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Comparative Typing of *Pseudomonas aeruginosa* by Random Amplification of Polymorphic DNA or Pulsed-Field Gel Electrophoresis of DNA Macrorestriction Fragments

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Eighty-seven strains of *Pseudomonas aeruginosa* were typed by random amplification of polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) of macrorestriction fragments. Strains were clustered on the basis of interpretative criteria as presented previously for the PFGE analysis. Clusters of strains were also defined on the basis of epidemiological data and subsequently reanalyzed by RAPD. It was found that in an RAPD assay employing the enterobacterial repetitive intergenic consensus sequence ERIC2 as a primer, single band differences can be ignored; in this case, clonally related strains could be grouped as effectively and reliably as with PFGE. These data could be corroborated by the use of other primer species. However, some primers either showed reduced resolution or, in contrast, identified DNA polymorphisms beyond epidemiologically and PFGE-defined limits. Apparently, different primers define different windows of genetic variation. It is suggested that criteria for interpretation of the ERIC2 PCR fingerprints can be simple and straightforward: when single band differences are ignored, RAPD-determined grouping of *P. aeruginosa* is congruent with that obtained by PFGE. Consequently, this implies that RAPD can be used with trust as a first screen in epidemiological characterization of *P. aeruginosa*. The ability to measure the rate of molecular evolution of the *P. aeruginosa* genome clearly depends on the choice of restriction enzyme or primer when RAPD or PFGE, respectively, is applied for the detection of DNA polymorphisms.

Molecular typing of microbial pathogens is of pivotal importance in the elucidation of transmission routes. By closely monitoring genetic variability, phylogenetic distances can be measured, and these data can give insight into the interrelationship of bacterial, protozoan, or fungal isolates (17). Detailed genetic analysis at the species level gives insights into the variability within a bacterial population and generates evidence on genome plasticity and evolution, which in turn leads to bacterial adaptation to various environmental conditions. This type of information can be used in clinical settings to discriminate ongoing epidemics of an infectious agent from incidentally increased infection rates. Various molecular strategies have been adapted to an experimental format such that the data obtained can help the clinical microbiologist to indicate potential risk factors and to track down sources of epidemic strains (16). Besides the technical point of view, several major questions still exist, however. First, there is no general agreement on the optimal typing strategy to be used for a given pathogen (31, 32). Second, although there is a general concordance among typing procedures when comparative analyses are performed, sometimes discrepancies are obvious (22). The assessment of such discrepant results seems to be possible only when further molecular details about the respective organisms are made available. It has been suggested that combining data obtained by different typing procedures will give optimal insight into strain relatedness (32). However, only a small number of studies describe in detail the basis of the variability observed between different typing techniques.

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The aim of the present study was to determine to what degree two frequently used genetic typing procedures give concordant results, using clinical and environmental strains of *Pseudomonas aeruginosa*. Since standardization of restriction site variation, as detected by pulsed-field gel electrophoresis (PFGE) and annealing site variation in random amplification of polymorphic DNA (RAPD), has not been discussed before, sets of clonally related and unrelated isolates of this opportunistic bacterial pathogen were compared in detail.

MATERIALS AND METHODS

Bacterial strains. Strains were selected on the basis of their PFGE-determined genotypes, the determination of which has been described in previous publications (24, 25, 27) (see below for technical details and Table 1 for a survey of strain characteristics). Four groups of strains were gathered. First, the entire Z group ($n = 24$) belonged to a single clonal type (PFGE C type). PFGE banding patterns differed by up to six new restriction fragments. Strains derived from environmental and clinical sources and various subtypes were represented. The G group ($n = 16$) was comprised of clearly different strains; seven different American Type Culture Collection strains were included as well. PFGE banding patterns displayed gross differences, always exceeding the minimum number of six differently oriented DNA restriction fragments. One of the strains in this group was identical to a member of the Z group (internal control duplicate). In the R group ($n = 25$), several small clusters of identical pairs or triplets were mixed. The strains in this group were epidemiologically unrelated but showed similar PFGE patterns. Finally, the B group ($n = 22$) contained several sets of strains with identical PFGE patterns.

RAPD analysis. DNA was isolated in accordance with the Celite affinity chromatography protocol as described previously (4). The DNA was stored in a buffered solution (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) at −20°C. RAPD was performed on 50 ng of template DNA as presented before (23, 33). For each strain of *P. aeruginosa*, two RAPD assays were performed. Either primer ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) or primer 1290 (5′-TACATTCG AGGACCCCTAAGTG-3′) was employed. Because of the complexity of the R group, several strains from within this cluster were also analyzed with other primers. These primers were ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′), RAD1 (5′-GGTTGGGTGAGAATTGCACG-3′), RAPD7 (5′-GTGGATG CGA-3′), 325 (5′-TCATGATGCA-3′), 327 (5′-CCTGCTTTGAACACTCTAA

TTT-3′), 44 (5′-CGCTACCAAGCAATCAAGTTGCCC-3′), and 70 (5′-CATC GTCGCTATCGTCTTCACCAC-3′). Using this same set of primers, some of the strains defined as PFGE-identical or -related strains were reexamined as well. After electrophoresis in 1% agarose gels, the ethidium bromide-stained DNA fragments were photographed with Polaroid equipment. Banding patterns were analyzed by two independent researchers, and (sub)types were assigned on the basis of single or multiple band differences.

PFGE analysis. PFGE was performed as described previously (9, 26). *P. aeruginosa* cells were embedded in agarose blocks and treated with proteinase K, *N*-lauroylsarcosine, and EDTA. Before electrophoresis, the DNA was digested with the restriction enzyme *Spe*I (New England Biolabs, Hertfordshire, United Kingdom), and after PFGE, banding patterns were visualized by ethidium bromide staining and then photographed. Interpretation was also performed in accordance with previously determined standards implying that separate types should differ by more than six DNA fragments. Each type was coded with a capital letter, and subtypes were identified by numbers.

RESULTS

PFGE data interpretation. The PFGE patterns for all of the strains were determined in previous studies (24, 27) and are summarized in Tables 1 and 2. Strains from the Z group were considered clonally related (C type; subtypes as indicated in Table 1), although the individual electropherograms may differ in up to even six DNA macrorestriction fragments (25). The G, R, and B groups are more heterogeneous, although clusters of related and sometimes even identical (by PFGE) strains may be discerned (see, for instance, strains B1 to B7 or B22 to B24).

Integrated analysis of the PFGE and RAPD data. All RAPD-derived banding patterns were indexed with capital letters. This is exemplified in Fig. 1 and 2, and data are summarized in Tables 1 and 2 in a schematic format as well.

The number and sizes of DNA fragments generated by RAPD are clearly primer dependent. As can be deduced from Fig. 1, when the ERIC2 primer is employed, between 8 and 15 DNA fragments ranging from 100 to 2,500 bp are synthesized by the *Taq* polymerase. When RAPD1 is used, approximately 17 fragments are generated, while for primer 325, between 16 and 19 DNA molecules can be seen after electrophoretic separation (data not shown). Sometimes smearing is observed when multiple DNA fragments which differ slightly in length are visible.

The members of the PFGE-homogeneous Z group were shown to generate individually similar RAPD banding patterns. From the banding patterns it was concluded that for the entire group only five RAPD (sub)types could be observed. When the ERIC2 primer was applied, for instance, the two subtypes A1 and A2 differed by the presence or absence of only a single DNA fragment when compared with the A type. This may imply that single band differences in the banding patterns generated in this way do not represent epidemiologically relevant genetic differences among related clusters of strains (see also Discussion). The only aberrant strain in the Z group is Z27, which was included as a control sample in this group (PFGE type CO). The distribution of fragment sizes shown by this strain was similar to that of genuine clone C isolates (24).

Data obtained for members of the G group corroborated the PFGE findings. Major differences in banding patterns were observed; only in the case of strains G1 and G3 were somewhat similar patterns documented (they still differed at two positions, but no subtypes were thus identified). Both the ERIC2 and 1290 primers generated concordant results in this respect. Note that the RAPD type for strain G5, which is of the PFGE C type, is identical to the RAPD types established for the majority of the Z strains.

As can be seen in Table 1, data obtained for the B group by ERIC2 RAPD show excellent agreement with the PFGE codes. All the clusters enclosed are adequately recognized by the ERIC2 typing results. In some instances, the banding patterns generated with primer 1290 identified additional heterogeneity among the related strains (for instance, type f for B8 and type g for B9). This indicates that this particular primer may give rise to an overestimation of the actual number of distinct types that can be distinguished in a given collection of *P. aeruginosa* strains. These RAPD fragments may be reminiscent of DNA loci displaying a high speed of alteration due to a high frequency of mutation or rearrangements caused by intra- or interstrain exchange of genetic material.

The most complex set of data was obtained for the R group of strains. In this group, several (sub)clonally related strains are present, as determined by PFGE. In Table 2, the data obtained by RAPD are summarized; strains are ordered with respect to the initially assigned PFGE type. As such, it can be deduced that the ERIC2 RAPD tests are in reasonable agreement with the PFGE data; again, the 1290 fingerprints show more variability. For this reason, other primer species were evaluated for typing efficacy. These experiments resulted in a number of interesting observations. It appeared that application of the primers 70, RAPD7 (which is very well suited for typing of staphylococci [32]), 327, and ERIC1 did not generate interpretable results. Either the DNA banding patterns were identical for all strains or no DNA was amplified whatsoever. Data obtained with the primers that could be applied successfully are summarized in Table 2 and illustrated in Fig. 2. From these data, it can be concluded that RAPD analysis generates results that compare very well with those obtained by PFGE. The RAPD-based grouping is a clear reflection of the PFGE-related clusters. Depending on how the data are interpreted, it is evident that PFGE subtypes may sometimes be defined as different clonal types by RAPD. Strains R10, R19, and R24, which are PFGE subtypes M3, M3, and M5, respectively, are grouped into two RAPD types. The ERIC2, RAPD1, 325, and 44 data are precisely concordant; only primer 1290 gives rise to an overestimation of the number of types that can be distinguished. The latter primer also shows overdiscrimination with strains R11 and R20. Also, the latter phenomenon can be observed in some of the other groups displayed in Table 2.

DISCUSSION

P. aeruginosa is a common pathogen in cystic fibrosis (CF) patients (2, 6, 13). By applying molecular typing procedures, it has been demonstrated that the clinical problems caused by *P. aeruginosa* may result from its capacity to also colonize inanimate surfaces for prolonged periods of time (5, 10). Although the relevance of molecular typing for CF patients may not be as obvious as it possibly should be, several reports of studies employing molecular typing of *P. aeruginosa* were recently published. Striking examples were the proof of existing cross-contamination among neonates in certain clinical settings (34) and a study showing the usefulness of molecular typing in gaining insight into the putative pseudomonad exchange between CF patients spending time in summer holiday camps (14). Reservoirs could thus be identified, and the nosocomial ecology of the microorganism could in some instances be unravelled in great detail. In order to perform such studies, the clinical laboratory should have appropriate technical means at its disposal. Presently the number of typing systems described for *P. aeruginosa* is large (19–21, 28, 29), but recently, newly developed procedures such as PFGE of DNA macrorestriction fragments (9, 26, 30) and RAPD analysis (3, 7, 18) have been used for detailed comparisons of clinical and environmental strains of *P. aeruginosa*. However, in only a limited set of studies were the efficacies of the typing strategies compared.

The most elaborate multicentered comparative typing effort

TABLE 1. Compilation of PFGE and RAPD typing data for strains of *P. aeruginosa*[a]

Strain no.	PFGE type	Source of isolation[b]	Date of isolation (mo/yr or yr)	RAPD type (ERIC2/1290)[c]
Z1	C	P8, CF, Hannover	8/86	A/A
Z2	C1	P8, CF, Hannover	4/87	A/A
Z4	C3	P8, CF, Hannover	11/89	A/A
Z6	C5	P9, CF, Hannover	11/87	A/A
Z7	C6	P9, CF, Hannover	6/88	A/A
Z8	C7	P10, CF, Hannover	5/92	A/A
Z10	C9	P4, CF, Hannover	4/86	A/A
Z11	C10	P4, CF, Hannover	4/87	A/A
Z12	C11	P11, CF, Hannover	Not known	A/A
Z13	C12	P11, CF, Hannover	7/84	A/A
Z14	C13	P12, CF, Hannover	1/85	A/A
Z15	C14	P12, CF, Hannover	12/85	A1/B
Z16	C15	P12, CF, Hannover	3/87	A/A
Z17	C16	P12, CF, Hannover	5/87	A1/A
Z18	C17	Clinical environment, Hannover	12/89	A2/A
Z19	C18	Clinical environment, Hannover	12/89	A2/A
Z20	C19	P1, CF, Hannover	2/89	A/A
Z21	C20	Butchery, tap water, Muelheim	92	A/A
Z22	C21	River, Muelheim	92	A/A
Z23	C22	Swimming pool, Muelheim	92	A/A
Z24	C23	Ear isolate, Heidelberg	92	A/A
Z25	C21	River, Muelheim	92	A/A
Z26	C	P8, CF, Hannover	1/86	A/A
Z27	CO	ATCC 33351, serotype 4	Not known	B/C
G1	CP	ATCC 14886, soil	Not known	C/D
G2	CQ	ATCC 33348, serotype 1	Not known	D/E
G3	CR	Patient, Heidelberg	Not known	E/F
G4	CS	Outer ear infection, DSM 1128	Not known	F/G
G5	C	P8, CF, Hannover (same as Z26)	1/86	A/A
G6	AK	Burn wound, Hannover	1989	G/−
G7	CT	ATCC 10145, neotype	Not known	H/H
G8	M	P18, CF, Hannover	6/91	I/I
G9	BB	Clinical environment, Hannover	12/89	J/J
G10	CU	ATCC 33818, mushroom	Not known	K/K
G11	DM	CF, not from Hannover	1984	L/L
G12	PAK	Reference laboratory strain, Hannover	Not known	M/H
G13	PAO	Reference strain, wound, Melbourne, Australia	1955	N/−
G14	CV	ATCC 15691	1950	O/M
G15	CW	ATCC 21776, soil, Japan	Not known	P/N
G16	CX	ATCC 33356, serotype 9	Not known	Q/−
B1	I	P6, CF, Hannover	1/90	R/O
B2	I	P6, CF, Hannover	1/90	R/O
B3	I	P6, CF, Hannover	11/90	R/O
B4	I	P6, CF, Hannover	3/91	R/O
B5	I	P6, CF, Hannover	3/91	R/O
B6	I	P6, CF, Hannover	9/91	R/O
B7	I	P6, CF, Hannover	9/91	R/O
B8	C	P9, CF, Hannover	4/89	A/P
B9	C	P9, CF, Hannover	7/89	A/Q
B10	C	P9, CF, Hannover	8/90	A/−
B11	F	P2, CF, Hannover	8/90	S/−
B12	C	P9, CF, Hannover	8/90	A/P
B13	C	P9, CF, Hannover	5/91	A/Q
B14	G5	P3, CF, Hannover	10/89	T/R
B15	G5	P3, CF, Hannover	7/90	T/R
B16	G5	P3, CF, Hannover	2/91	T/R
B19	F	P2, CF, Hannover	8/90	S/S
B20	C	P9, CF, Hannover	8/90	A/−
B21	F	P2, CF, Hannover	3/91	S/S
B22	CY	Brass tube	92	U/T
B23	CY	Sink, private household	92	U/T
B24	CY	Sink, private household	92	U/T
R10	M3	Clinical environment, Hannover	11/93	V/W
R19	M3	Clinical environment, Hannover	12/89	W/b
R24	M5	Clinical environment, Hannover	12/89	W/d

Continued on following page

TABLE 1—*Continued*

Strain no.	PFGE type	Source of isolation[b]	Date of isolation (mo/yr or yr)	RAPD type (ERIC2/1290)[c]
R11	K	P11, CF, Hannover	7/85	X/X
R20	K2	P11, CF, Hannover	2/91	X/C
R3	J10	Pond, Muelheim	92	Y/P
R12	J1	P7, CF, Hannover	9/85	Y/Y
R21	J8	P7, CF, Hannover	7/89	Y/Y
R25	J7	P12, CF, Hannover	11/89	Y/Y
R5	A	P13, CF, Hannover	12/85	Z/R
R14	A	P13, CF, Hannover	1/86	Z/Z
R7	AH	P24, CF, Hannover	84	a/T
R16	AH	P23, CF, Hannover	84	a/Z
R22	AH	P4, CF, Hannover	84	a/R
R6	B	P15, CF, Hannover	5/86	b/S
R15	B1	P15, CF, Hannover	12/86	b/a
R8	F	P2, CF, Hannover	2/85	S/S
R17	F2	P2, CF, Hannover	5/92	C/b
R23	F	P2, CF, Hannover	5/92	S/S
R13	CZ1	Clinical environment, Muelheim	92	d/−
R4	CZ	Sink, private household, Muelheim	92	d/Q
R9	G	P3, CF, Hannover	4/86	T/V
R18	G4	P3, CF, Hannover	5/92	T/V

[a] Subtypes are indicated by affixed Arabic numbers; in the case of PFGE, this may be reminiscent of differences in the positions of six DNA fragments, while in case of RAPD, the cutoff was at more than a single band difference. In each of the RAPD tests a single primer was included (either ERIC2 or primer 1290).
[b] For the source of isolation, patients are identified by a capital P; patients 8, 9, and 10 are siblings. All strains, except for the reference and ATCC strains, derive from Germany.
[c] —, not done.

TABLE 2. Comparative analysis of PFGE-typed, (sub)clonally related strains of *P. aeruginosa* by multiple RAPD assays

Strain no.	RAPD type determined with primer:		
	RAPD1	325	44
R10	A	A	A
R19	B	B	B
R24	B	B	B
R11	C	C	C
R20	C	C	C
R3	D	D	D
R12	D	D1	D
R21	D	D1	D
R25	D	D1	D
R5	E	E	E
R14	E	E	E
R7	F	F	F
R16	F	F	F
R22	F	F	F
R6	G	G	C
R15	G	G	C
R8	H	H	G
R17	I	I	H
R23	H	H	G
B21	H	H	G
R13	J	J	I
R4	J	J	I
B14	K	K	K
R9	K1	K1	L
R18	K1	K1	L

for *P. aeruginosa* was presented 3 years ago (15). This study, which essentially lacked molecular analyses, suggested that serological typing of the lipopolysaccharides in the outer surface of *P. aeruginosa* provides an efficient means of bacterial typing, especially because it is simple and efficient. CF isolates of *P. aeruginosa* are not typeable by this method because of their rough phenotype. Due to aberrant phenotypic characteristics, CF strains can be reliably typed only by molecular methods. This was recently confirmed in a study of the colonization of patients with bronchiectasis in which the conventional methods proved ineffective (12). An even more recent study included PFGE typing (11). The experimental results demonstrated that the resolution of PFGE exceeded that of restriction fragment length polymorphism analysis with ribosomal or toxin A DNA probes. Nevertheless, strains of the same type were found in hospitals at different geographic locations. Finally, these authors emphasize that typing data

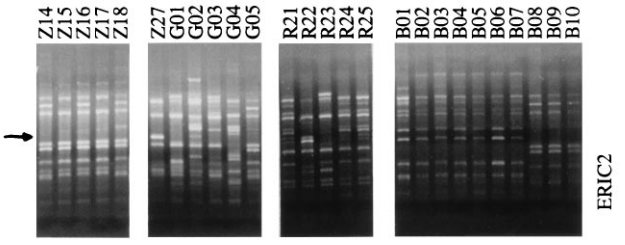

FIG. 1. Examples of RAPD-generated DNA fingerprints for strains of *P. aeruginosa* genetically clustered in four different groups (Z, G, R, and B). The primer used was ERIC2. Strains Z14 to Z18 are part of the clonally related C cluster as defined by PFGE. Note that only single-band differences are observed. The second panel from the left shows strains belonging to different clonal entities (Z27 to G05); all the banding patterns are clearly different. In the third (R21 to R25) and fourth (B01 to B10) panels, some of the epidemiologically clustered strains are on display. Note that B08 to B10 are identical to the C-type strains showed in the panel on the left. For a detailed description of the data, see Table 1. The arrow on the right indicates a molecular length of 800 bp.

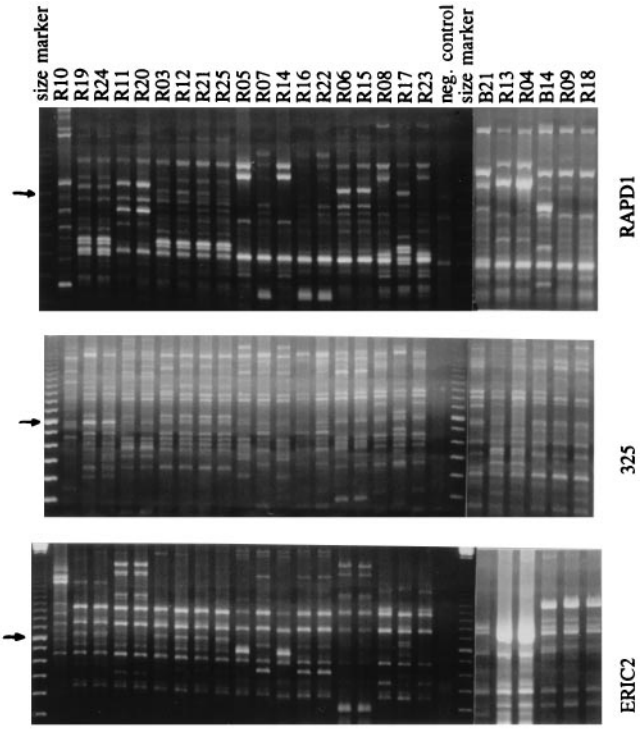

FIG. 2. Comparative analysis of the R group strains of *P. aeruginosa* by using different RAPD primers. Clonal relatedness was defined on the basis of PFGE, which is summarized in Table 2. Note that the schematic interpretation of data as presented in this figure is given in the same table. Lanes labeled size marker contain 1-kb ladder DNA. The arrow on the left indicates an 800-bp DNA molecule. In the lane labeled neg. control, amplimers derived from mixtures containing no extraneous DNA were analyzed. Patterns displayed in the panels, going from top to bottom, were generated with the help of primers RAPD1, 325, and ERIC2. Note that there is identity among the ERIC2 fingerprints that are shown in both Fig. 1 and this figure.

should be interpreted only in the context of sound epidemiological data because, otherwise, unequivocal conclusions with respect to strain persistence or transmission cannot be drawn. There is a recent (and still singular) publication that discusses the relationship between data obtained by PFGE and that obtained by RAPD for the same set of strains of *P. aeruginosa* (18). The present data indicate that RAPD should serve as a first screen for *P. aeruginosa* typing because of the simplicity and high speed of this technique and that the bacterial grouping results attained coincide with those of PFGE analysis. The authors of reference 18 do not fully discuss the relationship between the two sets of experimental data; neither do they define strict interpretative criteria for the PFGE and RAPD DNA banding patterns. The present communication indicates that if single band differences between RAPD-derived fingerprints are ignored, there is excellent agreement of the RAPD results with the PFGE-based grouping of clonally related *P. aeruginosa* strains.

The interpretation of data generated by PFGE was the general subject of a recent and timely discussion (31). In this paper, which tried to define guidelines for the interpretation of the DNA banding patterns in the absence of a generally accepted technologically standardized approach, it was suggested that a difference in the electropherogram of more than three bands should lead to the definition of another, new bacterial clone. Subclones are identified on the basis of smaller numbers of differences. Such a rigid definition does not take into account biological properties such as different degrees of variability in different species. This type of information can be gathered, for example, by studying the results of comparative physical mapping of bacterial genomes (8) and should be included in epidemiological evaluations when available. In the present paper, we show that these criteria may vary by microorganism and that measurement of the speed of genomic evolution heavily depends on primer choice or choice of restriction enzyme, respectively, when either RAPD or PFGE is involved. In the case of *P. aeruginosa*, the existence of as many as six differences between the PFGE-generated DNA banding patterns may not rule out clonal relatedness. This is confirmed by the data obtained with RAPD primer ERIC2, even if very stringent interpretation criteria are used (only single band differences are ignored). Detailed studies of DNA typing and the standardization thereof should involve, in the case of the interpretative analysis of RAPD and PFGE, multiple restriction enzymes for PFGE and multiple primers for RAPD. These should be optimized for all of the medically important microorganisms. This would allow the following typing scheme: by screening by RAPD, clonal relatedness can be determined at a high speed and a relatively low cost. This would enable clinical microbiologists to unravel most of the nosocomial epidemics. In a second stage, PFGE could be used for confirmation of the RAPD data and for fine-tuning the sanitary or clinical measures already taken on the basis of the RAPD data. The primary criterion for the selection of the restriction enzyme to be used for PFGE pretreatment should be the presence of a sufficient number of restriction sites to allow adequate discrimination and resolution.

In conclusion, it can be stated that RAPD provides an excellent first screen for typing of *P. aeruginosa*, and this is supported by data obtained by others (11, 15, 18, 31). The interpretation of data obtained with a single primer, as described in this communication, is straightforward: when single band differences are neglected, full concordance with data obtained by PFGE may be expected. This makes interpretation of the experimental results simple, especially when automated analysis is feasible. The application of RAPD in multicentered studies, however, should be subjected to thorough research, since it has been demonstrated before that RAPD, although highly reproducible within a single laboratory, may generate different experimental outcomes when performed in different laboratories (33). Although it was recently demonstrated that ribotyping may be as discriminative as PFGE (1), in the case of large (inter)national studies, PFGE may still be the method of choice. On the other hand, a simple single-primer RAPD test, as described in the present paper, may be amenable to multicentered standardization, especially in the context of epidemiological investigations by reference labs, and requires a lower level of expenditure than PFGE.

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