Immunochemical reactivity of *Aspergillus fumigatus* antigens from different sources

Marta-E. García, José L. Blanco and Viswanath P. Kurup

Allergy-Immunology Division, Department of Medicine, Medical College of Wisconsin and Zablocki Veterans Administration Medical Center, Milwaukee, Wisconsin, USA.

Summary

We have studied the immunochemical and biochemical differences in 12 *Aspergillus fumigatus* strains isolated from different sources. The enzymatic activity of all these strains were studied by a rapid enzyme detection method (API-ZYM). One of 12 strains studied produced alkaline phosphatase, while two produced chymotrypsin, and three produced trypsin.
By SDS-PAGE we studied proteins present in the antigen extracts from all 12 strains. Several of the protein bands were unique and may be used to differentiate the strains. One such protein is the 58 kDa band present in the mycelial extract and the 33 kDa in the culture filtrate.
By crossed immunoelectrophoresis, differentiation of the strains isolated from cystic fibrosis patients can be made based on a few specific precipitin arcs developed against anti-*Aspergillus* rabbit serum.

Key words

Aspergillus fumigatus, Enzymatic activity, SDS-PAGE, Crossed immunoelectrophoresis, Antigens

Reactividad inmunoquímica de antígenos de *Aspergillus fumigatus* aislados de diferentes orígenes

Resumen

En el presente trabajo hemos estudiado las diferencias inmunoquímicas y bioquímicas en 12 cepas de *Aspergillus fumigatus* aislados de diferentes orígenes. La actividad enzimática de estas cepas fue estudiada usando un método rápido de detección enzimática (API-ZYM). Una de las 12 cepas produjo fosfatasa alcalina, dos produjeron quimotripsina, y tres produjeron tripsina.
Por SDS-PAGE, estudiamos las diferentes proteínas presentes en los extractos antigénicos de las 12 cepas. Algunas de estas bandas proteicas fueron únicas, y podrían ser usadas en la diferenciación de cepas de *Aspergillus*. Una de tales proteínas corresponde a la banda de 58 kDa presente en el extracto miceliar, y otra de 33 kDa presente en el filtrado de cultivo.
Por inmunoelectroforesis cruzada, se puede llegar a una diferenciación de las cepas aisladas de pacientes con fibrosis quística basándose en unos pocos específicos arcos de precipitación desarrollados frente a suero hiperinmune anti-*Aspergillus* obtenido en conejo.

Palabras clave

Aspergillus fumigatus, Actividad enzimática, SDS-PAGE, Inmunoelectroforesis cruzada, Antígenos

Aspergillus fumigatus is an ubiquitous thermotolerant fungus associated with a number of diseases in humans and animals. Variations among the strains of *A. fumigatus* have been reported, although no confirmed association of strains of *A. fumigatus* and clinical forms of disease has been established. Delineation of isolates of *A. fumigatus* would be useful in ascertaining the source of outbreaks of infection, and the predictability of isolates recovered from different clinical diseases caused by *A. fumigatus*. However, no such acceptable method for distinguishing strains yet exists.

Dirección para correspondencia:
Dr. José L. Blanco
Dpto. Patología Animal I, Facultad de Veterinaria,
Universidad Complutense, 28040 Madrid, Spain.
Tel. (+34 1) 394 3717, Fax: (+34 1) 394 3908
E-mail: jlblanco@eucmvx.sim.ucm.es

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Although individual isolates of *A. fumigatus* are distinguishable by phenotypic characteristics, most of these are not suitable for development as a typing system [1].

Phenotypic variability within isolates of *A. fumigatus* has been demonstrated in terms of gross morphology [2], susceptibility to applied *Hansenula* killer toxins [3], isozyme electrophoresis with respect to esterase and phosphatase mobility [4], and immunoblot fingerprinting [5]. None of these techniques has been developed as a formal typing system because either the degree of discrimination is low or standardization is difficult [6]. Ubiquinone systems and electrophoretic comparison of enzymes have also been used as aids in the precise identification of members of the genus *Aspergillus* [7].

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 [8].

A number of fingerprinting systems based on genomic differences have been applied to the fungi, including restriction fragment length polymorphism (RFLP) patterns [1], electrophoretically separated chromosome patterns [9], randomly amplified polymorphic DNA patterns [8], Southern blots probed with ribosomal [10] or mitochondrial [11] DNA, and Southern blots probed with moderately repetitive, nonribosomal genomic sequences [12].

A number of biotyping methods have been used to asses the relatedness of strains of *A. fumigatus*: separating polypeptides by polyacrylamide gel electrophoresis, using Western blots [5], and determining sensitivity to yeast killer factor [3]. These biotyping methods have proven relatively ineffective since they rarely provide the level of discrimination necessary for assessing strain relatedness [13].

Variation in the antigenic components was described with different batches of antigens prepared from the same strain [14]. In contrast, Odds *et al.* [15] reported qualitative similarity of all batches of antigens from *A. fumigatus* but considered that the variability was only quantitative in nature. From the results of a number of studies it has been concluded that simultaneous use of several different *A. fumigatus* antigen preparations and serological methods might help eliminate both false-positive and -negative results, although such procedures are cumbersome and time-consuming [16].

Because of the tremendous variations detected among the various strains of *A. fumigatus*, we decided to investigate the immunochemical differences among the strains. With this in view, we have studied 12 strains of *A. fumigatus* isolated from different sources for their enzyme profile by a rapid method of enzymatic determination (API-ZYM), protein profile by SDS-PAGE, reactivity with anti-*A. fumigatus* rabbit serum by crossed immunoelectrophoresis.

MATERIALS AND METHODS

Strains of *A. fumigatus*. In the present study, we used 12 strains of *A. fumigatus* from different origins: three from patients with invasive aspergillosis (AF-102, AF-103, AF-125), two from patients with aspergilloma (AF-118, AF-120), two from environment (AF-101, AF-126), and five from children with cystic fibrosis (CF-001, CF-014, CF-020, CF-039, CF-044). These strains have been maintained in long term storage in our culture collection.

Enzymatic activity of the strains. Enzymatic activity was assayed using API-ZYM strips (Analytab Products, USA.). Each *A. fumigatus* strain was grown in Czapek-Dox Broth medium (Difco Laboratories, USA) at 37°C for 24 h in aerated culture. By this time, spores had germinated and minute granular colonies had formed. We took liquid directly from this medium to inoculate API-ZYM strips which were incubated at 37°C for 4 h. The results were evaluated following the recommendations of the manufacturer.

***A. fumigatus* antigen**. *A. fumigatus* antigen were prepared as previously described [17, 18].

Both culture filtrate and mycelial extract were prepared as follows:

Culture filtrate antigens (CF): The organism was grown in synthetic broth (1/2 AOAC Broth (Difco Laboratories, USA) + 1/2 Czapek-Dox Broth (Difco Laboratories, USA) + 0,7 % Dextrose) for three weeks at 37°C in stationary culture. The broth was separated from the mycelium after the incubation period, dialyzed extensively against deionized water, filtered through a 0,45 µm membrane and finally freeze dried.

Mycelial extracts antigens (ME): The organism was grown in the same synthetic broth for 96 h in aerated culture. After the incubation period, mycelium was obtained by filtration, washed three times with PBS and homogenized in a french press (S.L.M. Instruments, USA) at 10.000 p.s.i. Microscopic examination of the preparation revealed less than 80% breakage of the hyphae. The extract obtained was centrifuged at 10.000 rpm for 30 min, and the supernant collected and dialyzed extensively against deionized water. The retentate was filtered through a 0,45 µm membrane and freeze dried.

Characterization

1. *Protein content*. Both antigens were evaluated for their protein content by the BCA protein assay (Pierce, USA).

2. *Protein in sodium-dodecyl-sulphate polyacrylamide gel electrophoresis*. A 15% polyacrylamide gel of 0,75 mm thickness was used to study the protein profile using the Mini Protean II electrophoresis system (BioRad, USA). Samples were boiled in a sample buffer before applying to the gel and running the gel at 200 V for 45 min. The gel was stained with Coomassie blue and dried as described previously [19].

AMBIS Optical imaging system was used to acquire the SDS-PAGE profile via a CCD camera before analyzing the protein bands using a software package (AMBIS, USA).

The different lanes were scanned into the data file and normalized. The band positions were identified and the molecular weight automatically calculated by comparison with the lane representing known molecular weights markers.

3. *Crossed immunoelectrophoresis.* Crossed immunoelectrophoresis was carried out in 1% agarose gel cast on gel bound film (Marine Colloids, USA) as described previously [20]. Briefly, antigens were electrophoresed at 10 V/cm and 20 mA/gel for 1h. Rabbit anti-*A. fumigatus* serum was mixed with agarose to a final concentration of 10%, and second dimension electrophoresis was carried out at 3 V/cm and 7 mA/gel overnight at 18°C. The gels were transfered to saline solution and washed for 24 h. The gels were then washed with distilled water, then pressed between filter papers, and stained with Coomassie brilliant blue. The developed precipitin arcs were compared.

RESULTS

Enzyme profile

The enzyme profiles of the different *A. fumigatus* strains are shown in table 1. All the strains studied were positive for esterase, esterase lipase, valine arylamidase, acid phosphatase and α-galactosidase by the API-ZYM method. One strain gave positive alkaline phosphatase reaction. This had been isolated from a case of cystic fibrosis.

Protein profile

The proteins in the mycelial extract antigens and the SDS-PAGE profiles are shown in table 2. The following Coomassie stained bands were seen in all the strains: 100, 80 and 35 kDa, while 70 kDa appeared in all except CF-044, 27 kDa in all except AF-126 and 18 kDa in all strains except AF-102. Based on the intensity of the bands, three major antigens were detectable in the present

Table 1. Enzymatic activity detected by API-ZYM in twelve strains of *A. fumigatus.*

Enzymes	AF-101	AF-102	AF-103	AF-118	AF-120	AF-125	AF-126	CF-001	CF-014	CF-020	CF-039	CF-044
Phosphatase alkaline	-	-	-	-	-	-	-	+	-	-	-	-
Lipase (C 14)	+	+	-	-	+	+	-	-	+	+	-	-
Leucyne arylamydase	+	+	+	-	+	+	+	+	+	+	+	+
Valine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+
Cystine arylamidase	-	-	+	-	-	+	+	-	+	+	+	+
Trypsin	-	-	-	-	-	-	-	+	+	-	-	+
Chymotrypsin	-	-	-	+	-	-	-	+	-	-	-	-
Naphtol-AS-BI-phosphohidrolase	+	+	+	+	+	+	+	+	-	+	-	+
Beta-galactosidase	+	+	-	+	+	+	+	+	+	+	-	+
Beta-glucuronidase	+	-	+	+	+	+	+	-	-	+	+	-
Alfa-glucosidase	-	-	+	-	+	-	-	-	+	-	-	-
Beta-glucosidase	-	+	+	+	-	-	-	+	+	+	+	+
N-acetyl-beta-glucosaminidase	+	+	+	+	+	+	+	+	+	-	+	+
Alfa-mannosidase	-	-	-	-	-	+	-	-	-	-	+	-
Alfa-fucosidase	+	-	-	+	-	-	-	-	-	-	-	-

Table 2. Different protein bands detected in the mycelial extract of 12 strains of *A. fumigatus* by SDS-PAGE.

Band	AF-101	AF-102	AF-103	AF-118	AF-120	AF-125	AF-126	CF-001	CF-014	CF-020	CF-039	CF-044
100 KDa	+	+	+	+	+	+	+	+	+	+	+	+
80 KDa	+	+	(+)	+	(+)	(+)	(+)	(+)	(+)	+	(+)	(+)
75 KDa	-	-	+	-	-	-	-	-	-	-	-	-
70 KDa	+	+	+	+	+	+	+	+	+	+	+	-
58 KDa	-	-	+	-	+	+	-	+	(+)	(+)	-	(+)
45 KDa	+	(+)	+	-	+	+	(+)	+	+	-	(+)	
35 KDa	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
30 KDa	-	-	-	+	-	-	-	-	-	-	-	-
27 KDa	+	+	+	(+)	(+)	(+)	-	(+)	(+)	(+)	+	+
25 KDa	-	-	+	-	-	-	-	-	-	-	-	-
22 KDa	(+)	+	-	-	+	+	+	+	-	-	-	-
20 KDa	+	-	-	-	-	-	-	-	-	-	-	-
18 KDa	(+)	-	(+)	+	+	+	+	+	+	+	+	+
15 KDa	+	(+)	(+)	-	-	-	+	-	-	+	+	+
6 KDa	-	-	+	-	-	-	+	-	-	-	+	+
4,5 KDa	-	-	-	-	-	-	-	-	-	-	-	+
3,5 KDa	-	-	-	-	-	-	-	-	-	-	+	+

(+): Dominant band

study which include 35, 27 and 80 kDa proteins.

In table 3 are shown the protein bands present in culture filtrate antigen of *A. fumigatus* strains. The following bands were present in antigens from all strains: 150, 100, 80 and 18 kDa. The 58 kDa was present in all strains except CF-014, 33 kDa in all strains except AF-103, and 15 kDa in all except AF-126.

Crossed immunoelectrophoresis

The results of crossed immunoelectrophoresis are shown like arbitrarily enumerated bands, in table 4 by mycelial extract and in table 5 by culture filtrate. In figure 1 we show one example of the peak obtained in one ME and in figure 2 to CF. Two precipitin arcs were visible with all strains when reacting with rabbit antiserum. There was a quantitative difference as evidenced by the peak heights.

DISCUSSION

The enzyme profiles obtained by the API-ZYM method were statistically analyzed using a value of 1 to a positive and 0 to a negative reaction. Using Student's t test analysis, the 12 strains could be divided into three groups:

Group I: AF-101, AF-103, AF-118, AF-120, CF-020, CF-044, Group II: AF-102, AF-126, CF-039, and Group III: Af-125, CF-001, CF-014.

Of the several enzymes detected in *A. fumigatus* antigens, chymotrypsin-like activity was reported to be specific for diagnosis of aspergilloma [21]. This enzyme is an exocellular fibrinogenolytic enzyme [22], that produce circulating antibodies in the sera of aspergilloma patients [23,24]. This enzyme could be detected in two of the 12 strains investigated: one came from a patient with aspergilloma and the other from cystic fibrosis. All three strains producing trypsin, namely CF-001, CF-014 and CF-044, were isolated from cystic fibrosis, which suggested that this enzyme might play a role in the development of ABPA in cystic fibrosis.

Thus, the use of API-ZYM could be useful in the detection of pathogenic strains in the *A. fumigatus* complex. However, additional strains need to be studied before a final conclusion can be made.

In our studies of the protein profiles, the dominant bands present in culture filtrate antigen that we consider specially interesting are 18 kDa and 20 kDa. On comparison of CF and ME protein profile, no distinguishable groups were detected.

Fratamico *et al.* [25] described a dominant 58 kDa band in the mycelial extract that may be present in the *A. fumigatus* cell walls. This may be a glycoprotein or some form of glycogalactomannan-protein conjugate.

A band of 33 kDa in the culture filtrate preparation has been described as a prominent band in *A. fumigatus* species, may probably represent chymotrypsin [22]. Nevertheless, Hearn [26] indicated that this band is not

Table 3. Different protein bands detected in the culture filtrate of 12 strains of *Aspegillus fumigatus* by SDS-PAGE.

Band	AF-101	AF-102	AF-103	AF-118	AF-120	AF-125	AF-126	CF-001	CF-014	CF-020	CF-039	CF-044
150 KDa	+	+	+	+	+	+	+	+	+	+	+	+
100 KDa	+	+	+	+	+	+	+	+	+	+	+	+
80 KDa	+	+	+	+	+	(+)	(+)	(+)	+	+	+	+
65 KDa	-	+	-	-	-	-	-	+	-	-	-	-
58 KDa	+	+	+	+	+	(+)	+	(+)	-	(+)	(+)	(+)
42 KDa	-	-	-	-	-	-	-	-	-	+	-	-
40 KDa	+	-	-	-	+	+	-	+	+	(+)	+	-
35 KDa	-	-	-	-	(+)	-	-	-	+	-	-	-
33 KDa	+	+	-	(+)	+	+	(+)	+	(+)	+	+	+
27 KDa	+	-	+	+	+	(+)	+	+	+	(+)	-	+
26 KDa	+	-	-	-	-	-	+	+	-	-	+	-
22 KDa	+	-	-	-	+	-	-	-	-	-	-	-
21 KDa	+	+	-	-	+	+	+	+	+	+	-	+
20 KDa	+	(+)	(+)	+	-	-	-	+	(+)	(+)	(+)	(+)
18 KDa	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
17 KDa	-	-	-	(+)	(+)	+	-	-	-	-	-	-
15 KDa	(+)	+	(+)	(+)	(+)	(+)	-	(+)	+	(+)	(+)	(+)
14 KDa	-	-	(+)	+	-	-	-	+	+	(+)	+	+
12 KDa	+	-	-	-	+	-	-	-	-	-	-	-
10 KDa	(+)	-	+	-	+	(+)	(+)	(+)	+	-	+	-
9 KDa	-	-	-	-	-	-	-	+	(+)	+	-	+
8 KDa	(+)	+	+	(+)	-	-	+	+	-	-	-	-
6 KDa	+	-	(+)	-	-	+	-	-	-	-	-	-
5 KDa	-	-	-	-	-	+	-	+	-	+	+	-
4 KDa	(+)	+	+	+	+	-	+	-	-	+	+	+
2 KDa	+	(+)	+	-	-	-	+	+	-	+	+	-

(+): Dominant band

Table 4. Different peaks detected in the mycelial extract of 12 strains of *A. fumigatus* by crossed-immunoelectrophoresis.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
AF-101	+	+	+	+	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-
AF-102	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-
AF-103	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-
AF-118	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+
AF-120	+	+	+	-	-	-	+	+	-	+	-	+	+	-	+	-	-	-	-
AF-125	-	+	+	-	+	-	+	+	+	-	-	+	-	-	+	-	+	-	+
AF-126	+	+	+	+	-	+	+	+	-	-	-	+	+	-	+	-	-	-	-
CF-001	-	+	+	+	+	-	+	+	+	-	-	-	-	-	+	-	-	+	+
CF-014	-	+	+	-	+	-	+	+	+	-	+	+	+	-	-	-	+	-	-
CF-020	-	+	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-	+	-
CF-039	-	+	+	-	+	-	+	+	-	-	-	-	-	-	+	-	-	+	-
CF-044	+	+	-	+	-	-	+	+	-	-	+	+	-	-	-	-	+	-	-

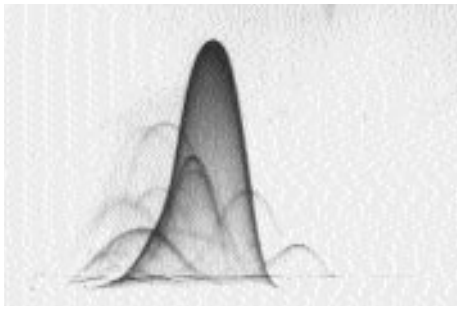

Figure 1. Crossed immunoelectrophoresis gel of the mycelial extract of the strain AF-118.

Table 5. Different peaks detected in the culture filtrate of 12 strains of *A. fumigatus* by crossed-immunoelectrophoresis.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AF-101	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-
AF-102	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-
AF-103	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-
AF-118	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-
AF-120	+	+	+	+	-	-	+	+	+	-	-	+	+	-	-	-
AF-125	+	+	+	+	+	+	-	+	+	-	-	+	-	-	-	-
AF-126	+	+	+	+	-	-	+	+	+	-	+	+	+	-	-	-
CF-001	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+
CF-014	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-
CF-020	+	-	-	+	+	+	+	-	+	-	+	+	-	-	-	-
CF-039	+	+	+	-	-	-	+	+	-	-	-	+	+	+	+	-
CF-044	+	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-

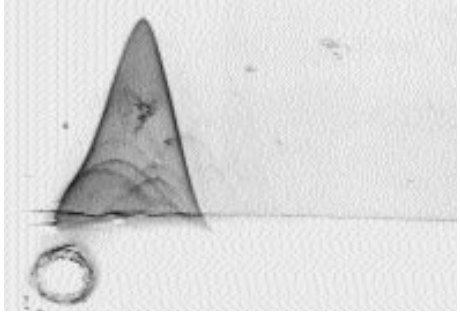

Figure 2. Crossed immunoelectrophoresis gel of the culture filtrate of the strain CF-020.

present in all the strains of *A. fumigatus*. In the present study, we noticed this as a dominant band in most of the strains studied.

Lamy *et al.* [27] demonstrated a mitogillin secreted by *A. fumigatus* in urine of patients with invasive aspergillosis. This correspond with a band size of 18 kDa, detected in most strains of *A. fumigatus* and may contribute towards the pathogenicity of the *A. fumigatus* strain [28].

Crossed immunoelectrophoresis is a very simple methodology and can be used to asses the antigenicity of strains and together with other findings may be of use in typing strains from different sources.

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