Specific Immunological Test for the Rapid Identification of Members of the Genus *Histoplasma*

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A sensitive and specific immunological method was developed for rapid identification of the mycelial forms of *Histoplasma capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. farciminosum* and for separation of these pathogenic fungi from morphologically similar hyphomycetes and other fungal pathogens. This method is based on the fact that all of the *Histoplasma* spp. produce H and M histoplasmin antigens, whereas the other fungi do not. Inocula consisting of heavy mycelial growth from a pure, full-grown culture were transferred into flasks containing small volumes of brain heart infusion broth. These cultures were placed on a shaker and grown at 25 C. Using the micro-immunodiffusion technique and antisera containing antibodies to H and M precipitinogens, we detected exoantigens in 3-day-old brain heart infusion culture supernatants concentrated 25 and 50 times. The ability of the procedure to identify *Histoplasma* spp. was evaluated by testing 96 unknown mycelial cultures that grossly or microscopically resembled *Histoplasma* spp. Three- and six-day-old concentrated culture supernatants prepared from each unknown were tested against rabbit anti-*Arthroderma tuberculatum*, *Chrysosporium keratinophilum*, *H. capsulatum* var. *duboisii*, and *Corynascus (Thielavia) sepedonium* sera and human histoplasmosis case serum. Each unknown was also identified by conventional laboratory procedures involving cultural and, where necessary, in vivo studies. In the comparative evaluation the immunological test was observed to be 100% sensitive. It permitted the accurate generic identification of the *Histoplasma* spp. within 5 days, in contrast to the average of 33 days required by the routine mycological procedure.

Histoplasmosis is one of the most common systemic mycotic infections. Its unequivocal diagnosis rests upon isolation of the etiological fungus *Histoplasma capsulatum* from clinical material and its subsequent identification, both of which can be difficult at times to accomplish. Successful isolation of a hyphomycete that bears tuberculate conidia provides data which is only highly suggestive that the fungus is a pathogen, since a number of species of saprophytes in the genera *Arthroderma*, *Chrysosporium*, *Corynascus (Thielavia)*, and *Sepedonium* grossly and microscopically resemble the mycelial form of *H. capsulatum*. To further confuse matters, these saprophytes are occasionally isolated from clinical specimens. Atypical *H. capsulatum* isolates that produce only microconidia may also be confused with the mold form of *Blastomyces dermatitidis*. Differentiation of *H. capsulatum* from morphologically similar saprophytes requires conversion from its mycelial to its yeast form. The sapro-

phytes are not dimorphic. Unfortunately, in vitro conversion of *H. capsulatum* to the yeast form cannot always be achieved and in vivo conversion must be accomplished. The latter process is time consuming, and expensive animals must be used. It is obvious that an accurate and rapid immunological method for identifying the mycelial form of *H. capsulatum* would be a valuable asset in the diagnostic laboratory.

Kaufman and Brandt (8), using the fluorescent antibody test, and Li et al. (11), using the complement fixation and immunodiffusion (ID) tests, attempted to immunologically distinguish *H. capsulatum* from the saprophytes. They were unsuccessful, however, because they were unable to demonstrate species-specific antigens.

Manych and Sourek (12) attempted to use a serum-agar technique for identification of fungal pathogens. Their preliminary report indicated that such fungi might be rapidly identi-

fied in reactions with homologous hyperimmune sera. However, additional studies were necessary since some diagnostic precipitin bands did not appear until after 3 weeks of incubation, the occurrence of cross-reactions was widespread, reference systems for specifically identifying bands were lacking, and only one or two representative isolates of selected pathogens were used in the study.

The aim of the present study was to determine whether the histoplasmin H and M antigens were specific for *H. capsulatum* and whether they could be used in double diffusion tests to rapidly and accurately distinguish the mold form of *H. capsulatum* from morphologically similar saprophytes and pathogens.

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MATERIALS AND METHODS

Cultures. The following isolates were used to prepare precipitinogens and precipitins: *H. capsulatum* var. *capsulatum* 6623, B-884, B-1342; *H. capsulatum* var. *duboisii* 936, 1085; *Arthroderma tuberculatum* 1593, 3147; *Chrysosporium keratinophilum* B-1512, B-1513; and *Corynascus sepedonium* 1106, 9787, and 11814 (15). The isolates of the *Histoplasma* spp. were maintained in their yeast form on Pine's agar (BBL) and in the mold form on Sabhi agar (Difco). The nonpathogenic hyphomycetes were maintained on Sabouraud dextrose agar (Difco).

Inocula. All mold form inocula were obtained from a Sabouraud dextrose or Sabhi agar slant. Standardized mold form inoculum consisted of 0.5 cm² of mold form inoculated into 125 ml of Smith's asparagine broth (6). Routine mold form inoculum consisted of at least 2 to 4 cm² of growth. Routine inoculum for yeast form cultures consisted of one 3-mm loopful of growth from an agar slant.

Antigen production and testing. Routine mold form inoculum from a pure full-grown unknown fungus culture was transferred in duplicate into 30 ml of brain heart infusion broth in 125-ml flasks. After 3 and 6 days of incubation at 25 C on a gyratory shaker rotating at 150 rpm, the cultures were killed by adding merthiolate to a concentration of 1:5,000. Incubation was continued for an additional 24 h. Then the cultures were centrifuged at $1,050 \times g$ for 20 min, the mycelial growth was removed, and each of the supernatants was concentrated 25 $\times$ and 50 $\times$ with Amicon Minicon Macrosolute concentrators B-15 (Amicon Corp., Lexington, Mass.). For the micro-ID test, the 25 $\times$ and 50 $\times$ -concentrated supernatants from each broth culture were placed in adjacent lateral wells. The formation of H or H and M precipitin bands after 24 h constituted a positive reaction.

Antisera production. Ten times-concentrated supernatant of 2- and 6-week-old Smith's asparagine broth cultures of the saprophytes and 10 $\times$ -con-

centrated supernatants of 6-week-old Smith's asparagine broth cultures of *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii* were each used to immunize three albino rabbits. Each rabbit was injected intramuscularly with 0.5 ml of supernatant antigen and 0.5 ml of Freund incomplete adjuvant (Difco) on days 1, 2, and 3 and 0.5 ml of supernatant antigen intravenously on days 7 and 14. On day 21 the rabbits were bled and their sera were tested in micro-ID tests against the homologous immunizing antigens to determine whether precipitins were elicited. If the precipitin response was weak or negative, two additional intravenous injections consisting of 0.5 ml of supernatant antigen were administered 7 and 14 days after the bleeding date. Injections were continued until the harvested sera demonstrated intense precipitin bands after reaction with the homologous antigens. All sera were also tested against heterologous antigens. In addition, uninoculated brain heart infusion broth and Smith's asparagine broth concentrated 25 $\times$ and 50 $\times$ were tested against all the sera used in this study. The absence of precipitin bands with these controls revealed that no precipitins due to medium ingredients were present in the antisera studied.

ID and counterelectrophoresis tests. A modification of Busey and Hinton's (2) micro-ID procedure was used. Six and one-half milliliters of ID (7) agar was poured into a plastic petri dish (15 by 100 mm) and allowed to solidify for 30 min; then 3.5 ml of agar was poured as an overlay, and a plastic matrix containing 17 seven-well patterns (L.L. Pellet Co., Dallas, Tex.) was immediately placed in the liquid agar. Using this procedure and human histoplasmosis case serum (CDC lot 10716) containing H and M antibodies, we tested the concentrated supernatants in parallel with control histoplasmin antigen (CDC lot 7 or 9) for H and M precipitinogens. The antiserum was placed in the central well and preincubated for 1 h at 25 C. Reference antigens were then placed in the upper and lower wells of each pattern, and unknown antigens were placed in duplicate in the lateral wells. Each plate was incubated in a moist chamber at 25 C for 24 h.

The counterelectrophoresis technique of Kleger and Kaufman (10) was used.

RESULTS

Studies were performed to determine whether 6-week-old cultures of *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and some morphologically similar saprophytes incubated at 25 C produced H or M antigen, or both, in Smith's asparagine broth after inoculation with standardized mold form inocula. The results of these studies are shown in Table 1. It is evident from the data presented that H or H and M precipitin bands were produced only when 10 $\times$ -concentrated *Histoplasma* spp. exoantigens or the reference histoplasmin reacted with serum from humans with histoplasmosis or rabbit antisera to the *Histoplasma* spp. No reactions were observed between the exoanti-

TABLE 1. Immunodiffusion reactions of 10×-concentrated supernatant antigens of pathogenic and nonpathogenic hyphomycetes with homologous and heterologous antisera

Fungus supernatants	Control human histoplasmosis anti-serum	Precipitin bands formed ^a upon reactions with:						
		Rabbit antisera to:						
		<i>H. capsulatum</i> var. <i>capsulatum</i>		<i>H. capsulatum</i> var. <i>duboisii</i>		<i>A. tubercu-</i> <i>latum</i> ^b	<i>C. ker-</i> <i>atino-</i> <i>phil-</i> <i>um</i> ^b	<i>Cory-</i> <i>nascus</i> <i>sepe-</i> <i>don-</i> <i>ium</i> ^c
		6623	B-884	936	1085			
Control histoplasmin	HM	HM	HM	HM	HM	0	0	0
<i>H. capsulatum</i> var. <i>capsulatum</i> 6623	HM	HM	H	H	HM	0	0	0
<i>H. capsulatum</i> var. <i>capsulatum</i> B-884	H	H	H	H	H	0	0	0
<i>H. capsulatum</i> var. <i>capsulatum</i> B-1342	HM	H	H	H	H	0	0	0
<i>H. capsulatum</i> var. <i>duboisii</i> 936	H	H	H	H	H	0	0	0
<i>H. capsulatum</i> var. <i>duboisii</i> 1085	HM	H	H	H	H	0	0	0
<i>A. tuberculatum</i> 1593	0	0	0	0	0	1-2	0	0
<i>A. tuberculatum</i> 3147	0	0	0	0	0	1	0	0
<i>C. keratinophilum</i> B-1512	0	0	0	0	0	0	2-3	0
<i>C. keratinophilum</i> B-1513	0-1 ^d	0	0	0	0-1 ^d	0	2	0
<i>Corynascus sepedonium</i> 1106	0	0	0	0	0	0	0	2-3
<i>Corynascus sepedonium</i> 9787	0	0	0	0	0	0	0	1-2
<i>Corynascus sepedonium</i> 11814	0	0	0	0	0	0	0	1-3

^a Numerals represent number of bands formed.^b Antiserum pooled from two strains.^c Antiserum pooled from three strains.^d Band of nonidentity inconsistently produced.

gens of the *Histoplasma* spp. and the rabbit antisera produced against the nonpathogens. All of the 10×-concentrated supernatant from the nonpathogenic hyphomycetes demonstrated one to three antigens upon reaction with homologous antisera. Except for one isolate of *C. keratinophilum* (B-1513), which inconsistently produced an antigen not identical to H or M (Fig. 1), none of the nonpathogenic hyphomycetes produced antigens reactive with *Histoplasma* spp. antisera. Studies with 10×-concentrated 1-, 2-, and 4-week-old culture supernatants produced with standardized inocula indicated that H and M exoantigens could be produced with 2-week-old cultures.

The value of routine yeast and mold form inocula in Smith's asparagine broth and the effects of incubation at 25 and 37 C were also examined for each of the *Histoplasma* spp. cultures. Our studies indicated that the mold form inocula of *Histoplasma* spp. incubated at 25 C produced H or H and M antigens more rapidly

(within 1 week) than did the other combinations of inocula and incubation temperatures.

Although specific H or H and M antigens could be consistently produced by the *Histoplasma* spp., the prolonged incubation time necessary for exoantigen formation limited its diagnostic value. Additional studies were therefore conducted to determine if the antigens could be produced more rapidly by using heavy inocula (routine inocula) in small volumes of a dehydrated, commercially available medium. Accordingly, 30 ml of brain heart infusion broth in 125-ml nalgene flasks were each inoculated with mold form routine inocula of all stock cultures. These cultures were incubated at 25 C on a gyratory shaker rotating at 150 rpm, and merthiolate was added at 3, 6, and 10 days. Supernatants were concentrated 25× and 50× in attempts to obtain rapid results. The 25×- and 50×-concentrated 3-, 6-, and 10-day antigen preparations produced similar results. The modified procedure permitted the early de-

FIG. 1. Formation of a precipitin line by *C. keratinophilum* B-1513 unrelated to either the H or M bands. Wells 1 and 4 contained positive histoplasmin control antigen lot 9, and well 6 contained supernatant antigen prepared from a 4-week-old culture of *C. keratinophilum* B-1513. Center well contained rabbit anti-*H. capsulatum* var. *duboisii* 1085 serum.

tection of H or H and M antigens in 3-day-old BHI broth supernatants of *Histoplasma* spp. (Table 2). No apparent antigenic crosses were observed between the 25 $\times$ -concentrated supernatants from the *Histoplasma* spp. and the antisera to the nonpathogens. The concentrated supernatants from the 3-day-old nonpathogens produced precipitin bands only when they were tested against the homologous antisera.

A study was carried out to determine the sensitivity and specificity of the newly developed immunological technique and to compare the times necessary to identify *Histoplasma* spp. immunologically and by routine cultural methods. The immunological and conventional identification procedures were performed in parallel on 96 unidentified fungi that grossly or microscopically resembled *Histoplasma* spp. These unknowns were received from many areas of the world. They had been isolated from human, animal, and environmental sources, and their identity was unknown to us. Forty-eight of the 96 unknowns produced H or H and M antigens when tested against a histoplasmo-

sis case serum. Of these 48 immunologically positive isolates, 41 were identified as *H. capsulatum* var. *capsulatum* and seven as *H. capsulatum* var. *duboisii* on the basis of their morphology either in vitro or in vivo or both. Five of the 41 cultures morphologically identified as *H. capsulatum* var. *capsulatum* were received as *H. farciminosum* from reliable sources. All but two of the *Histoplasma* isolates produced H or H and M antigens within 3 days, and the latter cultures produced H antigen in 6 days. None of the supernatants from these 48 fungi reacted with antisera to *A. tuberculum*, *C. keratinophilum*, or *Corynebacterium sepeodonium*. Figure 2 shows the typical ID reactions when supernatant antigens of the unknown *Histoplasma* spp. were reacted with *Histoplasma* antiserum.

Supernatants from five of the unknowns produced a band that was unrelated to the H and M reference bands. Immunologically these were not identified as *Histoplasma* spp. Morphologically they were identified as *Coccidioides immitis*. Forty-three of the unknowns did not produce H or M antigens (Fig. 2), and

TABLE 2. Immunodiffusion reactions of 25×-concentrated 3-day-old supernatant antigens of pathogenic and nonpathogenic hyphomycetes with antisera to *H. capsulatum* var. *capsulatum* and nonpathogenic hyphomycetes

Supernatant antigens of:	No. of cultures	Precipitin bands formed ^a upon reaction with antisera to:			
		<i>H. capsu-</i> <i>latum</i> var. <i>capsula-</i> <i>tum</i>	<i>A. tubercu-</i> <i>latum</i> ^b	<i>C. keratin-</i> <i>ophilium</i> ^b	<i>Corynas-</i> <i>cus sepe-</i> <i>donium</i> ^c
<i>H. capsulatum</i> var. <i>capsulatum</i>	3	H or HM	0	0	0
<i>H. capsulatum</i> var. <i>duboisii</i>	2	H or HM	0	0	0
<i>A. tuberculatum</i>	2	0	2-3	0	0
<i>C. keratinophilum</i>	2	0	0	1	0
<i>Corynascus sepedonium</i>	3	0	0	0	2-3

^a Numerals represent number of bands formed.
^b Antiserum pooled from two strains.
^c Antiserum pooled from three strains.

FIG. 2. Immunological identification of unknown *Histoplasma* spp. cultures and differentiation of *Histoplasma* spp. from morphologically similar nonpathogens. Wells 1 and 4 contained positive control histoplasmin lot 9, well 2 contained supernatant antigen produced from *S. chrysospermum* ATCC 13378, well 3 contained supernatant antigen produced from *C. keratinophilum* ATCC 14803, and wells 5 and 6 contained histoplasmin produced from two unknown *Histoplasma* spp. cultures. Center well contained human histoplasmosis case serum containing H and M antibodies.

they were not immunologically identified as *Histoplasma* spp. Morphological studies on these 43 unknowns confirmed the immunological test results and showed them to be either

saprophytes or pathogens other than the *Histoplasma* spp. It is of interest to note that, of the 48 *Histoplasma* spp., 25 (52%) failed to produce conidia (macro- and microconidia).

Of the 43 fungi whose supernatants did not react with the antiserum to H or M antigens, only five produced bands upon reaction with antisera to the saprophytes: one with *A. tuberculatum* antiserum, two with the *C. keratinophilum* antiserum, and two with the *Corynascus sepedonium* antiserum. In contrast, the morphological studies indicated that 10 of the 43 fungi were members of the aforementioned genera.

DISCUSSION

ID studies with *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii* mycelial-form exoantigens showed them to share H or H and M precipitinogens (Tables 1 and 2). These findings are in agreement with those of Ehrhard and Pine (5), who reported that *H. capsulatum* var. *duboisii* produced H and M antigens. Antigenic analysis of the supernatants of *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii* suggest that the exoantigens are identical. Attempts to demonstrate species-specific precipitinogens were unsuccessful. A new and interesting finding was that *H. farciminosum* exoantigens reacting with human histoplasmosis case sera also produced H or H and M bands (Table 3).

The exoantigens of the nonpathogenic hyphomycetes *A. tuberculatum*, *C. keratinophilum* and *Corynascus sepedonium* did not produce H or M bands upon reaction with rabbit or human *Histoplasma* spp. antiserum (Tables 1-3), nor

did the exoantigens of the nonpathogens elicit production of H or M antibodies after injection into rabbits (Tables 1 and 2). These results are not consistent with the findings of Li et al. (11), who reported that rabbit anti-*C. keratinophilum* ATCC 14803 and anti-*Sepedonium chrysospermum* ATCC 13378 produced M bands after reacting with histoplasmin produced from one isolate of *H. capsulatum* but did not produce precipitins when reacted with histoplasmin produced from eight other *H. capsulatum* isolates. Because of such reactions, these two saprophytic fungi were included in this investigation (Table 3; Fig. 2). The present study did not substantiate the implications of Li et al. (11) that *C. keratinophilum* and *S. chrysospermum* shared antigens with *H. capsulatum*. The exoantigens produced by the isolates used by Li et al. (11) did not produce M precipitin bands or any other bands when tested against the *H. capsulatum* reference antiserum used in this study (Fig. 2).

The precipitin bands observed by Li et al. (11) could be similar to the band noted earlier with *C. keratinophilum* B-1513 reacting with *H. capsulatum* antiserum (Fig. 1). The line of nonidentity noted with the supernatants of *C. keratinophilum* B-1513 and that noted with some of the *C. immitis* isolates (Table 3) represent the only apparent exoantigen cross-reactions occurring with the *H. capsulatum* antisera. The difference in the results obtained by Li et al. (11) and by us might be due to the fact that homoge-

TABLE 3. Evaluation with 96 unknown hyphomycetes of the accuracy of the immunological procedure for identifying *Histoplasma* spp. as compared to conventional methods

Type of precipitin reactions	No of unknown fungus supernatants producing reaction	Identification by immunological test	Identification by conventional test	No. identified	% Agreement
H or HM bands	48 ^a	<i>Histoplasma</i> spp.	<i>H. capsulatum</i> var. <i>capsulatum</i> ^b <i>H. capsulatum</i> var. <i>duboisii</i>	41 ^c 7	100
Band noted, but not H or M	5	Not <i>Histoplasma</i> spp.	<i>C. immitis</i>	5	100
No bands	43	Not <i>Histoplasma</i> spp.	<i>B. dermatitidis</i> <i>Chrysosporium-Sepe-donium</i> complex ^d <i>C. immitis</i> <i>Mycelia sterilia</i> <i>P. brasiliensis</i> Saprophytes	9 10 6 2 9 7	100

^a All but two supernatants showed H or M bands in 3 days.

^b An average of 33 days was required for identification.

^c Five of the 41 cultures identified as *H. capsulatum* were received as *H. farciminosum*.

^d Includes ATCC cultures *C. keratinophilum* 14803 and *S. chrysospermum* 13378.

nate antigens were used in the former study and supernatant antigen was used in the latter. The differentiation of fungi producing such antigens from *Histoplasma* spp. poses no problem where the immunological test is used, since none produce H or M antigens.

In studies with histoplasmin skin test antigens, Palmer et al. (13), Asgari and Conant (1), and Edwards et al. (4) implied that, in certain human and animal cases, false positive delayed hypersensitivity reactions occurred and might be attributed to encounters with saprophytic fungi sharing common antigens with *H. capsulatum*, rather than to encounters with the latter pathogen. The results presented in our investigation revealed extrageneric reactivity between *H. capsulatum* and some isolates of *C. keratinophilum* and *C. immitis*. None of these reactions was due to H and M antigens. To obviate false positive skin test reactions with histoplasmin, it is recommended that purified histoplasmin containing only H and M antigens and no common antigens be used for skin testing (9).

White fluffy fungal isolates that do not sporulate are frequently obtained by diagnostic mycology laboratories. These nonsporulating fungi could be atypical *H. capsulatum* isolates and might be erroneously reported as contaminants. Fifty-two percent (25 of 48) of the unknown *Histoplasma* isolates used in this study were nonsporulating. Some of the pathogens studied were not primary isolates. Conventionally, the only way these nonsporulating *Histoplasma* spp. cultures could be correctly identified was by persistent in vitro or in vivo efforts to convert them to their yeast form. In those instances where conversion attempts proved unsuccessful, the diagnosis would be equivocal. The immunological test eliminates the need for conversion and reduces the time necessary for identifying *Histoplasma* spp. to 5 days, as compared with the average of 33 days required by routine morphological procedures.

The reagents developed in this study proved to be of little value for the immunological identification of the saprophytic *Arthroderma* spp., *Chrysosporium* spp., and *Corynascus* spp. Application of the antisera prepared against the stock cultures of the nonpathogenic hyphomycetes to concentrated supernatant antigens prepared from numerous isolates of *Arthroderma* spp., *Chrysosporium* spp., and *Corynascus* spp. received during the study failed in most cases to produce precipitin bands. Serologically, this group of fungi appeared to be very heterogeneous. Consequently, the accurate identification of these fungi must still be based upon morphological criteria (3).

None of the supernatant antigens of the mycelial form of the pathogens *B. dermatitidis*, *C. immitis*, and *Paracoccidioides brasiliensis* shared H or H and M antigens with the *Histoplasma* spp. (Table 3). This observation is interesting in light of the recent studies of Restrepo and Moncada (14). They noted that some paracoccidioidomycosis case sera, when reacted with paracoccidioidin, formed three bands designated 1, 2, and 3. Band 3, which was closest to the serum well, was considered to be identical to the histoplasmosis M band. However, of 35 patients' sera producing band 3, only eight cross-reacted with histoplasmin. In the present study, no cross-reactions were noted when concentrated supernatant antigens from 3- and 6-day cultures grown from 21 isolates of *P. brasiliensis* were tested against *H. capsulatum* antisera. The consistent absence of M antigens in the *P. brasiliensis* supernatant antigens presently studied and the fact that M precipitins were not consistently demonstrated in the paracoccidioidomycosis case sera studied by Restrepo and Moncada (14) suggest that the M bands noted in the latter study were not produced by infection with *P. brasiliensis* but by coincidental or earlier infection with *H. capsulatum*.

By using a dense mycelial mass for inoculum and small volumes of brain heart infusion broth as substrate, we developed a procedure that permitted the rapid production of H or H and M antigens by *Histoplasma* spp. The immunological procedure for identification of *Histoplasma* spp. based upon detection of H or H and M antigens was 100% sensitive and specific when compared with the results obtained by the conventional laboratory procedures (Table 3). Ninety-six unknown cultures that consisted of 48 *Histoplasma* spp. and 48 morphologically similar hyphomycetes and other fungal pathogens were used in evaluating this immunological procedure. Of the latter 48 fungi, none produced H or M antigens and none was reported as *Histoplasma* spp. However, five supernatants from what proved to be *C. immitis* cultures did react with *Histoplasma* spp. antisera, producing precipitin bands that were nonidentical to the H or M reference bands. This did not pose any diagnostic problem. The five *C. immitis* supernatants also produced lines of nonidentity with all the saprophyte ID references used in this study. These results indicate the need for establishing lines of identity in making an immunological diagnosis.

The immunological method does not permit the delineation of *Histoplasma* spp. Although representative isolates of the two species and their varieties occasionally produced bands

other than H or M, they were not consistently observed. This study indicates that the culture supernatants of the mycelial forms of *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, and *H. farciminosum* consistently contained the common antigens H or H and M. The strong antigenic and morphological similarities displayed by these fungi appear to justify their being classified together as species of *Histoplasma* or varieties of *H. capsulatum*.

The fact that two *Histoplasma* spp. cannot be differentiated immunologically does not pose a diagnostic problem in the Americas, since the only *Histoplasma* spp. found here is *H. capsulatum* var. *capsulatum*. In Africa, immunological identification of a fungus as a *Histoplasma* spp. would only signify that the agent was one of the two varieties of *H. capsulatum* or the species *H. farciminosum*. In such a case, in vivo and morphological studies would be needed for human isolates to distinguish *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii* from each other. If the fungus was isolated from a soliped, morphological and clinical manifestations would be necessary to distinguish *H. capsulatum* var. *capsulatum* and *H. farciminosum*.

It is obvious from the data presented that the new immunological procedure will provide the laboratory with an additional tool for rapidly identifying atypical *H. capsulatum* isolates that fail to sporulate or convert to the yeast-form. If this test is used, fewer false negatives will be reported. Preliminary studies have shown that the time required for the immunological identification (5 days) can be shortened by almost 24 h by using counterelectrophoresis in lieu of ID techniques (10).

For performance of the immunological test, it is recommended that two flasks of brain heart infusion broth be inoculated for each isolate. Then, if the 3-day culture supernatant is negative for H or H and M antigens, merthiolate treatment of the second culture supernatant would be carried out in 6 days, and then the supernatant could be concentrated and tested. Supernatants should be concentrated 25 $\times$ and 50 $\times$. For the most part, in the present study the 25 $\times$ and 50 $\times$ concentrates yielded equally potent precipitogens. In rare cases weak M bands were demonstrated in 50 $\times$ supernatant antigens but not in the 25 $\times$ concentrates. H bands, however, were seen in both 25 $\times$ and 50 $\times$ concentrates of *Histoplasma* spp. In some cases, 50 $\times$ concentration was excessive and tended to drive the precipitin reaction into the center well of the ID pattern, whereas the 25 $\times$ concentration yielded well-separated precipitins.

In performing the immunological test, it is recommended that supernatants of only two suspected *Histoplasma* spp. isolates be placed in each ID pattern (Fig. 1 and 2). Since some *Histoplasma* spp. produce potent antigens in 3 days, the bands produced may extend and run in front of adjacent wells containing negative supernatants. Such a phenomenon could be misinterpreted and be a source of false positives. To avoid such false results, we recommend that the 25 $\times$ - and 50 $\times$ -concentrated supernatants from each unknown isolate be placed in the adjacent wells on either side of the reference wells. For example, the supernatants of one isolate should be run in wells 2 and 3 and the supernatants of the other isolate in wells 5 and 6 (see numbered patterns in Fig. 1 and 2).

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