Application of Polymerase Chain Reaction to Fingerprinting *Aspergillus fumigatus* by Random Amplification of Polymorphic DNA

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A new method for fingerprinting *Aspergillus fumigatus* by random amplification of polymorphic DNA (RAPD) by using single primers with arbitrary sequences is described. Five primers were examined with 19 isolates from six patients with aspergilloma as well as with *A. fumigatus* NCPF 2109. Two of the primers (GCT GGT GG and GCG CAC GG, 5' to 3') gave adequate discrimination between isolates, generating five and six types, respectively. Combination of the results obtained with each of these two primers generated 12 types. This compares very favorably with immunoblot fingerprinting and *Xba*I-generated restriction fragment length polymorphisms on the same isolates. Typeability and reproducibility were good with RAPD, and RAPD was less labor-intensive than immunoblot fingerprinting. RAPD typing results suggested that aspergillomas sometimes contain isolates of more than one type.

Molds of the genus *Aspergillus* mainly inhabit the natural environment, but some species, most commonly *Aspergillus fumigatus*, are opportunistic pathogens, causing diseases such as invasive aspergillosis, aspergilloma, and allergic bronchopulmonary aspergillosis in humans (22). Study of the epidemiology of *A. fumigatus* has been hampered by the lack of simple, reproducible typing methods. Conventional bacteriological techniques such as phage typing, antibiograms, and plasmid profiles are clearly not applicable.

Phenotypic variability within isolates of *A. fumigatus* has been demonstrated in terms of gross morphology (11), susceptibility to applied *Hansenula* killer toxins (7), isozyme electrophoresis with respect to esterase and phosphatase mobility (10), and immunoblot fingerprinting (3). None of these techniques has been developed as a formal typing system because either the degree of discrimination is low or standardization is difficult. The ubiquinone systems and electrophoretic comparison of enzymes have been used as aids in the precise identification of members of the genus *Aspergillus* to the species level rather than as a typing system (12). Immunoblot fingerprinting has the advantages that all isolates are typeable and reproducibility is acceptable for the 16 antigenic bands on which the system is dependent. The major disadvantages are that the method depends on gene expression, it is laborious, and it is not easy to standardize the procedure between different laboratories.

Genetic fingerprinting of *Aspergillus* species by restriction endonuclease analysis of genomic DNA is less discriminatory than immunoblot fingerprinting (2). When the DNA was digested with *Eco*RI, all isolates examined from eight patients with aspergilloma were indistinguishable. The enzyme *Xba*I subdivided the 21 isolates into six types, but four of the types were represented by a single isolate. In that study (2), differences were determined by variations in intense bands at 1.8, 2.0, 3.3, 3.8, 4 to 4.2, and 16 kb. In contrast, Denning et al. (5, 6), digesting *A. fumigatus* genomic DNA with *Sal*I

and *Xho*I, classified isolates according to the number of bands above a heavy band at 23 kb (*Sal*I) or between 21 and 23 kb (*Xho*I) and the number of bands down to a constant region at 12 kb.

The polymerase chain reaction (PCR) has been adapted for fingerprinting microorganisms by using paired primers derived from previously characterized sequences for PCR amplification. This technique has been applied to bacteria, (9, 14, 19), parasites (16), viruses (1, 15, 17), and fungi (18). More recently, a new DNA polymorphism assay has been developed. The assay is based on PCR amplification of random DNA fragments with single short primers made up of arbitrary nucleotide sequences (20, 21). No prior sequence information is required. These polymorphisms have been termed random amplified polymorphic DNA markers (21). The method has been applied to two fungal plant pathogens, *Fusarium solani* and *Leptosphaeria maculans* (4, 8).

Here we describe the application of the random amplification of polymorphic DNA (RAPD) assay to the human pathogen *A. fumigatus*. Genomic DNAs from 19 isolates from six patients with aspergilloma were amplified with five different oligonucleotide primers under conditions which favor the production of multiple products. The results of this method for typing *Aspergillus* species was compared with those obtained by silver staining of protein profiles after gel electrophoresis, immunoblot fingerprinting, and *Xba*I-generated restriction fragment length polymorphism (RFLP) analysis.

MATERIALS AND METHODS

Isolates. Nineteen isolates from sputa were examined from six patients with aspergilloma (patients 1 to 6 in Table 1). They were identified as *A. fumigatus* by their cultural characteristics and microscopic appearances. *A. fumigatus* NCPF 2109 was the control isolate.

Preparation of DNA. DNA was prepared as described previously (2). Briefly, each isolate was subcultured onto

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TABLE 1. Results of RAPD by using primers 2 and 5 in
comparison with those of three other typing systems
for *A. fumigatus*

Isolate no.	Patient no.[a]	Silver stain type	Immunoblot type	RFLP type	PCR type primer 2	PCR type primer 5
NCPF 2109		A	1	4	1	1
1	1	B	3	3	1	2
2	1	B	3	3	3	3
3	2	A	4	3	2	4
4	2	A	4	3	3	2
5	2	A	4	3	1	2
6	3	C	5	6	4	2
7	3	D	6	6	3	2
8	3	D	6	6	3	2
9	4	C	7	6	3	2
10	4	C	7	6	3	2
11	4	D	6	6	3	2
12	5	E	8	3	1	2
13	5	E	8	3	1	2
14	6	A	9	3	5	5
15	6	A	9	3	5	6
16	6	A	10	2	5	4
17	6	F	11	5	2	1
18	6	A	9	3	3	5
19	6	A	9	3	3	4

[a] Patients 1 to 6 correspond to patients 3 to 8 in previously published reports (2, 3) because of the death of two isolates from patients 1 and 2.

TABLE 2. PCR fingerprinting of *A. fumigatus* with primer 5

Band (kb)	Result for type no.[a]:					
	1 (n = 2)	2 (n = 11)	3 (n = 1)	4 (n = 3)	5 (n = 2)	6 (n = 1)
2.036	+	+	−	−	+	+
1.018	+	+	−	−	−	−
0.890	+	+	+	−	+	−
0.690	−	±	−	−	−	−
0.642	+	±	−	−	−	−
0.603	+	+	+	+	+	+
0.565	−	±	−	−	−	−
0.457	+	±	−	−	−	−
0.384	−	+	−	−	−	+

[a] +, band present; −, band absent; ±, variable band. n indicates number of isolates.

Sabouraud's dextrose agar, grown at 30°C for 48 h, and then inoculated into Sabouraud's dextrose broth and grown for a period of 3 to 5 days at 30°C in a rotating orbital shaker. The mycelium was harvested by filtration and was freeze-dried overnight at 4°C prior to adding liquid nitrogen to fragment the *Aspergillus* isolates. Ten milliliters of extraction buffer (made up of 20 ml of 1 M Tris [pH 8], 25 ml of 1 M sodium chloride, 5 ml of 0.5 EDTA [pH 8.5], 2.2 ml of 20% sodium dodecyl sulfate, 47.5 ml of sterile distilled water) was added per g of *Aspergillus* isolates, mixed, and stored on ice for 30 to 60 min. This mixture was extracted twice with phenol-chloroform and was then treated with RNase as described previously (2). After a further phenol-chloroform extraction and ethanol precipitation overnight, the dried DNA precipitate was resuspended in 3 ml of TE buffer (10 mM Tris hydrochloride buffer [pH 7.5], 1 mM EDTA [pH 7.5]) and was stored at −20°C until required.

Oligonucleotide synthesis. Oligonucleotide primers were synthesized by standard phosphoramidite chemistry on a PCR-MATE (Applied Biosystems) DNA synthesizer. Primer sequences (5′ to 3′) were as follows: primer 1, AAT GCA GC; primer 2, GCT GGT GG; primer 3, CGC GGC CA; primer 4, CAG GAC GG; primer 5, GCG CAC GG.

DNA amplification. DNA amplification was carried out in 50 mM KCl–1.5 mM MgCl₂–10 mM Tris HCl (pH 8.8) containing 200 μM (each) dATP, dCTP, dGTP, and dTTP (Promega), 1 μg of primer, 2.5 U of *Taq* DNA polymerase (Northumbria Biologicals Ltd.), and 5 μl of template DNA in a final volume of 100 μl. Amplification was performed in a Perkin-Elmer Cetus DNA thermal cycler. All manipulations were carried out with dedicated DNA-free pipettes in a sterile field to minimize the risk of contamination.

All reagents were added together except for the *Taq* polymerase. The reaction mixture was overlaid with 50 μl of sterile mineral oil and was incubated in the DNA thermal cycler at 95°C for 10 min. Then, *Taq* polymerase was added and amplification was carried out over 45 cycles, as follows:

96°C for 1 min, 30°C for 2 min, 72°C for 2 min, and finally, an additional 72°C for 2.5 min. The reaction mixture was held at 4°C until required. Blank control tubes containing all reagents except the template DNA or primer were also run.

Gel electrophoresis. The amplified products were electrophoresed at 80 V in a horizontal 300-ml, 1.3% agarose gel in Tris-borate buffer containing 0.5 μg of ethidium bromide per ml, and the products were photographed under UV light. *Hae*III-digested φX174 DNA was used as a molecular mass marker, of which fragments at 1.353, 1.078, 0.872, 0.603, and 0.31 kb were used to determine the sizes of the PCR products.

RESULTS AND DISCUSSION

Primers 2 and 5 successfully typed all the isolates examined. Primer 5 generated six types (Table 2; Fig. 1), all with a conserved band at 0.603 kb. Type 2 was the most common, accounting for 11 of the 19 isolates examined; this was followed by type 4 (3 isolates). Types 3 and 6 were each represented by only one isolate. Among the type 2 isolates, some had bands at 0.690, 0.642, 0.565, and 0.457 kb (for example, lane 3 in Fig. 1), whereas with the other types, the bands were weak (for example, lane 7 in Fig. 1) or absent (for example, lane 14 in Fig. 1). Therefore, to avoid difficulties in interpretation, these were considered as one type. Primer 2 generated five types (Table 3; Fig. 2 to 4), with a conserved band at 1.353 kb. Type 3 was the most common (9 of 20 isolates), type 1 was the next most common (5 isolates), and the remaining types were represented by up to three isolates each. Types 1 and 4 were characterized by a double band at 0.975 kb.

The results of RAPD with primers 2 and 5 were compared with those obtained previously (2, 3) with the same isolates by silver staining after gel electrophoresis, immunoblot fingerprinting, and RFLP (Table 1). The isolates were typed by all of the methods, and the reproducibilities were comparable among the methods. Reproducibility between the RAPD assays was good. This is illustrated in Fig. 3 and 4; different batches of DNA were prepared from isolates 14, 16, 18, and 19, and the DNAs were amplified by PCR on two different occasions. RAPD had the advantages of being the least labor-intensive and relatively easy to standardize between laboratories.

Two of the 21 isolates described previously (2, 3) were lost on subculture. Silver staining yielded 6 types (types A to F), immunoblot fingerprinting with rabbit antiserum to *A. fumigatus* NCPF 2109 yielded 10 types, and RFLP generated by
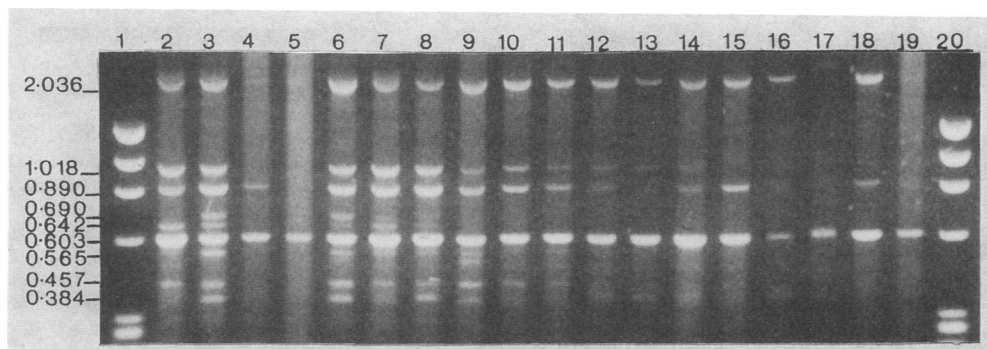
FIG. 1. RAPD generated with primer 5 showing type 1 (*A. fumigatus* NCPF 2109, lane 2), type 2 (isolates 1 and 4 to 12, lanes 3 and 6 to 14, respectively), type 3 (isolate 2, lane 4), type 4 (isolates 3, 16, and 19, lanes 5, 17, and 19, respectively), type 5 (isolates 14 and 18, lanes 15 and 18, respectively), and type 6 (isolate 15, lane 16). *Hae*III-digested ϕX174 DNA markers were run in lanes 1 and 20. The sizes of the PCR products (in kilobases) are given on the left.

*Xba*I yielded 5 types (Table 1). Discrimination was greatest by immunoblot fingerprinting, with the most common type containing four isolates. All five typing systems failed to distinguish between isolates 7, 8, and 11. Isolates 9 and 10 (from patient 4) were also identical, as were both isolates (isolates 12 and 13) from patient 5. The degree of discrimination by RAPD increased to 12 separate types when the results for the two primers were considered together.

An added advantage of RAPD over RFLP is the small amount of DNA required. When applied to monitoring changes in the hyperviable L-S junction of the cytomegalovirus genome, PCR required only 0.1 µg of viral DNA, whereas 6 µg of viral DNA is required for Southern blotting (15). Typing by PCR can be performed with a pair of primers derived from a known sequence. Variation then depends on the size of the PCR product produced, the presence or absence of a particular restriction site, or differences in the sequence itself. In some cases, as with *Chlamydia* species, changes can be detected in the DNA coding for structures, such as outer membrane proteins, which are known to be phenotypically variable (9, 14). This method of typing by PCR looks at changes within only a small part of the genome and may sometimes be the result of genetic drift within an isolate rather than the presence of two distinct strains. This then raises the problem of how much genetic difference between two subclones from the same isolate is needed before these, in effect, become separate strains. It is also dependent on the sequence being sufficiently conserved for the PCR to work and sufficiently variable to produce differ-

ent types. If the gene is vital to the survival of the microorganism, it will tend to be conserved, whereas if it is relatively unimportant, it may be deleted and so not all isolates will be typeable.

RAPD has the advantage of requiring little knowledge of the molecular biology of the species being studied, and no sequence information is necessary for RAPD (20, 21). Each primer gives a different pattern of PCR products, each with the potential of detecting polymorphisms between strains. RAPD may, in some instances, detect single-base changes in genomic DNA (21). Other sources of polymorphisms include deletions of a priming site and insertions that either increase the size of the DNA segment, giving a larger PCR product, or render the priming sites too distant to support amplification. RAPD can be used not only to distinguish strains within a species but also to build a genetic map of the degree of relatedness (4, 8, 20).

Primers 1, 3, and 4 produced disappointing results and therefore were not tested with every isolate (data not shown). Primer 1 produced a single bright band at 0.396 kb with isolates 6, 12, and 18 and no bands with isolates 15 and 16. Primer 4 produced no bands with any of these same isolates. Primer 3 produced numerous bands with *A. fumigatus* NCPF 2109 and isolates 1 to 5, 10, 12, 16, and 18 but failed to produce reproducible differences between isolates. Since the pattern produced with primer 3 was unique to *A. fumigatus* when compared with the pattern produced with DNAs from *Candida albicans*, *Staphylococcus aureus*, and *Cryptococcus neoformans*, it might form the basis of a

TABLE 3. PCR fingerprinting of *A. fumigatus* with primer 2

Band (kb)	Result for type no[a]:				
	1 (n = 5)	2 (n = 2)	3 (n = 9)	4 (n = 1)	5[b] (n = 3)
1.353	+	+	+	+	+
1.078	+	−	+	+	+
0.975	DB	−	+	DB	+
0.679	±	−	±	+	±
0.631	−	−	−	+	−
0.535	±	−	±	+	−
0.485	±	−	±	+	−
0.360	+	−	+	+	−

[a] +, band present; −, band absent; DB, double band; ±, variable band. *n* indicates number of isolates.
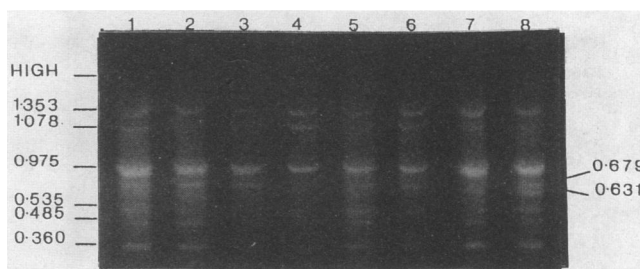[b] Variable extra band of high molecular mass.

FIG. 2. RAPD generated with primer 2 showing type 1 (*A. fumigatus* NCPF 2109 and isolates 1, 5, and 13, lanes 1 to 3 and 7, respectively), type 3 (isolates 7, 9, and 11, lanes 4 to 6, respectively), and type 4 (isolate 6, lane 8). The sizes (in kilobases) are given on the left.
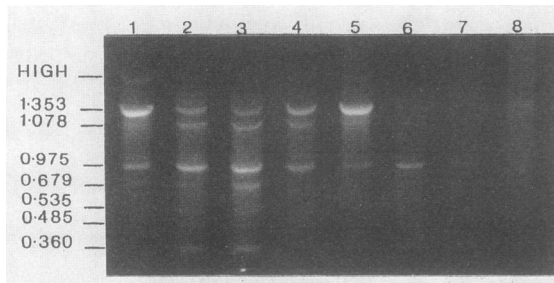
FIG. 3. RAPD generated with primer 2 showing type 2 (isolate 3, lane 8), type 3 (isolates 18, 19, 4, and 2, lanes 2, 3, 6, and 7, respectively), and type 5 (isolates 14, 16, and 15, lanes 1, 4, and 5, respectively). Sizes (in kilobases) are given on the left.

diagnostic test or species identification assay (unpublished data). Riley et al. (13) showed that, with primers from a variable DNA repeat from *Trichomonas vaginalis*, species-specific patterns were created with DNAs from *Giardia lamblia*, *Leishmania donovani*, three species of *Trypanosoma*, two species of *Acanthamoeba*, and *Saccharomyces cerevisiae*.

In some cases, isolates from the same patient (isolates 7 and 8 from patient 3, isolates 9 and 10 from patient 4, and isolates 12 and 13 from patient 5) were indistinguishable from each other by all five typing systems. Isolates 7 and 8 were also indistinguishable from isolate 11. In other cases, isolates from the same aspergilloma were phenotypically identical but genotypically distinct by RAPD (patients 1 and 2), phenotypically distinct but genotypically indistinguishable (patient 4), or both phenotypically and genotypically distinct (patients 3 and 6). Two of the three isolates from patient 3 were indistinguishable phenotypically and genotypically, but the third isolate was different. One of the three isolates from patient 4 was phenotypically different, but all three isolates produced the same genotype. This raises the question of whether the initial infection involved exposure to multiple strains or sequential exposure that led to superinfection with a second strain, or whether phenotypic and genotypic variation is induced in vivo in response to therapy or the immune response. This and other questions concerning the pathogenesis and epidemiology of aspergillosis can now be investigated by RAPD.
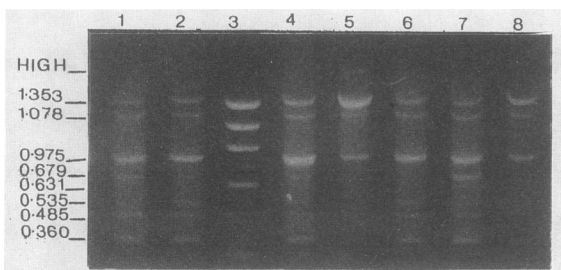

FIG. 4. RAPD generated with primer 2 showing type 1 (isolate 12, lane 4), type 3 (isolates 8, 10, 18, and 19, lanes 1, 2, 6, and 7, respectively), and type 5 (isolates 14 and 16, lanes 5 and 8, respectively). *Hae*III-digested ϕX174 DNA markers were run in lane 3. The sizes of the PCR products (in kilobases) are given on the left.

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