ORIGINAL ARTICLE

Characterization of a possible nosocomial aspergillosis outbreak

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Objective To study the epidemiologic aspects of a suspected outbreak of nosocomial invasive aspergillosis.

Methods Sixteen *Aspergillus fumigatus* strains were isolated from bronchoalveolar washings or sputa of 10 patients during a 9-month period. Furthermore, two environmental samples, isolated in a microbiological screening of the hospital, were also available for analysis. Random amplified polymorphic DNA analysis (RAPD) was carried out.

Results The analysis performed by RAPD clearly demonstrated substantial genetic variation among the isolates. Both of the two different primers selected for RAPD analysis (R-108 and AP12h) were able to demonstrate that the strains isolated from all patients infected with the same fungal species and the environmental samples were genotypically distinct. The results by RAPD typing demonstrated that this technique could detect variability among isolates of *Aspergillus fumigatus* from different patients and even from the same patient.

Conclusions RAPD genotyping proved that the outbreak of invasive aspergillosis consisted of a series of events, non-related, and probably not coming from the same source within the hospital. This type of analysis is an easy, quick and highly discriminatory technique that may help in planning epidemiologic studies of aspergillosis.

Keywords *Aspergillus*, typing, RAPD, outbreak

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INTRODUCTION

Aspergillus fumigatus is found universally in soil, water and decaying vegetation. The small size of the spores and their high concentration in the air make *Aspergillus* the most threatening aerial fungal pathogen, especially in those patients undergoing immunosuppressive therapies. Inhalation of conidia and penetration to the distal lung seem to be the primary means of acquiring pulmonary infection [1]. The first immunologic line of defense against *Aspergillus* in the lungs comprises the macrophages, which are capable of ingesting and killing spores, while hyphae are primarily killed by neutrophils. Corticosteroids considerably impair the macrophage killing of *A. fumigatus* spores and the neutrophil and mononuclear cell killing [2]. Because *Aspergillus* is ubiquitous in the air and environment, the primary means of control is by filtering the air to remove all the *A. fumigatus* spores. Although this could be possible within the hospital, this measure is expensive and does not generally include all patients at risk, such as those with AIDS and those receiving corticosteroid treatment [3].

Typing of clinical and ambient isolates could help to provide hints about environmental sources of strains causing outbreaks as well as to provide guidelines for patient management. For epidemiologic studies, the subspecific characterization of isolates is as important as the species-level identification. *A. fumigatus* differentiation by phenotypic methods has a low discriminatory power and is difficult to standardize [4,5]. Since 1990, all efforts have been focused on trying to demonstrate variability at the DNA level. Many DNA-based methods have been developed as an aid to the identification of the source of infection and the means of transmission. These include restriction fragment length polymorphism (RFLP) [5–8], southern hybridization with different repetitive

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sequence-based probes [5,9–15], random amplification of polymorphic DNA using arbitrary primers (RAPD) [5,7,8,13,16–22], isoenzyme analysis (IEA) [5,7], and PCR amplification of ribosomal intergenic spacer sequences [23]. Among these, RAPD has proved to be a quick and easy procedure used in many laboratories with different primer sets [5,7,8,13,16–19,21,22,24].

In our study, 18 *A. fumigatus* isolates from a pneumology unit (clinical and environmental) and eight collection strains of different geographic origin were compared by using RAPD. The genetic relatedness between strains and the possibility of using these techniques in the management of possible hospital outbreaks were assessed. (Preliminary data from this work were presented at the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy.)

MATERIALS AND METHODS

Strains

Sixteen strains of *A. fumigatus* were cultured from bronchoalveolar lavage (BAL) fluid or sputa of 10 patients from a hospital pneumology unit at one hospital in Madrid, obtained during a 9-month period (between September 1996 and June 1997). Two additional strains were obtained from air sampling at the same unit. Eight strains belonging to the mold collection at the Mycology Unit (Instituto de Salud Carlos III), all of them of different geographic origin, were used as control samples for the typing methods. All the strains were identified as *A. fumigatus* by their culture characteristics in appropriate media after growth at 37 °C for 72 h and on the basis of conidiophores and conidia morphologies.

Patients

Eight patients were receiving corticosteroid treatment on the basis of chronic obstructive lung disease. The remaining two had diagnoses of tuberculosis and extrinsic bronchial asthma, respectively. Six of the patients died with the diagnosis of invasive aspergillosis, despite intensive antifungal therapy. Four patients had more than one isolate: patients 1 and 9 had two isolates each, and patients 6 and 10 had three different isolates each.

DNA extraction

After identification, conidia from each strain were inoculated into 3 mL of GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) and they were grown overnight at 37 °C. Mycelial mats were recovered and subjected to a DNA extraction protocol described previously [25]. After RNase treatment, the samples were treated with proteinase K (Sigma, Madrid, Spain), and DNA was then purified by phenol/chloroform extraction and ethanol precipitation. The integrity of the DNA was verified and its concentration determined by running serial dilutions of the DNA in 0.8% agarose gels in comparison with a commercial bacteriophage lambda DNA of known concentration (Pharmacia, Barcelona, Spain). The DNA concentration was adjusted to 5 ng/mL prior to using it for typing methods.

RAPD typing

Primer sequences were as follows: primer R-108 (5′-GTA TTG CCC T-3′) [16] and primer AP12h (5′-CGG CCC CTG T-3′) [26]. The final RAPD reactions included 10 mM Tris-HCl, pH 8, 50 mM KCl, 200 μM each of dATP, dTTP, dCTP and dGTP, 2.5 mM $MgCl_2$, 1 μM primer, 2.5 U of *Taq* polymerase (Perkin-Elmer, Madrid, Spain) and 25 ng of template DNA in a final volume of 50 μL. Amplifications were performed in a thermal cycler (Perkin-Elmer). PCR was performed by using a 40-cycle program consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 36 °C (27 °C for primer R-108) and 2 min of primer extension at 72 °C. The reaction mixtures were held at 4 °C until required. A negative control with all the PCR reagents, except DNA, was always included and used to standardize the conditions of the assay (mainly related to cycle number and annealing temperature). Each amplification was repeated at least twice in order to verify the presence or absence of the scored bands.

Genotyping analysis

The amplified products were electrophoresed through 1.3% agarose gels and stained with ethidium bromide. After intensive washing with distilled water, gels were photographed under UV light. Pictures were digitized into the database of the software package LaneManager (TDI, Madrid, Spain) using a Scanjet scanner with the transparency option (Hewlett Packard, Madrid, Spain). Patterns were unwrapped and normalized against the marker for cross-comparisons. Similarity coefficients (S_{AB}) were computed for each pair of lanes (A and B), based on band positions alone according to the Dice formula $S_{AB} = 2E/2E + (a + b)$, where E is the number of bands in pattern A and B sharing the same positions, a is the number of bands in pattern A with no correlates in pattern B, and b is the number of bands in B with no correlates in pattern A [27]. Dendograms based on S_{AB} values were generated using the unweighted pair-group method with averages (UPGMA) to visualize the relationships between isolates [28].

RESULTS

RAPD typing analysis

Fourteen oligonucleotide primers, composed of 10 bases with a G + C content ranging from 40% to 87%, were tested for their ability to discriminate between genomic DNAs of *A. fumigatus* strains of different geographic origin (control strains). Primers R-108 and AP12h yielded the best RAPD patterns with respect to number, spreading and intensity of the bands. Consequently, they were chosen to be used with all strains for typing analysis. An example of RAPD profiles obtained with primer AP12h with some clinical isolates compared to control strains is given in Figure 1.

Primer AP12h generated some conserved bands that could be detected in the patterns from clinical, environmental and control strains (Figures 1 and 2). The remaining bands showed enough variability to distinguish 14 different genetic profiles out of the 18 strains from the same ward at the hospital (Figure 2 and Table 1). By using primer R-108 to compare the same 18 strains, 14 different types were obtained (Figure 3 and Table 1). Moreover, primer R-108 distinguished both environmental strains (strains 47 and 48, Figure 3) that could not be differentiated by primer AP12h (Figure 2). In contrast, two strains isolated from different patients (strains 34 and 35) were genotypically identical by primer R-108 (Figure 3), but could be differentiated by primer AP12h (Figure 2). Furthermore, the genotypes assigned to the two environmental strains did not match any of the genotypes assigned to the clinical isolates, with either of the two primers used. Therefore, the combination of the results obtained by using both primers showed that all patients were infected or colonized by genotypically different *A. fumigatus* strains.

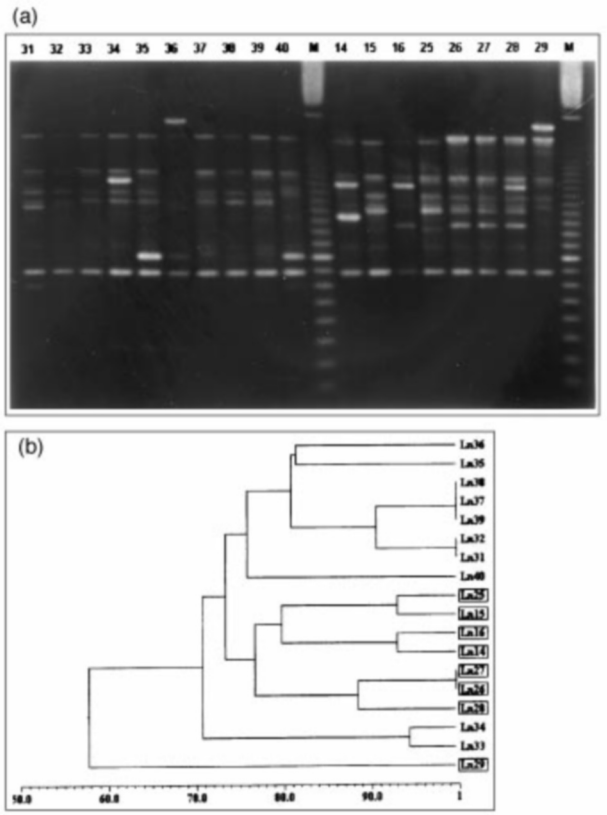

Figure 1 (a) 1.3% agarose gel of the RAPD profiles obtained with AP12h primer from the *Aspergillus fumigatus* isolates (patients 1–7, lanes 31–40) and the control strains of different geographic origin (lanes 14–16 and 25–29). M, molecular markers (100-bp ladder). (b) Dendogram of the RAPD profiles included above. Boxed numbers represent control strains. Percentages of similarity coefficient (S_{AB}) are given below.

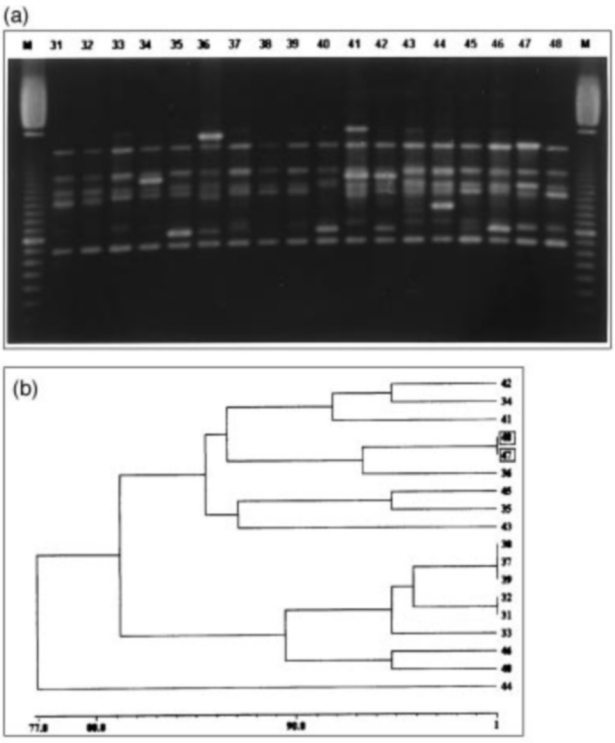

Figure 2 (a) 1.3% agarose gel of the RAPD profiles obtained with primer AP12h of the entire collection of isolates included in this study (lanes 31–48). M, molecular markers (100-bp ladder). (b) Dendogram of the 18 strains based upon the similarity coefficient (S_{AB}) derived from the RAPD profiles generated with primer AP12h. Boxed numbers represent environmental strains. Percentages of similarity coefficient (S_{AB}) are given below.

Table 1 Summary of the PCR profiles of *Aspergillus fumigatus* isolates obtained by RAPD fingerprinting

	RAPD types			
Patient/Isolate	**AP12h**	**R-108**	**Overall types**	
1/31	A	A	AA	I
1/32	A	A	AA	I
2/33	B	B	BB	II
3/34	C	C	CC	III
4/35	D	C	DC	IV
5/36	E	D	ED	V
6/37	F	E	FE	VI
6/38	F	E	FE	VI
6/39	F	E	FE	VI
7/40	G	F	GF	VII
8/41	H	G	HG	VIII
9/42	I	H	IH	IX
9/43	J	I	JI	X
10/44	K	J	KJ	XI
10/45	L	K	LK	XII
10/46	M	L	ML	XIII
Ambient/47	N	M	NM	XIV
Ambient/48	N	N	NN	XV
Total types	14	14	15	

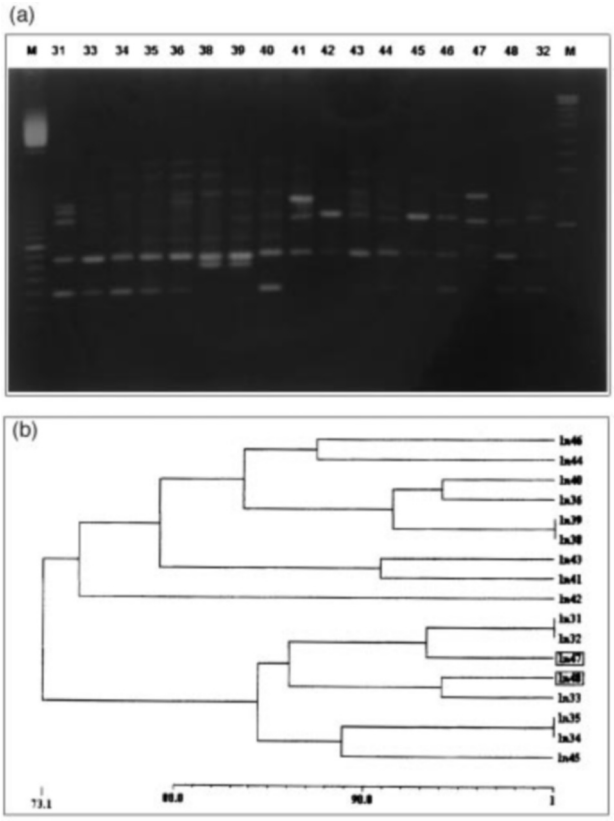

Figure 3 (a) 1.3% agarose gel of the RAPD profiles obtained with primer R-108 of clinical isolates from the 10 patients and both environmental isolates included in the study (lanes 31–48). M, molecular markers (100-bp ladder and 1.0-kb ladder, respectively). (b) Dendogram derived from the RAPD profiles included above. Boxed numbers represent environmental strains. Percentages of similarity coefficient (S_{AB}) are given below.

Four patients had sequential isolates. Strains 31 and 32 from patient 1 exhibited identical patterns, as showed by primer AP12h (Figure 1) and by primer R108 (Figure 3). The same result was obtained for strains 37, 38 and 39 from patient 6 (Figures 2 and 3; the pattern for strain 37 is not shown in Figure 3). The other two patients with repeated sampling harbored sequential strains which were clearly different, as shown by the two primers used (strains 42 and 43 from patient 9, and strains 44, 45 and 46 from patient 10) (Figures 2 and 3).

Table 1 summarizes the types assigned to each of the 18 isolates obtained by the two primers used in the RAPD analysis. The highest level of discrimination was achieved by combining the results of primers R-108 and AP12h (15 types out of 18 strains).

Genetic relationship analysis

The genetic relationship among the strains of this study was analyzed by the Lanemanager software package (TDI), which computed the similarity coefficients (S_{AB}) for every pair of isolates on the basis of its RAPD patterns. A combined dendrogram showing the genetic distance among 10 isolates from patients in the hospital (same geographic origin) and the eight control strains (different geographic origins) generated on the basis of the similarity between the RAPD patterns obtained with primer AP12h is included in Figure 1b. With the exception of strain 29, the control strains were clustered with a mean S_{AB} of 77%. The strains from the same hospital were also clustered, with a mean S_{AB} of about 76%; only two strains from patients at the same hospital co-clustered with the group of control strains. These data showed that the same genetic diversity was obtained for strains isolated in the same hospital and for strains of different geographic origin. Similar results were obtained when primer R-108 was used with the control strains (data not shown). Once it was established that the RAPD method with primers AP12h and R-108 has a high potential to discriminate between strains, the 18 isolates included in the possible outbreak were further analyzed for their genetic relatedness by the software package on the basis of the data from RAPD patterns. The branch points at which the 18 isolates were connected were $S_{AB} = 77\%$ when we used primer

AP12h, and $S_{AB} = 73.1\%$ when the primer used was R-108 (see dendrograms of Figures 2b and 3b). The isolates obtained from a single patient that were considered identical by visual inspection (strains 31 and 32 from patient 1, and strains 37, 38 and 39 from patient 6) were clustered at an $S_{AB} = 100\%$ in all of the dendrograms shown. These data indicated a considerable degree of genetic diversity among the isolates from the same ward at the hospital.

DISCUSSION

Among several other methods, RAPD typing has long been used, with several advantages, such as the small amount of DNA required and the lack of need for sequence information. When using the appropriate primers, this procedure yields different profiles of PCR products, each of them with the potential of detecting polymorphism between strains. The issues of reproducibility and the difficulty in the interpretation of the band patterns are the major limitations generally attributed to RAPD. However, here we have used two primers that partially overcome these problems. The primer R-108 has been widely used before with *A. fumigatus*, and its value in the molecular characterization of strains of this species has been described elsewhere [7,8,13,16,19]. Nevertheless, there is no consensus concerning the PCR profiles obtained with this primer when used by different authors in different laboratories. For this specific primer, we lowered the annealing temperature to 27 °C in an attempt to obtain a pattern of DNA fragments that could differentiate between isolates. Most other laboratories used temperatures between 30 °C and 36 °C, and this might be the explanation for the differences found between our results and those published by others [7,8,13,16,19]. The primer AP12h [26] has never been used before, either with *A. fumigatus* or with any other filamentous fungi. It was selected on the basis of its discriminatory power and because it yields patterns with a range of DNA fragment sizes amenable to computer-aided analysis. These two primers showed a similar discriminatory power, but the highest discrimination was achieved by a combination of data generated by both of them. By using this approach we could assign different DNA types to each of the strains included in the suspected outbreak. Therefore, we emphasize the convenience of using at least two primers for RAPD typing.

Furthermore, none of the patients shared the same *A. fumigatus* type with any of the two environmental isolates. Because of the retrospective nature of this study and the reduced number of environmental samples, we cannot exclude a nosocomial origin for some strains isolated from patients. Air-contaminating conidia were probably the source of infection, but is difficult to know whether it was a community-acquired or a nosocomial infection. Since the concentration of specific strains may fluctuate rapidly, a hospital-acquired aspergillus infection cannot be excluded, even if the infected strain is not found in the hospital environment [21,22]. Some studies of air contamination have shown that *A. fumigatus* spores change dramatically between each measurement, and a transient increase of the spores may be missed even under weekly sampling [22].

The availability of knowledge of the genetic relatedness between clinical and environmental isolates, early in the course of a possible outbreak of invasive aspergillosis, will help in the search for a common source. The computer-assisted method also showed that the genotypes of the clinical isolates were not more closely related to each other than the genotypes of strains of different geographic origin. Previous epidemiologic studies have investigated this matter, and the results pointed to the absence of clustering of the strains on the basis of their geographic origin [14,29]. The high diversity of *A. fumigatus* has been described before [12–15] in studies in which practically any new case of aspergillosis appeared to be due to a different isolate. The high intraspecies variability seen at the genomic level and by different techniques had led to the suggestion that any strain could be a pathogen, whether it comes from the environment or from within the hospital. As more typing methods become available and more strains of *Aspergillus* are typed, some questions are being answered. While the majority of patients with aspergilloma and/or those with aspergillosis are probably infected by a single strain, those patients with cystic fibrosis seem to be colonized by more than one single strain [3,11,12,14,18]. In our study, although a reduced number of patients was available, it was found that patients harboring sequential isolates could have the same or different strain. Interestingly, the patients with fatal outcomes always had the same strain, whereas those surviving had different ones. The interpretation of this fact is very difficult, mainly because of the retrospective nature of the investigation and the small number of patients. However, this approach should be investigated in prospective studies.

We conclude that this technique has a considerable level of reproducibility within laboratories and can be a reliable typing tool for detecting possible outbreaks, particularly due to its simplicity and speed. From the results obtained in the present work, we inferred that the use of one primer alone (even if its discriminatory power is very high) could yield confusing results, as two strains could be considered the same when this is not the case. Therefore, the use of at least two RAPD primers should give enough discriminatory power, as variations in different regions of DNA are explored. RAPD genotyping suggested that the outbreak of invasive aspergillosis consisted of a series of events, non-related, and probably not originating from the same place within the hospital. However, the absence of identity between genotypes found in patients and those found in their environment should not exclude a nosocomial origin for an infection. Data involving typing of environmen-

tal aspergillosis indicated that each patient is surrounded by an extremely different population of strains, and therefore, during outbreaks of aspergillosis, multiple patients are rarely infected by the same strain [15].

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