Revista Mexicana de Micología 10, 49-62, 1994

HISTOPLASMOSIS IN THE STATE OF GUERRERO, MEXICO: A BIOLOGICAL APPROACH[1]

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HISTOPLASMOSIS EN EL ESTADO DE GUERRERO, MÉXICO: ENFOQUE BIOLÓGICO

RESUMEN

Con el fin de establecer un modelo de investigación de la histoplasmosis en poblaciones susceptibles y estudiar el agente etiológico, *Histoplasma capsulatum*, en la naturaleza, se desarrolló un proyecto en uno de los estados de México referido como de alta prevalencia para esta micosis. Éste se llevó a cabo en los municipios de Juxtlahuaca y Olinalá, Guerrero. Se encontraron niveles elevados de reactores positivos a la prueba cutánea con histoplasmina (87% en Juxtlahuaca y 80% en Olinalá). Los individuos estudiados refirieron actividad ocupacional relacionada con excretas de aves o de murciélagos. Se aislaron tres cepas del hongo a partir de muestras de suelo contaminado con guano y excretas de ave, y otras tres de especímenes de murciélagos insectívoros identificados como *Myotis californicus*, *Mormoops megalophyla* y *Pteronotus parnelli*. La infección de las dos primeras especies de murciélagos es considerada como primer registro en el mundo y la de *P. parnelli* como primer registro para México. Los hongos fueron caracterizados por macro y micromorfología, así como por la producción de exoantígenos específicos. La respuesta en el ratón, a la inoculación de muestras de suelo contaminado con el hongo, reveló altos niveles de anticuerpos anti-*H. capsulatum* en el suero, determinados por ELISA, en 26 de un total de 80 muestras de guano, así como en 3 muestras de excretas de aves de un

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Received: April 2, 1994. Accepted: June 24, 1994.
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total de 13. La alta incidencia de reacciones positivas a la histoplasmina en la población estudiada, aunada a la presencia del hongo revelada por aislamientos positivos a partir de guano de murciélagos, excretas de aves y de murciélagos infectados, así como los altos títulos de anticuerpos anti-*H. capsulatum* encontrados en el suero de ratones inoculados con muestras de suelo contaminado, demuestran la gran prevalencia de la infección por *H. capsulatum* en los municipios estudiados.
PALABRAS CLAVE: *H. capsulatum*; histoplasmina; murciélagos; suelo contaminado.

ABSTRACT

A pilot project has been developed in the state of Guerrero, Mexico, to establish a model of epidemiological, immunological, and biological research for histoplasmosis in susceptible populations, and to characterize the causative agent, *Histoplasma capsulatum*, in nature. The Mexican state selected for this work shows records with a great prevalence for this mycosis, and two areas, Juxtlahuaca and Olinalá, were chosen. High levels of positive histoplasmin-skin test (87% in Juxtlahuaca and 80% in Olinalá) were found in both areas. Individuals studied referred occupational activities related with bats and birds excreta. Three *H. capsulatum* isolates were obtained from excreta-contaminated soil, and three isolates from insectivorous bats identified as *Myotis californicus, Mormoops megalophyla* and *Pteronotus parnelli*. The fungal infection of the first two bat species is considered as the first record in the world, whereas *P. parnelli* infection is a new record for Mexico. The fungus was identified by its macro and microscopic characteristics, as well as by its exoantigens. Mice inoculated with different soil samples revealed high anti-*H. capsulatum* antibody titers in sera, determined by ELISA in 26 out of 80 soil samples with bat guano, as well as in 3 out of 13 bird droppings samples. The high incidence of positive histoplasmin-skin test in the studied population, the isolation of the fungus from bird droppings and infected bats, and the high specific antibody titers from mice sera inoculated with soil samples, demonstrate the great prevalence of histoplasmosis in the studied areas of Guerrero state.
KEY WORDS: *H. capsulatum*; histoplasmin; bats; contaminated soils.

INTRODUCTION

Histoplasmosis is a common disease of worldwide distribution, specially in tropical and subtropical regions of America, caused by the dimorphic fungus *Histoplasma capsulatum* var. *capsulatum* Darling 1906. Epidemic outbreaks have been reported from most states of the Mexican Republic, and those with the highest frequency possess great number of mines and caverns, such as Guerrero, Michoacán, Querétaro, Hidalgo, Jalisco, San Luis Potosí, Guanajuato, Tamaulipas, Morelos, Yucatán, and Chiapas (González-Ochoa, 1963a; Velasco-Castrejón and Fujigaki-Lechuga, 1984). The fungus can be found in sites with bat guano and bird droppings, which together with soil conditions, humidity and temperature, constitute the ecological niche of this microorganism (Rippon, 1988; Velasco-Castrejón, 1981). *Histoplasma capsulatum* isolation from bat guano had been reported previously (Aguirre-Pequeño, 1959; González-Ochoa, 1963b). Bats are the typically infected mammals that could be responsible for the maintenance of this fungus in nature, although infection has been reported from other mammals. Kunz, in 1988, integrated all *Histoplasma* isolates from bats reported in America (Table 1).

In natural conditions, the fungus develops a mycelial form, and infection is initiated after inhalation of conidia by individuals that penetrate contaminated sites. In spite of a severe form of the disease, which develops in some susceptible hosts, the asymptomatic and mild histoplasmosis is the rule (Rippon, 1988). Histoplasmosis in Mexico is considered an occupational disease, as stated in the list of Labor Diseases, Code number 130, Article 513, Title Nine of the Federal Labor Law (Climent-Beltrán, 1990). In Mexico, infection is acquired in close spaces through aerosols containing a high number of microconidia or hyphal microfragments, which increase the risk of a severe clinical form. As a consequence, primary pulmonary histoplasmosis shows a high death rate (Velasco and Fujigaki-Lechuga, 1984).

There are different microenvironments in areas where histoplasmosis is reported (Eissenberg and Goldman, 1991), and different phenotypes of *H. capsulatum* strains have been shown in the same geographical area (Spitzer *et al.*, 1990). Considering the great number of mines and caves in the state of Guerrero, Mexico, this study was aimed at: a) to detect the frequency of fungal immune contact in the populations living in the studied areas; b) to characterize the peculiarities of the soil studied, which might favor strains or variants growth; c) to identify the precise sites of fungus harboring in soil conditions, and d) to study the bat's species involved in natural infection and to characterize their habits.

MATERIALS AND METHODS

Area of study: This work was performed in a geographical zone of the country with a great number of mines, probably endemic for histoplasmosis. This zone includes two specific areas of the state of Guerrero (Gro.) and the localities studied were Juxtlahuaca and Olinalá.

Population: Histoplasmin-skin test (ST) was performed in 128 consenting individuals. A control group constituted by researchers working with *Histoplasma* or bats was included.

Antigen: Histoplasmin obtained from the synthetic Smith's medium (1948) culture filtrate of strain EH-53 of *H. capsulatum* (Laboratorio de Micología Básica, Facultad de Medicina UNAM, México, D.F.) was used for all immune tests. Antigen was standardized by protein (Lowry *et al.*, 1951) and carbohydrate determinations (Dubois *et al.*, 1951), as well as by its reactivity in positive ST volunteer reactors.

Skin test (ST): Histoplasmin at 10 µg protein/0.1 ml was injected intradermally in the left forearm. Positive response was considered when an erythematous and induration reaction was observed, and the induration area was greater than 8 mm. Reactions were recorded at 24, 48 and 72 h.

Fungal isolation and identification: a) From guano, bird droppings and soil. Eighty samples were taken from guano in close spaces, 10 from soil mixed with guano used as fertilizer, and 13 from bird droppings. These samples were collected in sterile bottles and sent to the laboratory for processing. From each sample, 1 g was mixed with 10 ml phosphate-saline buffer, pH 7.2, supplemented with 50-100 µg/ml streptomycin and 50-100

U/ml penicillin (Lakeside Laboratory, Mex. D.F.). The supernatant was separated by centrifugation at 300 g, and then 0.1 ml inoculated in Petri dishes with mycobiotic agar, Sabouraud agar, and brain heart infusion agar (Bioxon, Mex. D.F.). A duplicate of each medium was made including bengal rose to reduce contamination growth, and all media were incubated at 28 and 37°C, respectively. All plates were checked daily, and after 3 to 4 weeks, *Histoplasma* was searched micro and macromorphologically in suspected colonies. After microscopic identification of *H. capsulatum*, the exoantigen test of Kaufman *et al.* (1983) was performed in double immunodiffusion (Ouchterlony and Nilsson, 1978). The positive reference antigen was histoplasmin from the laboratory characterized strain EH-53, and reference sera were a positive human histoplasmosis serum and a negative one from human volunteers, previously standardized. One ml of the previously obtained supernatant from each sample was inoculated intraperitoneally to BALB/c male adult mice. These were observed during 15-30 days and bled to separate sera samples. Sera were processed for the determination of anti-*H. capsulatum* antibodies by ELISA (Voller *et al.*, 1979) with 100 µg protein/ml of histoplasmin per well. The revealing system was biotinylated/streptavidin-peroxidase mice anti-gammaglobulin (Sigma Chemical Co., St. Louis, Mo.). **b) From bat specimens.** A total of 105 bats were captured and placed in dry ice at -70°C for transportation. Important data for taxonomic classification were recorded from each animal by the mastozoologist members. Autopsies were performed within 1-2 days after capture and samples of intestine, lungs, liver, and spleen were processed together in sterile conditions and cultured as mentioned above.

Collection and identification of bats from their natural refuges (mines and caves): Bats were captured with traps and nylon nets (Hall, 1981; Handley, 1988). All collected specimens were assigned a code number, and all data such as sex, somatic measures, reproductive condition, weight and age, determined by the nature of the hair and the ossification of their phalanges, were recorded. Material was prepared as described by Handley (1988) and taxonomic determination was performed according to Hall (1981) and Willson and Reeder (1993).

Relative humidity, ambient and soil (superficial to 10 cm depth) temperatures were recorded from each dwelling.

Statistical analysis: Mean and standard deviation for skin test results were calculated. A significant difference of $P \leq 0.01$ was considered when $\alpha \leq 0.01$ by Mann-Whitney U-test (Siegel, 1977).

RESULTS AND DISCUSSION

A high positive percentage of histoplasmin-skin test (ST) response was found in Juxtlahuaca (87%) and Olinalá (80%). The control group showed a low percentage of positivity (22.2%) to histoplasmin-ST (Fig. 1), and a significant difference was observed between the ST responses of the population from studied areas (Juxtlahuaca and Olinalá) when they were

compared with the control group ($p < 0.0001$). The low histoplasmin-ST response of the control group is related to the absence of contact with the fungus in its natural habitat, with the exception of the bat specialists. The high positivity of the histoplasmin response in Juxtlahuaca and Olinalá is a strong evidence to consider the studied areas as endemic for histoplasmosis, and shows its relationship with occupational risk factors, because it is associated with people who work in close spaces like miners, cave tourist guides, peasants who use bat guano as fertilizer, game-cock handlers, etc. These high ST percentages are remarkable when compared to a bare 2% in 98 individuals from the state of Tlaxcala, which belong to another geographical zone from Mexico, where no caves or mines were referred (Pedroza-Serés *et al.*, 1994).

Three fungal isolations were obtained from guano and bird droppings, and other three from bat specimens. The fungus was identified by its macro and micromorphology (Figs. 2a and 2b), as well as by the production of specific exoantigens (Figs. 3a and 3b). Figure 2a shows the typical colony morphology of *H. capsulatum* from one of the isolates of an infected bat named M-168, moderately grown, white-to-buff-brown colony, with sparse aerial hyphae. An unusual isolate (S-005) bearing red diffusible pigment was obtained from soil samples. Figure 2b shows the characteristic thick-walled, spherical, macroconidia with finger-like projections (tuberculate conidia). Other isolates presented also small, oval microconidia with smooth to finely roughened walls. Exoantigens production confirmed the identification of isolates, by the observation of H and M specific precipitation lines of *H. capsulatum*. Figures 3a and 3b show these lines among game-cock excreta SE-1, bat M-168 isolates, and the reference antigen with the antiserum positive system in gel immunodiffusion, as well as the negative result with a normal control serum.

The low isolation frequency of *H. capsulatum* from soil could be explained by the deficiencies in the methods of fungus isolation from soil, already described by Menges *et al.* (1967). However, high titers of ELISA test in sera of mice injected with soil samples suggest indirect evidence of the fungus.

Natural conditions of the fungus habitat correspond to an ambient temperature within a 24-32°C range, with a soil temperature of 23.4-31.2°C and a relative humidity of 75-100% in close spaces. Fungal isolations were obtained mainly from superficial soil and/or from soil down to 4-5 cm depth (Tables 2 and 3). This fungus has been isolated down to 38 cm beneath the soil surface (Mahvi, 1970) with a vegetative growth equally common and abundant. Edaphologic data as well as the mycobiota from guano or bird dropping-contaminated soil, associated to *H. capsulatum*, are still being studied.

Until the present time the bats from which the fungus was isolated correspond to male and female adults, insectivorous, identified as *Myotis californicus* (1 out of 3), *Mormoops megalophyla* (1 out of 17) and *Pteronotus parnelli* (1 out of 16) (Table 4). The first two bat species are not reported in the table of Kunz (1988), and they may be considered as the first records for bat *H. capsulatum* infection in the world, whereas *P. parnelli* infection is a new record for Mexico. Although bat infection has not been reported in Mexico, González-Ochoa

(1963b) referred that 94% of the epidemic outbreaks of histoplasmosis were associated with guano of *Tadarida brasiliensis* and *Desmodus rotundus*; furthermore, he also reported negative results in *D. rotundus* experimental infection.

Alimentary habits of bats are particularly interesting, and probably related with the infection route of these bat species. In spite of the variety of outdoor insects used as food by insectivorous bats, when the bat gathers some of the abundant insects from the caves' soil with *H. capsulatum* contaminated guano, they probably produce aerosols containing fungal spores, which are inhaled by themselves; moreover conidia have been found in the topsoil by Goodman and Larsh (1967). Another less probable bat infection route could be the ingestion of soil insects covered by fungal spores, since many insect species are present in the superficial guano found in caves. Considering that 105 bats were processed, this finding outstands due to the fact that the fungus was isolated only from the insectivorous species, although other frugivorous, polliniferous and even 25 hematophagous *D. rotundus* were investigated. These data suggest a possible relationship between alimentary habits and infection, and it might also be probable that these insectivorous species act as a fungal dispersion vehicle in nature due to their migratory habits, as already suggested for some bat species by Di Salvo *et al.* (1969).

Sera obtained from mice infected with all processed samples showed high anti-*H. capsulatum* antibodies titers by ELISA, in 26 out of 80 soil and guano samples (Table 2), as well as in 3 out of 13 bird dropping samples (Table 3). These data account for the spreading of *Histoplasma* to different sites; diverse isolates from these samples are still being characterized.

The high percentage of histoplasmin-ST response, the presence of *H. capsulatum* isolated from bat guano, bird droppings and infected bats, as well as the high antibody titers found in most sera obtained from inoculated mice, demonstrate the health hazard for individuals attending the studied sites, as well as the high incidence of histoplasmosis in the studied areas from Guerrero. This is the first multi-disciplinary study of histoplasmosis in Mexico, contemplating at the same time biological aspects of the fungus, its relationship to the bat's habitat, as well as the activities of the populations, which influence the high prevalence of the disease in this area. The results obtained, together with the health services support from the Guerrero State Government will help to integrate an educational-preventive histoplasmosis control program in the future.

ACKNOWLEDGMENTS

Authors gratefully thank the Guerrero State Government for the technical and economical supports for the present research as well as the partial support of DGAPA IN203294; they also thank Ingrid Mascher for editorial assistance, and Dr. M. L. Taylor acknowledges the extraordinary help of the cave guides Enrique Ortega Jiménez and Cayetano Morales Hernández. This paper received a Glaxo Foundation award in epidemiological research.

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Table 1. *Histoplasma capsulatum* isolates from bats.

FAMILY\GENERA-SPECIES	COUNTRY
Noctilionidae	
Noctilio labialis	Panama
Mormoopidae	
Mormoops blainvillii	Cuba
Pteronotus parnellii	Panama, Belize
Pteronotus suapurensis	Panama
Phyllostomidae	
Artibeus jamaicensis	El Salvador, Cuba
Brachyphylla cavernarum	Puerto Rico
Brachyphylla nana	Cuba
Carollia perspicillata	Panama, Colombia, Ecuador
Desmodus rotundus	Panama, Colombia
Glossophaga soricina	Panama, Colombia
Leptonycteris sanborni	U.S.A
Lonchophylla robusta	Panama
Lonchorhina aurita	Panama
Micronycteris megalotis	Panama
Phyllostomus discolor	Panama, El Salvador
Phyllostomus hastatus	Panama
Tonatia bidens	Panama
Vespertilionidae	
Eptesicus brasiliensis	Colombia
Eptesicus fuscus	U.S.A., Cuba
Myotis austroriparius	U.S.A.
Myotis grisescens	U.S.A.
Myotis lucifugus	U.S.A
Myotis myotis	Israel
Myotis sodalis	U.S.A.
Nycticetus humeralis	U.S.A.
Pipistrellus subflavus	U.S.A
Molossidae	
Molossus molossus	Panama, Ecuador
Molossus sp.	Panama
Nyctinomops laticaudatus	Panama
Tadarida brasiliensis	U.S.A.

Modified from Kunz (1988).

Table 2. Data from bat guano samples with direct and indirect evidences of *Histoplasma capsulatum*.

CODE Nº	SITE*	T°C SOIL	T°C AMBIENT	DEPTH (cm)	HUMIDITY (%)	FUNGAL ISOLATE	ELISA[1] TITERS
S-005	Z1	23.8	24.8	4	75	*H. capsulatum*	1:20480
S-006	Z1	23.4	24	10			1:20480
S-008b	Z1	24.8	25.4	sup			1:5120
S-008d	Z1	24.8	25.4	sup			1:20480
S-009	Z2	31.2	32	sup			1:10240
S-016b	Z2	31.2	31.2	4			1:20480
S-017	Z2	31.2	31.2	5			1:20480
S-019	Z2	31.1	34	sup	100		1:5120
S-022	Z2	25.8	28	2	100		1:2560
S-023	Z2	29.8	28	4	100	*H. capsulatum*	1:20480
S-025	Z2	26	31	sup			1:5120
S-026	Z2	25.8	31	2			1:2560
S-032	Z3	30	32	15			1:20480
S-033	Z3	30.2	32	sup			1:10240
S-043	Z4	29.1	31	2			1:20480
S-046	Z5	28	28	sup			1:10240
S-047	Z6	28	28	sup			1:10240
S-048	Z6			sup			1:5120
S-051	Z6			sup			1:5120
S-055	Z6			sup			1:5120
S-058	Z5	28.3	28	3			1:5120
S-064	Z8	29.2	31	sup			> 1:640
S-067	Z8			sup			> 1:640
S-068	Z8			4			> 1:640
S-069	Z8			6			> 1:640
S-071	Z8			5			> 1:640

Sup: Superficial. *Z1: Gallery "Beso Milenario", JUXTLAHUACA CAVES, GRO., *Z2: Gallery "Infierno", JUXTLAHUACA CAVES, GRO., *Z3: Gallery "El Sótano", ZINACATLAN CAVES, GRO., *Z4: QUECHULTENANGO CAVES, GRO., *Z5: CHEUTZINGO CAVES, GRO., *Z6: DIABLO CAVE, OLINALÁ, GRO., *Z8: TEMALACATZINGO MINE, OLINALÁ, GRO. [1]ELISA test was performed in sera from mice infected with the abovementioned samples. Mice bleeding was performed 20 days after infection. Total number of samples = 80.

Table 3. Data from bird dropping samples with direct and indirect evidences of *H. capsulatum*.

CODE Nº	T°C SOIL	T°C AMBIENT	DEPTH (cm)	FUNGAL ISOLATE	ELISA1 TITERS
SE-1*	28	28	sup	*H. capsulatum*	1:10240
SE-2*	28.5	28	sup		1:10240
SE-3*	28.2	30	sup		1:2560

*SE-1: Soil mixed with game-cock droppings, OLINALÁ, GRO., *SE-2: Chicken droppings used as fertilizer, OLINALÁ, GRO., *SE-3: Dove droppings, OLINALÁ, GRO. [1]ELISA test was performed in sera from mice infected with the above-mentioned samples. Mice bleeding was performed 20 days after infection. Total number of samples = 13.

Table 4. Data from infected *Histoplasma capsulatum* bats

CODE Nº	SITE*	SEX	AGE	BAT'S SPECIES
M-013	Z1	Female	Adult	*Myotis californicus* (1/3)**
M-015	Z1	Male	Adult	*Pteronotus parnelli* (1/16)
M-168	Z9	Female	Adult	*Mormoops megalophyla* (1/17)

*Z1: Gallery "Beso Milenario", JUXTLAHUACA CAVES, GRO., *Z9: COPALA CAVE, OLINALÁ, GRO. **Numerator indicates number of infected bats and denominator total number of bat's species studied. Total number of bats processed = 105.

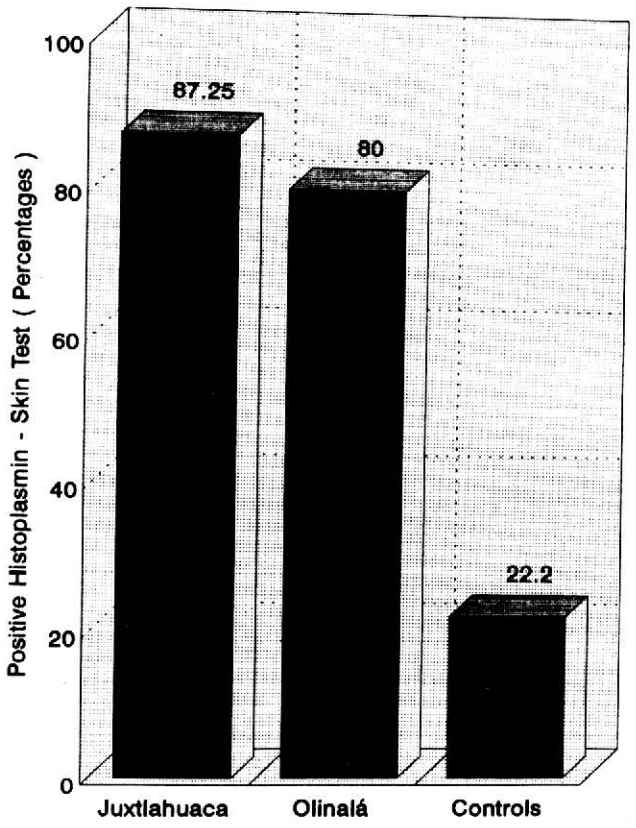

Fig. 1. Positive histoplasmin-ST percentages in the studied population. Histoplasmin-ST was performed in 128 individuals. Positive ST response was considered when the induration inflammatory area was greater than 8-mm in diameter, after 24, 48 and 72 h of antigen application. See details under Materials and Methods.

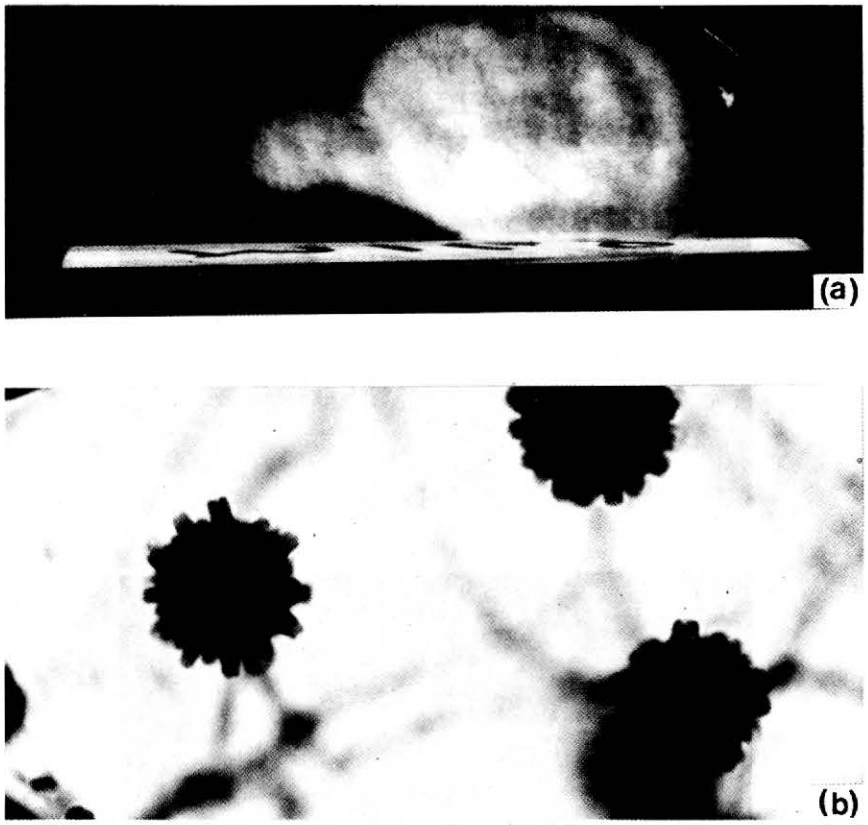

Fig. 2. Macroscopic (a) and microscopic (b) morphology of the mycelial infecting phase of *H. capsulatum* isolated from an infected bat (strain M-168). (a) White-to-buff-brown colony with sparse aerial hyphae. (b) Characteristic thick-walled spherical macroconidia with finger-like projections, 2100 X. Photos by Calixto Benavides.

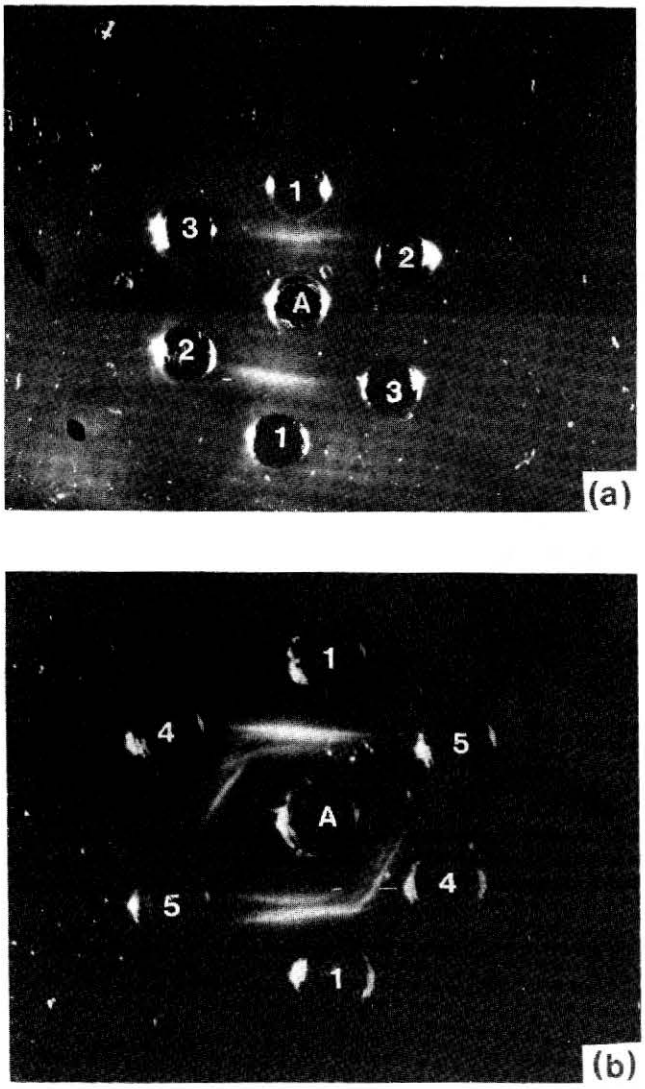

Fig. 3 (a) and (b). *H. capsulatum* isolates identification by exoantigens production. A = Positive reference serum from a patient with histoplasmosis; **1** = *H. capsulatum* strain EH-53 reference exoantigen; **2** = isolate SE-1; **3, 5** = negative controls; **4** = isolate M-168. Exoantigens were produced by the method of Kaufman *et al.* (1983).