

Immunohistologic Identification of *Aspergillus* spp. and Other Hyaline Fungi by Using Polyclonal Fluorescent Antibodies

LEO KAUFMAN,* PAUL G. STANDARD, MAXINE JALBERT,
AND DOROTHY E. KRAFT

Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases,
Centers for Disease Control and Prevention, Atlanta, Georgia 30333

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Isolation and identification of pathogenic *Aspergillus* and *Fusarium* spp. from clinical materials provide the most accurate means for establishing a diagnosis of infections by these molds. Such efforts, however, are not always successful. Histologic diagnosis also has its limitations. In vivo the hyphae of *Aspergillus* and *Fusarium* spp. are very similar and their in situ manifestations are not pathognomonic. To improve the histologic diagnosis of infections by *Aspergillus* and *Fusarium* species, we developed polyclonal fluorescent-antibody reagents to *Aspergillus fumigatus* and *Fusarium solani* and evaluated their diagnostic utilities. Our studies revealed that *A. fumigatus* and *F. solani* share epitopes not only with one another but also with other *Aspergillus* and *Fusarium* spp. as well as with *Paecilomyces lilacinus* and *Pseudallescheria boydii*. Adsorption of the *A. fumigatus* conjugate with cells of *Fusarium proliferatum* and *F. solani* and *F. solani* antiserum with cells of *Aspergillus flavus* resulted in reagents that distinguished *Aspergillus* spp. from *Fusarium* spp. but that still cross-stained *P. lilacinus* and *P. boydii*. Adjunctive use of a specific *P. boydii* conjugate enabled the identification of *Aspergillus* spp., *Fusarium* spp., *P. lilacinus*, and *P. boydii* in formalin-fixed tissue sections from 19 humans with culture-proven cases of mycotic infection.

Infections caused by *Aspergillus* and *Fusarium* spp. are being seen with increasing frequency among immunocompromised patients. Patients with these diseases demonstrate lesions that are clinically and histopathologically similar (8). Accurate diagnosis is essential for delivery of appropriate therapy (12), but diagnosis of these infections can be difficult. Isolation and identification of pathogenic *Aspergillus* and *Fusarium* spp. from clinical materials provide the most accurate means of establishing a diagnosis. Such efforts, however, are not always successful. Immunodiagnostic methods for aspergillosis are available but suffer from limited sensitivity or have not been sufficiently evaluated. Established serodiagnostic tests are not available for infections caused by *Fusarium* spp. Histologic diagnosis also has its limitations. The hyphae of *Aspergillus* and *Fusarium* spp. are very similar in tissues, and specific histologic identification can be problematic. Since 1987, numerous studies have reported the development of tests for identifying *Aspergillus* spp. in tissues and to distinguish them from various hyaline filamentous fungi. These tests, based on immunoperoxidase staining, involved the use of *Aspergillus fumigatus* monoclonal antibodies (10, 11) which, although useful, shared epitopes with morphologically divergent fungi or adsorbed polyclonal antibodies (3) that were apparently specific. In an attempt to improve the immunohistologic diagnosis of these diseases, we produced polyclonal fluorescent-antibody (FA) reagents to *A. fumigatus* and *Fusarium solani* and evaluated their diagnostic utilities.

MATERIALS AND METHODS

Fungal strains and antigen production. All isolates used in this study were obtained from the Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention. *A. fumigatus* B-1172 and *F. solani* B-4421 were

used to produce antisera. Hyphal antigens were prepared as follows. The isolates were grown on potato dextrose agar slants for 1 week at 37°C. Growth was transferred from the slants to duplicate tubes containing 10 ml of brain heart infusion broth (BHIB), and the tubes were incubated at 37°C for 1 week. Four 1-liter flasks containing 500 ml of BHIB were prepared. Duplicate flasks were each inoculated with 10 ml of the *A. fumigatus* and *F. solani* BHIB cultures. The inoculated flasks were incubated at 37°C in a gyratory shaker-incubator for 7 days while rotating at 150 rpm, after which the cultures were killed with 0.5% formalin. After appropriate sterility checks, the cells were recovered by centrifugation at $3,000 \times g$ for 30 min and washed three times with phosphate-buffered saline (PBS; pH 7.2). A portion of the washed mycelium was suspended in 0.5% formalin-treated PBS and was stored at 4°C for preparation of smears to evaluate the FA reagents and for adsorbing antisera or conjugates. For use as smears, mycelium was partially disrupted by adding 1-mm-diameter glass beads, followed by agitation in a vortex mixer. Another portion of the washed mycelium was diluted 1:4 by adding 1 part of packed mycelium to 3 parts of 0.5% formalin-treated PBS and then was disrupted in a Braun MSK mechanical cell homogenizer for 11 min so that the hyphal fragments easily passed through a 25-gauge needle. The vaccines for immunization were prepared by adjusting the turbidities of the homogenates with 0.5% formalinized PBS so that they were equivalent to that of a McFarland no. 5 nephelometer standard and were stored at 4°C.

Antisera. Two mature albino female New Zealand White rabbits were used for each immunizing strain. The rabbits were bled before immunization for preimmunization sera. Antisera against the homogenate antigens were prepared by injecting the rabbits subcutaneously in the dorsal area with an emulsion consisting of 1.0 ml of the homogenate and 1.0 ml of Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.). After 1 week, each rabbit was injected intravenously with 1, 2, and 2 ml of antigen without adjuvant on 3 consecutive days for 3 weeks. Six weeks after the initial immunization, the rabbits were injected intravenously with 1 ml of the homogenate antigen. During the 5th, 6th, 8th, and 9th weeks, each rabbit was test bled and its serum was screened along with its corresponding preimmunization serum for antibody reactivity against homologous antigens in indirect FA (IFA) tests. Each serum sample was tested undiluted and diluted twofold to 1:32 for evidence of antibody reactivity in the IFA test. Moderate to strong staining occurred at titers of 1:8 to 1:16 only with sera obtained in the 8th and 9th bleedings. These sera were pooled and fractionated with 35% saturated ammonium sulfate, and their immunoglobulin fractions were conjugated with fluorescein isothiocyanate.

Conjugation procedure. Conjugates were prepared as described by Jones et al. (5). Accordingly, rabbit immunoglobulin to *A. fumigatus* B-1172 was obtained by precipitation of the antiserum with 35% saturated ammonium sulfate. The precipitated immunoglobulin was recovered by centrifugation, dissolved in distilled water, and then dialyzed against 0.85% saline solution. A biuret protein determination was made, and the immunoglobulin was labeled with 0.05 mg of fluorescein isothiocyanate per mg of protein.

* Corresponding author. Mailing address: Division of Bacterial and Mycotic Diseases, MS-G11, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Ga. 30333. Phone: (404) 639-3547. Fax: (404) 639-3546.

TABLE 1. Staining titers of unadsorbed and adsorbed fluorescein isothiocyanate-labeled rabbit *Aspergillus fumigatus* B-1172 antiglobulin with homologous and heterologous fungi

Fungus species (no. of strains)	Staining titers of antiglobulins	
	Unadsorbed	Adsorbed with <i>Fusarium proliferatum</i> and <i>Fusarium solani</i>
<i>Aspergillus flavus</i> (8)	1:32–1:64	1:8–1:16
<i>Aspergillus fumigatus</i> (7)	1:16–1:64	1:8–1:16
<i>Aspergillus nidulans</i> (4)	1:32–1:64	1:8–1:16
<i>Aspergillus niger</i> (5)	1:8–1:16	1:8
<i>Aspergillus terreus</i> (4)	1:8–1:32	1:8–1:16
<i>Fusarium dimerum</i> (2)	1:32	0
<i>Fusarium moniliforme</i> (1)	1:2	0
<i>Fusarium oxysporum</i> (1)	1:8	1:1
<i>Fusarium proliferatum</i> (1)	1:32	0
<i>Fusarium solani</i> (1)	1:8	0
<i>Paecilomyces lilacinus</i> (1)	1:32	1:16
<i>Paecilomyces varioti</i> (1)	1:1	1:2
<i>Pseudallescheria boydii</i> (2)	1:64	1:8
<i>Cunninghamella bertholletiae</i> (1)	NT ^a	0
<i>Rhizopus arrhizus</i> (3)	1:4–1:16	0–1:1
<i>Alternaria alternata</i> (1)	NT	0

^a NT, not tested.

Adsorption procedure. Two volumes of antiserum or conjugate were mixed with 1 volume of the packed cell mass, and the mixture was incubated for 2 h in a 37°C water bath. After each adsorption, the FA titers were determined against homologous and heterologous fungi.

Immunofluorescence staining. IFA tests were conducted by the procedure described by Kaufman et al. (7). Rabbit anti-*F. solani* serum was applied to heat-fixed smears of suspensions of formalin-killed cultures, and the smears were incubated for 45 min at 37°C in a moist chamber. The smears were rinsed with PBS, after which fluorescein isothiocyanate-conjugated goat anti-rabbit globulin (Centers for Disease Control and Prevention) diluted 1:100 was added. The direct staining procedure was performed by the method of Kaufman and Kaplan (6). Heat-fixed smears treated with the conjugates were examined with a Leitz Ortholux II incident-light fluorescence microscope. The intensity of staining was arbitrarily graded. Results were recorded as 0 for negative, 1+ to 2+ for moderate staining, and 3+ to 4+ for strong staining. Both the IFA and direct staining procedures also were applied to deparaffinized tissue sections.

For FA studies, smears of washed mycelia of the *Aspergillus* spp., *Fusarium* spp., and morphologically similar heterologous fungi were prepared from formalin-killed cultures grown at 37°C in BHIB for 1 week.

A conjugate developed by Jackson et al. (4) was used to identify *Pseudallescheria boydii* (anamorph, *Scedosporium apiospermum*) in culture or tissue. The fluorescein isothiocyanate-labeled *P. boydii* antiglobulin was adsorbed with *A. fumigatus* and *Fusarium oxysporum* to eliminate cross staining with species of *Aspergillus*, *Fusarium*, and *Paecilomyces*. Cells that stained 1+ or greater were considered positive.

Preparation and staining of infected tissues. Deparaffinized formalin-fixed tissue sections from 19 humans with cases of culture-proven hyalohyphomycosis were studied. All of the tissues were stained with Gomori methenamine-silver to verify that each contained hyphal elements of *Aspergillus* spp., *Fusarium* spp., *Paecilomyces lilacinus*, *P. boydii*, or *Cunninghamella bertholletiae*. Replicate unstained sections for FA studies were heated, deparaffinized, and treated with 1% trypsin for 45 min just before staining (9).

RESULTS

The sensitivity of the *A. fumigatus* conjugate was determined with smears of suspensions from cultures of 28 strains of 5 pathogenic *Aspergillus* spp., whereas the specificity was ascertained with smears of cultures of heterologous fungi with morphologically similar tissue forms representing 15 strains of 11 species in 6 genera (Table 1). The unadsorbed conjugate stained all the *Aspergillus* spp. with titers ranging from 1:8 to 1:64. Unfortunately, intensive cross staining in the same titer range occurred with the *Fusarium* spp. as well as with *P. lilacinus*, *P. boydii*, and *Rhizopus arrhizus*. Adsorptions, once with

TABLE 2. IFA staining titers of unadsorbed and adsorbed *Fusarium solani* B-4421 antiserum with homologous and heterologous fungi

Fungus species (no. of strains)	Staining titer	
	Unadsorbed antiserum	Antiserum adsorbed with <i>Aspergillus flavus</i>
<i>Aspergillus flavus</i> (8)	1:50–1:120	0
<i>Aspergillus fumigatus</i> (7)	0–1:120	0
<i>Aspergillus nidulans</i> (4)	1:100–1:120	0
<i>Aspergillus niger</i> (5)	1:50–1:120	0
<i>Aspergillus terreus</i> (4)	1:50–1:120	0
<i>Fusarium dimerum</i> (2)	1:120	1:100
<i>Fusarium moniliforme</i> (1)	1:120	1:120
<i>Fusarium oxysporum</i> (1)	1:120	1:120
<i>Fusarium proliferatum</i> (1)	1:120	1:120
<i>Fusarium solani</i> (1)	1:120	1:120
<i>Paecilomyces lilacinus</i> (1)	1:120	1:100
<i>Paecilomyces varioti</i> (1)	0	0
<i>Pseudallescheria boydii</i> (2)	1:120	1:100
<i>Cunninghamella bertholletiae</i> (1)	0	0
<i>Rhizopus arrhizus</i> (3)	0	0
<i>Alternaria alternata</i> (1)	0	0

cells of *Fusarium proliferatum* M5115 and once with cells of *F. solani* B-4421, significantly diminished or eliminated the staining of the *Fusarium* spp. Cross staining, ranging from 1 to 3+, however, still persisted with *P. lilacinus* and *P. boydii*. Additional adsorptions with cells of the latter two fungi resulted in a conjugate that stained the aspergilli weakly and that still exhibited cross staining.

The *A. fumigatus* conjugate adsorbed with *F. proliferatum* and *F. solani* and diluted 1:8 showed good sensitivity and staining intensity (2 to 4+) and was selected for further evaluation as a diagnostic reagent.

Preliminary studies to develop a conjugate for the direct staining of *Fusarium* spp. were not successful. The conjugates poorly stained the *Fusarium* spp. and demonstrated extensive cross staining with the heterologous fungal isolates. Adsorptions with *A. fumigatus* or *A. flavus* resulted in weakly staining reagents with titers of 1:1 to 1:2.

Rabbit *F. solani* antiserum was diluted 1:20 to 1:240 and was tested against a variety of *Aspergillus* and *Fusarium* spp. as well as other filamentous fungi by the IFA test (Table 2). The reagent intensely stained all the *Fusarium* spp. when it was diluted up to 1:120. However, it also exhibited similar staining with the filaments of the *Aspergillus* spp., *P. lilacinus*, and *P. boydii*. The most intense cross staining occurred with the strains of *Aspergillus flavus*. A single adsorption of the antiserum with homogenized cells of *A. flavus* 88-002730 eliminated the cross staining with the *Aspergillus* spp., but not with *P. lilacinus* or *P. boydii*. The *Fusarium* spp. stained with a 2 to 3+ intensity, whereas *P. boydii* and *P. lilacinus* stained with a 1 to 2+ intensity. An additional adsorption of portions of the adsorbed antiserum with cells of *A. flavus* or *P. boydii* diminished the homologous staining without eliminating the cross-reactions. The *F. solani* antiserum, adsorbed once with *A. flavus* and diluted 1:100, proved reliable as a reagent for distinguishing isolates of *Fusarium* from those of *Aspergillus*. The hyphal walls and septae of the *Fusarium* spp. stained at levels of 2 to 3+, while the conidia generally stained more brightly.

The specificity of the *P. boydii* conjugate (4) was verified in studies with smears of a variety of *Aspergillus* and *Fusarium*

TABLE 3. FA staining patterns of the adsorbed *A. fumigatus*, *F. solani* and *P. boydii* reagents with smears of *Aspergillus* spp., *Fusarium* spp., *P. lilacinus*, and *P. boydii*

Fungus	FA staining reactions with reagents for the following ^a :		
	<i>A. fumigatus</i> ^b	<i>F. solani</i> ^c	<i>P. boydii</i> ^b
<i>Aspergillus</i> spp.	+	—	—
<i>Fusarium</i> spp.	—	+	—
<i>P. lilacinus</i>	+	+	—
<i>P. boydii</i>	+	+	+

^a +, positive staining; —, negative staining.

^b Direct FA reagent.

^c Indirect FA reagent.

spp., plus *P. boydii*, *P. lilacinus*, and *Paecilomyces varioti*. The conjugate stained only the *P. boydii* strains. The staining patterns noted after application of the three adsorbed reagents to smears of *Aspergillus* and *Fusarium* spp., *P. lilacinus*, and *P. boydii* are presented in Table 3. Specific staining patterns were evident for each of the groups of fungi studied.

The value of the three conjugates for diagnosing aspergillosis and hyalohyphomycosis caused by *Fusarium* spp. and *P. boydii* was ascertained in studies with 19 formalin-fixed tissue sections from humans with a variety of culturally proven mycotic diseases (Table 4). Hyphal elements in all of the 13 sections from patients with aspergillosis caused by *A. flavus*, *A. fumigatus*, *Aspergillus terreus*, and *Aspergillus ustus* stained intensely with the *A. fumigatus* conjugate. None stained with the *F. solani* or *P. boydii* reagents. Hyphal elements of *F. proliferatum* and *F. solani* in three tissue sections stained only with the *F. solani* reagent. Hyphal elements in cutaneous tissue from a patient infected with *P. lilacinus* stained only with the *Aspergillus* and *Fusarium* sp. conjugates, whereas hyphae in lung tissue from a patient with *P. boydii* infection stained with all three conjugates. None of the reagents reacted with short hyphal fragments in lung tissue from a patient with zygomycosis caused by *C. bertholletiae*.

From 1990 to 1996 we received tissue sections from 75 patients with suspected aspergillosis or infections caused by *Fusarium* spp. For none of these patients was the diagnosis confirmed by culture. These tissues contained hyaline hyphae with or without dichotomous or right-angle branching. Our three diagnostic reagents were applied to these sections, and they helped to presumptively identify or exclude infections caused by *Aspergillus* or *Fusarium* spp. and, where applicable,

allowed for the selection and delivery of appropriate antifungal therapy.

DISCUSSION

Our FA studies revealed that the hyaline filamentous pathogenic *Aspergillus* spp., *Fusarium* spp., *P. lilacinus*, and *P. boydii* are not only morphologically similar in tissue but share epitopes (Tables 1 and 2). Fluorescein isothiocyanate-conjugated rabbit anti-*A. fumigatus* globulins cross stained the pathogenic *Aspergillus* spp., *Fusarium* spp., *P. boydii*, *P. lilacinus*, *P. varioti*, and *R. arrhizus* (Table 1). Similar cross-reactions with *Fusarium anthophilum* and *R. arrhizus* were noted by Fukuzawa et al. (3) in immunoperoxidase studies with rabbit antisera to *A. fumigatus* conidia. Those workers did not study strains of *P. boydii* or *Paecilomyces* spp. Adsorption of our conjugate with *F. proliferatum* and *F. solani* cells and dilution of the conjugate 1:8 resulted in a reagent that allowed for the differentiation of *Aspergillus* spp. from *Fusarium* spp. Although the adsorbed reagent did not stain the *Fusarium* spp. studied, it still cross stained *P. lilacinus* and *P. boydii* (Table 1). In contrast, Fukuzawa et al. (3) reported that they were able to eliminate the cross-reactivity of their *A. fumigatus* antiserum by adsorption with cells of *Candida albicans*.

Rabbit anti-*F. solani* sera contain antibodies that reacted not only with other *Fusarium* sp. antigens but also with those of the *Aspergillus* spp., *P. lilacinus*, and *P. boydii*. Unlike *A. fumigatus*, *F. solani* does not share antigens with *P. varioti* or *R. arrhizus*. Adsorption of the *F. solani* antiserum with cells of *A. flavus* eliminated the staining of the aspergilli, but not that of the cells of *P. lilacinus* or *P. boydii*. Interestingly, Fukuzawa et al. (3) noted that their rabbit antiserum to *F. anthophilum* conidia cross stained *A. fumigatus* and *R. arrhizus* in immunoperoxidase studies. Their reagent, as stated above, was rendered specific by adsorption with *C. albicans* cells.

Invasive pseudallescheriasis, like aspergillosis and fusariosis, is a problem in immunosuppressed patients. *P. lilacinus* infections are uncommon in immunosuppressed patients (2), but can cause localized disease in nonimmunocompromised patients. To enable the specific immunohistologic diagnosis of aspergillosis or *Fusarium* sp. infections in which the possibility of a *P. boydii* or *P. lilacinus* infection exists, we recommend the adjunctive use of the *P. boydii* conjugate with the adsorbed *A. fumigatus* and *F. solani* reagents (Table 3). Use of the three reagents also permits the identification of *P. lilacinus* and *P. boydii*. After prior review of a clinical history, each tissue was stained with hematoxylin and eosin and Gomori methenamine-

TABLE 4. FA staining of fungal elements in 19 tissues from humans with culture-proven mycotic infections by adsorbed polyclonal antibodies to *Aspergillus fumigatus*, *Fusarium solani*, and *Pseudallescheria boydii*

Culture isolated	Tissue and no. of patients	FA staining for the following:		
		<i>Aspergillus</i> spp.	<i>Fusarium</i> spp.	<i>Pseudallescheria boydii</i>
<i>Aspergillus fumigatus</i>	Lung (6)	4+	0	0
<i>Aspergillus fumigatus</i>	Brain (1)	4+	0	0
<i>Aspergillus fumigatus</i>	Kidney (1)	4+	0	0
<i>Aspergillus flavus</i>	Maxillary sinus (3)	4+	0	0
<i>Aspergillus terreus</i>	Lung (1)	4+	0	0
<i>Aspergillus ustus</i>	Skin (1)	3+	0	0
<i>Cunninghamella bertholletiae</i>	Lung (1)	0	0	0
<i>Fusarium proliferatum</i>	Lung (1)	0	2+	0
<i>Fusarium solani</i>	Lymph node (1)	0	4+	0
<i>Fusarium solani</i>	Maxillary sinus (1)	0	2+	0
<i>Paecilomyces lilacinus</i>	Skin (1)	3+	2+	0
<i>Pseudallescheria boydii</i>	Lung (1)	1+	1+	1+

silver to localize and morphologically characterize the fungal elements and to ascertain whether they were hyaline. Application of the three reagents allowed for the specific identification of the *Aspergillus* spp. in 13 formalin-fixed, paraffin-embedded tissue sections from patients with culture-proven cases of aspergillosis and infections caused by *Fusarium* spp. in 3 tissue sections from patients with culture-proven cases of fusariosis (Table 4). Our results, although based on the study of a limited number of tissues from patients with culturally proven mycotic infections, suggest that immunohistologic studies can be helpful when the histologic results are not pathognomonic. Use of the three conjugates permits the specific in situ identification of *Aspergillus* spp., *Fusarium* spp., *P. lilacinus*, and *P. boydii* in culture or formalin-fixed tissue sections. Although tissues were not available from patients with *Aspergillus niger* infections, the adsorbed *A. fumigatus* conjugate was capable of staining *A. niger* filaments (Table 1). In situations in which *P. lilacinus* or *P. boydii* can be excluded from the differential diagnosis, use of the aspergillosis direct and fusariosis indirect FA reagents would suffice to distinguish the respective etiologic agents.

Although our reagents have proven diagnostic value, the method recommended here is complicated by the need for three reagents whose production requires time-consuming immunization schedules and adsorptions. Reports suggest that the identification of *Aspergillus* and *Fusarium* spp. in tissue may be simplified through the use of genus-specific polyclonal antibodies produced against conidial antigens adsorbed with *C. albicans* (3) or by monoclonal antibodies (10, 11). As the number of opportunistic filamentous fungi increases, the conventionally used reagents will have to be evaluated further to verify their specificities. Molecular probes may provide another means for the rapid, specific, and reliable identification of fungal elements in tissue. A polymerase chain PCR-based test that targets *Fusarium* sp. DNA from formalin-fixed ocular tissue has already been reported by Alexandrakis et al. (1).

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