d	d	d	d	d	d
17	e	e	e	e	e	e	e
18	c	c	c	c	c	c	c
19	a	a	a	a	a	a	a
20	b	b	b	b	b	b	b
21	c	c	c	c	c	c	c
22	d	d	d	d	d	d	d
23	e	e	**d**	e	e	e	e
24	**ug**[b]	c	c	c	c	c	c
25	ug	ug	**e**	ug	ug	ug	ug
26	b	b	b	b	b	b	b
27	d	d	d	d	d	d	d
28	e	e	e	e	e	e	e
29	c	c	c	c	c	c	c
30	f	f	**ug**	f	f	f	f
31	a	a	a	a	a	a	a
32	a	a	a	a	a	a	a
33	a	a	a	a	a	a	a
34	f	f	**c**	f	f	**a**	f
35	a	a	a	a	a	a	a
36	c	c	**ug**	c	c	c	c
37	f	f	**ug**	f	f	f	f
38	d	d	d	d	d	d	d
39	a	a	a	a	a	a	a
40	a	a	a	a	a	a	a

[a] Results deviating from the consensus result are indicated in boldface. Laboratory 7 used ALF analysis of PCR end products; the other six laboratories analyzed PCR end products on agarose gels.

[b] ug, isolate ungrouped with any other isolates.

RESULTS

Interpretation of isolate groupings obtained by the participating laboratories. The initial part of the study was designed simply to examine whether the overall isolate grouping (i.e., typing and epidemiological) conclusions reached by the participating laboratories were reproducible. Table 2 indicates the isolate groupings assigned by the different laboratories on the basis of all the fingerprinting data available. There were no significant differences between the isolate grouping results obtained with the different primers. Of the seven laboratories participating in the trial, six laboratories analyzed the isolate groupings following agarose gel electrophoresis of the PCR end products, while one laboratory, indicated in Table 2, used ALF analysis. The consensus isolate groups (i.e., those recognized by the majority of the participating laboratories) are summarized in Table 3. When the grouping results obtained by the seven participating laboratories were compared with the consensus view, 10 deviations (3.6% of the total isolate group-

TABLE 3. Consensus isolate groupings obtained by comparing the isolate groupings allocated by the participating laboratories

Group	Isolates included in group	Origin[a]
a	1, 7, 13, 19	Freiburg, GE
	31, 33, 35, 39, 40	Venlo, NL
	32	Copenhagen, DK
b	2, 8, 14, 20, 26	Ghent, BE
c	3, 9, 15, 21	Cologne, GE
	6, 12, 18, 24, 29, 36	Nottingham, UK
d	4, 10, 16, 22, 27, 38	Odense, DK
e	5, 11, 17, 23, 28	Nijmegen, NL
f	30, 34, 37	Copenhagen, DK
ug[b]	25	Freiburg, GE

[a] See footnote *b* of Table 1 for definitions of country abbreviations.
[b] ug, isolate ungrouped with any other isolates.

ing allocations) were shared between three laboratories; the results obtained by the other four laboratories were in complete agreement. Although several different models of thermal cycler were used, these did not appear to influence the results obtained by the standardized protocols and with the standardized reagents.

Two anomalies were identified when the consensus isolate groupings were compared with the isolates' geographical origins (and presumed epidemiological relationships) listed in Table 1. First, isolate 32, from Denmark, did not group with the other Danish isolates. This isolate was isolated from the same burns unit as isolates 30, 34, and 37. However, following retrospective clinical inquiries, it was determined that this patient had acquired the acinetobacter while hospitalized in Turkey and was already colonized when he was transferred to the Danish burns unit. Second, isolate 25, from Freiburg, Germany, did not group with the other isolates from Freiburg. However, although the isolate was obtained from the same intensive care unit, its antibiogram was found on retrospective testing to be different from those of the other Freiburg isolates. While antibiograms are not an accurate means of distinguishing strains, this observation provided an additional indication that the strain was a sporadic acinetobacter that was isolated by chance in the middle of an ongoing outbreak. Importantly for this molecular typing study, these two anomalies were recognized by all seven participating laboratories.

Effects of different primers on pattern complexity. Figure 1 provides examples of the fingerprint patterns obtained on agarose gels following duplicate PCRs with the four primer sets with isolates 1 to 6 in a single laboratory. Similar findings were obtained by all six participating laboratories that analyzed fingerprints on agarose gels. Simple visual comparison indicated that the duplicate samples (generated in a single PCR run) yielded fingerprint patterns that were reproducible, albeit with some variations in particular band intensities. Of the four different primer sets, the M13 primer generated the most distinct fingerprints, with only a relatively small number of secondary or faint bands. These patterns were amenable to central analysis following scanning of the gel photographs submitted by the participating laboratories (see below). The other three primer sets generated more complex fingerprints with a large number of closely spaced bands, some of which were faint, albeit reproducible. The results obtained with these three primer sets were analyzed only by the ALF technique.

Analysis of M13 fingerprint reproducibilities on agarose gels. The reproducibilities of two sets of fingerprint data (for isolates 1 to 10 and standard DNA extracts A to J, respectively) were examined by scanning photographs of the PCR fingerprints obtained on agarose gels by six independent laboratories with the M13 primer. The 10 isolates contained four epidemiologically related isolate pairs (isolates 1 and 7, isolates 2 and 8, isolates 3 and 9, and isolates 4 and 10) and two isolates (isolates 5 and 6) that were not related epidemiologically to the other isolates (Table 1). In addition, DNA extracts A to J were prepared from isolates 1 to 10, but all of these facts were unknown to the participating laboratories at the time of the trial. All PCRs were performed in duplicate, yielding 120 fingerprints produced from DNA extracts prepared by the individual laboratories themselves and 120 fingerprints produced from the centrally prepared standard DNA extracts.

Of the 120 fingerprints produced from the DNA preparations prepared by the individual laboratories, 110 (91.6%) were defined as concordant at the practical level in that they were correctly grouped with the other epidemiologically related isolates at an S_{AB} value of ≥ 0.7 following alignment and cluster analysis of corresponding isolate fingerprints from different gels by the DENDRON program (see Materials and Methods). The 10 fingerprints that did not fall within the definition of concordant still formed distinct clusters with the fingerprints derived from the same isolate by the other participating laboratories, but with an S_{AB} value of <0.7, as illustrated in Fig. 2. Of the 120 fingerprints obtained from the standard DNA extracts, 106 (88.3%) were similarly defined as concordant. The

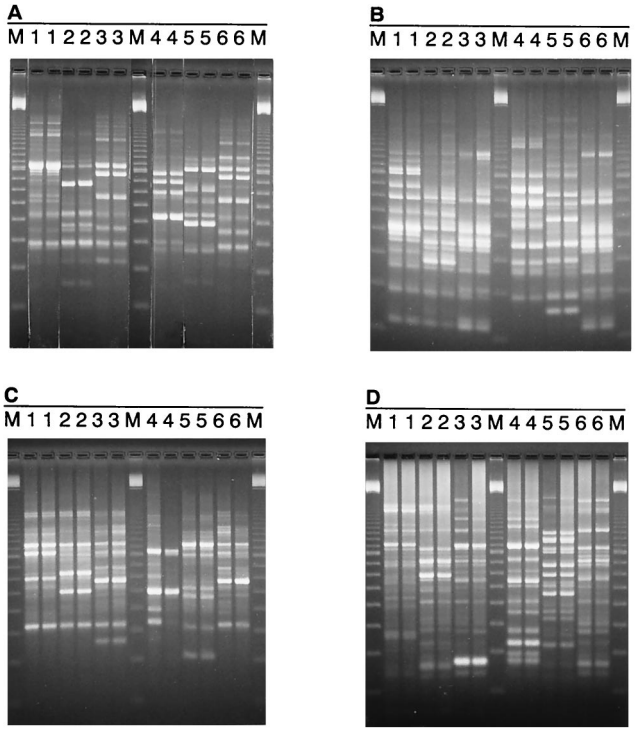

FIG. 1. Examples of fingerprint patterns obtained for isolates 1 to 6 on agarose gels following PCR amplification with M13 primer (A), DAF4 primer (B), ERIC-2 primer (C), and REP1 + REP2 primers (D). Lanes: M, 100-bp ladder; 1 to 6, isolates 1 to 6, respectively (groups a, b, c, d, e, and c, respectively; Table 2).

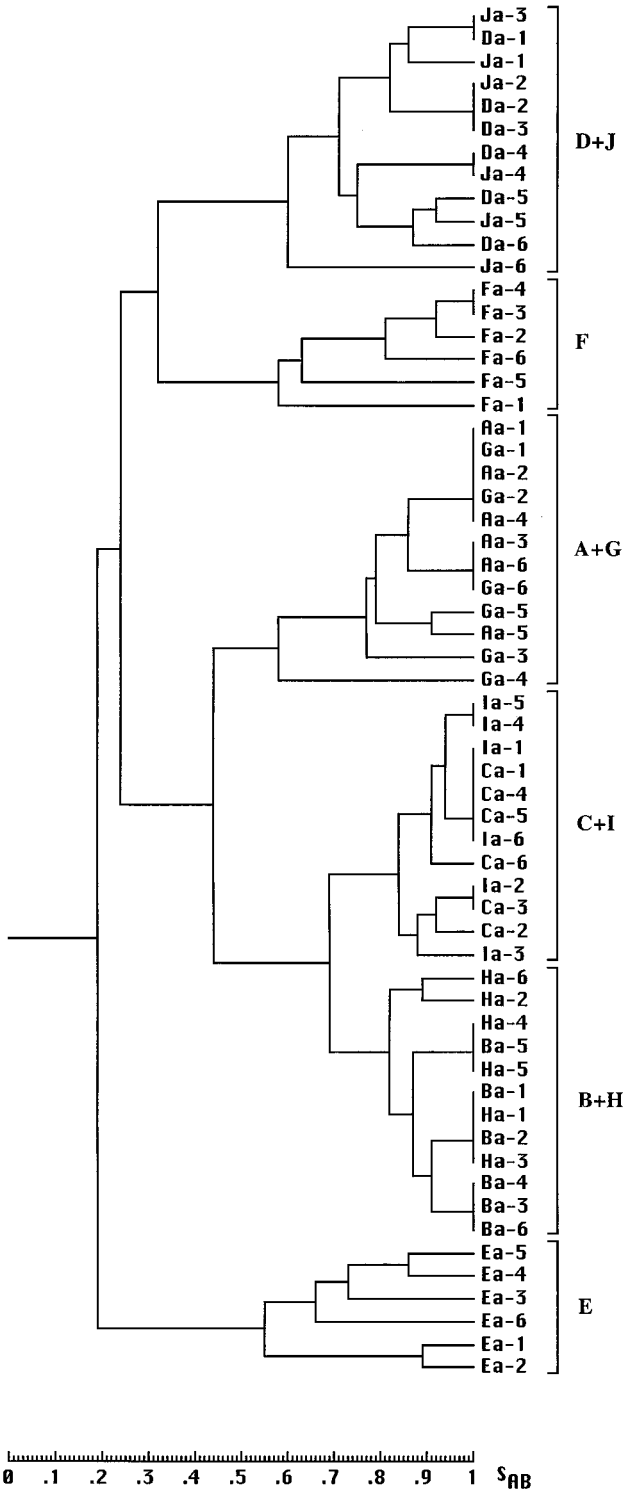

FIG. 2. Sample dendrogram showing the cluster analysis results for one of the duplicate set of fingerprints obtained by the six participating laboratories using agarose gel analysis in conjunction with the M13 primer and the centrally produced DNA extracts. Each fingerprint is designated by a letter indicating the DNA extract (A to J), the set of results (for the a set), and the laboratory (1 to 6).

14 fingerprints derived from standard DNA extracts that did not fall within the definition of concordant also formed distinct clusters with the fingerprints derived from the corresponding DNA extracts by the other participating laboratories. As an illustration of these results, Fig. 2 presents the dendrogram constructed for one of the duplicate sets (the a set) of fingerprints derived from standard DNA extracts A to J. Seven fingerprints (derived from standard DNA extracts Ja-6, Fa-5, Fa-1, Ga-4, Ea-6, Ea-1, and Ea-2) had S_{AB} values of <0.7 in comparison with the fingerprints obtained by other participating laboratories from the same standard DNA extract. Similar results were obtained with the duplicate b set of fingerprints.

ALF analysis of fingerprint reproducibility. Computerized ALF analysis offered the possibility of rapid automated assessment of pattern reproducibility for a larger number of samples. Of the 560 samples analyzed in Freiburg (280 fingerprints produced from DNA extracts prepared by the individual laboratories themselves and 280 fingerprints produced from the centrally prepared standard DNA extracts), the results for 7 (5 from independent preparations and 2 from standard extracts) were not interpretable; i.e., they did not give readable results on the ALF equipment. Thus, the overall interpretability for the samples was 98.8%. Of the 553 interpretable samples, 547 (98.9%) yielded concordant results ($r = \geq 70$) in that they were correctly grouped with the other epidemiologically related isolates. When the individually prepared extracts were compared with the standard DNA extracts, 10 of 280 (3.6%) and 3 of 280 (1.1%) samples were either uninterpretable or not concordant with the fingerprints of isogenic isolates, respectively. Table 4 presents the concordance obtained with each individual primer set. Concordance was best with the DAF4 and REP1 + REP2 primers (these were the most complex fingerprints when visualized on agarose gels), followed closely by the M13 primer. The lowest concordance was obtained with the ERIC-2 primer. Overall, the results obtained with the standard DNA extracts were slightly more concordant than those obtained with the individually prepared DNA extracts (98.9 versus 96.4%; not statistically significant).

ALF analysis of pattern correlation. An exact measure of the interlaboratory reproducibility of a given amplification profile is the degree of similarity (pattern correlation) between PCR fingerprints generated independently for the same isolate. The overall pattern correlation was calculated as the arithmetic mean of Pearson's product moment correlation coefficients of fingerprints for all identical isolates after parallel analysis of the PCR products by ALF analysis on sequencing gels. Table 5 presents the pattern correlation results obtained with each individual primer set over the 120- to 800-bp size range, excluding the uninterpretable fingerprints. Overall, the pattern correlation was good (range, 83.3 to 86.6%), but it was

TABLE 4. ALF analysis of concordance obtained with different primers

Source of DNA extract and primer(s)	No. (%) of concordant samples ($r > 70$)	No. of noncordant samples ($r < 70$)	No. of noninterpretable samples
DNA extracts prepared by individual laboratories			
M13	67 (95.7)	2	1
DAF4	69 (98.6)	0	1
ERIC-2	65 (92.9)	3	2
REP1 + REP2	69 (98.6)	0	1
DNA extracts prepared centrally			
M13	69 (98.6)	1	0
DAF4	70 (100)	0	0
ERIC-2	68 (97.1)	0	2
REP1 + REP2	70 (100)	0	0

TABLE 5. ALF analysis of pattern correlation obtained with different primers in the size range of 120 to 800 bases, excluding the seven noninterpretable samples

Primer(s)	% correlation (mean ± SD) for the following type of DNA sample:	
	Prepared by individual laboratories	Prepared centrally
M13	82.8 ± 10.6	88.6 ± 7.8
DAF4	87.9 ± 8.5	86.6 ± 8.5
ERIC-2	84.7 ± 10.7	86.3 ± 12.5
REP1 + REP2	83.5 ± 8.6	86.8 ± 17.5
Mean	84.7	87.1

slightly better for the standard DNA extracts (87.1%) than for the individually prepared DNA extracts (84.7%), although this difference was again not statistically significant.

DISCUSSION

A large number of related methods use PCR to generate DNA fingerprints from microorganisms for typing and epidemiological studies (8, 14). All of these methods use slightly different approaches, but the underlying principle is the same in that a single primer is used to target recurrent genomic DNA motifs to generate a fingerprint profile. Amplification can be conducted at relatively low annealing temperatures that allow mismatches and a degree of nonspecific binding of the primer to the template. Alternatively, known conserved regions that occur in multiple copies in the template can be targeted with specific primers. Repetitive sequences are particularly amenable to this approach (rep-PCR), and several highly conserved intergenic repetitive consensus nucleotide sequences have been targeted for this purpose (16). In either case, amplimers are generated whenever two correctly orientated copies of the primer are close enough for the PCR to proceed efficiently (15). The main advantages of these techniques over more traditional phenotypic typing methods are their speed and general applicability to a wide range of bacteria in most routine microbiology laboratories. However, doubts have been raised as to the reproducibility of these techniques, particularly in and between laboratories that use slightly different equipment and protocols (7, 8, 12, 13), and most studies have concentrated on investigating relatively small local outbreaks in which direct, same-gel comparison of different isolates has been possible.

Several recent reports have outlined the numerous factors that may influence the reproducibility of these techniques (7, 8, 12, 13) and that have been reported to make interlaboratory comparisons difficult or impossible. The aim of the present study was to examine whether these problems could be overcome at the practical level by simply defining appropriate standardized PCR reagents, conditions, primers, and analysis methods so that reliable, discriminatory, and reproducible results could be achieved in different laboratories. As an initial step, standardized DNA extraction procedures and PCR reagents were used, with the latter based around the availability of PCR beads that are supplied in a quality-controlled, premixed, predispensed, room temperature-stable format containing Ampli-*Taq* DNA polymerase and the Stoffel fragment and all necessary buffer ingredients and nucleotides. The four different amplification protocols, chosen on the basis of previously published results (5, 10, 17–19), included two primers (primers M13 and DAF4) that targeted conserved sequences at relatively high annealing temperatures and two primers (primers ERIC-2 and REP1 + REP2) that targeted enterobacterial repetitive sequences at lower annealing temperatures to allow for mismatches in *Acinetobacter* spp. The standardized protocols were then used by the seven participating laboratories to generate PCR fingerprints for 40 isolates of *Acinetobacter* spp. with known epidemiological relationships.

The overall epidemiological conclusions reached by the participating laboratories were substantially correct, with 96.8% of the total isolate grouping allocations agreeing with the consensus view (Tables 2 and 3). Each of the participating laboratories was successful in identifying the main epidemiological clusters, and all laboratories identified two non-outbreak-related isolates as not belonging to the expected clusters. These findings are in agreement with the general view that PCR-based fingerprinting techniques can produce useful results when performed in a single laboratory over a limited time period. More important is the evidence that independently produced PCR fingerprint patterns can achieve a high degree of reproducibility at the practical level if (i) standardized reagents (quality controlled), (ii) standardized extraction of DNA, and (iii) standardized amplification conditions are used. In contrast to previous suggestions (12, 13), other factors (e.g., the use of crude whole-cell DNA extracts as templates and the precise model of thermal cycler and the gel running conditions used) did not seem to be important, provided that the aforementioned factors were standardized. Indeed, the standardized technique seemed to be remarkably robust, even when used by workers with limited previous experience with PCR-based fingerprinting techniques, and the crude DNA extracts, whether prepared locally or on a central basis, seemed to be remarkably stable. Minor variations in fingerprint profiles were detected by both gel and ALF analyses, but these did not affect the isolate grouping relationships obtained at the cutoff similarity level of 70% shown previously (5, 18) to distinguish unambiguously between unrelated isolates of *Acinetobacter* (Fig. 2). This is in line with the general consensus that several band differences (depending on the total number of bands) in the PCR-based fingerprint profiles are required before two isolates may be considered different (11). Further studies with defined isolates belonging to other species of bacteria will be required to define whether different cutoff values are appropriate for use with other organisms.

So far as the typing of *Acinetobacter* spp. is concerned, there was no significant difference between the isolate grouping results obtained with the different primers. However, the M13 primer generated the most distinct fingerprints on agarose gels, with only a relatively small number of secondary or faint bands. The other three primer sets generated more complex fingerprints with a large number of closely spaced bands, some of which were faint, albeit reproducible. Two of these primer sets (primers DAF4 and REP1 + REP2) yielded the best reproducibility following ALF analysis. The lowest reproducibility was obtained with ERIC-2, probably reflecting the low annealing temperature (25°C) required to use this primer to fingerprint *Acinetobacter* spp. (10).

Identification and typing of microorganisms are extremely important in efforts to monitor the geographical spread of pathogens. However, central analysis of an ever growing number of important microorganisms at a national or an international level imposes an unmanagable workload on central reference facilities, with a concomitant delay in obtaining results, and involves the undesirable procedure of shipping pathogens by air or surface carriers. Meaningful understanding and sensible intervention by local health authorities to prevent the national and international spread of disease can only be achieved

by extensive and rapid communication of data between different local laboratories. Therefore, local isolate analysis combined with central data collection offers a more efficient and safer alternative. The design of new fingerprinting methods for analysis of microbial DNA means that such typing strategies can potentially be applied to many microorganisms of public health significance (11). Use of the combination of quality-controlled reagents, ALF analysis of PCR fingerprints, and digital data communications may bring new opportunities for the comparison of typing data generated in different facilities. Such data could be made accessible to other laboratories via an Internet-based database. In places where automated sequencers are not available, DNA extracts could easily be prepared and shipped, without the risk accompanying the shipping of pathogens, to sentinel surveillance laboratories with appropriate equipment for immediate analysis. The increasing public health problem resulting from the national and the international spread of pathogenic microorganisms means that it is now timely for further studies on establishing standardized methodologies and systems for use in conjunction with this technology.

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