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Comparison of Three Typing Methods for Clinical and Environmental Isolates of *Aspergillus fumigatus*

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To evaluate procedures used for epidemiologic analysis of outbreaks of aspergillosis, we analyzed a collection of 35 *Aspergillus fumigatus* isolates using three typing methods: isoenzyme analysis (IEA), random amplified polymorphic DNA (RAPD) analysis, and restriction endonuclease analysis (REA). Twenty-one isolates were from a single hospital, with four isolates coming from different patients. Three clinical isolates came from a different hospital, and 11 clinical or environmental isolates were derived from a culture collection. With IEA, the patterns of alkaline phosphatase, esterase, and catalase discriminated nine types. In contrast, 22 types were obtained with five different RAPD primers, and 21 types could be detected with three of these (R108, R151, and UBC90). Restriction endonuclease analysis of genomic DNA, digested with either *Xba*I, *Xho*I, or *Sal*I, detected 3, 17, and 13 different REA types, respectively, and 22 types were identified by combining the data from the *Xho*I and *Sal*I REAs. Twenty-eight types were obtainable with a combination of REA, IEA, and RAPD patterns. Overall, the results pointed to substantial genetic variation among the isolates. Though two isolates had markedly distinct genotypes, their morphologic features and exoantigens were consistent with their being *A. fumigatus*. The analysis will help in planning epidemiologic studies of aspergillosis.

Aspergillus fumigatus is a haploid filamentous fungus. It is ubiquitous in the environment, especially in decaying plant matter and soil. Of all the species of *Aspergillus*, *A. fumigatus* is the organism most frequently isolated from human and animal infections (17). The number of reported invasive infections caused by this opportunistic fungus has been rising, in proportion to the significant increase in the size of the immunocompromised, especially neutropenic, population (2, 28).

Better understanding of the environmental sources of *A. fumigatus* strains causing infections would facilitate prevention measures. However, epidemiologic investigations have been hampered by the lack of a simple and rapid typing system for this fungus. Typing of clinical and environmental isolates could help to provide the answer to several medically relevant problems, such as pinpointing the environmental sources of strains causing outbreaks of aspergillosis, determining the existence of pathogenic strains and their natural habitats, improving guidelines for patient management, and determining the purity of stock cultures. Several methods have been used for phenotypic identification of *A. fumigatus* isolates, including methods based on immunoblotting (6), susceptibility to killer toxins (10), patterns of cellular proteins produced following electrophoresis (16), and cultural and morphologic characteristics (20). Unfortunately, these methods can be time-consuming and labor-intensive, and they may lack sufficient discriminatory power. Also, the types may reflect phenotypic variability. For some procedures, the reagents are currently available in only one laboratory. These difficulties, therefore, limit the use of phenotypic identification for routine typing of isolates from epidemiologic studies.

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To overcome the problems resulting from phenotypic variability, several molecular genetic typing methods have been applied to *A. fumigatus*. Isoenzyme analysis (IEA) was the first such method to be used (23), and a combination of IEA and an analysis of an isolate's ubiquinone system has been utilized by Matsuda et al. (22). A second molecular genetic typing method was the analysis of restriction fragment length polymorphisms of rRNA genes by hybridization of *A. fumigatus* genomic DNA blots with a 1.2-kb DNA fragment containing the rRNA intergenic spacer region from *Aspergillus nidulans* (26). Likewise, *A. fumigatus* isolates have been typed by identifying differences in the pattern of bands generated by hybridization of genomic blots with dispersed repetitive DNA probes (11). Restriction endonuclease analysis of genomic DNA (REA), with both *Sal*I and *Xho*I, allowed detection of 24 REA types among 31 unrelated isolates of *A. fumigatus* that had been obtained from three continents (7). Similarly, using *Xba*I, Burnie et al. (5) detected six REA types among 21 isolates. Two independent investigations, in which different sets of oligonucleotide primers were used, demonstrated that the random amplified polymorphic DNA (RAPD) method generated a relatively high degree of discrimination among isolates (1, 21). Regrettably, these typing methods are not ideal. Both the ubiquinone and the rRNA gene restriction fragment length polymorphism analyses showed a low degree of intraspecies discrimination, and procedures relying on hybridization to Southern blots may be too technically demanding and tedious for routine use in many hospital laboratories. Also, questions concerning reproducibility and the amount of discrimination associated with these methods still remain.

Few investigators have directly compared the results for typing isolates of *A. fumigatus* by several different methods. Such studies have used small numbers of isolates, and the division of strains into groups may have been influenced by phenotypic variability (5, 21). In contrast, our investigation directly compares the epidemiologic utilities of three genomic

TABLE 1. Profiles of *A. fumigatus* isolates obtained by IEA, RAPD analysis, and REA

Isolate	Source/geographic location	Types obtained by:		
		IEA[a]	RAPD[b]	REA[c]
A1[d]	Air sample/Pa.	E.A.B	A.E.A.C.B	3.17.3
A2[d]	Air duct surface/Pa.	E.A.B	A.E.A.C.B	3.17.3
A3[d]	Environment/Pa.	E.A.B	A.C.A.C.B	3.1.1
A4[d]	Environment/Pa.	E.A.B	A.C.A.C.B	3.1.1
A5[d]	Air sample/Pa.	E.A.B	A.C.A.C.B	3.1.1
A6[d]	Air duct surface/Pa.	E.A.B	A.C.A.C.B	3.1.1
A7[d]	Air sample/Pa.	E.A.B	A.C.A.C.B	3.1.1
A8[d]	Thoracic fluid/Pa.	E.A.B	A.C.A.C.B	3.1.4
A9[d]	Environment/Pa.	E.A.B	A.C.A.C.B	3.14.1
A10[d]	Air sample/Pa.	E.A.B	A.C.A.D.B	3.1.1
A11[e]	Stool/Washington, D.C.	E.A.B	A.C.A.A.B	3.1.1
A12[d]	Air duct surface/Pa.	E.A.B	A.E.A.D.B	3.5.3
A13[d]	Air sample/Pa.	E.A.B	A.E.A.D.B	3.17.3
A14[d]	Air sample/Pa.	E.A.B	A.E.A.D.B	3.15.3
A15[e]	Nasal swab/Washington, D.C.	E.A.B	A.I.A.D.B	3.1.1
A16[d]	Abdomen/Pa.	E.A.B	A.E.C.D.B	3.1.5
A17[f]	Environment/France[g]	E.A.B	A.J.E.F.A	3.7.8
A18[d]	Air sample/Pa.	E.A.B	A.G.H.F.A	3.3.13
A19[f]	Lung/Fla.	E.A.B	A.J.E.G.A	3.6.11
A20[d]	Mediastinum/Pa.	E.B.B	A.D.A.D.B	3.2.2
A21[d]	Air duct surface/Pa.	E.B.B	A.D.A.D.B	3.2.2
A22[d]	Left ankle/Pa.	E.B.B	A.D.A.D.B	3.2.2
A23[d]	Air sample/Pa.	E.B.B	A.C.A.C.B	3.1.1
A24[e]	Sputum/Washington, D.C.	E.B.B	A.H.A.D.A	3.4.1
A25[d]	Air sample/Pa.	E.B.B	A.C.A.C.B	3.1.17
A26[f]	Bronchial wash/Fla.	G.A.B	D.J.E.F.A	3.6.11
A27[f]	Sputum/Pa.	G.A.B	A.M.G.B.A	3.9.10
A28[f]	Bronchial wash/Calif.	A.A.B	A.A.A.A.A	3.10.12
A29[f]	Bronchial wash/Calif.	A.A.B	B.B.B.B.A	3.11.12
A30[f]	Lung/Honduras	G.C.C	A.K.E.F.A	3.7.11
A31[f]	Environment/France[g]	G.C.C	A.J.G.F.A	3.6.11
A32[f]	Bronchial wash/Fla.	F.A.B	D.L.A.H.A	3.8.X[h]
A33[f]	Air sample/Ind.	A.B.B	A.L.A.D.A	3.16.X[h]
A34[f]	Sputum/Ala.	G.E.E	E.N.F.I.D	1.12.9
A35[f]	Air sample/Pa.	I.G.G	C.F.D.E.C	7.13.6

[a] IEA types obtained for EST, ALP, and CAT, respectively (left to right).
[b] RAPD types obtained with primers RP4-2, R108, R151, UBC90, and UBC69, respectively (left to right).
[c] REA types obtained with *Xba*I, *Xho*I, and *Sal*I, respectively (left to right).
[d] Obtained from hospital A in Pennsylvania.
[e] Obtained from hospital B in Washington, D.C.
[f] Obtained from Centers for Disease Control and Prevention culture collection.
[g] Isolated by Weems et al. (30).
[h] X, type not determined.

typing methods, REA, IEA, and RAPD analysis, applying them to a large collection of *A. fumigatus* clinical and environmental isolates. The advantages and disadvantages of each typing method, and of combinations of typing methods, have been evaluated by three major criteria: typeability, reproducibility, and discriminatory power (13).

MATERIALS AND METHODS

Strains. The 35 *A. fumigatus* isolates used in this study are listed in Table 1. Species identification was based on colonial characteristics and the microscopic morphology of the conidia and conidiophores. Cultures were maintained on Sabouraud agar (10 g of neopeptone, 40 g of glucose, and 15 g of agar per liter) slants and stored at 4°C. Of the isolates, 21 were obtained from either patients or environmental sources in hospital A in Pennsylvania. Three isolates were obtained from patients in hospital B in Washington, D.C., and an additional 11 isolates were taken from cultures of stock strains in the fungal culture collection maintained in the Emerging Bacterial and Mycotic Diseases Branch at the Centers for Disease Control and Prevention.

IEA. Conidia of *A. fumigatus* were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of yeast nitrogen base without amino acids–0.1 M sucrose–0.03 M mannitol broth and incubated in a rotatory shaker (7 days, 28°C, 150 rpm). Mycelia were washed twice with 100 mM Tris hydrochloride, pH 8.0, and then ground three times in a mortar under liquid nitrogen in the presence of 1.0 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged (13,000 × *g*, 5 min), and the supernatant was assayed for its protein content (3). About 200 μg of protein was applied to native discontinuous polyacrylamide gels. After electrophoresis, the gels were washed twice in buffer and stained for enzyme activity, as described previously (15, 18). Six isoenzyme activities were analyzed in 7.5% (wt/vol) polyacrylamide gels: glucose-6-phosphate dehydrogenase (EC 1.1.1.49), malate dehydrogenase (EC 1.1.1.37), α-glucosidase (EC 3.2.1.20), β-glucosidase (EC 3.2.1.21), superoxide dismutase (EC 1.15.1.1), and esterase (EST) (EC 3.1.1.1), which was detected with 4-methylumbelliferyl acetate. The enzymes analyzed in 5.0% (wt/vol) polyacrylamide gels were α-mannosidase (EC 3.2.1.24), detected with 4-methylumbelliferyl-α-mannoside (50 μg/ml of 0.1 N sodium acetate buffer, pH 5.5); catalase (CAT) (EC 1.11.1.6); alkaline phosphatase (ALP) (EC 3.1.3.1); and acid phosphatase (EC 3.1.3.2).

Isolation of *A. fumigatus* genomic DNA. A loopful of conidia was used to inoculate 40 ml of YPD broth (1% glucose, 1% yeast extract, 2% peptone) in a 250-ml Erlenmeyer flask. After incubation on a rotatory shaker (36 to 48 h, 37°C, 150 rpm), mycelia were harvested by filtration, washed once with sterile distilled water, and frozen in liquid nitrogen. Genomic DNA was then purified from the lysate by repeated phenol-chloroform extractions as described by Buffington et al. (4). Purified genomic DNA was resuspended in TE buffer (10 mM Tris hydrochloride, 1 mM Na_2-EDTA [pH 8.0]) and stored at −70°C. The integrity of the genomic DNA samples was analyzed by electrophoresis of 0.5-μg samples of genomic DNA through 0.4% agarose gels and subsequent staining with ethidium bromide: degraded samples showing smearing were excluded from further analysis.

REA. Approximately 1 μg of *A. fumigatus* genomic DNA was digested with 10 U of *Xba*I, *Xho*I, or *Sal*I at 37°C for 4 to 6 h in the buffers recommended by the supplier (New England Biolabs, Beverly, Mass.). The restriction endonuclease digests were electrophoresed through 0.8% agarose gels for *Xba*I-digested samples or through 0.5 or 0.7% agarose gels for the *Xho*I- and *Sal*I-digested samples, as described by Denning et al. (7). Following electrophoresis, gels were stained for 20 min in 1 μg of ethidium bromide per ml. Gels were destained in several changes of distilled water for 1 h, placed on UV transilluminator, and then photographed. Isolates were typed on the basis of differences in the banding patterns as described elsewhere (7).

The patterns of ethidium bromide-stained bands were compared visually on prints (20 by 25 cm) of the negative image. Because of differences in the number and relative migration of bands compared with those reported previously (7), and because of the lack of isolates common to that study and ours, the REA types were not designated by the nomenclature used by Denning and colleagues. Instead, each isolate was assigned to a type on the basis of differences in the number and size of restriction fragments for the *Xho*I, *Xba*I, and *Sal*I digests. Comigrating bands with differing staining intensities were assumed to be identical. When required, samples containing a similar pattern of bands were loaded in adjacent lanes of the same gel for direct comparisons. Each designated number for a given restriction endonuclease pattern, as listed in Table 1, reflects a distinct and recognizable pattern of bands, or the REA type for a particular enzyme digest. *Xho*I and *Sal*I REA types were designated by different numbers, ranging from 1 to 17 and 1 to 13, respectively.

RAPD analysis. Primers that were evaluated included R151 (5′-GCTGT AGTGT) and R108 (5′-GTATTGCCCT), previously demonstrated to show a high degree of discrimination among a panel of nine *A. fumigatus* isolates (1). Primer RP4-2 (5′-CACATGCTTC) was also evaluated since it demonstrated a high degree of intraspecies and interspecies discrimination among several species of *Candida* (19). The remaining primers, UBC69 (5′-GAGGGCAAGA) and UBC90 (5′-GGGGGTTAGG), were purchased from J. R. Hobbs, University of British Columbia, Vancouver, Canada. Primers RP4-2, R108, and R151 were synthesized at the Centers for Disease Control and Prevention as described earlier (19). RAPD reaction conditions were as described previously (19). RAPD profiles were compared visually, and those isolates giving patterns with identical bands were recorded as the same type.

Discriminatory power. The discriminatory power of a typing method, *D*, is the mathematical probability that two unrelated strains, chosen at random from a test population, can be shown to belong to different groups. Discriminatory power was calculated in the manner described by Hunter and Gaston (14).

RESULTS

IEA. Of the isoenzyme activities examined, only ALP, EST, and CAT discriminated among the 35 isolates. Gels showing examples of the heterogeneity of enzyme patterns are included in Fig. 1. Figure 1 shows three types for ALP, based on three upper band positions; two types for EST, where extra bands are seen in lanes 2, 3, and 5; and two types for CAT. The patterns of EST, ALP, and CAT allowed detection of totals of five, five, and four types, respectively, among all the isolates.

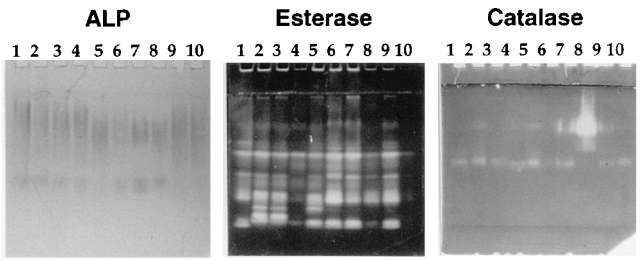

FIG. 1. Heterogeneity of enzyme patterns shown in IEA gels from different experiments. IEA patterns for ALP were as follows: lanes 1 to 4, type A (isolates A10, A4, A17, and A2, respectively); lanes 5 to 8, type B (A24, A25, A20, and A22, respectively); lanes 9 and 10, type C (A30 and A31, respectively). Patterns for EST were as follows: lanes 1, 4, and 6 to 9, type E (A19, A23, A24, A22, A16, and A9, respectively); lanes 2, 3, and 5, type A (A28, A29, and A32, respectively). Patterns for CAT were as follows: lanes 1 to 7 and 9 and 10, type B (A2, A18, A17, A27, A4, A28, A23, A14, and A26, respectively); lane 8, type G (A35).

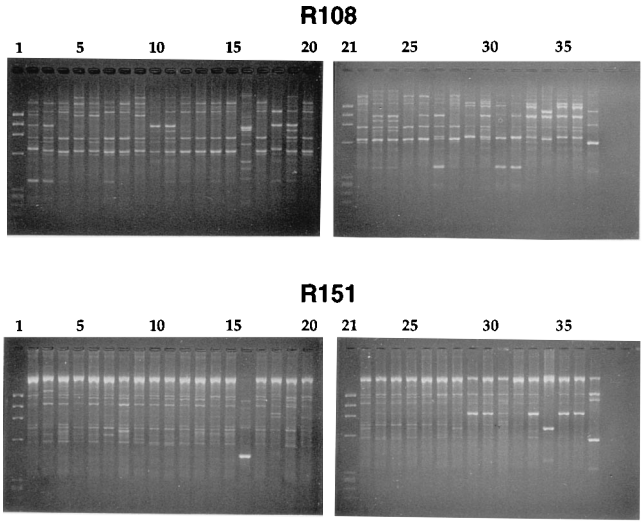

FIG. 2. RAPD patterns produced with R108 and R151 as primers. Lanes 1 and 21, ϕX174 DNA cut by *Hae*III; lane 2, isolate A28; lane 3, A29; lane 4, A8; lane 5, A20; lane 6, A22; lane 7, A16; lane 8, A6; lane 9, A21; lane 10, A12; lane 11, A2; lane 12, A9; lane 13, A3; lane 14, A4; lane 15, A7; lane 16, A35; lane 17, A5; lane 18, A18; lane 19, A13; lane 20, A23; lane 22, A10; lane 23, A1; lane 24, A14; lane 25, A25; lane 26, A24; lane 27, A15; lane 28, A11; lane 29, A26; lane 30, A30; lane 31, A32; lane 32, A32; lane 33, A19; lane 34, A27; lane 35, A31; lane 36, A17; lane 37, A34. RAPD patterns are listed in Table 1.

Although each single isoenzyme reaction demonstrated a low degree of discrimination, the discriminatory power of IEA was enhanced by combining the isoenzyme data, which allowed nine IEA types to be discriminated ($D = 0.682$) (Tables 1 and 2). The first and second most common types, E.A.B and E.B.B, contained 19 and 6 isolates, respectively, whereas the remaining seven types contained only 1 or 2 isolates per group. Good reproducibility of the patterns for ALP and EST was observed by running different preparations of the same isolate in multiple gels. The pattern of CAT, however, varied in intensity in different preparations, and only very marked differences were accepted as discriminatory.

RAPD analysis. The discriminatory power varied greatly according to the individual RAPD primer used. For instance, primer R108 demonstrated the highest degree of discriminatory power ($D = 0.886$) and detected 14 types, whereas primer RP4-2 showed the lowest degree of discriminatory power ($D = 0.267$), detecting only 5 types (Table 1). RAPD patterns obtained with primers R108 and R151 are shown in Fig. 2. As expected, the highest degree of discrimination was observed by combining the results for all RAPD primers: 22 different types were thus obtained ($D = 0.928$). A comparable degree of discrimination was obtained by using only three primers, R151, R108, and UBC90. With this primer set, 21 types were obtained, with a comparable degree of discrimination ($D = 0.926$) (Table 2). Combining these three primers with RP4-2 resulted in an additional type being observed, while combination with UBC69 gave no additional discriminatory power. The most common RAPD type, A.C.A.C.B, consisted of nine isolates. The next most frequent types, A.D.A.D.B and A.E.A.D.B, each contained three isolates. Each of 18 RAPD types was represented by a single isolate. The RAPD patterns were generally reproducible between runs when samples were run a second time. However, occasionally, the interpretation of some bands was difficult because of changes in staining intensity between runs.

TABLE 2. Discriminatory powers of typing methods and combinations of methods for 35 isolates of *A. fumigatus*

Typing method	No. of types	No. of isolates with same type[a]	No. of types represented by single isolate	*D* value
IEA	9	19, 6, 2, 2, 2	4	0.682
RAPD[b]	21	9, 3, 3, 2, 2	16	0.926
RAPD[c]	22	9, 3, 3, 2	18	0.928
REA[d]	21	9, 3, 3, 3	17	0.928
IEA + RAPD[c]	23	7, 3, 3, 2, 2	18	0.941
IEA + REA[d]	24	8, 3, 3	21	0.943
RAPD[c] + REA[d]	27	6, 3, 2	24	0.968
IEA + RAPD[c] + REA[d]	28	5, 3, 2	25	0.976

[a] Each value is for a separate type.
[b] RAPD types were obtained with primers R108, R151, and UBC90.
[c] RAPD types were obtained with primers R108, R151, UBC90, RP4-2, and UBC69.
[d] REA types were obtained with *Sal*I and *Xho*I.

REA. Initially, genomic DNAs were digested with *Xba*I and assigned to different REA types by using the scheme of Burnie et al. (5). Thirty-three isolates were type 3, isolate A34 was type 1, and isolate A35 had an *Xba*I pattern not previously described and therefore designated type 7 (Table 1).

To better discriminate among isolates, the REA typing method of Denning et al. (7) was evaluated. Representative gels are shown in Fig. 3. The most common *Xho*I REA type, type 1, consisted of 12 isolates. *Xho*I REA types 2, 6, and 17 were each represented by three isolates. Twelve *Xho*I REA types were represented by single isolates. Following *Sal*I digestion, the most frequent REA type was type 1, which consisted of 11 isolates. The next most frequent *Sal*I REA types, types 3 and 11, consisted of five and four isolates, respectively. Eight *Sal*I REA types were represented by single isolates.

After combination of the data for REA types that had been generated by using *Xba*I, *Xho*I, and *Sal*I, 22 types were distinguished. The most common combined REA type, 3.1.1, consisted of nine isolates, and 17 of the types obtained by combined REA were represented by single isolates. The discriminatory power for this combined REA was identical to that of RAPD analysis with the set of five primers ($D = 0.928$) (Table 2). The reproducibility of the *Xba*I, *Sal*I, and *Xho*I REA patterns was investigated by two methods. By the first method, digestion of the same preparation of DNA for several isolates on different days produced an identical pattern of bands. By the second, independent DNA preparations from the same isolate also gave an identical pattern of bands.

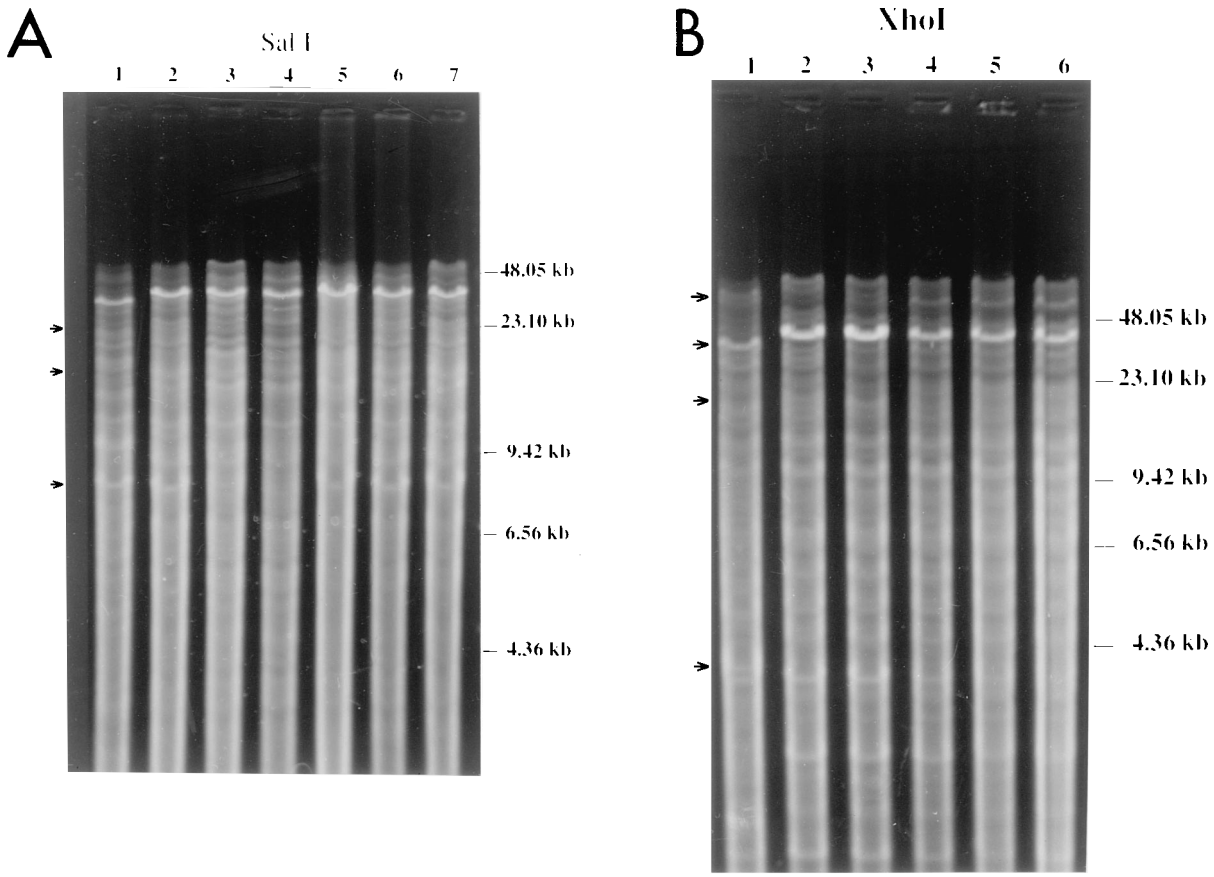

FIG. 3. REA with *Sal*I and *Xho*I. (A) *Sal*I-digested samples. Lane 1, A26 (REA type 11 [as shown in Table 1]); lane 2, A30 (type 11); lane 3, A32 (type X); lane 4, A8 (type 4); lane 5, A16 (type 5); lane 6, A19 (type 11); lane 7, A34 (type 9). (B) *Xho*I-digested samples. Lane 1, A34 (type 12); lane 2, A29 (type 11); lane 3, A28 (type 10); lane 4, A9 (type 14); lane 5, A8 (type 1); lane 6, A10 (type 1). Undigested bacteriophage lambda DNA and lambda DNA digested with *Hin*dIII molecular size markers are indicated on the right. Arrows on the left point to DNA fragments that were useful in typing.

Combination of methods. IEA, RAPD analysis, and REA showed 100% typeability, the proportion of isolates that can be typed in a population. When the analyses were combined, more types could be discriminated. As shown in Table 2, combination of RAPD analysis and IEA identified 23 types (D = 0.941) and the combination of REA and IEA identified 24 types (D = 0.943), while RAPD analysis and REA together identified 27 types (D = 0.968). Twenty-eight types were obtained by combining all three typing methods, which, when combined in this manner, gave a slightly higher degree of discrimination (D = 0.976) than was observed with the combination of RAPD analysis and REA.

DISCUSSION

IEA detected several monomorphic enzymes. While these may be useful for species identification, they were not suitable for typing *A. fumigatus*. In contrast, three enzymes, CAT, EST, and ALP, demonstrated interstrain variation similar to that reported by Nealson and Garber (23). If more variable enzyme activities were identified, the discriminatory power of IEA might be raised; however, the discriminatory power of the current method was only moderate.

The RAPD and REA patterns offered a greater degree of discrimination for the panel of isolates than did IEA (Table 2). The RAPD method offers several advantages for typing studies. It is quick to perform, so that large numbers of isolates can be analyzed in a few days (19); it demonstrates 100% typeability; and it can be applied to other types of study. For example, RAPD analysis has been used to generate species-specific probes for typing isolates of *Aspergillus flavus* (4).

The reproducibility of the RAPD patterns was demonstrated by repeated runs from the same DNA preparation; occasionally, however, we observed bands with different staining intensities, which made the interpretation of the banding patterns more difficult and complex. Variability in staining intensities is most likely due to the relative efficiency of the RAPD primer to anneal with non-perfectly complementary nucleotide sequences during different runs, or even during different PCR cycles, influencing which DNA fragments are preferentially amplified. Reproducible RAPD patterns have been observed for a number of microorganisms (12, 29), but the procedure requires rigorous control of the reaction conditions (9, 31, 32). Likewise, while interlaboratory reproducibility of RAPD analysis results has been little examined, it may be possible to generate reproducible patterns provided that the conditions are strictly controlled (24).

A disadvantage of the RAPD method is that each individual primer may display only moderate to low discriminatory power. Primer R108 showed the greatest degree of discriminatory power (D = 0.866), identifying 14 types, consistent with the degree of discrimination observed by Aufauvre-Brown et al. (1). Analysis of the banding patterns obtained with several RAPD primers is recommended because the discriminatory

power of the RAPD method may be increased by using more primers. However, the analysis becomes more complicated by the larger numbers of bands and gels, and complication also arises because the pattern reproducibility is more difficult to control with the greater number of reactions. For economy, we suggest using the three-primer set (R108, R151, and UBC90) instead of our five-primer set, since addition of the two remaining primers resulted in only a modest increase in discrimination ($D = 0.926$ versus $D = 0.928$) (Table 2).

Burnie et al. previously used REA to type 21 *A. fumigatus* isolates into six *Xba*I REA types (5). In our investigation, we observed only three of these types, with 33 of 35 isolates being classified as type 3. This result suggests that the method used by Burnie et al., or our interpretation of its application, was not sufficiently discriminatory to distinguish among our cluster of isolates. Alternatively, the lack of discrimination may be due to a geographic clustering of type 3 isolates in our investigation.

With the combined data obtained with three restriction endonucleases, we observed 22 different REA types among the 35 isolates, and 17 types were represented by a single isolate. This degree of discrimination by REA was equivalent to that obtained with five RAPD primers and is in good agreement with the high degree of discrimination observed by Denning et al. (7, 8). Also in agreement with these researchers, we observed a high degree of reliability and reproducibility for the *Xho*I and *Sal*I REA patterns.

One disadvantage of REA was the difficulty in interpreting the complex pattern of bands. This was due to the large number of faint bands detected following digestion of DNA by *Sal*I or *Xho*I. Other disadvantages of REA included the observer subjectivity in comparison of patterns between gels, lack of standardization of the methods and strains, and the labor involved in isolating highly purified DNA from a large number of isolates. The subjective error in analyzing DNA types may be reduced by some methods we employed in this study, also used by Denning et al. (7); these included the examination of samples in adjacent lanes, the use of a variety of different restriction endonucleases for analysis, and the analysis of ethidium bromide-stained bands on enlarged prints.

As expected, combination of data obtained by more than one typing method led to an increase in strain discrimination. While good agreement between methods was generally observed, the concordance among methods was not always obvious. This result suggests a substantial degree of genetic variation among our isolates, as well as subclasses of isolates. For instance, the number of test differences required to classify isolates as separate strains may be heuristic, since REA data cannot be used to estimate genetic relatedness. Since no sexual stage has been identified for this fungus, mitotic recombination and spontaneous mutations may be responsible for strain diversity, which suggests that the population structure of *A. fumigatus* is represented by independent genotypes. Substantial genetic variation in *A. fumigatus* has been implied both by our study, since we observed 28 different types by combining three different typing methods, and by other studies, which showed that each new case of aspergillosis appeared to be due to a different isolate (11, 27). Proper sampling of patient isolates for laboratory analysis may also be important, since patients harboring multiple isolates of *A. fumigatus* have been reported (7, 8). Finally, epidemiologic data are also an important consideration in interpretation of data acquired during typing analysis.

Of interest was the detection of two isolates, A34 and A35, with very distinct types determined by all molecular genetic methods (Table 1); however, their morphologic features and the results of an exoantigen test (25) were consistent with those of *A. fumigatus*. Therefore, the taxon *A. fumigatus*, while being morphologically homogeneous, may represent more than one genetically distinct organism, a finding that has been reported for other taxa, such as *Trichosporon beigelii* (15).

In conclusion, we directly compared and examined the advantages and disadvantages of three genotypic typing methods used for analyzing the molecular epidemiology of a large set of *A. fumigatus* isolates. Strain variation and discrimination were best obtained by using a combination of typing methods. The combination of RAPD analysis and REA appears to provide the best discriminatory power, reliability, and reproducibility for typing *A. fumigatus*. Ideally, studies correlating the molecular genetic typing results with appropriate phenotypic characters may provide useful information regarding the epidemiology and pathogenesis of this important opportunistic fungus.

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