Identification of *Aspergillus fumigatus* by PCR

Marta Eulalia García[1], José Luis Blanco[1] and Viswanath P. Kurup[2]

[1]Dpto Patologia Animal I , Facultad de Veterinaria, U.C.M., 28040 Madrid , Spain and [2]Allergy-Immunology Division, Department of Medicine, Medical College of Wisconsin, and Zablocki Veterans Administration Medical Center, Milwaukee, Wisconsin, USA

Summary The specifity of gene encoding the ribotoxin protein in *Aspergillus fumigatus* was determined by PCR amplification of a portion of the gene. All *A. fumigatus* strains studied showed the presence of amplifiable ribotoxin product, while none of the other fungal species, with the exception of *Aspergillus restrictus,* showed this amplification product. Hence, this method may be used for rapid and specific identification of *A. fumigatus*.

Key words *Aspergillus fumigatus*, Mitogillin, Polymerase Chain Reaction, Ribotoxin

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Resumen En el presente estudio se determinó la especificidad del gen que codifica la proteína ribotoxina en *Aspergillus fumigatus* utilizando la amplificación de una parte del gen por la reacción en cadena de la polimerasa. Todas las cepas de *A. fumigatus* investigadas mostraron la presencia del producto amplificado, mientras que ninguna de las otras especies fúngicas lo mostró, con la excepción de *Aspergillus restrictus*, que también presentó el mencionado producto de amplificación. Por tanto, esta metodología podría ser usada para la identificación rápida y específica de *A. fumigatus*.

Palabras clave *Aspergillus fumigatus*, Mitogilina, PCR, Ribotoxina

Aspergillus fumigatus, an ubiquitous fungus that can survive all climates and extremes of temperatures, is frequently implicated in a number of diseases in humans [1,2]. Several recent studies emphasized the existence of strain differences and pathogenic propensities among these fungi. Diversity of antigens in different strains of *A. fumigatus* have been reported and probably play a role in inducing allergic reactions [1]. The enhanced pathogenicity of certain strains of *A. fumigatus* associated with invasive aspergillosis has been attributed to the production of proteolytic enzymes by these strains [3,4]. However, conclusive evidence to substantiating the involvement of specific *A. fumigatus* strains in different clinical conditions is lacking.

Several species belonging to the genus *Aspergillus* are morphologically indistinguishable. Currently, the identification of *A. fumigatus* is based on morphological characteristics. The occurrence of variants is of particular interest, which do not consistently show all the diagnostic characteristics of the species. Mutants of *A. fumigatus* lacking conidia formation and pigmentation have been described, thus presenting difficulties in the identification. By identifying unique genes encoding frequently secreted proteins, speciation of the organism can be achieved faster.

Several genes from *A. fumigatus* and related species have been cloned and sequenced. One gene encoding a ribotoxin has been isolated from several species of *Aspergillus*, including *A. fumigatus*, *Aspergillus restrictus*, and *Aspergillus giganteus* [5-8]. Using carefully designed primers, a fragment of the gene which encodes the species-specific riboproteins was amplified.

With this in view, we have studied the *Asp fI* gene in several strains of *A. fumigatus*, various species of *Aspergillus* and other fungi by PCR amplification and electrophoretic analysis. The gene *Asp fI* was previously cloned and sequenced [8,9].

MATERIAL AND METHODS

Strains and species. In the present study, 10 strains of *A. fumigatus* from different sources were used: two-from patients with invasive aspergillosis (AF-103, AF-125), one from a patient with aspergilloma (AF-118), two from the environment (AF-101, AF-126), and five from children with cystic fibrosis having allergic bronchopulmonary aspergillosis (CF-001, CF-014, CF-020, CF-039, CF-044). We have also studied additional *Aspergillus* species and other fungal species, including *A. giganteus*, *Aspergillus fischerii*, *A. restrictus*, *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, and *Penicillium notatum*. These strains were maintained in our culture collection and all strains demonstrated characteristic colo-

Dirección para correspondencia:
Dr. José L. Blanco Cancelo
Departamento Patología Animal I, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain
Tel.: +34 1 3943717; Fax: +34 1 3943908
E-mail: jlblanco@eucmax.sim.ucm.es

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nial and microscopic morphology in standard culture media.

DNA extraction. DNA was extracted according to established methods [10,11], using 3 to 4 days-old cultures of the fungi in Sabouraud's Dextrose Agar (Difco, USA) incubated at 37°C. One ml (10^6) conidial suspension of each strain in sterile PBS was inoculated into 125 ml Sabouraud's dextrose broth (Difco), in 250 ml Erlenmeyer flasks and shaken at 37°C at 150 rpm for 72 h. Mycelium was harvested by filtration, washed three times with cold PBS, and twice with cold distilled water. The mycelial mass was dried between filter papers and weighed. Ten ml of lysis buffer (10 mM Tris-HCl pH=7.4, 10 mM sodium chloride, 10 mM EDTA) per gram of mycelium, 1% sodium dodecyl sulphate, and 200 mg/ml proteinase K were added to the mycelium. This was mechanically ground in a mortar and transfered into a 50 ml polypropylene tube. The mixture was then incubated for 1 h at 50°C.

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the mixture centrifuged for 15 min at 16,000 g. The aqueous phase was collected, mixed with an equal volume of chloroform:isoamyl alcohol (24:1), and centrifuged. The aqueous phase was then mixed with 1/10 volume of 2 M sodium acetate and two volumes of cold ethanol. The DNA was precipitated overnight at -20°C, then collected by centrifugation at 16,000 g for 15 min at 4°C, and washed with 70% cold ethanol. The precipitate was dried at room temperature and resuspended in TE buffer (10 mM Tris-HCl pH=7.6, 1 mM EDTA). The quality of DNA was assessed by the absorbance ratio at 260/280 nm and the concentration estimated from the absorbance at 260 nm.

Gene amplification by polymerase chain reaction (PCR). The DNA extracted from the different strains of *A. fumigatus* and other fungi was used as PCR template for evaluating the specificity of the fragment of the gene encoding the ribotoxin protein.

Two primers amplifying a fragment of 315 base pair size of the *Aspf I* gene was designed. The primers are: Oligo 1, sequence 5' TGG ACA TGT ATA AAC CAG 3', and Oligo 2, sequence 5' GTC AAA CTT ATA GTC GTG 3'. The PCR was carried out in a thermocycler (Perkin-Elmer Corp., USA), using a step cycle program with the following temperature profile: 20 sec at 94°C, 20 sec at 63°C, and 20 sec at 72°C with one second increment on subsequent cycles.

Agarose gel electrophoresis of DNA. The amplified DNA was analyzed by electrophoresis on 2% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 20 mg ethidium bromide [11]. In a few instances, the Southern blots were probed with 32P labeled internal oligonucleotide CCA CTA CCT GCT GGA GTT CCC AAC in order to demonstrate the specificity of the amplified DNA fragment [11].

RESULTS

The presence of *Asp fI* gene was demonstrated in all the 12 strains of *A. fumigatus*, as evidenced by the appearance of a 315 base pair size DNA fragment in PCR products (Figures 1 and 2). The PCR product of the gene of one *A. fumigatus* strain from cystic fibrosis (CF-001) showed a weak band in the gel.

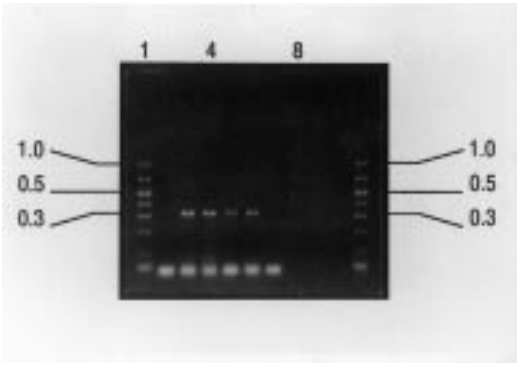

Figure 1. Agarose gel electrophoresis of PCR products. Lanes: [1] DNA marker; [2-6] *A. fumigatus* strains from cystic fibrosis: CF-001, CF-014, CF-020, CF-039, CF-044; [7] Blank.

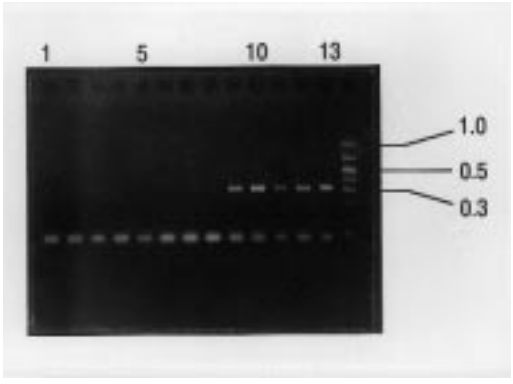

Figure 2. Agarose gel of PCR products. Lanes [1] *A. giganteus*; [2] *A. fischerii;* [3] *A. restrictus*; [4] *A. niger*; [5] *A. flavus*; [6] *C. albicans*; [7] *P. notatum*; [8] Blank; [9-13] *A. fumigatus* strains: AF-101 (environmental), AF-103 (invasive aspergillosis), AF-118 (aspergilloma), AF-125 (invasive aspergillosis), AF-126 (environmental).

DISCUSSION

It is interesting to note that *A. fischerii*, *A. niger*, and *A. giganteus* failed to show amplification of *Asp fI* gene. Although ribotoxins from *A. giganteus* showed DNA homology to *A. fumigatus*, no amplification of the PCR product was detected, while *A. restrictus* showed only a weak band. Thus, the results indicate that the *Asp fI* gene is an uniquely significant gene in *A. fumigatus* species and the amplified fragments is highly specific.

It is interesting to note that even though mitogillin gene is closely related to *Asp fI* gene, there was a significant difference in the amplified product using *Asp fI* primers.

Although PCR amplification of *A. fumigatus* DNA has been used for the diagnosis of invasive aspergillosis [12], this method has not been effectively used in the identification of the organism. This finding indicated that PCR can be of use in early identification of strains of *A. fumigatus*. However, the current study has limitations due to the fewer number of species investigated. More strains and species originating from different sources and clinical conditions may be studied before using the *Asp fI* gene as the marker gene for PCR studies.

Recently Makimura *et al.* [13] have described a

PCR methodology to detect a wide range of medically important fungi, and also the possibility to detect *A. fumigatus* infections [14].

In conclusion, PCR method will be of considerable value in the early specification and identification of *A. fumigatus.* Our results demonstrate that the used method is sensitive and rapid for the identification of all strains of *A. fumigatus* studied, irrespective of their origin.

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