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THE IMPORTANCE OF SEROLOGICAL ASSAYS IN DIAGNOSING ACUTE PULMONARY HISTOPLASMOSIS

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ABSTRACT: Histoplasmosis is a systemic mycosis caused by inhalation of *Histoplasma capsulatum* microconidia. The disease does not normally affect immunocompetent individuals after a single, transient inhalation exposure. However, longer exposure may cause chronic or disseminated acute pulmonary infection. Herein, we report the case of a 24-year-old immunocompetent patient, who presented fever, cough and dyspnea for one month. The chest radiography revealed interstitial infiltrate and diffuse micronodules. The patient reported having had close and prolonged contact with bats. Diagnosis was confirmed by positive double immunodifusion and immunoblotting assays. She was treated with ketoconazole (400 mg) and there was complete resolution of the disease.

KEY WORDS: histoplasmosis, *Histoplasma capsulatum,* immunocompetence, serodiagnosis, bats.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Histoplasmosis (HP) is a systemic mycosis provoked by the dimorphic fungus *Histoplasma capsulatum* that is endemic in the United States along the Ohio and Mississippi River valleys and many other parts of the world, particularly in Latin America (1-3). In Brazil, HP illness and/or infection have been reported in São Paulo, Rio de Janeiro, Minas Gerais, Mato Grosso do Sul and Rio Grande do Sul states (1, 4-9). Human infection usually occurs after inhalation of aerosolized microconidia released from disturbance of soil. Exposures may occur during activities such as building, remodeling, demolition, soil excavation, spelunking, camping, cutting sugar cane and/or wood as well as cleaning sites that harbor the fungus (2, 3). The risk of infection depends on the following factors: type of activity performed, activity duration, amount of dust containing microconidia or soil exposure and host immunological condition (2, 3, 10). Usually, longer and more intense exposures result in more severe acute pulmonary disease (2, 3).

In endemic areas, about 50% – in some cases, even more than 80% – of adults are infected with *H. capsulatum*, based on rates of histoplasmin skin-test positivity. Although fewer than 5% of individuals develop the symptomatic disease after low-level exposure, attack rates may exceed 75% following intense exposure (3, 10). The illness presents a wide spectrum and includes acute pulmonary histoplasmosis, which may ocasionally lead to adult respiratory distress syndrome or disseminated histoplasmosis; chronic pulmonary histoplasmosis; histoplasmoma and other clinical manifestations (3, 10, 11). According to Panackal *et al*. (2), acute pulmonary histoplasmosis in returning travelers is usually manifested as a flu-like disease characterized by high-grade fever, chlills, headache, nonproductive cough, pleuritic chest pain and fatigue. Chest radiographs often reveal diffuse reticulonodular infiltrates and mediastinal lymphadenopathy. In almost 80% of cases the infection is self-limited.

This report describes the high value of serological assay – mainly in cases with absence of *H. capsulatum* yeast form in direct examination – for diagnosing acute pulmonary histoplasmosis in an HIV-negative woman who contracted the disease after visiting a bat-infested cave.

MATERIALS AND METHODS

Case Report and Serum Samples

A 24-year-old woman, a veterinary physician, tobacco addict who was living in an urban area but exposed to some rural activities sought information at the Laboratory of Mycosis Immunodiagnosis, Immunology Section, Adolfo Lutz Institute, after a one-month history of irregular fever, asthenia, dyspnea, malaise, chest pain and mucopurulent sputum. Additionally, the woman presented episodes of gastric alterations with vomiting and weight loss. Two weeks before, she had visited caves seeking bats and arachnids in Bonito, Mato Grosso do Sul state, Brazil. She remained inside the cave for two to three hours. The laboratory test examination revealed leukocytosis among lymphocytes and monocytes, while HIV antibodies were negative. Chest radiograph displayed multiple nodules as infiltrate foci in the base of the lungs and hilar adenopathy. Sputum direct examination of three samples was negative for *H. capsulatum* yeast form. The hemoculture analysis was also negative. The patient was treated with ketoconazole 400 mg/day for 12 days. We evaluated three serum samples: before, during and 30 days after antimycotic therapy.

H. capsulatum Antigen

For *H. capsulatum* antigen preparation, the Kaufman and Standard's method was employed with some modifications (12, 13). Briefly, mycelial cells from isolate 200 of *H. capsulatum* were grown in solid Sabouraud dextrose medium (Difco Laboratories, USA) at 27°C for 33 days. After incubation, the cultures were treated with aqueous solution of thimerosal 1:5,000 (Sigma Chemical Co., USA) and left standing for 24 hours at room temperature. After this, the supernatants were filtered through Whatman® n. 1 paper (Whatman, UK) for the preparation of *H. capsulatum* antigen. Antigens were concentrated 20-fold by lyophilization. *H. capsulatum* antigens, purchased from Immuno-Mycologics (USA), were employed as control.

Serological Assays

Double immunodiffusion (DI) assay

The reactions were performed according to the modified method of Ouchterlony (14). Glass slides were covered with 3.0 mL of a gel composed of 1% agarose type II medium (Sigma Chemical Co., USA) in a buffered saline solution (pH 6.9) containing

0.4% sodium citrate and 7.5% glycine. Antigen (12 µL) was placed in the central well, while control and patient sera (12 µL) were put in surrounding wells. The slides were incubated in a humid chamber at room temperature for 48 hours. Then, they were washed with saline solution with several changes over a 24-hour period. Gels were dried and stained in 0.4% Coomassie brilliant blue R-250® (Sigma Chemical Co., USA) in an ethanol-acetic acid-water mixture as solvent.

SDS-PAGE and immunoblotting assay

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), *H. capsulatum* antigen was diluted in a buffer – 62 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 50 mM 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue – that was boiled for three minutes and centrifuged before application in gels. Antigen was then submitted to electrophoresis (20 mA at room temperature) on a 10% discontinuous SDS buffer system in a Mini-Protean II® electrophoresis cell (Bio Rad Laboratories, USA) and molecular mass was determined by the use of a 6.5-175 kDa standard prestained protein marker (New England BioLabs, UK) (15). Immunoblot assay was performed as previously described by Towbin *et al*. (16). Proteins from SDS-PAGE were electrotransferred onto 0.20-µm nitrocellulose membrane (Sigma Chemical Co., USA) in a Mini Trans-Blot Cell (Bio Rad Laboratories, USA), with 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol (v:v). The nitrocellulose membrane containing electrophoresed antigen was blocked with 5% non-fat dry milk in PBS, for one hour at room temperature. Membranes were incubated for two hours at room temperature with human sera diluted to 1:40 in PBS containing 0.05% Tween-20 (Sigma Chemical Co., USA) then were washed six times with PBS-T and developed with peroxidase conjugated goat of human IgG antibody (Sigma Chemical Co., USA) for two hours at room temperature. The reactions were observed with 4-chloro-1-naphtol substrate (Sigma Chemical Co., USA).

RESULTS

The three serum samples from this patient showed positive results through DI assay against both *H. capsulatum* antigens. Figure 1 reveals the representative scheme of the qualitative DI assay. Semiquantitative DI assay disclosed that the titer of circulating anti-*H. capsulatum* antibody – obtained from isolate 200 of *H. capsulatum* – was 1/16 (Figure 2). Through the immunoblotting assay, a strong reaction of patient

sera to a protein with molecular mass of 74 kDa (M fraction) was observed, while a weak response against a protein of 109 kDa (H fraction) was registered (Figure 3).

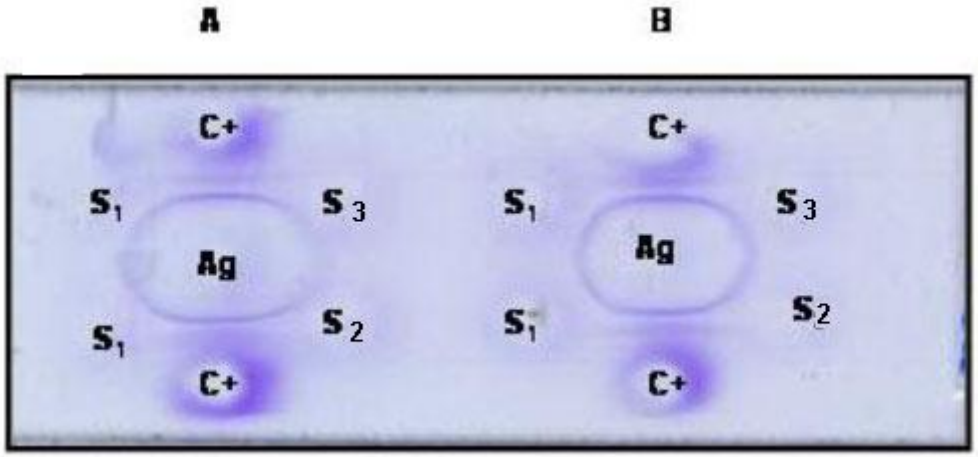

Figure 1. Qualitative immunodiffusion assay. Central wells present the antigen obtained from isolate 200 of *H. capsulatum* (**A**) and *H. capsulatum* reference antigen (**B**); peripheral wells have (C+) *H. capsulatum* control serum with bands H and M. Wells S_1, S_2 and S_3 contain patient serum samples evaluated before, during and 30 days after antimycotic therapy, respectively.

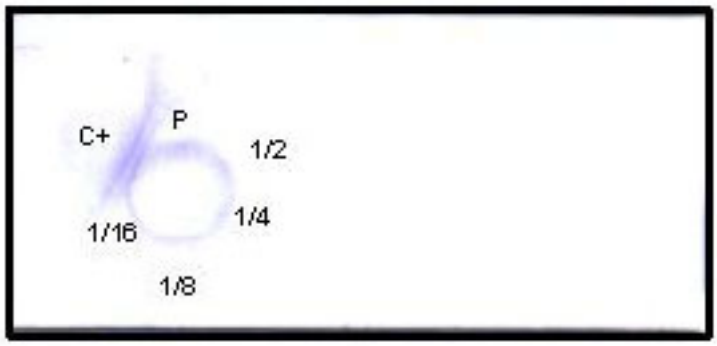

Figure 2. Semiquantitative immunodiffusion assay. The circulating anti-*H. capsulatum* antibody titer observed was 1/16.

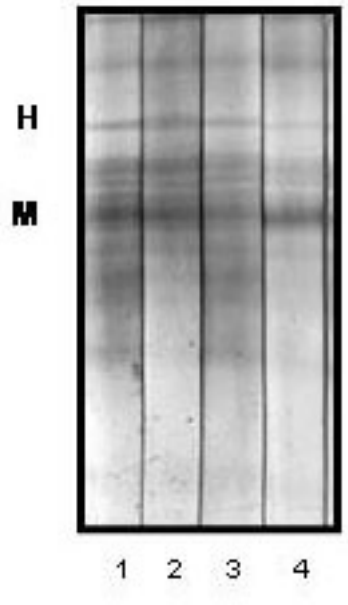

Figure 3. Antibody recognition of the H and M fractions of *H. capsulatum* antigen. Acute pulmonar histoplasmosis patient sera, concentrated to 10-fold, was evaluated by immunoblotting assay. Lane 1: patient serum before treatment; lane 2: patient serum during treatment; lane 3: patient serum 30 days after antimycotic therapy; lane 4: histoplasmosis control serum.

DISCUSSION

Histoplasmosis is an inhalation-acquired mycosis (3). *H. capsulatum* can be found either in confined spaces where bird and bat feces are abundant or in open spaces such as public parks and home yards, where bird droppings are frequent (3, 17). Excreta from these animals are rich in nutrients necessary for fungal growth and, together with specific soil characteristics (acidity and porosity) and environmental conditions (humidity and temperature), constitute the *H. capsulatum* ecologic niche (3, 17). After inhalation and deposition of microconidia in host alveolar spaces, they must be converted to the yeast form to become pathogenic, a process completed within a range from several hours to a few days (3, 10). The pathogenesis is similar to tuberculosis. The granulomatous inflammatory response to *H. capsulatum* in immunocompetent hosts generally heals the infection over several weeks (3, 10). Once exposed, individuals become sensitized to the fungus and the skin test becomes positive.

Only in a few cases can the disease become disseminated, usually in individuals with some associated risk factors including advanced age, lymphoma,

immunosuppression or chronic disease (3). Several human activities are also associated with high exposure to histoplasmosis, like spelunking, mining, building and agriculture (2, 3). Bats are among the few infected mammals that contribute to the maintenance of this fungus in a natural environment, in addition to some gregarious birds such as blackbirds and chickens (17).

Emmons (18) first described the association between bats and pathogenic fungi, after observing the isolation of *H. capsulatum* from soil contaminated by bat guano in Maryland, USA. *H. capsulatum* var. *capsulatum* was isolated by Zancopé-Oliveira and Wanke (19) in the soil of Rio da Prata, Rio de Janeiro state, Brazil. Their analysis of 111 soil samples revealed eight (7.2%) contaminated foci, all related to chicken habitats with one infected focus enriched by both chicken and bat feces. In Mexico, of 208 captured bats, *H. capsulatum* was found in the gut, lung, liver or spleen of 17 animals (17).

Ashford *et al.* (20) reported an outbreak of histoplasmosis during the National Speliological Society Convention in Texas, USA, in which acute histoplasmosis was associated with exposure to two bat-infested caves. This infection has long been recognized as common recreational disease among spelunkers in North America, 60 to 64% of whom presented positive skin test against histoplasmin (2). Additionally, outbreaks of histoplasmosis were reported among tourists after their spelunking trips to bat-infested caves in Central and South America. The occurrence of acute histoplasmosis among Japanese travelers who were exposed to bat guano in a cave near Manaus, Amazonas state, Brazil, was reported by Suzaki *et al.* (21). Similarly, in 1997 a sudden outbreak of histoplasmosis was spotted in Pedro Leopoldo, Minas Gerais state, Brazil, after four individuals had been in contact with a bat-inhabited cave (5). Nasta *et al.* (22) reported four cases of acute pulmonary disease in Italian cave explorers returning from Mato Grosso.

A cluster of cave-associated acute histoplasmosis that occurred among college students returning from Ecuador was reported by Valdez and Salata (23). During the spring of 2001, students from Pennsylvania reported an acute febrile respiratory illness after returning from spring break vacation in Acapulco, Mexico. Acute pulmonary histoplasmosis was presumptively diagnosed (24). Lyon *et al.* (25) registered that between October 1998 and April 1999, 51 persons belonging to two separate groups developed acute pulmonary histoplasmosis after visiting a cave in Costa Rica. The first group consisted of 61 children and 14 adults from San Jose,

Costa Rica; 44 (72%) were diagnosed with acute histoplasmosis. The second group comprised 14 tourists from the United States and Canada; 9 (64%) were diagnosed with histoplasmosis.

Epidemiological surveys carried out in Brazil indicated that histoplasmosis is endemic in all studied areas. Skin tests with paracoccidioidin, histoplasmin and sporotrichin were applied to 417 workers of Morro Velho Mining, Minas Gerais state, Brazil, with the main purpose of detecting the prevalence of paracoccidioidomycosis, histoplasmsis and sporotrichosis infections. The rates of positivity were 13.43% for paracoccidioidin, 17.50% for histoplasmin and 13.67% for sporotrichin (26). Silva-Vergara and Martinez (9), analyzed 109 individuals in Ibiá city, Minas Gerais state, Brazil, who showed 44 and 49.5% reactivity, respectively, for paracoccidioidin and histoplasmin intradermal antigens. In Brazil, acute pulmonary histoplasmosis is the most frequent clinical form reported, and presents a dry cough, malaise and diffuse pulmonary infiltrates associated with hilar lymphadenopathy (8).

Histoplasmosis diagnosis is based on clinical, radiological and epidemiological aspects. Laboratory tests include culture, fungal detection in stained smears or tissue sections, and detection of both antibodies and antigens. Isolation of *H. capsulatum* by culture of clinical specimens is a standard method of microbe identification, however the process of isolating *H. capsulatum* is expensive and time-consuming (11, 27, 28). Many HP cases are serologically diagnosed, a technique that ia a rapid alternative to microbiological procedures (11, 27, 28). The detection of circulating *H. capsulatum* antibodies by immunodiffusion assay is frequently used in immunocompetent individuals with histoplasmosis (11, 27, 28).

According to both studies by Wheat (28) and Wheat and Kauffman (3), DI is helpful to confirm the diagnosis, particularly in individuals with the mild disease by collection of sera from both acute and convalescent patients. This is possible because antibodies usually manifest at least one month after the initial exposure. All serum samples from the current case of histoplasmosis tested positive through DI and immunoblotting assays. When culture filtrate was used as the antigen, two major precipitin bands could be detected by DI. The M band typically can be detected in up to 75% of acute histoplasmosis cases but can persist for many months after the initial infection. The H band is specific to the acute disease; however, it only occurs in 10 to 20% of cases (11, 28). In our case, we could recognize both fractions in the patient sera.

Outbreaks of histoplasmosis have been increasingly reported among individuals involved in adventure tourism, ecotourism and recreation activities. Nevertheless, it is difficult to prevent sporadic exposure to sources of *H. capsulatum* in areas where it is endemic. If exposure cannot be avoided, individuals should be advised to wear masks and special protective equipment (1, 2). Transportation of soil, feces and other potential fomites should be avoided and public health authorities must place warning signs at known high-risk locations.

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