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Molecular Typing by Random Amplification of Polymorphic DNA and M13 Southern Hybridization of Related Paired Isolates of *Aspergillus fumigatus*

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Three forms of DNA-based typing procedures for *Aspergillus fumigatus* isolates have been developed over the last five years. The procedures are random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) detection, and Southern hybridizations with various repetitive sequence-based probes. Using two of these procedures, we compared 16 selected isolates, grouped into eight pairs on the basis of epidemiology or previously assigned RFLP types. RAPD with four primers (R108, RC08, 2, and 4), including three previously used with *A. fumigatus*, showed that one primer, R108, gave the best discrimination (8 types). Southern hybridization of total genomic DNA digested with *Hin*dIII and probed with the total bacteriophage M13 genome resulted in the highest overall level of discrimination. Combination of the RAPD and Southern hybridization with the previously assigned RFLP types discriminated 10 isolates of 16. Isolates closely linked epidemiologically could not be distinguished from each other. In addition, three pairs of isolates previously unlinked by epidemiology had the same overall types. Two pairs were obtained from the same hospital within 2 years of each other, whereas the third pair were isolated from California and Germany. A full understanding of the epidemiology and ecology of *A. fumigatus* requires multiple discriminatory typing procedures.

Members of the genus *Aspergillus* are ubiquitous in the environment, especially near decomposing vegetation. Their numerous conidia are dispersed on air currents, and it is assumed that all terrestrial animals, including humans, are constantly exposed to and are breathing in *Aspergillus* spores. Despite this constant exposure, only a few species of *Aspergillus* are capable of causing infection, of which *Aspergillus fumigatus* is the most common. In animals and rarely in humans, *A. fumigatus* is a primary pathogen; most cases of *A. fumigatus* infection occur in hosts with already-damaged tissues or already in an immunocompromised state. Diseases caused by *A. fumigatus* include allergic, saprophytic, and invasive aspergillosis. Invasive pulmonary aspergillosis is the most common form of life-threatening infection and can lead to dissemination to other organs, such as the heart, kidney, and brain. Hospital outbreaks of aspergillosis can occur, for instance, in transplant wards and during construction work.

As well as the identification of the species, it has been necessary for epidemiological studies to differentiate between strains of *A. fumigatus*. Techniques are available for typing *A. fumigatus* on the basis of a variety of markers. Over the past five years, research has concentrated on developing genotypic typing methods. These methods have included the direct detection of restriction fragment length polymorphisms (RFLPs) on ethidium bromide-stained gels (6) and the detection of RFLPs by Southern hybridization in combination with various probes, such as the intergenic spacer region from the ribosomal RNA gene complex of *Aspergillus nidulans* (17), a moderately repetitive sequence from *A. fumigatus* (8), and a telomeric sequence from *Fusarium oxysporum* (18). The other genotypic technique investigated, which generates polymorphic bands between strains, has employed PCR in combination with single short primers. This random amplification of polymorphic DNA (RAPD) procedure has been studied in several laboratories, each using different primer sets (2, 11, 12).

The typing of *A. fumigatus* strains has permitted the investigation of nosocomial outbreaks to ascertain whether patients have acquired the same strain and to attempt to identify the source of the outbreak (4, 10, 18). Typing has enabled the identification of isolates from different organs in a single patient (4) and investigations into the nature of aspergillomas (7, 9).

All of these DNA-based techniques have proved to be sufficiently discriminatory to be useful in answering specific questions, but care has to be taken when studying unrelated isolates by only one method. This study compares three different genotypic typing techniques and demonstrates that each technique has a similar level of discrimination. The combined consensus type of the three techniques provides a high level of discrimination and permits the definition of discrete strains of *A. fumigatus.*

MATERIALS AND METHODS

Isolates. A summary of isolate origins and types is given in Table 1. Two isolates (232AF and 235AF) were obtained from N. Nolard (Institute of Hygiene and Epidemiology, Brussels, Belgium) (IHEM 5452 and 5455). Two isolates (209AF and 210AF [NCPF 7101]) were obtained from a patient at Hope Hospital (Salford, United Kingdom), four isolates (1AF, 5AF, 20AF, and 71AF [NCPF 7098]) were from Stanford University Hospital (SUH) (Stanford, Calif.) patients, one isolate (10AF [ATCC 90240]) was from a Los Angeles, California, patient and two isolates (90AF and 91AF [NCPF 7100]) were from a Davis Medical Center (Davis, Calif.) patient. One isolate (58AF) was donated by H. Seeliger (Würzburg, Germany), one isolate (70AF) was donated by T. Chase (Rutgers University, Camden, N.J.), and one isolate (82AF) was donated by G. Shankland (Glasgow University, Glasgow, United Kingdom). Two isolates

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TABLE 1. Summary of epidemiology and previously assigned DNA types for isolates of *A. fumigatus*[a]

Isolate no.	Source	Underlying disease and/or condition	Geographical location	Culture collection no.	Date of isolation (mo/day/yr)	DNA type	
						*Sal*I	*Xho*I
232	BAL	IPA	Grenoble, France	IHEM 5452	11/1990	7, 11A	12, 7B
235	Outdoor air		Grenoble, France	IHEM 5455	11/1990	7, 11A	12, 7B
209	Liver	Laparostomy	Hope Hospital, Salford, United Kingdom		5/20/1991	9, 10A	12, 7A
210[b]	Colon	Laparostomy	Hope Hospital, Salford, United Kingdom	NCPF 7101	5/21/1991	9, 10A	12, 7A
90	Percutaneous lung aspiration	AIDS pulmonary disease	Davis Medical Center		6/1989	ND	ND
91[c]	Sputum	AIDS pulmonary disease	Davis Medical Center	NCPF 7100	12/1989	ND	ND
10	Lung	IPA, steroid therapy	UCLA	ATCC 90240	1986	3, 9A	5, 13B
82	Aspergilloma		Dundee, United Kingdom		12/1988	3, 9A	5, 13B
61	Cerebrospinal fluid culture contaminant		SUH		1988	4, 9A	5, 10A
62	Laboratory contaminant		SUH		1988	4, 9B	4, 10B
5	Trachea	α_1-Antitrypsin deficiency	SUH		1988	3, 8A	5, 8B
58	Trachea	Postsurgical colon resection	Würzburg, Germany		1988	3, 8A	5, 8B
1	Bronchopleural fistula		SUH		1988	4, 9A	5, 10A
20	Trachea	Leukemia	SUH		1987	4, 9A	5, 10A
70	Air sample near sewage		Camden, N.J.		before 1983	4, 11C	5, 9A
71	Percutaneous lung aspiration	Bone marrow transplant	SUH	NCPF 7098	1989	4, 11C	5, 9A

[a] BAL, bronchoalveolar lavage; IPA, invasive pulmonary aspergillosis; IHEM, Institute of Hygiene and Epidemiology, Brussels, Belgium; NCPF, National Collection of Pathogenic Fungi, Bristol, United Kingdom; UCLA, University of California at Los Angeles, Calif.; ND, not done.
[b] Isolate taken from same patient as for 209.
[c] Isolate taken from same patient as for 90.

(61AF and 62AF) were laboratory contaminants from the Clinical Microbiology Laboratory at SUH.

DNA extraction. Conidia were inoculated into 50 ml of Vogel's minimal medium (19) and grown for 24 to 28 h at 37°C. Mycelia were harvested and freeze-dried. The dried mycelia (0.25 to 0.5 g) were ground in a mortar and transferred to two or three microcentrifuge tubes. Extraction buffer (0.6 ml) [0.7 M NaCl–0.1 M $Na_2(SO_3)$–0.1 M Tris-Cl–0.05 M EDTA–1% (wt/vol) sodium dodecyl sulfate (SDS), pH 7.5 (at 25°C)] at 65°C was added, mixed thoroughly and the tubes incubated for 20 min at 65°C. An equal volume of chloroform-isoamyl alcohol (24:1) was added, and then the mixture was subjected to vortexing and incubation on ice for 30 min. After a high-speed 30-min centrifugation, the aqueous phase was transferred to a new tube and an equal volume of isopropanol was added. After a 10-min room temperature incubation, the DNA was precipitated by centrifugation and resuspended in 200 μl of water. One-half volume of 7.5 M ammonium acetate was added, and the tubes were placed on ice for 1 h. After a high-speed centrifugation for 30 min, the supernatant was transferred to a new tube and 0.54 volume of isopropanol was added. After a 10-min room temperature incubation, the DNA was precipitated, washed with 70% ethanol, and resuspended in 100 μl of TE buffer (10 mM Tris-Cl–1 mM EDTA, pH 7.5). The integrity of the DNA was verified on an agarose minigel, and the concentration was determined by measuring the A_{260} after RNase A treatment.

RAPD. Four primers were used in the RAPD analysis: primer R108 (2), GTATTGCCCT; primers 2 and 5 (12), GCTGGTGG and GCGCACGG, respectively; and primer RC08 (5), GGATGTCGAA. Fifty-microliter reactions were set up with 100 ng of genomic DNA, 2.5 U of *Taq* DNA polymerase (Advanced Biotechnologies, Leatherhead, Surrey, United Kingdom, and Boehringer Mannheim, Lewes, East Sussex, United Kingdom), 20 mM $(NH_4)_2SO_4$, 75 mM Tris-Cl (pH 9.0 [at 25°C]), 0.01% (wt/vol) Tween, and 200 μM (each) the four deoxynucleoside triphosphates overlaid with 50 μl of paraffin. Optimal primer and $MgCl_2$ concentrations were determined by titration: 0.5 μM primer R108 and 2.5 mM $MgCl_2$; 0.5 μM primer 2 and 2.5 mM $MgCl_2$; 1.0 μM primer 5 and 2.5 mM $MgCl_2$; and 1.0 μM primer RC08 and 4.0 mM $MgCl_2$. PCRs with primers R108 and RC08 were carried out with the following temperature profile: 4 min at 94°C and then 30 cycles of 1 min at 94°C, 1 min at 36°C, 1 min at 72°C, and 5 cycles of 1 min at 94°C, 1 min at 36°C, 5 min at 72°C. PCRs with primers 2 and 5 were performed as described above except that the annealing temperature was 30°C. One-fifth volume was loaded onto 1.8% agarose gels and then run at 2.5 V/cm in 1× TPE buffer (90 mM Tris-phosphate–2 mM EDTA), stained in ethidium bromide, and photographed.

M13 Southern hybridization. Ten micrograms of genomic DNA was digested with 50 U of *Hin*dIII (Boehringer Mannheim) overnight at 37°C. The DNA was loaded onto a 0.8% agarose gel and electrophoresed in 1× TPE for 20 h at 1.3 V/cm. The gel was treated and blotted onto positively charged nylon membrane (Boehringer Mannheim) in the standard manner with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The DNA was cross-linked onto the membrane by UV irradiation. Southern hybridization was done with a digoxigenin labelling and chemiluminescence detection system (Boehringer Mannheim). Double-stranded M13 bacteriophage DNA was linearized with *Bam*HI before labelling with digoxigenin. Membranes were hybridized in Easy-Hyb buffer (Boehringer Mannheim) with 2 ng of labelled M13 per ml for 16 h at 37°C. Membranes were washed twice in 2× SSC–0.5% (wt/vol) SDS for 15 min at 55°C before being processed for chemiluminescence detection.

RESULTS

Isolates. The eight pairs of isolates were chosen to represent a range of clinical and environmental isolates. Each pair consisted of two isolates, related either because they were epidemiologically linked or because they had been assigned the same type according to our previously published system based on *Xho*I and *Sal*I RFLPs (4, 6, 7) (Table 1).

RAPD. The sixteen isolates were typed together by using each of the four primers, R108, 2, 5, and RC08, separately (Fig. 1). Each amplification was repeated at least twice to verify the presence or absence of scored bands. Those isolates (62AF, 70AF, 90AF, 91AF, 209AF, and 210AF) which gave an RAPD pattern with a unique band were verified by regrowing the isolates from frozen conidial stocks, reextracting genomic DNA, and repeating the RAPD analysis. Variable bands, including a few faint bands, were scored if they were reproducible between runs, and types were assigned for each primer (Table 2). Primer R108 gave the highest level of variation, with eight types, of which two were limited to an isolate pair and two were unique to a single isolate. Primer 2 gave four types, with two again unique to an isolate. Interestingly, both primers differentiated the same two isolates, 62AF and 70AF. Primer RC08 gave a level of discrimination similar to that of primer 2 but differentiated two pairs of isolates from the rest (209AF and 210AF and 1AF and 61AF). The final primer, primer 5, had little discriminatory power, with only one variable band present.

Comparison of the RAPD patterns generated with the isolates in this study and the published patterns shows little agree-

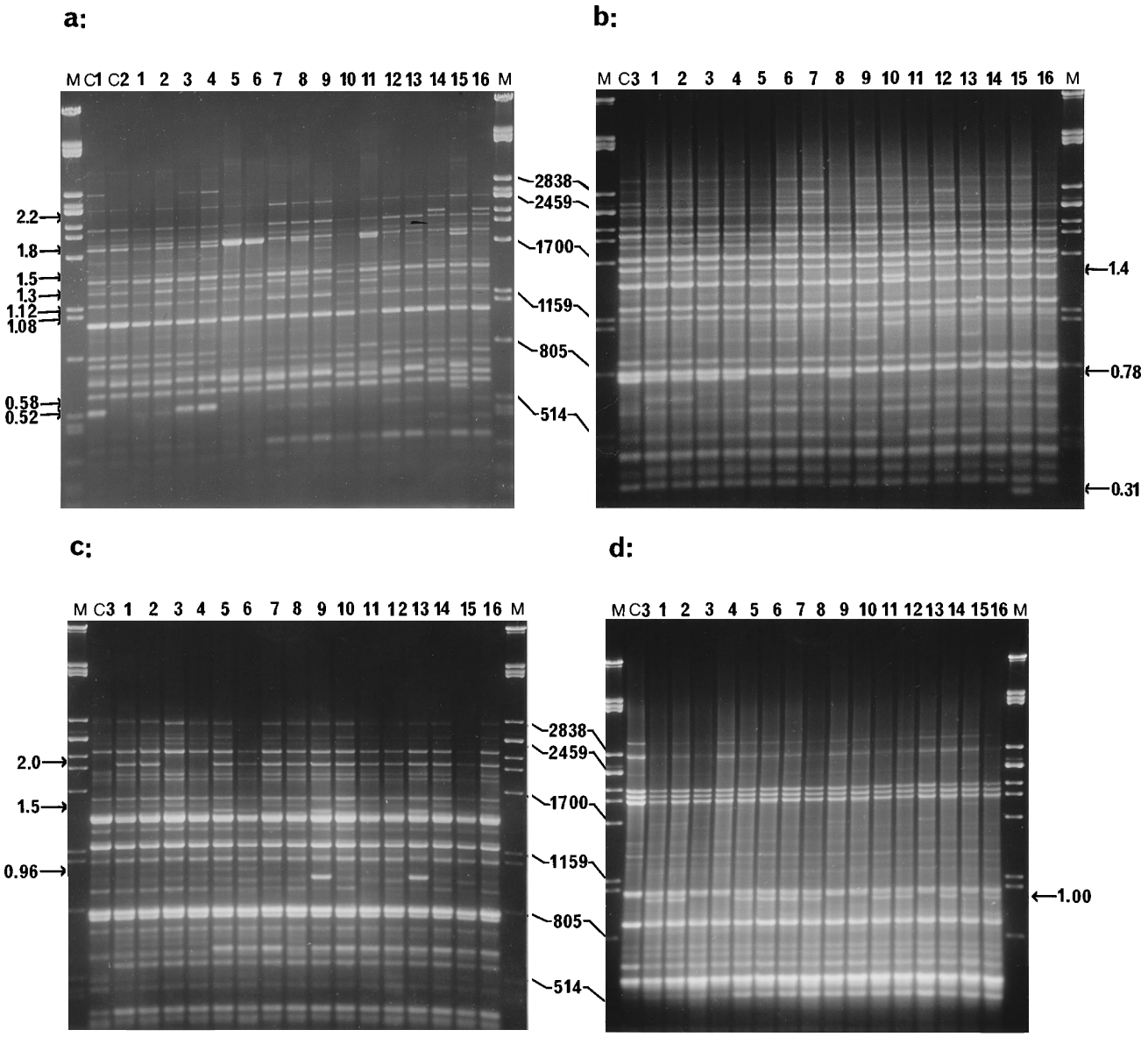

FIG. 1. RAPD typing with four primers. (a) Primer R108; (b) primer 2; (c) primer RC08; (d) primer 5. Samples were analyzed in 1.8% agarose gels run in 1× TPE at 2.5 V/cm. Markers (M) are lambda *Pst*I fragments, and selected sizes are indicated in base pairs. The control amplifications (lanes with C) used DNA extracted by another procedure (4): C1, 209AF; C2, 232AF; C3, 210AF. Negative reagent control amplifications without added DNA (not shown) resulted in either no bands, a background smear, or bands not in common with the isolate amplifications. The sizes of scored variable bands (in kilobases) are indicated by the arrows. Lane numbers for all amplifications: 1, 232AF; 2, 235AF; 3, 209AF; 4, 210AF; 5, 90AF; 6, 91AF; 7, 10AF; 8, 82AF; 9, 61AF; 10, 62AF; 11, 5AF; 12, 58AF; 13, 1AF; 14, 20AF; 15, 70AF; lane 16, 71AF.

ment. The primer R108 patterns of nine unrelated isolates in the study of Aufauvre-Brown et al. (2) have few bands in common, and it is difficult to match their bands with the patterns generated in our laboratory. The variable bands scored by Loudon et al. (12) in the primer 5 RAPD patterns are of similar size to the bands consistently present in all the isolates analyzed in our laboratory.

Southern hybridization with bacteriophage M13 DNA. Genomic DNA digested with *Hin*dIII was electrophoresed in 0.8% agarose gels, transferred onto nylon membranes, and hybridized with M13 DNA under nonstringent conditions (see Materials and Methods). A ladder of at least 32 fragments, ranging in size from 1.8 to >30 kb, was revealed, with differences in both band intensity and band position occurring between isolates (Fig. 2). Each isolate was assigned a type after scoring for the absence or presence of variable bands (Table 3). Only the absence or presence of a band was scored for cases in which it seemed that the band had shifted in size. For instance, the scored band at 2.2 kb in isolates 209AF and 210AF appears to have decreased slightly in size in comparison with the band present in the other isolates. The M13 hybridization gave a high level of discrimination with eight assigned types out of 16 isolates. An additional level of discrimination is possible with this probe if variations in band intensity are considered (for instance, those in the region of the 5.08-kb size marker). This level of resolution divides the four type A isolates (1AF, 61AF, 70AF, and 82AF) into three subtypes (i, ii, and iii), with only isolates 1AF and 61AF having an identical

TABLE 2. Analysis assignment of variable bands and types on the basis of RAPD data[a]

Isolate no.	Results for primer R108									Results for primer 2				Results for primer RC08				Scored 1.00-kb band by primer 5
	Scored band with size (kb)								Type	Scored band with size (kb)			Type	Scored band with size (kb)			Type	
	0.52	0.58	1.08	1.12	1.3	1.5	1.8	2.2		0.31	0.78	1.4		0.96	1.5	2.0		
232	0	0	0	0	1	1	0	0	A	0	1	0	B	0	0	1	A	1
235	0	0	0	0	1	1	0	0	A	0	1	0	B	0	0	1	A	1
209	1*	0	0	0	1	1	0	0	**B**	0	1	0	B	0	0	0	**C**	0
210	1*	0	0	0	1	1	0	0	**B**	0	1	0	B	0	0	0	**C**	0
90	0	0	0	0	1	1	1*	0	**C**	0	0	0	A	0	1	1	B	1
91	0	0	0	0	1	1	1*	0	**C**	0	0	0	A	0	1	1	B	1
10	0	0	0	0	0	1	0	0	D	0	0	0	A	0	0	1	A	1
82	0	0	0	0	1	1	0	0	A	0	1	0	B	0	0	1	A	1
61	0	0	0	0	0	0	0	0	E	0	0	0	A	1	1	1	D	0
62	0	0	0	1*	1	1	0	0	**G**	0	0	1*	**C**	0	0	1	A	0
5	0	0	0	0	1	1	0	0	A	0	0	0	A	0	0	1	A	1
58	0	0	0	0	0	1	0	0	D	0	0	0	A	0	0	1	A	1
1	0	0	0	0	0	0	0	0	E	0	0	0	A	1	1	1	D	0
20	0	0	0	0	0	1	0	1	F	0	0	0	A	0	0	1	A	1
70	0	1*	1*	0	0	1	0	0	**H**	1*	0	0	**D**	0	1	1	B	0
71	0	0	0	0	0	1	0	1	F	0	0	0	A	0	0	1	A	1

[a] 0, absence; 1, presence. Types limited to an isolate or isolate pair are shown in boldface. Bands present only in an isolate or isolate pair pattern are indicated with asterisks.

pattern. This subdivision concurs with the RAPD typing data, according to which isolates 70AF and 82AF differ from each other and from 1AF and 61AF. When variations in band intensity are considered, the M13 hybridization generates the highest level of discrimination (10 types) where in general a type is assigned only to single isolates or isolates already previously epidemiologically linked.

DISCUSSION

Sixteen isolates of *A. fumigatus* were typed by a combination of RAPD analysis and Southern hybridization with labelled bacteriophage M13 onto digested genomic DNA, and these results were compared with those from our previously published system based on *Xho*I and *Sal*I RFLPs (4, 6, 7).

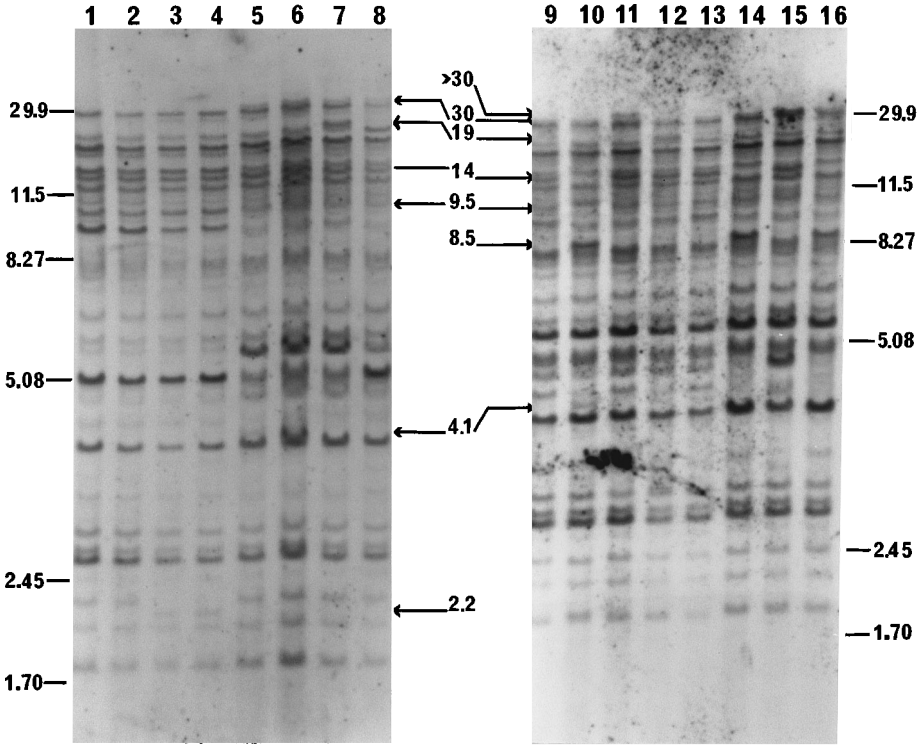

FIG. 2. Southern hybridization of *Hin*dIII-digested genomic DNA with the bacteriophage M13 genome. Markers are lambda *Pst*I fragments, and high-molecular-weight DNA markers (GibcoBRL, Paisley, United Kingdom) and selected sizes are indicated (in kilobases) on the outside edges of the autoradiographs. Sizes of the scored variable bands (in kilobases) are indicated by the arrows. Lane 1, 232AF; lane 2, 235AF; lane 3, 209AF; lane 4, 210AF; lane 5, 90AF; lane 6, 91AF; lane 7, 10AF; lane 8, 82AF; lane 9, 61AF; lane 10, 62AF; lane 11, 5AF; lane 12, 58AF; lane 13, 1AF; lane 14, 20AF; lane 15, 70AF; lane 16, 71AF.

TABLE 3. Assignment of variable bands and type by M13 hybridization to *Hin*dIII-digested genomic DNA[a]

Isolate no.	Scored band with size (kb)								Type
	2.2	4.1	8.5	9.5	14	19	30	>30	
232	0	0	0	1	1	0	0	0	**B**
235	0	0	0	1	1	0	0	0	**B**
209	1*	0	0	1	1	0	0	0	**C**
210	1*	0	0	1	1	0	0	0	**C**
90	0	1	0	1	1	0	1	0	**D**
91	0	1	0	1	1	0	1	0	**D**
10	0	0	0	1	1	1	1	0	E
82	0	0	0	1	1	0	1	0	Aii
61	0	0	0	1	1	0	1	0	Ai
62	0	1	1	0	0	0	1	0	**G**
5	0	0	0	1	1	0	1	1*	**H**
58	0	0	0	1	1	1	1	0	E
1	0	0	0	1	1	0	1	0	Ai
20	0	0	1	1	0	0	1	0	F
70	0	0	0	1	1	0	1	0	Aiii
71	0	0	1	1	0	0	1	0	F

[a] 0, absence; 1, presence. Types limited to an isolate or isolate pair are shown in boldface. Bands present only in an isolate or isolate pair pattern are indicated with asterisks. i, ii, and iii, subtypes based on variations in band intensity.

RAPD was tested as a typing system because its usefulness for *A. fumigatus* typing (2, 12) had already been demonstrated and because of its relative simplicity. The typing system for *A. fumigatus* previously used in our laboratory requires very high quality DNA of large molecular weight. Several disadvantages which require careful control and limit the utility of any comparison of results between research centers have been reported for RAPD analysis. Variations in the components of the reaction, especially magnesium chloride and primer concentrations and the supplier of the *Taq* DNA polymerase and reaction buffer, can have a significant effect on the results. In our laboratory, RAPD reactions were optimal at magnesium concentrations between 2 and 4 mM and primer concentrations between 0.5 and 1.0 μM. Variation in DNA concentration over a 20-fold range from 0.5 to 10 μg/μl had little effect on RAPD patterns (data not shown). When different commercial preparations of *Taq* DNA polymerase were used, only changes in band intensity were observed with primers R108 and 5 (data not shown). These data contrast with those from a published report in which little discrimination was possible with one commercial preparation of *Taq* DNA polymerase (13). Other factors such as buffer composition and thermal cycler performance probably also play roles in band patterns, as our data differ substantially from those from other reports in which the same primers were used (2, 12). Another problem with RAPD is reproducibility between amplifications. Some amplifications were less efficient and resulted in the gradual loss of larger fragments (Fig. 1a, lane 10, and 1c, lane 15). Such amplifications were easy to recognize and were repeated. Except in those instances of inefficient amplification, the presence or absence of scored variable bands and the presence of nonvariable bright bands were consistent.

RAPD analysis was carried out on the 16 isolates with four primers already shown to be useful in typing *A. fumigatus* and *Fusarium solani* (2, 5, 12). Primer R108 gave the highest level of discrimination (8 types), with four isolates present in the most common type, type A (Table 2). Of the type A isolates (5AF, 82AF, 232AF, and 235AF), two have been previously epidemiologically linked (232AF and 235AF) and 5AF is discriminated from the other three isolates by primer 2. On their own, primers 2, 5, and RC08 do not provide a sufficient level of discrimination to be of use in typing, but they could provide additional data to back up the differentiation of the R108 primer. The R108 primer should be useful for studying epidemiologically linked isolates, such as those obtained during a nosocomial outbreak, thus providing a relatively rapid and simple typing tool. We have demonstrated its use in distinguishing isolates from four Swedish sawmills (3).

The other DNA-based technique investigated for its potential as a typing tool was Southern hybridization with the bacteriophage M13 genome. The M13 genome has been used successfully as a hybridization probe to differentiate the strains of several fungal species of different genera (14). A basic hybridization pattern, with either variations in band intensity or changes in band size possible, was obtained. This basic pattern is represented by strains 1AF, 61AF, 70AF, and 82AF (type A), for which the only differences between the isolates are in band intensities. The other isolates vary from this pattern in either the loss and/or gain of bands. Type was assigned on the basis of the absence or presence of these variable bands (Table 3). When variation in band intensity was taken into consideration, the M13 hybridization resulted in the highest level of discrimination.

Table 4 summarizes the types assigned to each of the sixteen isolates by the four RAPD primers and the M13 hybridization. The highest level of discrimination provided by combining all the types divides the isolates into 10 types. This level of discrimination also occurs when the R108 RAPD primer and the M13 hybridization are considered together and when variations in band intensity are considered with the M13 hybridizations. The combination of the two most discriminatory RAPD primers (R108 and 2) generates nine different types, for which the only change from the R108 types is that a unique type is assigned to isolate 5AF. Only three of the original eight pairings, grouped together on the basis of epidemiology or RFLP type, remain paired when all the data are considered. The two pairings, 209AF and 210AF and 90AF and 91AF, represent two isolates from each of two different patients and therefore show that the same strain of *A. fumigatus* was isolated from two different organs in one patient and 6 months apart in the other patient. The other isolate pair that had the same overall type

TABLE 4. Summary of RAPD, M13 hybridization, and consensus types[a]

Isolate no.	RAPD type with primer				M13 hybridization type	Overall consensus type
	R108	2	RC08	5		
232	A	B	A	A	**B**	**1**
235	A	B	A	A	**B**	**1**
209	**B**	B	**C**	B	**C**	**2**
210	**B**	B	**C**	B	**C**	**2**
90	**C**	A	B	A	**D**	**3**
91	**C**	A	B	A	**D**	**3**
10	D	A	A	A	E	4
82	A	B	A	A	Aii	**7**
61	E	A	D	B	Ai	5
62	**G**	**C**	A	B	**G**	**8**
5	A	A	A	A	**H**	**9**
58	D	A	A	A	E	4
1	E	A	D	B	Ai	5
20	F	A	A	A	F	6
70	**H**	**D**	B	B	Aiii	**10**
71	F	A	A	A	F	6

[a] Types limited to an isolate or isolate pair are shown in boldface. i, ii, and iii, subtypes based on variations in band intensity.

was 232AF and 235AF. Again, these two isolates probably represent the same strain which was sampled from the hospital environment and which infected a patient at that hospital. The combined data corroborate the initial study done on the four isolates 209AF, 210AF, 232AF, and 235AF (4) and show that any of the three DNA-based typing techniques is useful in the study of hospital outbreaks or with respect to individual patients.

Three pairs of isolates, previously unlinked epidemiologically, have the same overall consensus type, and these isolates are identical when their overall R108 and M13 band patterns are compared. Of these, the pair 1AF and 61AF had been assigned the same *Sal*I and *Xho*I RFLPs. For the other two pairs, the original gels were checked to verify their RFLP patterns and it was concluded that the pair 10AF and 58AF and the pair 20AF and 71AF do in fact have the same patterns. The analysis of these six isolates by three different DNA-based typing systems was unable to differentiate each isolate from its pair mate. We propose, therefore, that each pair consists of representatives of a strain of *A. fumigatus*. These three genotypic typing systems investigate many regions of the genome at once, and as different generations of a strain have been shown to have stable genomes (8, 18) then representatives of a strain will maintain the same overall genomic structure. There potentially exists a RAPD primer or another typing system (such as multilocus-enzyme electrophoresis) which could differentiate between the isolates in these three pairs, but the appearance or disappearance of a single RAPD band or the change in mobility of a single enzyme could reflect the occurrence of a mutation within that strain or clone. Two of the strains were from the same hospital: in one case they were isolated in the same year (1AF and 61AF), and in the other case they were isolated within 2 years of each other (20AF and 71AF). In contrast to the localized distribution of these two strains, the isolates of the final strain were from California (10AF) and Germany (58AF). In addition, two of these isolates, 1AF and 61AF, are the only ones studied by our group so far that have a specific type of double-stranded RNA "plasmid" (1). One other isolate, 62AF, also has a similar type of plasmid of a different size. If the situation is similar to that in the plant-pathogenic fungus *Cryphonectria parasitica*, these plasmids could be double-stranded RNA viruses which, as they lack an extracellular stage, are only transmissible through the mycelium (15). Since, unlike *C. parasitica*, *A. fumigatus* is not thought to form heterokaryons outside the laboratory, these plasmids will remain within a single clone.

Previous research by this and other groups has established the benefits of using DNA-based typing techniques for the study of the epidemiology of *A. fumigatus*. These techniques have included RAPD and RFLPs detected directly by ethidium bromide staining or by Southern hybridization with various probes. Any of these techniques should provide sufficient discrimination for the epidemiological study of nosocomial outbreaks or of isolates from individual patients. The RAPD technique has the advantage of being relatively straightforward and quick but has a major disadvantage in not being transferable between research centers. The use of primer R108 in combination with either primer RC08 or 2 should provide sufficient discrimination. The use of the R108 RAPD primer on its own has proved sufficient for discrimination in three hospital outbreaks studied (reference 16 and 18 and our unpublished data). The detection of RFLPs by Southern hybridization has been shown to provide the highest level of discrimination. A probe based on a moderately repetitive sequence from *A. fumigatus* has been used in the study of nosocomial invasive aspergillosis and to study isolates from aspergilloma patients (9, 10). This probe gave distinct and unique patterns for 70 unrelated strains. The telomeric sequence from *F. oxysporum* has also been used as a probe in the study of an invasive aspergillosis outbreak in a renal transplantation unit and was shown to provide sufficient discrimination to separate all the environmental isolates (18). In this study we have shown that the M13 bacteriophage genome can be used as a probe in Southern hybridizations and that this RFLP analysis provided the highest level of isolate discrimination. For use in multicenter studies, the M13 probe has an advantage over the other two probes since it is readily available from commercial suppliers. This study has shown that the use of the M13 probe in combination with primer R108 RAPD gave the same level of discrimination as the consensus of three DNA-based typing techniques, and therefore these two typing techniques should be useful for the general study of *A. fumigatus* isolates and the definition of strains.

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