ENVIRONMENTAL CONDITIONS FAVORING BAT INFECTION WITH
HISTOPLASMA CAPSULATUM IN MEXICAN SHELTERS


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Abstract. Histoplasma capsulatum was isolated from gut, lung, liver, and spleen of 17 of 208 captured bats belonging to 6 different genera and species. Three of the 17 infected bats were from the State of Guerrero and 14 were from the State of Morelos. All were adult bats: 6 males (1 Pteronotus parnelli, 2 Natalus stramineus, 2 Artibeus hirsutus, and 1 Leptonycteris nivalis) and 11 females (1 Myotis californicus, 1 Mormoops megalophylla, 8 A. hirsutus, and 1 L. nivalis). High rates of bat infection with H. capsulatum were found in the monitored sites of the State of Morelos. Histoplasma infection of N. stramineus, A. hirsutus, and L. nivalis should be considered as the first records in the world. The fungus isolated from infected bats was identified by its typical mycelial-phase morphology and by its yeast-phase conversion. Exoantigen production confirmed the fungal identification by the presence of specific precipitation lines in double immunodiffusion assays using human immune serum. Histopathologic studies showed intracellular yeast-like cells compatible with H. capsulatum yeast-phase in tissues of several bats, especially in pulmonary (intra-alveolar and septal) macrophages, with none or minimal tissue reaction. In contrast to past reports, present data support a high risk of bat infection with H. capsulatum in Mexican cave environments.

Bat guano and bird droppings have been shown to be the most common sources of the pathogenic fungus Histoplasma capsulatum var. capsulatum Darling, 1906, the causative agent of the deep mycosis histoplasmosis, which has a worldwide distribution.1 The fungus is found either in confined spaces where bat guano is abundant or in open spaces such as public parks and home yards, where bird droppings are frequently found. Excreta from these animals are rich in nutrients necessary for fungal growth and together with soil and environmental conditions, humidity and temperature, constitute the ecologic niche of this microorganism.14

Bats are among the few infected mammals that contribute to the maintenance of this fungus in natural foci, in addition to some gregarious birds such as starlings, black birds, chickens, oil birds, and pigeons. Aguirre-Pequeño in 1959 and González-Ochoa in 1963,5 defined the habitat of bats related to the isolation of H. capsulatum in Mexico, and the latter tested experimental infection of Desmodus rotundus in the laboratory without success. The number of bat species in which H. capsulatum has been isolated, particularly in America, is increasing. Kunz7 in 1988 reported bat species from which the fungus had been isolated from 1970 to 1981. Subsequently, new H. capsulatum bat infections in North America were reported. Fernández-Andreu in 1988 reported infection of Macrotus waterhousii minor in Cuba,2 and Taylor and others in 1994 isolated in Mexico 3 H. capsulatum strains from insectivorous bats identified as Myotis californicus, Mormoops megalophylla, and Pteronotus parnelli.4 Fungal infection of Myotis californicus and Mormoops megalophylla is considered the first record in the world, whereas P. parnelli infection is new for Mexico.

Although the environmental factors promoting H. capsulatum growth in shared ecologic niches have long been known, the circumstances that favor bat infection have been poorly studied. This paper presents data that might contribute to the understanding of the conditions that foster bat infection with this fungus.

MATERIALS AND METHODS

Bats capture sites. Bat specimens were captured from 11 different sites located in 2 states in Mexico: Guerrero and Morelos. Both states are characterized by a great number of mines and caves, and by a high endemicity for human histoplasmosis.4,9,10 Nine caves, 1 orchard, and 1 mine were monitored. In Guerrero, these included the caves of Juxtlahuaca, Chichicastenango, and Zacataltepec in the Quechultenango Municipality; Diablo, Coapala, and Chiautzingo in the Olinalá Municipality; and the orchard named La Chulada in the Coyuca de Benítez Municipality. In Morelos, these included the mine of El Clarín in the Tlahuitoltepec Municipality; and the caves El Salitre in the Tlalaltipan Municipality and El Diablo in the Tepoztlán Municipality. Details and specific locations of the municipalities are shown in Figure 1.

Bat specimens. Two hundred eight bats were captured. Most were netted in their roosts during the day; others were netted free flying at night. They were selected in situ; pregnant females were released. Animals were brought to the laboratory preferentially alive, dead specimens were placed in dry ice at −70°C. All captured specimens were assigned a code number. Data such as sex, somatic measures, reproductive condition, weight and age, determined by the nature of the hair and ossification of their phalanges, were recorded. Material was prepared as described by Hänle11 and taxonomic determination was performed according to Hall12 and Wilson and Reeder.13 Relative humidity and temperature of the bats’ capture sites were recorded. The topography and distance between roof and floor of the sites, where each bat colony was dwelling, and guano deposits heights were estimated.

Fungal isolation from bats. At least 1–2 days after capture, bats were killed and samples of their guts, lungs, livers, and spleens were separated for histopathology and processed for fungal isolation. Briefly, each organ sample was fixed in 10% buffered formalin and processed for the routine paraffin

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embedding technique. Sections of 3 μm thickness were prepared for staining with hematoxylin and eosin and Gomori's methenamine. Simultaneously, homogenates of each duplicate organ sample were obtained in sterile conditions, and mixed with 5 ml of phosphate-saline buffer, pH 7.2, supplemented with 50–100 μg/ml of streptomycin and 50–100 U/ml of penicillin. Each tissue homogenate was centrifuged at 400 g, and 0.1 ml of supernatant was inoculated in mycelial cultures by their colonial morphology and further serially diluted to separate them. After microscopic identification, the exoantigen test of Standard and Kaufman was performed in double immunodiffusion assay. Carbohydrate and protein determinations were previously made to normalize their respective concentrations in each assay. A positive human histoplasmosis immune serum was used to detect exoantigens tested. Reference antigen of *H. capsulatum* was prepared from the EH-53 strain isolated from a Mexican patient with histoplasmosis, as standardized elsewhere, and saline solution was used as negative antigen control.

The fungal mycelium-to-yeast-phase conversion was processed at 37°C in synthetic-liquid medium, under mild shaking conditions for 1–2 weeks. The yeast-phase of each *H. capsulatum* isolate was grown at 37°C in supplemented BHI medium. Yeast-cells were harvested by centrifugation at 400 × g and suspended in 1 ml of a 9:1 (v/v) mixture containing fetal bovine serum (Gibco-BRL, Gaithersburg, MD) and dimethyl sulfoxide. They were stored in vials at −196°C.

**RESULTS**

A total of 208 bats, 103 males and 105 females, belonging to 5 families, 13 genera, and 18 species, were captured in the sites indicated in Figure 1. Captured bats are listed in Table 1. From these 6 bat species, 3 had been reported previously as infected by *H. capsulatum*. From the 17 infected

**Identification of *H. capsulatum***. Suspected colonies of *H. capsulatum* were first selected from the organ homogenate cultures by their colonial morphology and further serially diluted to separate them. After microscopic identification, the exoantigen test of Standard and Kaufman was performed in double immunodiffusion assay. Carbohydrate and protein determinations were previously made to normalize their respective concentrations in each assay. A positive human histoplasmosis immune serum was used to detect exoantigens tested. Reference antigen of *H. capsulatum* was prepared from the EH-53 strain isolated from a Mexican patient with histoplasmosis, as standardized elsewhere, and saline solution was used as negative antigen control.

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bats, 3 were from the State of Guerrero (2 from the Juxtlahuaca cave and 1 from the Coapala cave), and the remainder from the State of Morelos (2 from the El Clarín mine, 10 from the El Salitre cave, and 2 from the El Diablo cave). All were adult bats: 6 males (1 Pteronotus parnaellii, 2 Natalus stramineus, 2 Artibeus hirsutus, and 1 Leptonycteris nivalis), 11 females (1 Myotis californicus, 1 Mormoops megalophylla, 8 A. hirsutus, and 1 L. nivalis). The relationship of infected/captured bats was determined considering the sites of their captures (Table 2). The highest infection rates of 40% (2 of 5) and 66% (10 of 15) were detected in N. stramineus and A. hirsutus, respectively. Most infected bats presented colonial habits, possibly spending a long time inside their shelters. Bat species were non-migratory, except for L. nivalis, and their diets are specified in Table 2.

Relevant characteristics related to the high rate of *H. capsulatum* infection in the capture sites were analyzed, such as, guano deposits, floor surface, ceiling-floor distance, as well as their environmental humidity and temperature (Table 3). Capture sites showed from scarce (< 3 cm depth) to extremely abundant guano deposits (> 20 cm depth), which accumulated reaching up to 1 meter in some sites, e.g., the Juxtlahuaca cave, where 16 *P. parnaellii* specimens were captured. The floor surface was flat or irregular, with a ceiling-to-floor distance that ranged between 2 and 25 meters; humidity and temperature averages varied from 60% to > 75% and from 24.8°C to 32°C, respectively.

Histopathologic examination of the lung, gut, liver, and spleen showed that inflammatory tissue reaction was minimal or absent. The presence of numerous intracellular yeast-like cells, compatible with *H. capsulatum* yeast-phase, was observed within intra-alveolar and septal pulmonary macrophages as shown in a lung section of *L. nivalis* (Figure 2).

Although, no parasitized macrophages or granulomas were microscopically evident in liver and spleen, cultures of *H. capsulatum* isolates from these organs were obtained from *A. hirsutus* and *L. nivalis*. Fungal isolates from gut, liver, and spleen from *M. megalophylla*, *A. hirsutus*, *N. stramineus*, and *L. nivalis* suggest a probable disseminated infection in these bat specimens (Table 4).

Twenty *H. capsulatum* isolates were recovered from 17 infected bats; 2 isolates, 1 from *P. parnaellii* and the other from *Myotis californicus*, were lost due to contamination with other fungi. The number of each *H. capsulatum* isolate, the bat species, and the corresponding organ(s) from which the fungus was cultured of the remaining 18 isolates are shown in Table 4. Eight isolates were recovered from the lung, 7 from the gut, 2 from the liver, and 1 from the spleen. Isolates were firstly characterized by their mycelial-phase morphology with thin-septate-hyphae, microconidia, and thick-walled spherical macroconidia with finger-like-projections were observed in all *H. capsulatum* isolates, as shown in Figure 3. All studied isolates were converted to their yeast-phase in special culture conditions, as described in the Materials and Methods.

Table 2
Relevant data of bat species with isolation of *Histoplasma capsulatum* in Mexico

<table>
<thead>
<tr>
<th>Species</th>
<th>Capture sites</th>
<th>Sex*</th>
<th>Diet†</th>
<th>Migration</th>
<th>Relation of infected/captured bats‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pteronotus parnaellii§</td>
<td>Juxtlahuaca cave</td>
<td>Male</td>
<td>I</td>
<td>Non-migratory</td>
<td>1 (16)</td>
</tr>
<tr>
<td>Myotis californicus§</td>
<td>Juxtlahuaca cave</td>
<td>Female</td>
<td>I</td>
<td>Non-migratory</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Mormoops megalophylla§</td>
<td>Coapala cave</td>
<td>Female</td>
<td>I</td>
<td>Non-migratory</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Natalus stramineus</td>
<td>El Clarin mine</td>
<td>Male</td>
<td>I</td>
<td>Non-migratory</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Artibeus hirsutus</td>
<td>El Salitre cave</td>
<td>Female (8)</td>
<td>F</td>
<td>Non-migratory</td>
<td>10 (15)</td>
</tr>
<tr>
<td>L. nivalis</td>
<td>El Diablo cave</td>
<td>Male (1)</td>
<td>P, N, F</td>
<td>Migratory</td>
<td>2 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17 (55)</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis represent specimens studied in the referred capture sites.
† I = insectivorous, F = frugivorous; P = pollinivorous; N = nectarivorous.
‡ I = In the same capture site.
§ Previously reported in Taylor and others. ¹

Table 3
Physical characteristics of bat capture sites with isolation of *Histoplasma capsulatum*

<table>
<thead>
<tr>
<th>Capture sites</th>
<th>Guano deposits*</th>
<th>Floor surface</th>
<th>Ceiling floor distance (m)</th>
<th>Humidity/temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juxtlahuaca cave</td>
<td>++ + +++</td>
<td>Flat</td>
<td>25</td>
<td>75%/24.8°C</td>
</tr>
<tr>
<td>(Pteronotus parnaellii)</td>
<td>++ +</td>
<td>Flat</td>
<td>25</td>
<td>75%/24.8°C</td>
</tr>
<tr>
<td>Coapala cave</td>
<td>+++</td>
<td>Irregular</td>
<td>10</td>
<td>60%/30°C</td>
</tr>
<tr>
<td>El Clarin mine</td>
<td>+++</td>
<td>Flat</td>
<td>2</td>
<td>&gt;75%/32°C</td>
</tr>
<tr>
<td>El Salitre cave</td>
<td>+++</td>
<td>Irregular</td>
<td>7</td>
<td>75%/28°C</td>
</tr>
<tr>
<td>El Diablo cave</td>
<td>+ +</td>
<td>Irregular</td>
<td>4</td>
<td>75%/28°C</td>
</tr>
</tbody>
</table>

* +++ = <3 cm depth (scarce); +++ = 3–6 cm depth (regular); ++++ = 6–20 cm depth (abundant); +++++ = >20 cm depth (extremely abundant).
Exoantigen production confirmed identification of all *H. capsulatum* isolates by the presence of an identity reaction among precipitin lines in the double immunodiffusion assay using a human histoplasmosis immune serum (Figure 4).

**DISCUSSION**

Human histoplasmosis has been reported in a variety of environments, which could be related to different phenotypes of *H. capsulatum* strains isolated from several sources, such as contaminated soil, infected animals, and human patients. Fungal isolation in nature is an unusual finding and, in general, the rate of *H. capsulatum* isolated from contaminated soil, as well as from infected bats, is very low, as described elsewhere. This disease in Mexico has been frequently associated with enclosed spaces where bats roost, and 60 of the 134 bat species in this country roost in caves, as reported by Arita. Eighteen bat species were identified in the 208 specimens captured; some were more abundant than others, i.e., *M. megalophylla*, *P. parnellii*, *A. jamaicensis*, *Glossophaga soricina*, and *D. rotundus* each had more than 20 specimens (Table 1).

The size of the bat populations is may be related to anthropogenic activities. However, bat infection or illness must be considered as another factor responsible for the decrease in their population. The present study sought to determine the bat species involved in natural *H. capsulatum* infection and identify their behavior and habits, as well as the characteristics of their shelters, which increase their natural risk of infection. Our data show a high frequency of *H. capsulatum* infection in bats randomly captured in caves, suggesting a high infection risk in bats in these specific environments.

Bats are one of several vertebrates that have been infected with the pathogenic fungus *H. capsulatum*, and new reports from this study include *H. capsulatum* infection of *N. stramineus*, *A. hirsutus*, and *L. nivalis*, which are the first reports of these infections. Most bat species infected with *H. capsulatum* exhibit colonial habits and a variety of diets: insectivorous, pollinivorous, nectarivorous, frugivorous, hematophagous, and piscivorous, which would suggest that the diet is not associated with the occurrence of the fungus infection in bat species.

Experimental studies have shown that *Artibeus lituratus* can be intranasally infected with only 100 viable yeast-fungal cells. Inhalation of high fungal spore concentrations is a relevant factor that should be considered as a risk factor for bat infection with *H. capsulatum*. The fungal affinity for the respiratory tract suggests that this is a natural route of infection and dissemination into the lungs and other organs. Histopathologic studies of bat organs revealed a low inflammatory tissue reaction in experimentally infected *A. lituratus*, as described by McMurray and Greer. Tissue reaction of naturally

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**TABLE 4**

*Histoplasma capsulatum* isolates from infected bats in Mexico

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Bat species</th>
<th>Organ with fungal isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EH-315</td>
<td>Mormoops megalophylla</td>
<td>Gut</td>
</tr>
<tr>
<td>EH-365</td>
<td>Artibeus hirsutus</td>
<td>Gut</td>
</tr>
<tr>
<td>EH-366</td>
<td>Artibeus hirsutus</td>
<td>Gut</td>
</tr>
<tr>
<td>EH-367</td>
<td>Artibeus hirsutus</td>
<td>Lung</td>
</tr>
<tr>
<td>EH-368*</td>
<td></td>
<td>Gut</td>
</tr>
<tr>
<td>EH-370</td>
<td>Natalus stramineus</td>
<td>Lung</td>
</tr>
<tr>
<td>EH-371</td>
<td></td>
<td>Gut</td>
</tr>
<tr>
<td>EH-372</td>
<td>A. hirsutus</td>
<td>Gut</td>
</tr>
<tr>
<td>EH-373</td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>EH-374</td>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>EH-375</td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>EH-376</td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>EH-377</td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>EH-378*</td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>EH-383P*</td>
<td>Leptonycteris nivalis</td>
<td>Lung</td>
</tr>
<tr>
<td>EH-383H*</td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>EH-383I*</td>
<td></td>
<td>Gut</td>
</tr>
<tr>
<td>EH-391</td>
<td></td>
<td>Liver</td>
</tr>
</tbody>
</table>

*H. capsulatum* isolated from different organs of the same bat species.
infected bats, as observed in this study, also showed a low inflammatory response in the lungs despite the presence of intracellular yeast cells, especially in interstitial macrophages (Figure 2). Although no evidence of tissue reaction was observed in the liver and spleen of several bats, cultures of *H. capsulatum* isolates were obtained in 2 livers and 1 spleen of infected specimens. Negative fungal isolation from bat feces directly separated by gut washings was observed in repetitive assays, whereas *H. capsulatum* isolates were recovered from guts of 7 bats. Histopathologic studies of guts referred no inflammatory reactions; however, alterations in the mucous membrane of the intestine were hardly observed.

Although infection of bats with *H. capsulatum* was well documented in 1988 by Kunz, based on the corresponding literature, a low infection rate (1% to 12%) is usually reported. However, a higher rate of infection (57.1%) was observed in *Brachyphylla cavernarum* captured in the Aguas Buenas caves in Puerto Rico. The present study detected the highest infection rates in *N. stramineus* (40%) captured in the El Clarín cave and *A. hirsutus* (66%) captured in the El Salitre cave. Both of these caves are located in Morelos and share the characteristic ceiling-to-floor distance of 2–7 meters (Table 3), which probably favors high spore concentrations within enclosed spaces, especially under dark conditions.

It is important to emphasize that the infection rate of bats with *H. capsulatum* cannot be considered as a parameter to define bat susceptibility to fungal disease. Mortality rate or median lethal dose (LD₅₀) determinations are the most recommended methods to evaluate *H. capsulatum* virulence. However, this is difficult to determine experimentally, since bats maintained in captivity are not easy to manipulate, and these methodologies require a considerable number of specimens to ensure statistical accuracy.

The most critical circumstances that foster infection of bats with *H. capsulatum* have not been described. The present findings (Tables 2 and 3) show a close relationship bet-
between some bat population parameters and the physical characteristics of their roosts, which would be involved in fungal growth and dispersion of spores in these sites, exposing bats to infection. Although isolation of H. capsulatum from bat guano was not performed in the present study, results of bat infections suggest a high infection risk in the bat captured at the sites studied, which could be associated to air currents produced by animal movements or wing flapping of the bats, producing dispersion of fungal spores. In this way the spores reach the respiratory tract of bats, infecting them to different degrees, depending on their susceptibility. Smaller distances between the guano deposits and the ceiling increase the probability of inhaling a high concentration of fungal spores; consequently, physical conditions of shelters could influence the infection risk factor for humans and bats.

The present data support a close relationship among bat habitats, environmental conditions, and physical characteristics of roosting sites with the risk of infection with H. capsulatum. It is also probable that the high frequency of H. capsulatum infection in several bat species favor their role as a fungal dispersion vehicle in nature due to their migratory habits, as already suggested.

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