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Typing of Aspergillus Fumigatus and Aspergillus Niger Strains by Random Amplification of Polymorphic DNA Analysis Using a Six Primer Set.

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Abstract:

Differentiating and typing clinical and environmental isolates of Aspergillus strains are necessary for epidemiological studies and could contribute to the solution of several pertinent clinical problems. We investigated the utility of the RAPD-PCR technique for typing twenty-six strains of Aspergillus fumigatus and ten additional clinical and environmental Aspergillus fumigatus and Aspergillus niger isolates. Fungal genomic DNA was extracted using glass bead disruption and RAPD analysis was performed by using a six individual (R108, R151, UBC90) and combined (R108+R151, R108+UBS90, R151+UBC90) primer set. Primer pair R108+UBC90 demonstrated the highest discriminatory power with collection strains, whereas primer R151 displayed the highest degree of discrimination power with clinical and environmental isolates. Both primer sets detected seven types of strains.

It was found that primer R151, on its own and in combination with primer UBC90, shared a relative relationship between clinical and environmental A. fumigatus isolates. We found that although RAPD-PCR analysis can be applied as a simple, rapid, and very effective method for differentiating Aspergillus strains, selecting the best primers is a critical step to reach the desirable discriminatory power.

Introduction:

Members of the genus Aspergillus are filamentous fungi which are ubiquitous in the environment [2, 3], especially near decomposing plant matter, organic debris, and soil, where they can release a large number of airborne conidia.[2, 4] It is assumed that both animals and humans are constantly exposed to and breathing the Aspergillus conidia. Despite this constant exposure, only a few Aspergillus species are capable of causing infection, of which, Aspergillus fumigatus is the most common species.[1] Some characteristics which make it remarkable as a potent pathogen are its ability to grow at 37°C [5], and having conidia which are small enough (3-5µm) to reach the alveolar spaces of the lungs.[5, 6] Aspergillus is responsible for several pulmonary conditions, including allergic bronchopulmonary aspergillosis, aspergilloma, sinus infections, and invasive aspergillosis, a life-treating disease of immunocompromised patients [2, 4, 6, 7, 8] which may lead to dissemination to other organs such as heart, kidney, and brain. The frequency of reports of invasive aspergillosis caused by Aspergillus fumigatus has been gradually rising, due to the increase in immunocompromised individuals, especially neutropenic patients.[2]

A better understanding of the environmental sources of Aspergillus strains causing infection would facilitate prevention strategies.[9] Therefore, differentiating Aspergillus strains, along with identification of the species appears to be necessary for epidemiological studies.[1] Typing of clinical and environmental isolates could contribute to the solution of several relevant clinical problems such as the identification of the environmental source of Aspergillus strains causing outbreaks of aspergillusis, determining the existence of pathogenic strains and their natural habitants, and also providing a guide for patient management.[9]

Several techniques are available for phenotypic identification of Aspergillus strains. Briefly these methods include: susceptibility to killer toxins, patterns of cellular protein produced following electrophoresis, methods based on immunoblotting, culture, and morphologic characteristics.[9] However, these methods are relatively labor-intensive, time consuming, and may lack sufficient discriminatory power. Such difficulties limit the application of phenotypic identification for the routine typing of isolates.[9]

In recent years, there has been considerable interest in the molecular typing of Aspergillus strains for population studies and epidemiological purposes. These molecular typing methods include isoenzyme electrophoresis (IE), restriction fragment length polymorphism (RFLP) analysis, sequence-specific DNA primer (SSDP) analysis [2, 7, 8], analysis of polymorphic microsatellite markers (PMMs) [2], amplified fragment length polymorphisms (AFLPs) analysis, and analysis of single nucleotide polymorphisms (SNPs).[7,10] Unfortunately, the procedures may not be still ideal for epidemiological investigations [2, 7]; for instance, RFLP analysis followed by Southern blotting may be tedious and labor-intensive, or PMMs analysis requires specialist and expensive equipment.[2] RAPD analysis is considered as another method for typing isolates of the Aspergillus species. This

method relies on using primers of arbitrary sequence to amplify segments of genomic DNA in a polymerase chain reaction.[6]

The aim of the present investigation was to assess the utility of RAPD-PCR (Random Amplification of Polymorphic DNA) analysis as a technically simple and rapid method for typing clinical and environmental Aspergillus fumigatus and Aspergillus niger isolates.

Material and Method:

Isolates: Twenty-six strains of Aspergillus fumigatus were provided by the Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan. Ten additional clinical and environmental A. fumigatus and A. niger isolates were collected from patients with invasive aspergillusis from areas surrounding the hospital. The Aspergillus strains are listed in table 1. Species identification of the isolates was based on microscopic and macroscopic morphologic characteristics. All isolates were cultured on Glucose (4%) Peptone (1%) agar (1.5%) at 25°C for 48 hours and harvested mycelium was stored at 20°C until use.

Table 1. Aspergillus strains and their origins used in this study.

Species	Source	Strains No
A. fumigatus	TIMM*	2907, 2913, 2917, 2934, 2922, 2928, 2905, 2904, 2923, 29192908,2909
A. fumigatus	Collection Clinical isolates	025, 027, 030, 032, 033, 039,040, 041, 046, 048, 049, 047, 018, 008
A. fumigatus	New Clinical	1, 2
A. fumigatus	Environmental	3, 4
A. niger	New Clinical	5
A. niger	Environmental	6, 7, 8, 9, 10

*TIMM: Teikyo University Institute of Medical Mycology

DNA extraction: Fungal genomic DNA was extracted from mycelia using glass bead disruption.[11] Briefly, about 5 mm3 of mycelia was transferred to a 1.5ml microcentrifuge tube and 300 mg of 0.5 mm diameter glass beads, 300µg of lysis buffer (100mM Tris-Hcl pH 8, 10mM EDTA, 100mM NaCl, 1% sodium dodecyl sulfate, 2% triton X-100), and 300µl of phenol chloroform-isoamyl alchohol (25:24:1) was added. The samples were shacked vigorously for 5 min, centrifuged for 5 min at 5000 rpm, and the supernatant was transferred to a fresh tube. The supernatant was extracted again with chloroform and DNA was precipitated by adding the same volume of isopropanol and 0.1 volume of 3M sodium acetate (pH 5.2). The solution was vortexed and incubated for 10min at -20°C and centrifuged for 15min at 12000 rpm. The precipitant was washed with cold 70% ethanol, dried in air, dissolved in 50µl of double distilled water and stored at -20°C till used for PCR.

RAPD analysis: In total, three oligonucleotides i.e. primer R108 (5´-GTATTGCCCT-3´), primer R151 (5´-GCTGTAGTCT-3´), primer UBC90 (5´-

GGGGGTTAGG-3′), and a three-combined primer set, (R108 and R151), (R108 and UBC90) and (R151 and UBC90) were used in this study. Amplification reactions were performed in a final volume of 25µl containing 1µl of genomic DNA, 1.25U of Taq DNA polymerase, 0.1mM of each four deoxynucleoside triphosphate (dATP,dTTP, dGTP, dCTP), 2.5µl of MgCl2, 0.2µM of primer, and 2.5µl of 10X PCR buffer. PCR was carried out in a thermal cycler (Perkin Elmer, USA) with the following temperature profile: 1 cycle of 5 min at 94°C , followed by 40 cycles of 45s at 94°C, 2 min at 36°C and 1.5 min at 72°C and a final extension step at 72°C for 8 min. Next, 10 µl of amplification products were loaded onto 1.3% agarose gel and run in TBE buffer (0.09M Tris, 0.09 M Boric acid and 2mM EDTA, [pH 8.3]). The products were detected by staining with ethidium bromide (0.5µg/ml) and photographed.

Discriminatory power: the discriminatory power of a method is an estimate of its ability to place two unrelated isolates into two different groups. Discriminatory power was calculated according to Hunter's results [12] in which this estimate is calculated by following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j (x_j - 1)$$

Where s is the number of types, xj is the number of isolates belonging to the jth type and N is the total number of isolates. Thus, a value of 1.0 indicates that all isolates tested are in different groups and D is equal to 0.0 when all isolates belong to an identical type.

Results:

RAPD analysis of the collection strains: Several bands with different sizes ranging from less than 200 to 3500 (in basepair) were generated by using all six-primer sets. Variable electrophoretic patterns were observed, demonstrating a different discriminatory power for each primer. Of the six-primer set, primer pair (R108 and UBC90), displayed the highest degree of discriminatory power (D = 0.837), detecting seven electrophoretic types (A-G). Both primer pair R151+UBC90 and primer R151 detected seven types as well, however, the discriminatory powers were 0.794 and 0.714, respectively. Although primer R108, UBC90 and primer pair (R151 and R108) each produced five types (A-E), the lowest degree of discrimination power was demonstrated by primer pair (R151 and R108). RAPD electrophoretic patterns of the collection strains are shown in Fig. 1a, 1b, 1c and 1d. In total, 17 different types were obtained. Primer pair (R108 and UBC90) generated a banding pattern composing of five to 10 bands in various sizes ranging from about 250 to about 2000 and from 150 to 4000 basepair. Only primer R151 was able to differentiate two collection strains (strain 2907 and 2913) from other strains. Despite that, primer pair (R151 and UBC90) was not identified as the best primer set, as only one single strain (strain 2905) could be differentiated only by this set.

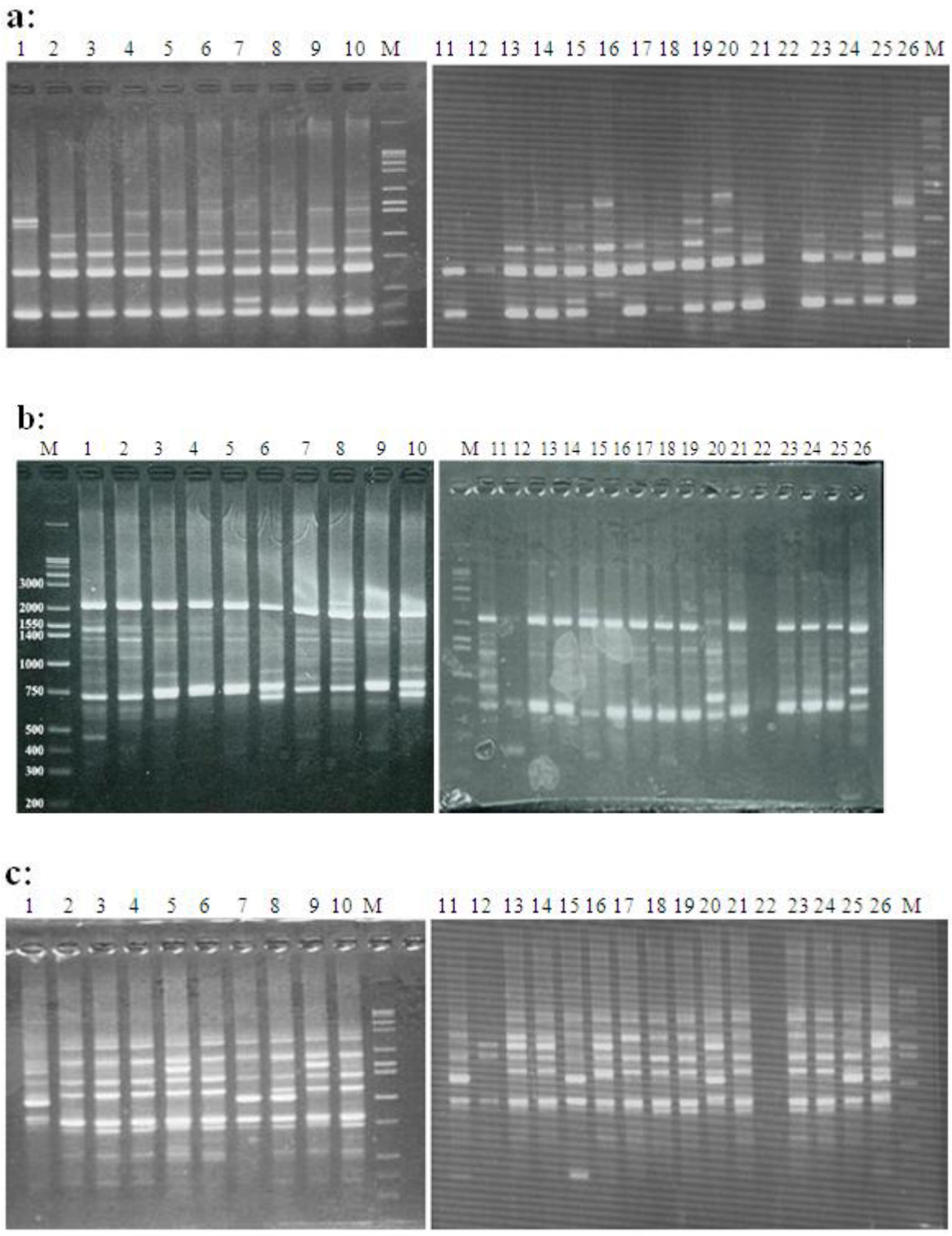

FIG 1a. RAPD typing with twenty-six collection strains by using three individual primers, (a) Primer R108; (b) Primer R151; (c) primer UBC90. The strains were analyzed in 1.3% agarose gel. Marker (M) was provided from Hi-LoTM DNA Marker and sizes are indicated in base pair. (a): type A: 1,20,26. Type B: 2, 3, 8,4,5,6,9,10. Type C: 18,25,7,15,19,25. Type D: 13, 14,17,21,22,23,24,11. Type E: 16. (b): type A: 1. type B: 20, 26. Type C: 6, 10. type D: 2,3,4,5,7,8,9,12,13,14,21,23 24,25. Type E: 16,17,18,19. Type F: 15. Type G: 11. (c): type A: 1. Type B: 2, 3, 4,610,17,18,19,21,23,24. Type C: 5, 9,13,14,16. Type D: 7, 8,25,15,11. Type E: 20, 26.

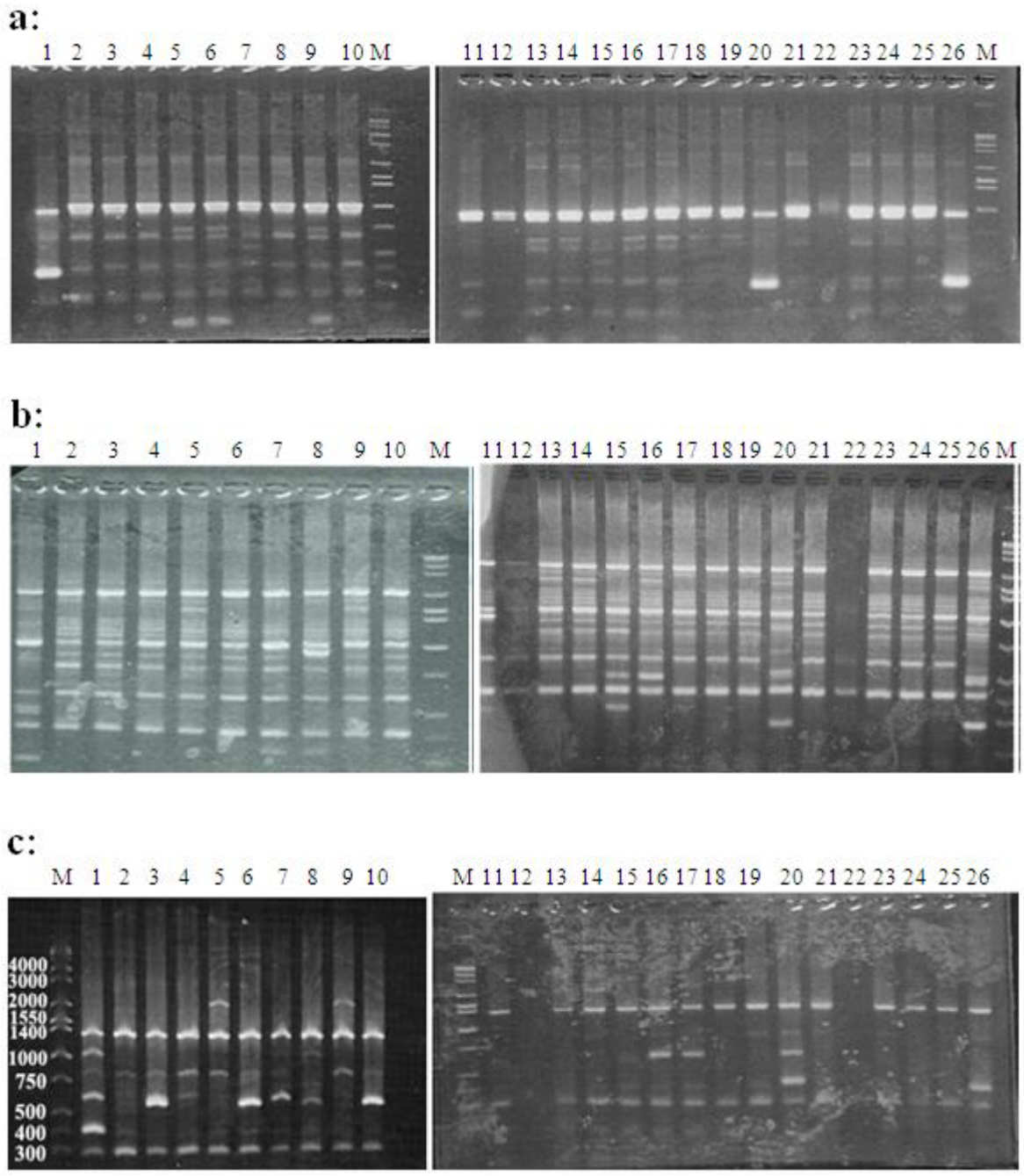

FIG 1b. RAPD typing of twenty-six collection strains by using three combined primers, (a) Primers (R108 and UBC90); (b) Primers (R151 and UBC90) ; (c) primers (R151 and R108). The strains were electrophoresed in 1.3% agarose gel. Marker (M) was provided from Hi-LoTM DNA Marker and sizes are indicated in base pair. (a): Type A: 1,20,26. Type B: 2, 3, 4,8,10,23,24,25. Type C: 16,18,19,21. Type D: 15. Type E: 5, 6, 9,13,14,17. Type F: 7. type G: 11, 12. (c): Type A: 1, 20, and 26. Type B: 2, 4. Type C: 3,6,7,8,10,16,17. Type D: 11, 12, 18,19,21,23,24,25,15. Type E: 5, 9, 13, 14.

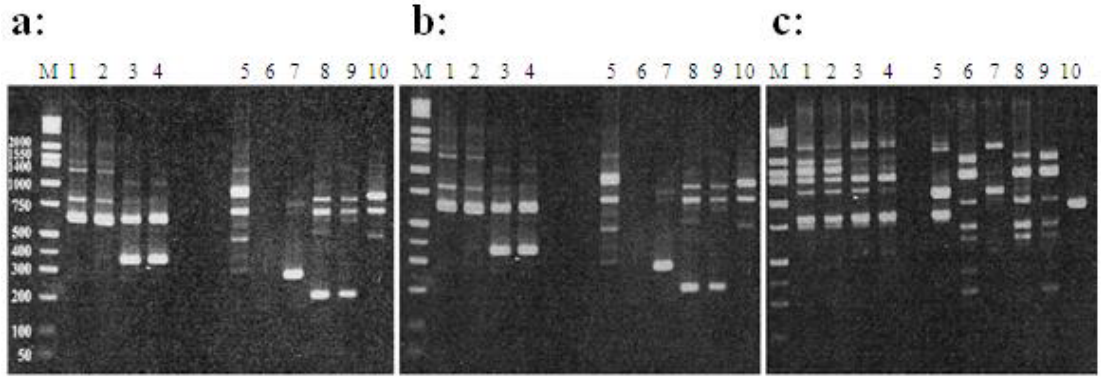

FIG 1c. RAPD typing with ten clinical and environmental isolates by using three individual primers, (a) Primer R108; (b) Primer R151; (c) primer UBC90. The strains were analyzed in 1.3% agarose gel. Marker (M) was provided from Hi-LoTM DNA Marker and sizes are indicated in base pair. Lane numbers: 1 and 2, Aspergillus fumigatus, clinical isolates. 3 and 4, Aspergillus fumigatus, environmental isolates. 5, Aspergillus niger, clinical isolates. 6, 7, 8, 9 and 10, Aspergillus niger, environmental isolates. According to picture C, clinical isolate NO. 1 and 3 gave the same patterns demonstrating that they are related to each other.

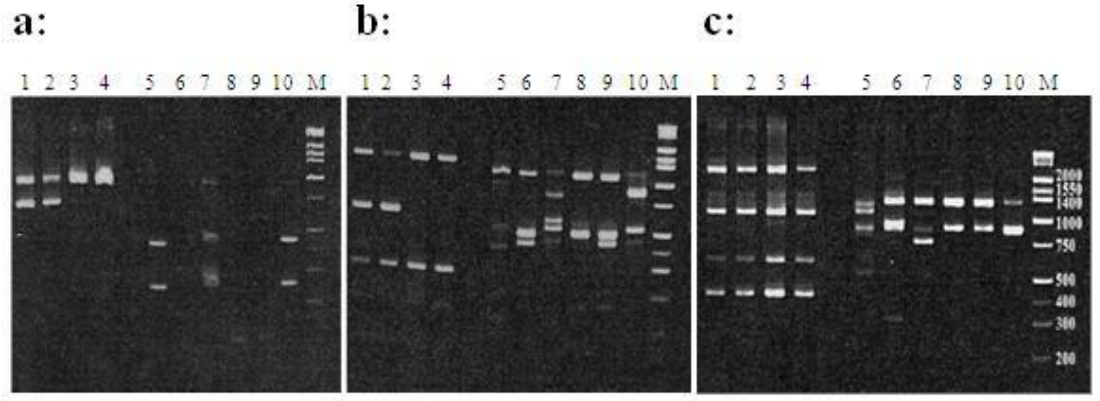

FIG 1d: RAPD typing ten clinical and environmental isolates by using three combined primers, (a) Primers (R151 and R108); (b) primers (R108 and UBC90); (c) primers (R151 and UBC90). The strains were analyzed in 1.3% agarose gel. Marker (M) was provided from Hi-LoTM DNA Marker and sizes are indicated in bare pair. Lane numbers: 1 and 2, Aspergillus fumigatus, clinical isolates. 3 and 4, Aspergillus fumigatus, environmental isolates. 5, Aspergillus niger, clinical isolates. 6, 7, 8, 9 and 10, Aspergillus niger, environmental isolates. Picture C shows four identical electrophoretic banding patterns indicating the relationship between environmental and clinical isolates.

The most common RAPD primer pair type by use (R108 and UBC90) was type B; and contained 13 of 25 strains (table 2a). For some uncertain reasons, no electrophoretic banding pattern was observed with strain 049.

Table 2a. Number of each type resulting from RAPD analysis with clinical and environmental isolates

Primers	Types						
	A	B	C	D	E	F	G
R108	2	2	1	1	2	1	
R151	2	1	1	1	2	1	1
UBC90	2	2	1	3	1	1	
R108 +R151	2	2	1	1	2		
R108 +UBC90	2	2	1	2	1	1	1
R151 + UBC90	4	1	4	1			

RAPD analysis of the environmental and clinical isolates: We also applied the six-primer set to type ten clinical and environmental A. fumigatus and A. niger isolates. All primers generated variable banding patterns in different sizes. However, with the exception of primer R151, none of them had sufficient discriminatory power to differentiate between the two clinical A. fumigatus isolates, nor to two environmental isolates within their own groups. Of the six-primer set, primer R151 demonstrated the highest discriminatory power (D = 0.955), detecting seven types (A-G), whereas primer R151, in combination with primer UBC90, displayed the lowest degree of discrimination (D = 0.734), detecting only four types (A-D). The discriminatory power for primer R108 and for both primer pairs, (R151 and R108) and (R108 and UBC90), were 0.933. Surprisingly, primer R151 in combination with primer UBC90 did not show sufficient discriminatory power, suggesting a relatively close relationship within clinical and environmental A. fumigatus isolates. According to these results, Type D was identified as the most frequent type. It is notable that without any known reason, no electrophoretic banding pattern was observed with A. niger isolates by using (R108 and R151) primer pair. The most frequent types with environment and clinical Aspergillus isolates have been listed in table 2b.

TABLE 2b: Number of each type resulting from RAPD analysis with collection isolates.

Primers	Types						
	A	B	C	D	E	F	G
R108	3	8	5	9	1		
R151	1	3	2	13	4	1	1
UBC90	1	12	5	5	2		
R108 + R151	3	2	7	9	4		
R108 + UBC90	3	8	4	1	6	1	2
R151 + UBC90	1	13	4	3	1	3	1

Reproducibility: Reproducible patterns were observed in both individual and combined primers with two collection strains (isolates 2907 and 2913 as random samples). Moreover, amplifications were repeated with four subcultured collection strains (2907, 2913, 2917 and 2934) to verify the presence or absence of scored bands. There was a slight variation in the intensity of major bands or a loss of faint band (data are not shown).

Discussion:

The benefits of using DNA-based typing techniques for the study of the genetic diversity of environmental and clinical isolates, revealing sources of infection, and for contributing to the solution of several relevant epidemiological problems of A. fumigatus linked isolates has

been established by several research groups.[7] An ideal technique should provide sufficient discrimination for the strains isolated from the epidemiological study of nosocomial outbreaks or from individual patients and their environment.[1] However, only a limited number of the methods are useful for type A. fumigatus isolates. RAPD-PCR has been examined as a typing system for A. fumigatus typing due to both its usefulness and its relative technical simplicity and speed.[1, 6, 13, 14] The major advantage of RAPD is that no previous sequence information is needed in contrast with other techniques like RFLP or microsatellite typing.[7] In previous studies, isolates of A. fumigatus were typed according to the RFLP patterns generated with one or two restriction enzymes.[2, 3, 4, 10, 15] 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" might have given different patterns if other areas of the genomes had been compared. Nevertheless, several disadvantages which limit the utility of any comparison of results between research centers have been reported for RAPD analysis. For example variations in the components of the reactions especially magnesium chloride, primer concentration, and reaction buffer may affect the results significantly.[1] In the present study, variations in primers, magnesium chloride, and template DNA concentrations had little effect on RAPD patterns (data not shown). Other factors such as thermal cycler function and buffer composition may also play roles in band patterns, since our results differ from those other reports in which the same primers were used. It has been reported [1, 8, 13] that combining methods provides a modest increase in discriminatory power. For example, applying the combination of SSDP analysis and PMM analysis gave a higher discriminatory power than the individual methods gave on their own.[2] We used RAPD analysis to type A. fumigatus collection, clinical and environmental, and also A. niger clinical and environmental isolates. According to research, [9] the three-primer set (R108, R151 and UBC90) had been used successfully to differentiate A. fumigatus isolates. We applied the three-primer set R108, R151 and UBC90 in addition to the three-combined primer set, UBC90 + R151, UBC90 + R108 and R151 + R108 for RAPD analysis of twenty-six A. fumigatus collection strains. All six-primers were able to discriminate A. fumigatus strains, as was demonstrated in our results. The efficacy of those six-primer sets allowed the A. fumigatus and A. niger isolates to be typed by this method. Although in our investigation primer R108, on its own, could provide a sufficient level of discrimination, it showed a greater degree of discrimination in combination with primer UBC90. This contradicts the results of previous studies, [1, 9, 16] in which primer R108 provided the greatest level of discrimination power. Interestingly, only primer R151 could differentiate two collection strains of A. fumigatus (2907 and 2913) from the other twenty-four strains.

One of the applications of a typing method, such as RAPD, is to establish whether both environmental and clinical isolates collected from one distinct area

are related. In our experiment, primer R151 demonstrated sufficient discriminatory power to differentiate between two clinical and two environmental isolates of A. fumigatus from each other, within their own groups. Moreover, two identical banding patterns were generated with clinical and environmental isolates of A. fumigatus (NO.1 and 3) by using primer R151. Primer R151, in combination with UBC90, demonstrated the lowest degree of discrimination. Nevertheless four identical electrophoretic banding patterns were observed with all clinical and environmental A. fumigatus isolates by using primer pair (R151 and UBC90). Therefore, it appears as if a close relationship exists between clinical and environmental isolates, suggesting that the environmental strains may responsible for causing invasive aspergillusis and that RAPD-typing can be a good approach for monitoring the source of infection (community acquired vs. nosocomial). Although the extensive genetic diversity within A. fumigatus isolates makes it difficult to ensure the determination of hospital sources of infection, the isolation of the identical strains from a patient and from the hospital environment indicates that the infection was nosocomially acquired.[7] RAPD-PCR analysis can be applied as a simple, rapid, and useful method, and can also play a remarkable role in typing and differentiating Aspergillus strains. It should be noted, however, that selecting the primers is a critical step to reach the highest level of achievable discriminatory power.

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