Evaluation of the Exoantigen Test for Identification of *Histoplasma* Species and *Coccidioides immitis* Cultures

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Recently it was demonstrated that certain fungal pathogens could be rapidly and accurately identified on the basis of their exoantigens. Mycelial-form cultures of the *Histoplasma* species consistently appear to produce either H or M antigens, or both, whereas cultures of *Coccidioides immitis* produce tube precipitinogen, heat-labile precipitinogen, or heat-labile F antigens. Three laboratories each evaluated 48 cultures by the exoantigen procedure for the identification of *Histoplasma* spp. and *C. immitis*. One hundred forty-four cultures were tested, and 143 (99.3%) were correctly identified as *Histoplasma* spp. or *C. immitis*, or neither. The simple and rapid exoantigen test appears to be a sensitive and specific method for identification of these fungi.

In 1976 Standard and Kaufman described a rapid and specific immunological test to identify *Histoplasma* spp. (7). Briefly, the method consists of inoculating test cultures into 30 ml of brain heart infusion broth and incubating them at 25°C for 3 and 6 days on a gyratory shaker. After Merthiolate is added, the culture supernatant is removed by centrifugation, concentrated, and allowed to react in a microimmunodiffusion system against human serum containing antibodies to H and M precipitinogens. A control antigen prepared from a mycelial-form culture of *Histoplasma capsulatum* is tested in parallel with the test culture concentrates. Either H or H and M bands were developed only in the presence of antigen concentrates from the *Histoplasma* spp. (*H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, or *H. farciminosum*).

Utilizing the same procedure, Standard and Kaufman also showed that the exoantigen test could be used for the serological identification of *Coccidioides immitis* with 100% sensitivity and 100% specificity (8). Huppert et al. confirmed the specificity of the exoantigen test for *C. immitis* by testing 49 isolates of arthroconidial fungi other than *C. immitis* in the immunodiffusion test and found them to be unreactive to *C. immitis* antiserum (1).

The exoantigen test for the identification of both *Histoplasma* spp. and *C. immitis* was later simplified. Seven- to 10-day-old Sabouraud dex-

trose agar cultures of a fungus are flooded with 8 to 10 ml of a 1:5,000 aqueous solution of Merthiolate. After overnight extraction, the supernatant is withdrawn, concentrated, and tested in the microimmunodiffusion test against appropriate positive control reagents (2). This method has been shown to be as specific and sensitive as the culture filtrate procedure for the identification of these fungal pathogens. The exoantigen test appears to be a rapid and reliable method for the identification of mycotic agents when performed in the test authors' laboratory. Therefore, we evaluated the procedure in a hospital diagnostic laboratory and two public health laboratories to determine if this procedure could be used in a variety of laboratories for the accurate identification of *Histoplasma* spp. or *C. immitis*, in either their typical or atypical mycelial forms. In this study the exoantigen test was found to be sensitive, specific, rapid, and simple to perform.

MATERIALS AND METHODS

Laboratories. The three laboratories that participated in the study were a hospital diagnostic laboratory and two public health laboratories (one in the United States and one in Canada). Each was large enough to have mycologists on the staff who were familiar with cultural and serological techniques for pathogenic fungi.

Cultures. Duplicate, coded, pure cultures of *H. capsulatum* var. *capsulatum*, *H. farciminosum*, *C. immitis*, and other dimorphic pathogens and saprophytes that resembled *H. capsulatum* and *C. immitis* morphologically were sent to each of the testing laboratories from the Center for Disease Control. Cultures were on Sabouraud dextrose agar slants in screw-

cap tubes (20 by 150 mm) containing 10 ml of agar per tube. Cultures were shipped to participating laboratories in groups of 10 over a 2-month period.

Control reagents. Coccidioidin and histoplasmin antigen, as well as the corresponding antisera, were supplied by the Fungus Immunology Branch, Mycology Division, Center for Disease Control.

Test procedures. Mature cultures with at least 15 by 30 mm of growth were covered with 8 to 10 ml of a 1:5,000 aqueous solution of Merthiolate. The cultures were usually 17 to 30 days old at the time of testing. The Merthiolate was permitted to react with the mycelial growth for at least 24 h at 25°C. Five milliliters of each cellular extract was then transferred to a Minicon B-15 macrosolute concentrator (Amicon, Lexington, Mass.) with a 22.5-cm Pasteur pipette. The concentration procedure took from 2 to 4 h. One drop of extract was also placed on a slant of Sabouraud dextrose agar to test the effectiveness of the Merthiolate. A wet mount of the mycelium in lactophenol cotton blue was prepared to study the microscopic morphology of each study culture. Cultures with tuberculate macroconidia were suspected of being *H. capsulatum*, and their extracts were concentrated 50×, whereas extracts from cultures with arthroconidia were suspected of being *C. immitis* and were concentrated to 25×. Cultures without characteristic spores were concentrated to 50× and tested against the control reagents for *H. capsulatum*. In addition, the 50× extract was diluted to 25× and tested as a suspected *C. immitis*. The microimmunodiffusion test was performed as described below.

Control antiserum was placed in the center well of a hexagonal pattern. The antiserum was allowed to diffuse for 1 h at room temperature before addition of the antigens. The control antigens were placed in the upper and lower wells. Unknown supernatant antigens were tested in duplicate in adjacent lateral wells (3). The charged immunodiffusion plates were placed in a moist chamber kept at 25°C. After 24 h, the immunodiffusion templates were removed, the agar surface was covered with distilled water, and plates were read on a view box with a dark background and oblique light (4). The unknown fungus was identified as *C. immitis* when its concentrated extract produced immunodiffusion lines of identity with the tube precipitinogen, the heat-labile precipitinogen, or the heat-labile F precipitinogen of the control reagents. Identification of a *Histoplasma* spp. was made when its culture extract contained either H or H and M precipitinogens determined with the appropriate control system.

RESULTS

The unknown fungi submitted for testing are listed in Table 1. Twenty of the cultures were species of *Histoplasma*. Only five of the 20 produced tuberculate macroconidia. However, this characteristic by itself is not diagnostic of the genus *Histoplasma* since such conidia are produced by several hyphomycetous genera. The remainder would have been identified as *Mycelia Sterilia*, as they were nonsporulating. Of the 17 isolates of *C. immitis*, 15 were suspicious

enough morphologically to warrant animal inoculation, but 2 isolates would have been described as *Mycelia Sterilia*.

Forty-eight cultures were tested with the exoantigen technique by each of the three laboratories, for a combined total of 144; 143 (99.3%) were correctly identified as either *Histoplasma* spp. or *C. immitis*, or neither (Table 2). One laboratory did not correctly identify one of the atypical *H. capsulatum* cultures. All isolates which were morphologically compatible with either a *Histoplasma* spp. or *C. immitis* were positive by the exoantigen test. There were no false-positive identifications, and only two of the exoantigen extracts developed lines of nonidentity with the control reagents. The nonspecific precipitations occurred when two isolates of *C. immitis* were reacted against the anti-*H. capsulatum* control serum.

There was no growth evident from any of the 24-h Merthiolate-treated extracts tested for fungal growth on Sabouraud dextrose agar after 32 to 50 days incubation at 25°C.

DISCUSSION

Histoplasmosis and coccidioidomycosis comprised 87% of 939 systemic mycotic infections

TABLE 1. *Unknown specimens submitted to three laboratories for testing*

Fungi	No. of iso-lates	No. having characteristic morphology
Histoplasma capsulatum var. *capsulatum*	18	5
Histoplasma farciminosum	2	0
Coccidioides immitis	17	15
Arachniotus reticulatus	1	0
Arthroderma tuberculatum	1	0
Chrysosporium keratinophilum .	1	1
Malbranchea sp.	2	1
Arthroconidia-producing saprophytes	3	3
Blastomyces dermatitidis	2	1
Paracoccidioides brasiliensis ..	1	0

TABLE 2. *Results of exoantigen studies utilizing antiserum to C. immitis and Histoplasma spp.*

Species studied	No. of iso-lates sub-mitted	No. (%) of isolates identified correctly		
		Labora-tory 1	Labora-tory 2	Labora-tory 3
Histoplasma spp.	20	19 (95)	20 (100)	20 (100)
C. immitis	17	17 (100)	17 (100)	17 (100)
Non-*Histoplasma* spp. or non-*C. immitis*	11	11 (100)	11 (100)	11 (100)

optionally reported to the U.S. Public Health Service in 1977 (10). The traditional morphological and cultural methods used to identify the respective pathogenic fungi, *H. capsulatum* and *C. immitis*, are time consuming. Definitive identification of these two dimorphic fungi requires their conversion from the mycelial form to the tissue form. The conversion of *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, or *H. farciminosum* may be accomplished in vitro by utilizing Pine's medium or in vivo by animal inoculation (5). *C. immitis* may sometimes be converted to the spherule form in vitro, utilizing the methods of Roberts et al. (6) or Sun et al. (9). However, intratesticular inoculation of the mycelial form into guinea pigs gives more consistent conversion to endosporulating spherules. These procedures for the definitive identification of either of these dimorphic pathogenic fungi are lengthy and cumbersome, often causing delays in initiation of specific therapy. A more reliable and rapid method for distinguishing these mycotic pathogens from morphologically similar saprophytic fungi, or a method which eliminates the need for animal inoculation, is desirable.

In this study, 143 of the 144 cultures submitted for exoantigen analysis were identified correctly when compared with identification by traditional morphological methods. The exoantigen procedure demonstrated a sensitivity of 99.3%. Furthermore, the test also proved to be specific for the identification of *Histoplasma* spp. and *C. immitis*, as it correlated 99.1% with expected results; i.e., 110 of 111 isolates were correctly identified. When the misidentified *H. capsulatum* culture was retested it was correctly identified, which indicates an error of technique rather than of the test system. It is common laboratory practice to repeat a test when the result is suspicious or equivocal or when the clinical findings do not concur with the laboratory data. Such practice ordinarily would be followed when performing a diagnostic immunodiffusion test, but it was not permitted in the protocol established for this study. Based upon our observations, we suggest that in routine use the test be performed on duplicate subcultures.

Appropriate positive controls are essential in performing a diagnostic immunodiffusion test for mycotic diseases (4). The development of any band(s) alone is not sufficient for a specific culture identification; the band(s) must form a line(s) of identity with the control band(s). For example, in the immunoidentification of *C. immitis*, the precipitin band for the tube precipitinogen antigen tended to stay close to the antiserum well and was often obliterated. Dilution of the culture extracts caused the movement of the precipitin band toward the center between the antigen and antiserum wells, where the tube precipitinogen band could easily be related to the tube precipitinogen reference band. When the immunodiffusion plates are examined, the bands produced by the control antigen should be studied very carefully. If the ends of an unknown band are straight and do not fuse with any of the bands from the control reagents, it is not a band of identity and the specimen is considered negative. At times, a reference line may demonstrate a slight bow toward the well containing unknown extract. This band frequently indicates a trace reaction, and in such instances the exoantigen supernatant should be further concentrated and retested.

It would be better practice to make the wet mount for microscopic examination of the test specimen before adding Merthiolate or immediately after its addition. After the 24-h extraction period, Merthiolate distorts the mycelial structure, which makes selection of the appropriate immunological system for specific testing more difficult.

The age of the culture before extraction of antigens also plays a major role in the reliability of this test, since it relates to the concentration of test antigen available. Previous experience has shown that a young culture may not produce sufficient antigen to form specific precipitin lines. However, after 3 to 5 days of additional growth, sufficient antigen will be produced and lines of identity with the control reagents are readily visible. If a submitted culture shows sufficient growth, it should be subcultured and the original culture should be extracted and tested. This approach makes the test relatively rapid and, therefore, particularly useful in public health and other reference laboratories where cultures rather than clinical material are received for identification of etiological agents.

The exoantigen test is based on the fact that cultures of *Histoplasma* spp. and *C. immitis* produce antigens that are different from those of morphologically similar fungi. In our tests with 11 isolates of fungi which morphologically resembled one or the other of these pathogens, none gave a line of identity with either specific antiserum. It is noteworthy that 17 isolates of the species of the two pathogenic genera studied would have been characterized by microscopic morphology as being sterile. Thus, their identity might have been overlooked unless the laboratory or attending physician was highly suspicious of the isolate based on the patient's clinical condition. In many cases, form conversion and further identification of these isolates might not be pursued.

Our results raise the question of the validity

of morphologically placing fungi into a form genus regarded by many as only having saprophytic species without some attempt being made to determine their potential pathogenicity. We feel that the exoantigen test offers the laboratory an opportunity to screen aconidial and atypical isolates for possible pathogens. Routine performance of the exoantigen test would decrease the likelihood of misidentifying a pathogenic fungus and, ultimately, of misdiagnosing a patient's illness.

In summary, there are nonpathogenic fungi that are morphologically similar to certain pathogens and there are atypical or morphologically aberrant isolates of the known pathogens. The procedure for the detection and identification of exoantigens, as developed by Standard and Kaufman (3, 4, 7, 8), provides an additional means for identifying mycelial forms of *C. immitis* and *Histoplasma* species. The test is recommended for routine use in those laboratories capable of handling the etiological agents of the deep mycoses.

ACKNOWLEDGMENTS

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