Serological diagnosis of pulmonary aspergillosis*

S. Sundaram[1], R. Kalyanasundaram[1]† & V. Rangaswamy[2]
[1]*Centre for Advanced Study in Botany, University of Madras, Madras-600 005, India*
[2]*Government Tuberculosis Sanatorium, Tambaram Sanatorium, Madras-600 047, India*

Abstract

Methods for the preparation of antigens from clinically isolated cultures of *Aspergillus* were standardized. Sera from 25 suspected cases of pulmonary aspergillosis were tested against antigens prepared by us, from 4 strains of *A. fumigatus* and one strain of *A. flavus*, using the Ouchterlony double diffusion and immunoelectrophoretic techniques.

Of the 25 sera tested, 18 reacted positively with antigens of *A. fumigatus*, one with *A. flavus* and 2 with both these species. Antigens of two non-pathogenic Aspergilli included in the study failed to react with any of the sera. Our antigen preparations gave more numerous as well as sharper precipitin lines than the commercial Bencard antigens which were used for comparison. Moreover, mycelial antigens from 48 to 96 h old cultures revealed precipitin lines comparable to that of the routine, 4 week old culture filtrate antigens, thus suggesting that the incubation period for obtaining antigens could be cut down considerably.

Introduction

Aspergillus organisms have been associated with human disease as tissue invaders, cavity colonizers and antigenic stimulators of the immune system leading to hypersentivity syndrome. Of the 150-odd species, relatively few have been ascribed to disease-producing organisms. *A. Fumigatus* Fresenius is the species usually associated with disease, though other *Aspergillus* species, such as *A. flavus* Link and the niger group (*'A. niger'*) have been described as possible causal organisms (3, 5, 13). Several cases of pulmonary aspergillosis reports from India show that *Aspergillus fumigatus, A. flavus* and *A. niger* are the dominant pathogenic species and among these *A. fumigatus* is considered to be the chief causal agent of this disease (3, 11, 12, 13).

In the diagnosis of aspergillosis, culture of the sputum may not always be reliable since there is often no communication between the aspergilloma and the bronchi. The presence of precipitating antibodies against extracts of organisms of the genus *Aspergillus*, and the species *A. fumigatus* in particular, in the sera of patients suffering from pulmonary aspergillosis was reported by Pepys *et al.* (9). With the advancement in serological methods, Ouchterlony's double diffusion test has been increasingly used in the diagnosis of pulmonary aspergillosis (1, 3, 6).

Even though pulmonary aspergillosis is apparently common in India, facilities for sero diagnosis are generally lacking. The required antigens have to be imported. The present study was therefore undertaken with a view to prepare standard antigens to test the sera of suspected cases of pulmonary aspergillosis. Strains of the species *A. fumigatus* and *A. flavus*, being known to be pathogenic, were mainly used. The antigens were compared with commercial Bencard antigens (Bencard, Brentford,

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Table 1. Source of fungi and types of antigens used.

No.	Fungi	Source		Type of antigen		% of protein	
		Accession No.	Obtained from	Isolated from	(CFT) 4 wk	(ME) 24 h	CFT	ME
1	*Aspergillus fumigatus* Fresenius (FA)	1	Tuberculosis Sanatorium, Tambaram, Madras	Sputum	CFT	ME	0.07	0.53
2	*Aspergillus fumigatus* Fresenius (FJ)	2	Tuberculosis Sanatorium, Tambaram, Madras	Sputum	CFT	ME	0.07	0.58
3	*Aspergillus fumigatus* Fresenius (FR)	3	Tuberculosis Sanatorium, Tambaram, Madras	Sputum	CFT	ME	0.05	0.18
4	*Aspergillus fumigatus* Fresenius (SP-285)	4	Vallabhbhai Patel Chest Institute, University of Delhi	Sputum	CFT	ME	0.06	0.20
5	*Aspergillus flavus* Link (A. fl)	5	Personal collection of Dr. Indira Kalyanasundaram	Sputum	CFT	ME	0.05	0.35
6	*A. candidus* Link (A. can)	MUBL 186	Culture Collection of Madras University Botany Laboratory, Madras-5.	Wheat	CFT	-	0.07	-
7	*A. chevalieri* (Mangin) (A. ch) Thom & Church	MUBL 372	Culture Collection of Madras University Botany Laboratory, Madras-5.	Rice	CFT	-	0.06	-
8	Bencard (B) *A. fumigatus* Fresenius	1	Commercially obtained from Bencard, Brentford, England	-	CFT	-	0.20	-
		2	Commercially obtained from Bencard, Brentford, England	-	CFT	-	0.30	-

England) by double diffusion and immuno-electrophoresis. In order to exclude errors owing to non-specific reactions, two non-pathogenic species of *Aspergillus* namely, *A. candidus* Link and *A. chevalieri* (Mangin) Thom and Church, isolated by our colleagues from stored grain, were also included in the study. Antigens were prepared by the standard method involving culture filtrates; since this proved time-consuming owing to the prolonged incubation required, an attempt was made with mycelial antigens, with a view to cut down the incubation time (4).

Materials and methods

Sources of antigens and sera

The fungi and their sources are presented in Table 1. Sera from suspected cases of pulmonary aspergillosis were obtained from Government Tuberculosis Sanatorium, Tambaram, Madras, and they are referred to in the text by arabic numerals, as serial numbers from 1 to 25.

Antigens from culture filtrate (CFT)

Glucose peptone broth (Glucose-20.0 g; Peptone-10.0 g and water – 1000 ml) was used for growing the fungi. Conidial suspension prepared from 3 day old cultures on glucose peptone agar slants was used for inoculation and the cultures were grown at 37 °C for 4 weeks in static condition (5). The culture fluid was freed from spores and mycelium by filtration through four layers of cheese cloth followed by centrifugation at 12 000 g for 30 minutes. The culture filtrate was then dialysed

against cold distilled water at 4 °C for 48 hours and lyophilized. The lyophilized powder was used as antigen for immunodiffusion and immunoelectrophoresis tests. Antigens prepared from this laboratory will be referred to in the text as MUBL antigens, to distinguish them from the commercially obtained Bencard antigens.

Antigens from mycelium (ME)

Cultures were grown as before but for shorter periods. The mycelial mat was initially harvested after 24 h in strains FA, FJ, FR & SP-285 of *A. fumigatus* and in *A. flavus* (Table 1), the antigens extracted and tested by double diffusion. In this preliminary test the largest number of precipitin lines was obtained with strain SP-285 and hence this strain alone was grown for different periods (24 h to 144 h) to standardize the optimum age level. Duplicate cultures were harvested at intervals of 24 h by filtering through four layers of cheese cloth and washed thoroughly with distilled water. The soluble mycelial proteins were extracted using cold Tris-HCl buffer (pH 8.0; 0.05 M) containing 17% sucrose, 0.1% ascorbic acid and 0.1% cysteine – hydrochloride. The amount of buffer added was three times (v/wt) that of the fungal mycelia. Initially the mat was ground to a paste using a pre-cooled pestle and mortar, with microglass beads twice the weight of the mat. The slurry thus obtained was homogenized in a 'VirTis 45' homogenizer for 20 min at 0 °C. The homogenate was then centrifuged at 12 000 g for 30 min at 0 °C. The resultant supernatant was dialysed against cold distilled water at 4 °C and the dialysate was lyophilized. The lyophilized powder was used as antigen. The mycelial antigens are referred to as ME to distinguish them from the culture filtrate antigens (CFT). The protein content of the antigens (CFT and ME), determined by the method of Lowry *et al.* (7) is presented in Tables 1 & 3.

Double diffusion

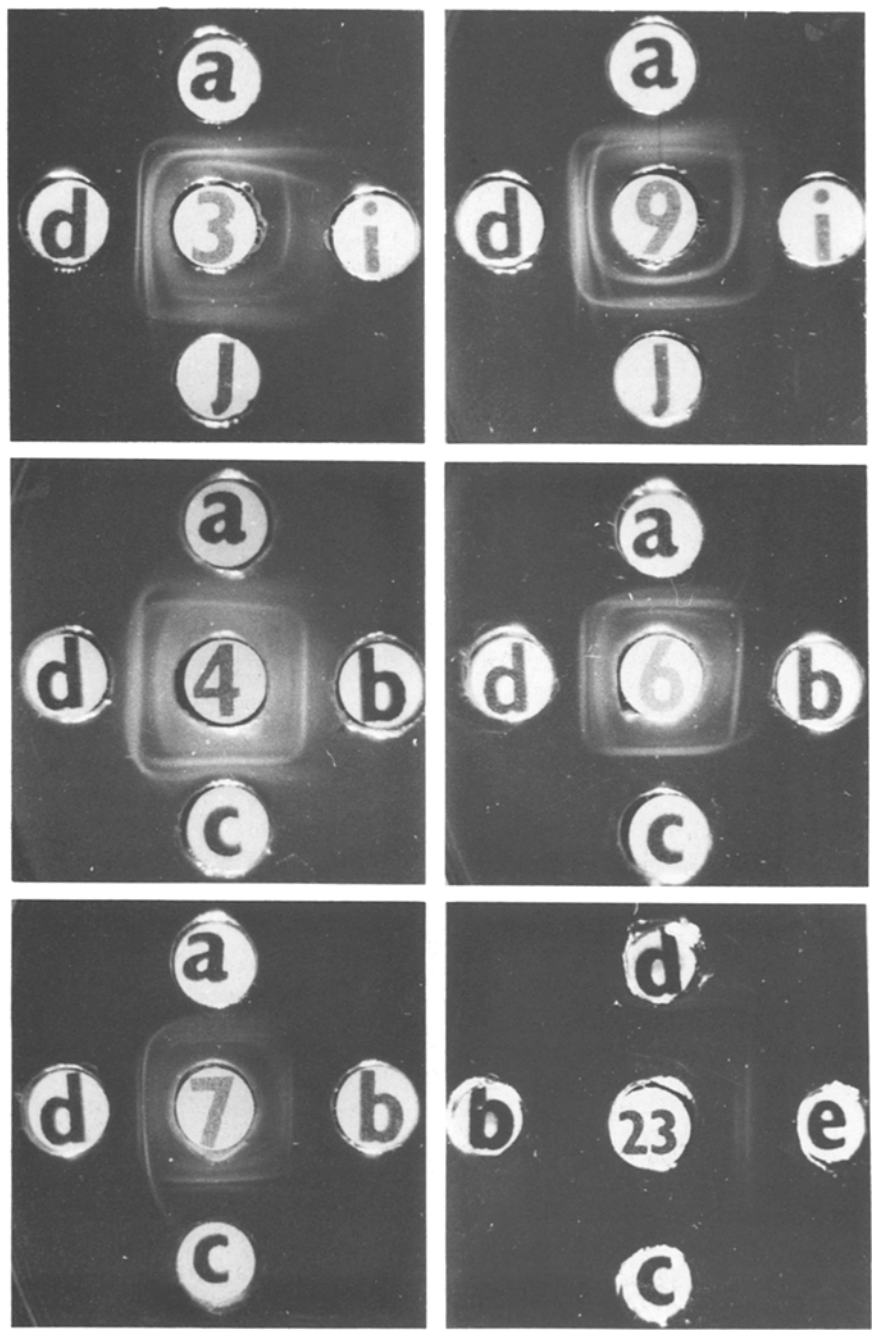
Agar gel double diffusion test of Ouchterlony was performed in plastic Petri dishes of 9 cms diameter. The medium for this test was prepared by dissolving Noble agar (Difco) at 1% concentration in 0.01 M phosphate buffered saline at pH 7.2. The plates were filled to a depth of 1.5 mm and a template was used to cut a pattern of peripheral wells of 6 mm diameter arranged 5 mm (edge to edge) around a central well of the same dimension. Lyophilized powder of MUBL antigens (CFT) was dissolved in sterile phosphate buffered saline (0.01 M, pH 7.2) to give a protein concentration of 20 mg/ml and the commercial Bencard antigens No. 1 & 2 were used at concentrations of 20 & 30 mg/ml. The plates were incubated in moist chambers at 25 ± 2 °C for up to one week. They were then washed with 0.01 M phosphate buffered saline and observed in a dark chambered box against diffuse lighting as suggested by Crowle (2). Photographs were made making use of the above box after the complete development of precipitin lines. Immunodiffusion test was also carried out in 1% Noble agar in 0.01 M McIlvaine citrate buffer pH 7.3, in order to eliminate possible non-specific reactions due to C-reactive proteins requiring Ca^{++} ions (13).

Immunoelectrophoresis

In the double diffusion studies, CFT antigens of the strains SP-285 and FR antigens reacted very well with sera 3 and 5. Therefore the immunoelectrophoretic technique described by Crowle (2) was employed for the above strains and sera in order to make a comparison. The test was performed on 7.5 by 2.5 cm glass slides in a 1 mm layer of 1% agarose in phosphate buffered saline pH 7.2. The gel was allowed to set and two wells 3 mm in diameter and 8 mm apart from each other were punched in the centre along the width of the slide. The wells were filled with SP-285 and FR antigens (20 mg/ml) and were electrophoresed with 10 mAmp/slide given for 2 hours in an electrophoretic apparatus containing phosphate buffered saline. After the run was over, a trench 1.5 mm wide and 55 mm long was made in the centre of the agar between the two wells and the trench was filled with antiserum. The slides were incubated in Petri dishes at 25 ± 2°C for one week and were observed in a dark chambered box as described earlier.

Results

Of the 25 cases tested, 18 patients' sera showed positive reactions with the CFT antigens of *A. fumigatus*. By contrast, only one patient's serum



Figs 1-6. Photographs from immunodiffusion plates showing antigen reactions with some of the sera tested. Figures in central well denote serial numbers of the sera of patients. Letters in peripheral wells denote antigens as follows: a - e: MUBL antigens (CFT); a - d: *Aspergillus fumigatus* strains FA, FJ, FR and SP-285 respectively. e: *A. flavus* ; i. & j: Commercial Bencard antigens 1 & 2.
Figs 1, 2. Comparison of MUBL antigens of *A. fumigatus* with Bencard antigens. *Figs 3, 4 & 5.* Antigens of 4 strains of *A. fumigatus* showing positive reaction with sera of patients 4, 6 & 7. *Fig. 6.* Antigenic comparison of *A. fumigatus* with *A. flavus.*

Table 2. Double diffusion data of suspected cases of Aspergillosis, with CFT antigens.

Case No.	Positive or negative	Strains and number of precipitin lines FA	FJ	FR	SP-285	A.fl.	A.ch.	A.can.	B1	B2
1	+	4	2	3	5	–	–	–	3	4
2	+	5	3	4	4	–	–	–	4	4
3	+	5	2	4	7	1	–	–	5	4
4	+	6	3	4	3	–	–	–	6	4
5	+	6	2	6	6	–	–	–	5	5
6	+	4	4	6	7	–	–	–	2	3
7	+	4	3	6	7	–	–	–	–	4
8	+	2	2	2	2	–	–	–	/	/
9	+	3	2	3	4	–	–	–	1	2
10	+	–	1	–	–	–	–	–	/	/
11	+	2	–	2	1	–	–	–	/	/
12	+	1	1	–	–	–	–	–	/	/
13	+	–	–	–	–	2	–	–	–	2
14	+	–	1	1	1	–	–	–	1	1
15	+	3	2	2	4	1	–	–	–	4
16	+	1	1	1	1	–	–	–	/	/
17	+	1	–	–	–	–	–	–	/	/
18	–									
19	–									
20	–									
21	–									
22	+	4	1	3	5	–	–	–	/	/
23	+	–	–	–	–	4	–	–	/	/
24	+	1	1	1	1	–	–	–	/	/
25	+	4	3	2	4	–	–	–	/	/

/ : Not done
– : No reaction

Table 3. Double diffusion data of suspected cases of Aspergillosis (ME).

Fungi	% of protein 24–144 h old mycelial extract (ME)						No. of detectable bands in double diffusion Case No. 9 ME of 24–144 h						Case No. 22 ME of 24 h	Case No. 23 ME of 24 h
	24	48	72	96	120	144	24	48	72	96	120	144		
FA	0.53	–	–	–	–	–	2	–	–	–	–	–	3	–
FJ	0.58	–	–	–	–	–	2	–	–	–	–	–	2	–
FR	0.18	–	–	–	–	–	2	–	–	–	–	–	3	–
SP-285	0.20	0.23	0.45	0.22	0.18	0.17	2	3	3	3	2	2	4	–
A.fl	0.35	–	–	–	–	–	–	–	–	–	–	–	–	4

reacted strongly with the antigens of *A. flavus* (Fig. 6). There were 2 sera showing positive reaction with the antigens of both *A. fumigatus* and *A. flavus* (Table 2; Figs. 1, 7). Out of the four strains of *A. fumigatus* (FA, FJ, FR & SP-285) strain SP-285 gave the maximum number of precipitin lines which varied from 2–7 among different sera (Figs. 1, 2, 3, 4 & 5). A comparison was made using Bencard and MUBL antigens for 11 cases. Of the 11 sera, 2 failed to react with Bencard antigen 1; the rest showed positive reaction against Bencard as well as MUBL antigens. However, MUBL antigens gave more numerous as well as sharper precipitin lines than the Bencard antigens (Figs. 1, 2).

In our preliminary studies, two sera (9 and 22) reacted strongly with *A. fumigatus* strain SP-285 antigen. Therefore, we tested these two sera against the mycelial extract antigens (ME) of this strain,

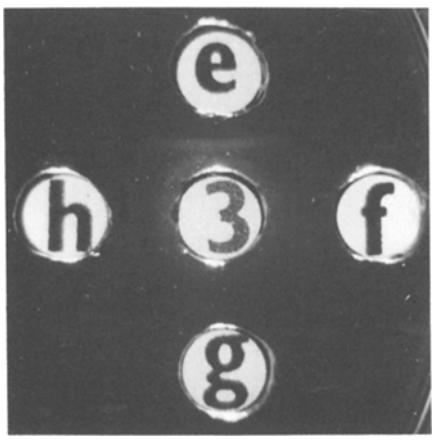
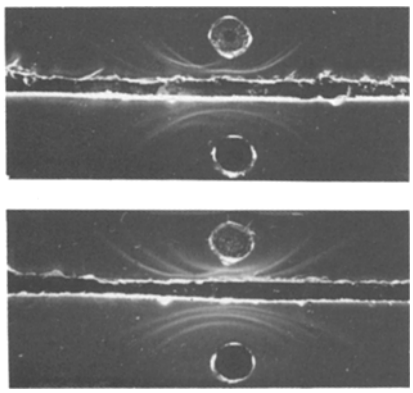


Fig. 7. Comparison of pathogenic and non-pathogenic MUBL antigens. e – *A. flavus;* f – *A. chevalieri;* g – Buffer; h – *A. candidus.*



Figs 8, 9. Photographs from immunoelectrophoretic plates to compare two strains of *A. fumigatus* (FR & SP-285) with two sera, i.e. 3 (Fig. 8) and 5 (Fig. 9).

and the results are presented in Table 3. With increasing age, there was a reduction in the protein content of mycelial extracts and consequently the number of precipitin lines. Thus, ME from 48, 72 and 96 h old cultures formed 3 lines, while that from 24, 120 and 144 h formed only 2 lines against serum 9 (Table 3). When ME antigens were cross reacted with the CFT antigens, there were 2 precipitin lines common to both the antigens.

The number of precipitin lines formed in agar prepared in phosphate buffered saline and agar in McIlvaine citrate buffer did not show any difference, but the lines were much sharper in the former buffer.

In immunoelectrophoresis serum 3 formed 6 to 7 arcs with the CFT antigen of *A. fumigatus* strain SP-285 and 4 arcs with that of strain FR while serum 5 formed 5 and 4 arcs with strains SP-285 and FR respectively (Figs. 8, 9).

Discussion

It is evident from the results that, in the serodiagnosis of pulmonary aspergillosis, the antigens prepared by us from *A. fumigatus* and *A. flavus* not only compared well with, but were actually superior to the Bencard antigens. This finding is in consonance with the observations of Philpot and Mackenzie (10) who recommended the use of antigens prepared in the laboratory.

Generally, antigens are prepared from 4-week old culture filtrates of *A. fumigatus* strains. As the fungus would have reached a lytic phase by that time, it was logical to assume that the mycelium of earlier age levels might contain the same antigens, which are released later into the medium. The presence of cross-reacting antigens between mycelial and culture filtrate preparations indicate that the CFT antigens were indeed lytic products of the mycelium. Positive results were obtained from cultures grown at 24, 48, 72, 96, 120 & 144 h. Mycelial antigens at earlier phases of growth can therefore be considered and may in fact be superior to conventional culture filtrate antigens for sero diagnosis. In a similar study, Kim & Chapras (4) have suggested that mycelial antigens are preferable, since culture filtrate antigens seem to undergo denaturation.

Sandhu *et al.* (13) have suggested the use of agar prepared in McIlvaine citrate buffer pH 7.3 to eliminate non-specific reactions due to C-reactive proteins requiring Ca^{++} ions. Our comparison of precipitin lines formed between phosphate buffered saline agar McIlvaine citrate buffered agar revealed that there was no difference in the number of precipitin lines formed. On the other hand, sharper precipitin lines were obtained in phosphate buffered saline agar. Hence we suggest that phosphate buffered saline might be used for immunodiffusion test.

Since none of the sera showed any cross reaction with the non-pathogenic species *A. candidus* and *A. chevalieri,* it is evident that the tests with our antigens are quite specific.

Despite the advances made in serological diag-

nosis of certain types of fungal diseases in Western countries, fungal serology is not much in vogue in India. In places where it is done, Bencard antigens are bought from the manufacturers abroad. These antigens are very expensive and as we see from our studies, their reaction may not be superior to those that can be easily prepared in a moderately equipped mycological laboratory. Since fungal diseases are now in the ascending order, the development of this area of research should fill a long felt need.

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