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Use of Randomly Amplified Polymorphic DNA Markers To Distinguish Isolates of *Aspergillus fumigatus*

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Forty-four oligonucleotide decamers were tested for their abilities to generate randomly amplified polymorphic DNA (RAPD) markers from genomic DNAs of three different isolates of *Aspergillus fumigatus*. Seven primers generated RAPDs that allowed the three isolates to be differentiated; one of the primers also yielded a unique RAPD pattern in each of an additional six fungal isolates, demonstrating the utility of this technique for distinguishing between *A. fumigatus* isolates.

Aspergillus fumigatus is a filamentous fungus which causes several pulmonary conditions, including allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis, a life-threatening disease of immunosuppressed patients (5). These conditions are usually acquired by the inhalation of fungal conidiospores, which exist in the air at concentrations of 2 to 15/m^3 (2) and are small enough (2 to 3 μm in diameter) to reach the alveolar spaces of the lungs.

Detailed epidemiological studies of *A. fumigatus* are hampered by difficulties in characterizing isolates. The ability to distinguish isolates of *A. fumigatus* would be useful in investigating the source of outbreaks of infection, the relatedness of isolates recovered from different patients, and the identities of multiple isolates from the same patient. The prevalence of *A. fumigatus* spores in the air and the similar colonial morphologies of many isolates can cause problems in laboratories where the fungus is being studied, as contamination of a culture by an environmental isolate may be impossible to detect. For these reasons, it would be useful to have a quick and easy means of distinguishing between fungal isolates that are morphologically identical.

Some isolates of *A. fumigatus* have been distinguished from each other on the bases of immunoblot fingerprinting (4), protein electrophoresis (9), and, more recently, restriction fragment length polymorphisms (3, 7, 11). In two of the restriction fragment length polymorphism based methods, high-molecular-weight DNA was prepared, and polymorphisms were detected among repeated DNA sequences by ethidium bromide staining of restriction endonuclease-cut DNA after fractionation through agarose gels (3, 7). In the other approach, Southern blots of DNA digests were probed with a region of the rRNA gene complex of *A. fumigatus* (11). Since all of these methods are relatively labor intensive and time-consuming and none of them distinguished between all of the isolates tested, we have investigated whether randomly amplified polymorphic DNA (RAPD) markers could be used to distinguish isolates from each other. This method, which relies on primers of arbitrary sequence to amplify segments of genomic DNA in a polymerase chain reaction (13, 14), has already been used successfully to differentiate between mating populations within *Fusarium solani* f. sp. *curcubitae* (6) and *Leptosphaeria maculans* (8). In both cases, polymorphisms could also be detected among isolates of the same population, suggesting that this approach may have a more general application in isolate typing of fungi.

A. fumigatus isolates 138, 201, 203, 234, and 324 were each cultured from bronchial washings from different patients with suspected or proven invasive aspergillosis over a 3-year period at Hammersmith Hospital. Isolate 237, obtained from M. Keaney, Hope Hospital, Manchester, United Kingdom, was from a patient with invasive aspergillosis. Isolate 268, obtained from J. Bille, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, was from an aortic-valve vegetation in a patient with disseminated aspergillosis. Isolates 170 and 254 are environmental isolates recovered from the Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School. Fungal isolates were classified as *A. fumigatus* on the basis of standard morphological features. Each isolate was purified by restreaking single colonies twice before being stored as a conidial suspension in water. DNA was extracted by a rapid small-scale method that allows several samples to be processed simultaneously (12). Briefly, 1.5 ml of 2% glucose–0.3% yeast extract–1% peptone in a 5-cm petri dish was inoculated with conidia and incubated at 37°C overnight. The mycelial mat was partially dried and then transferred to a 50-ml polypropylene screw-cap tube containing six glass beads (4-mm diameter). The tube was immersed in liquid nitrogen for 10 s and vortexed vigorously for 30 s. Then, 0.8 ml of 200 mM Tris-Cl (pH 8.0)–0.5 M NaCl–10 mM EDTA-1% sodium dodecyl sulfate was added, and the mixture was allowed to thaw. Phenol-chloroform (0.8 ml) was added with gentle shaking, and the emulsion was centrifuged at 15,000 × *g* for 15 min. The aqueous phase was extracted again with phenol-chloroform and with chloroform. DNA was collected by precipitation with ethanol and suspended at a concentration of 25 ng/μl in water containing 50 μg of RNase A ml^{-1}. DNA concentrations were determined by comparing the intensities of ethidium bromide-stained samples with those of known amounts of phage lambda DNA on agarose gels. Primers used for DNA amplification were a gift from J. Carlson and J. Kronstad, University of British Columbia Biotechnology Centre. Primers were 10 nucleotides in length, had a G+C content ranging from 50 to 90%, and did not contain any palindromic sequences. Amplification reactions were performed in 50-μl volumes containing 50 mM KCl; 10 mM Tris-Cl (pH 8.0); 1.5 mM $MgCl_2$; 100 μM

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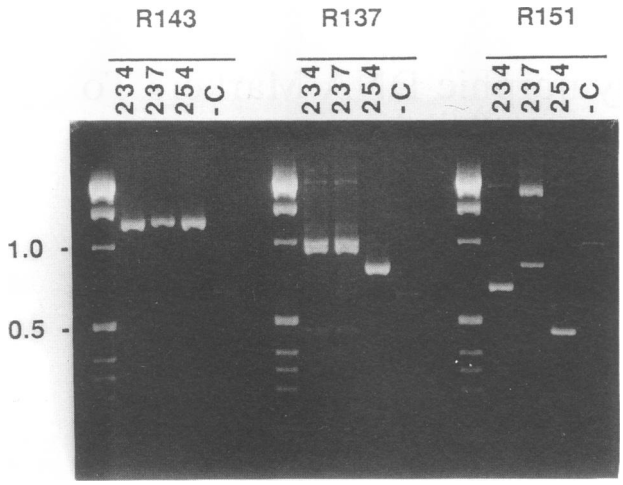

FIG. 1. DNA fragments amplified from genomic DNA samples from *A. fumigatus* isolates 234, 237, and 254 by using the decamer primers R143, R137, and R151. C indicates control amplifications in which target DNA was omitted from the reactions. Numbers on the left indicate sizes (in kilobases) of the components of a 1-kb DNA ladder (Gibco-BRL).

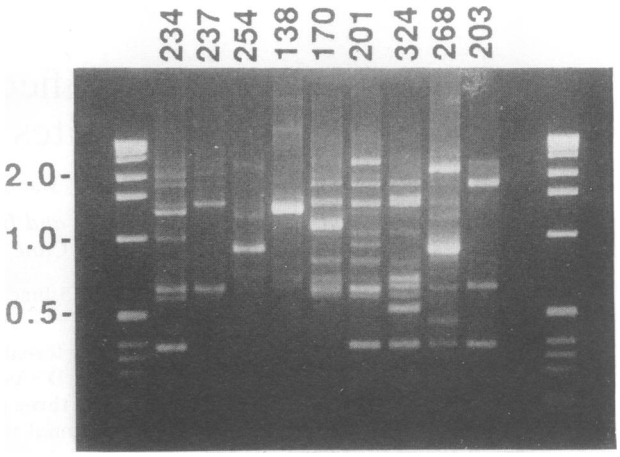

FIG. 2. DNA fragments amplified from genomic DNA samples from a series of *A. fumigatus* isolates by using primer R108. Numbers on the left indicate sizes (in kilobases) of the components of a 1-kb DNA ladder (Gibco-BRL).

each dATP, dCTP, dTTP, and dGTP (Pharmacia); 0.2 μM primer; 25 ng of genomic DNA; and 2.5 U of *Taq* DNA polymerase (Perkin Elmer Cetus). Amplifications were carried out in a thermal cycler (Hybaid) for 1 cycle of 5 min at 94°C to denature followed by 45 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C, with the fastest possible transitions between each temperature. Amplification products (10 μl) were fractionated by electrophoresis through 1.8% agarose gels and detected by staining with ethidium bromide.

Forty-four oligonucleotide decamers were tested for their abilities to generate RAPD markers from genomic DNAs of *A. fumigatus* isolates 234, 237, and 254. Twenty-one primers gave either multiple bands that were difficult to interpret or patterns that were not reproducible. One primer (R143; 5′-TCGCAGAACG) gave rise to a single band of the same size with DNAs of all three isolates (Fig. 1). A polymorphic DNA band was scored as such only if it was observed in repeat amplifications involving different DNA preparations of the same isolate and if its presence or absence was not affected when the amount of target DNA was increased to 50 ng per reaction. Twenty-two primers yielded clear and reproducible RAPDs, with sizes ranging from 300 bp to 3 kb, from genomic DNAs of the three isolates. Of these primers, 15 gave DNA fragment patterns that differentiated isolate 254 from isolates 234 and 237. The sequences of these primers were TGTACGTGAC (R109), AGTAGACGGG (R111), TGACCGAGAC (R114), TTCCGCGGGC (R115), TACGATGACG (R116), ATTGGGCGAT (R119), ATCTG GCAGC (R127), GAAACAGCGT (R131), GGTCTCTCCC (R137), GCTTCCCCTT (R138), TGTCGGTTGC (R145), GTGCGTCCTC (R147), TGTCCACCAG (R148), AGCAG CGTGG (R149), and GAAGGCTCTG (R150). Seven primers (R106) [CGTCTGCCCG], R108 [GTATTGCCCT], R120 [GAATTTCCCC], R128 [GCATATTCCG], R141 [ATCCT GTTCG], R142 [ATCTGTTCGG], and R151 [GCTGTAGT GT] differentiated all three isolates from each other. Examples of results with primers R137 and R151 are shown in Fig. 1. One primer (R108) from the group of seven differentiating all three isolates was then tested against six additional clinical and environmental isolates. R108 generated sufficient RAPDs to allow all of the isolates to be distinguished from each other (Fig. 2).

Little is known of the extent of genetic variation within *A. fumigatus*. This species appears to lack a sexual phase, and although the parasexual cycle has been demonstrated in the laboratory (1), it is not known whether any significant genetic exchange occurs between isolates in nature or whether heterokaryon incompatibility groups exist. It is assumed that *A. fumigatus* is propagated almost exclusively by the asexual (clonal) route via the production of conidiospores. Therefore, it is possible that there are genetically isolated subgroups of *A. fumigatus* which differ in their abilities to cause disease in humans.

In previous studies, isolates of *A. fumigatus* were typed according to the RFLP patterns obtained with one or two restriction enzymes (3, 7, 11). However, since only a small proportion of the genome of each isolate was analyzed in these studies, it is conceivable that isolates grouped together as a single "type" on the basis of identical patterns might have given different patterns if other areas of the genomes had been compared. In this study, of 22 primers generating RAPDs, 15 did not distinguish between isolates 234 and 237. If these primers alone had been considered, isolates 234 and 237 could have been classified as the same type. However, primer R108 clearly shows that they are not identical.

In addition to providing a means of distinguishing isolates from each other, RAPD analysis also indicates that isolates 234 and 237 are more closely related than either is to isolate 254. The data generated by this type of analysis can be used to determine the degree of genetic relatedness between different isolates on the basis of similarity coefficients (8, 10). Analysis of a larger number of RAPDs from a range of clinical and environmental isolates of *A. fumigatus* can be expected to provide useful information about the genetic relationships that exist within this clinically important species. This may help establish whether isolates from any one genetic subgroup are more frequently associated with the various types of pulmonary aspergillosis.

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REFERENCES

1. **Berg, C. M., and E. D. Garber.** 1962. A genetic analysis of color mutants of *Aspergillus fumigatus*. Genetics **47**:1139–1146.
2. **Bodey, G. P., and S. Vartivarian.** 1989. Aspergillosis. Eur. J. Clin. Microbiol. Infect. Dis. **8**:413–437.
3. **Burnie, J. P., A. Coke, and R. C. Matthews.** 1992. Restriction endonuclease analysis of *Aspergillus fumigatus* DNA. J. Clin. Pathol. **45**:324–327.
4. **Burnie, J. P., R. C. Matthews, I. Clark, and L. F. R. Milne.** 1989. Immunoblot fingerprinting *Aspergillus fumigatus*. J. Immunol. Methods **118**:179–186.
5. **Cohen, J.** 1991. Clinical manifestations and management of aspergillosis in the compromised patient, p. 117–152. *In* D. W. Warnock and M. D. Richardson (ed.), Fungal infection in the compromised patient, 2nd ed. John Wiley, Chichester, United Kingdom.
6. **Crowhurst, R. N., B. T. Hawthorne, E. H. A. Rikkerink, and M. D. Templeton.** 1991. Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Curr. Genet. **20**:391–396.
7. **Denning, D. W., K. V. Clemons, L. H. Hanson, and D. A. Stevens.** 1990. Restriction endonuclease analysis of total cellular DNA of *Aspergillus fumigatus* isolates of geographically and epidemiologically diverse origin. J. Infect. Dis. **162**:1151–1158.
8. **Goodwin, P. H., and S. L. Annis.** 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl. Environ. Microbiol. **57**:1482–1486.
9. **Kulik, M. M., and A. G. Brooks.** 1970. Electrophoretic studies of soluble proteins from *Aspergillus* spp. Mycologia **62**:365–376.
10. **Nei, M., and W.-H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**:5269–5273.
11. **Spreadbury, C. L., B. W. Bainbridge, and J. Cohen.** 1990. Restriction fragment length polymorphisms in isolates of *Aspergillus fumigatus* probed with part of the intergenic spacer region from the ribosomal RNA gene complex of *Aspergillus nidulans*. J. Gen. Microbiol. **136**:1991–1994.
12. **Tang, C. M., J. Cohen, and D. W. Holden.** 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol. **6**:1663–1671.
13. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18**:7213–7218.
14. **Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18**:6531–6535.