

Research Paper

Immunological assays employed for the elucidation of an histoplasmosis outbreak in São Paulo, SP

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Abstract

Several reports showed outbreaks of histoplasmosis acquired while bat-inhabited caves were visited by tourists, miners or researchers. We evaluated the performance of double immunodiffusion (DI) and immunoblotting (IB) assays, employed for the histoplasmosis outbreak elucidation occurred in Vale do Paraíba, São Paulo. The existence of epidemiologic link, four patients with clinical signs suggestive of histoplasmosis and mycological confirmation has made that all 35 individuals involved to the cave visit were subjected to serological evaluation. By DI, we observed reactivity against *H. capsulatum* antigen in a single serum examined nearly 20 days after exposure to fungal propagules. On the other hand, IB showed reactivity against H and M fractions in 50% of samples evaluated. The analysis of the second sample batch, collected two months after the exposure showed that 96.7% were reactive by DI with antibodies titers ranging from 1 to 16 and 100% of reactivity against H and M fractions, by IB, suggesting an acute infection. The analysis of the overall agreement between the methods showed to be reasonable ($\kappa = 0.37$). This study confirms the importance and efficacy of more sensitive methodologies, such as IB assay, to early elucidation of disease, especially in cases of patients without mycological information.

Key words: *Histoplasma*, histoplasmosis, immunoassay, double immunodiffusion, immunoblotting.

Introduction

Histoplasmosis is a systemic mycosis caused by *Histoplasma capsulatum*. The disease is autochthonous mainly in the American and African continents, however, it can be found in some European countries, especially in Italy. The few reported cases in Asia are associated with individuals who traveled to endemic areas and are therefore considered imported cases (Panackal *et al.*, 2002; Colombo *et al.*, 2011). The fungus grows in soil containing large amounts of bird or bat guano especially that found under blackbird roosts, next to chicken coops, in the land around trees that harbor birds and bats and inside and around caves

(Jülg *et al.*, 2008). *H. capsulatum* remains in the soil for many years and the draughts can spread conidia by miles, exposing individuals who have no direct contact with contaminated sites (Cano and Hajjeh, 2001; Kauffman, 2007; Jülg *et al.*, 2008). Several reports showed occasional and occupational histoplasmosis acquired while bat caves were visited by tourists, miners or researchers (Ashford *et al.*, 1999; Buxton *et al.*, 2002; Erkens *et al.*, 2002; Jülg *et al.*, 2008).

The infectious process begins when large amounts of microconidia or small hyphal elements are inhaled and convert to yeasts in the lungs, or when fungal cells in previous quiescent foci of infection are reactivated during immuno-

suppression (Wheat and Kauffman, 2003). There is a large spectrum of clinical forms of histoplasmosis, and the clinical presentation is strongly influenced by the extent of exposure to *H. capsulatum* propagules, age, immune status of the patient, and the presence of chronic pulmonary disease previous to fungal infection (Colombo *et al.*, 2011). The acute pulmonary form is characterized by respiratory symptoms that arise from 1 to 3 weeks after exposure to the pathogen (Goodwin and Des Prez, 1978; Buxton *et al.*, 2002).

The histoplasmosis diagnosis is based on the visualization of the fungus in organic fluids (sputum, blood, liquor) or tissues (histopathological evaluation) and by the culture of biological samples. Serologic analysis are highly useful for the immunodiagnosis of acute/subacute forms of disease (positive in greater than 90% of patients with pulmonary histoplasmosis), despite its wide applicability, serology has limitations especially in immunosuppressed patients with disseminated disease (Wheat and Kauffman, 2003; Ferreira and Borges, 2009). The double immunodiffusion (DI) assay assess qualitative and semi-quantitatively precipitating immunoglobulins and have highly specific for the detection of anti-M and anti-H antibodies, but shows variable sensitivity (70-100%). In general, DI is useful for detecting antibodies 4-6 weeks after infection (Guimarães *et al.*, 2006). Differently, the antibody response can be detected early by enzyme immunoassay. Immunoblotting (IB) assay has been employed successfully in order to diagnose histoplasmosis and evaluate epidemiologically the distribution of the disease (Fava Netto *et al.*, 1967; Kauffman, 2007). In acute histoplasmosis, antigen may be detected before antibodies appear, permitting early diagnosis and treatment (Guimarães *et al.*, 2006).

This study aims to evaluate the performance of two different immunoassays, double immunodiffusion (DI) and immunoblotting (IB), employed for the histoplasmosis outbreak elucidation occurred in Vale do Paraíba, São Paulo, Brazil.

Material and Methods

Patients and serum samples

In October 2007, the Vigilância Epidemiológica de Areais, SP, Brazil was informed that 4 patients were admitted to health care centers in Vale do Paraíba region, with suspicion of acute pulmonary histoplasmosis (APH) after had visited the same bat-inhabited cave located in the city of Arapeí, SP, Brazil, in September 2007. These patients looked for the health services 5 to 15 days after the visit. The clinical manifestations and cases evolutionary characteristics were described and published previously (Zöllner *et al.*, 2010). Due to a strong suspicion of an outbreak in the city, serological evaluation from all individuals that were involved in the cave visit was requested to the Laboratório de Imunodiagnóstico das Micoses, Instituto Adolfo Lutz,

São Paulo, Brazil. The group was composed of 35 individuals, 31 teenagers (14-16 years old) and 4 adults (mean 35 years); 22 (62.9%) male and 13 (37.1%) female. The laboratory received two batches for analysis: the first group was composed of 35 serum samples collected nearly 20 days after the cave visit and the second batch was composed of 31 serum samples, obtained approximately 60 days after the visit to the cave.

Antigen

For *H. capsulatum* antigen preparation, the Kaufman and Standard's method was employed with some modifications (Kaufman and Standard, 1978; Freitas, 2005). Briefly, mycelial cells from *H. capsulatum* 200, maintained at the Fungus Collection Institute of Tropical Medicine of the Laboratory Medical Mycology (Genbank Number DQ239887), isolate were grown in Sabouraud dextrose medium agar (Difco Laboratories, USA) at 27 °C for 30 days. After incubation, the cultures were treated with aqueous solution of thimerosal 1:5000 (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 antigen (AgHc200). Antigens were lyophilized and concentrated 20 times (2.77 µg/µL) for the DI and 10 times (1.39 µg/µL) for use in the IB.

Immunodiffusion

The reactions were performed according to the modified Ouchterlony's method (Ouchterlony, 1949). 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

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described by Laemmli (1970). Ag Hc200 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 3 min and centrifuged before gel application. Antigen (5 µg/mL/slot) 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). Immunoblot assay was performed as previously described by Towbin *et al.* (1979). Proteins from SDS-PAGE were electrotransferred onto 0.22 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 pH 7.4, for 1 hour at room temperature. Membranes were incubated for 2 hours at room temperature with human sera diluted to 1:100 in PBS pH 7.4, then were washed 6 times with PBS pH 7.4 containing 0.05% Tween-20 (Sigma Chemical Co., USA) and developed with peroxidase conjugated goat of human IgG antibody (Sigma Chemical Co., USA) for 2 h at room temperature. The reactions were observed with 4-chloro-1-naphthol substrate (Sigma Chemical Co., USA).

Analysis methods

The agreement between the two serological assays was determined by the Kappa index and interpreted according to Fleiss (1971) (8): $k = 0.00$ to 0.20 , poor; $k = 0.10$ to 0.20 , slight; $k = 0.41$ to 0.60 , moderate; $k = 0.61$ to 0.80 , substantial and $k = 0.81$ to 1.00 , almost perfect.

Ethics

This study was approved by Committee of Ethics in Research with Humans of Instituto Adolfo Lutz, protocol #31/2009.

Results

In the first set of samples, it was observed by DI quantitative test, reactivity against *H. capsulatum* antigen in a single serum, of which circulating antibodies were detected up, by semiquantitative DI assay, to dilution of 1:4. By IB methodology we observed in 21 (61.8%) sera the presence of reactivity, of these 17 (50%) presented anti- M (94 kDa) and H (120 kDa) fractions of *H. capsulatum* and 4 (11.8%) reacted only against to the M fraction. One sample has not been evaluated by IB due to insufficient material. The analysis of the second sample batch, collected two months after exposure showed that 96.7% were reagents to the *H. capsulatum* antigen with antibodies titers ranging from 1 to 16 by DI. When these sera were evaluated by IB, it was found that all of them (100%) specifically recognized the M and H fractions from *H. capsulatum* (Figure 1).

The performance of both methodologies was evaluated by comparing the results, as shown in Table 1. Overall, the sensitivity of the DI was 48% (31/65) and IB, 80% (52/65). The analysis of concordance between the methods showed to be reasonable and 59.6% of samples were co-positive (31/52). Considering only the samples of recent infection, the sensitivity of DI reduced to 3% (1/34) and of IB to 67% (21/34) and the methods showed only a slight agreement between them. However, evaluating the samples sent

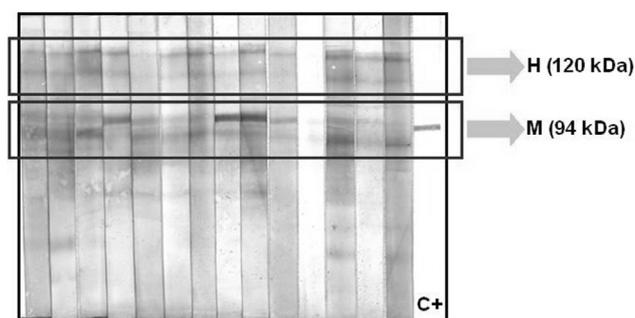


Figure 1 - Representative IB reactions for *H. capsulatum* antigen with serum samples obtained from individuals during an outbreak of acute pulmonary histoplasmosis, ~60 days after the exposure. C+: positive control.

later, the IB and DI methods showed sensitivity of 97% (30/31) and 100% (31/31), respectively, showing good agreement and co-positivity equal to 96.8%.

Discussion

H. capsulatum is a dimorphic fungus that causes respiratory or pulmonary infection after inhalation of infective fungal propagules by dispersion of dust or soil in humid atmospheres. Bats are among the few infected mammals that contribute to the maintenance of this fungus in a natural environment as they can host *H. capsulatum* in your gastrointestinal tract, spreading it from its habitat, which consists of caves, cellars and holes in trees (Wheat, 1994; Taylor *et al.*, 1999). Caves represent one of the mainly foci that facilitates the propagation of the fungus and bat-inhabited caves may harbor *H. capsulatum* even in areas where histoplasmosis is of low endemicity (Ashford *et al.*, 1999). Moreover, histoplasmosis also can be transmitted at entrances of bat caves and not only inside, since the draughts may be spread conidia by miles, thus exposing individuals who have no direct contact with contaminated sites (Cano and Hajjeh, 2001; Jülg *et al.*, 2008). Knowledge of the infection has been derived from descriptions of epidemics (Goodwin and Des Prez, 1978). Since 1958, 28 outbreaks of histoplasmosis involving 255 patients have been reported in Brazil, with the number of cases per outbreak ranging from 2 to 35 (Oliveira *et al.*, 2006; Unis *et al.*, 2005; Vicentini-Moreira *et al.*, 2008). In São Paulo State, only three outbreaks of histoplasmosis were reported and both happened in the city of Ubatuba in 1966 and 1971-1973 (Fava Netto *et al.*, 1967) and the last in city of Arapeí, Vale do Paraíba with the highest number of cases diagnosed in an outbreak of acute pulmonary histoplasmosis in Brazil (Vicentini-Moreira *et al.*, 2008). Knowledge of clinical syndromes and laboratory information available associated with the patient eco-epidemiological history may avoid empirical treatment. The anti-*H. capsulatum* antibod-

Table 1 - Agreement of samples results evaluated by double immunodiffusion and immunoblotting assays, according time of infection.

DI x IB	20 days of infection			~60 days of infection			All samples		
	R	NR	Total	R	NR	Total	R	NR	Total
R	1	0	1	30	0	30	31	0	31
NR	20	13	33	1	1 ^a	2	21	13	34
Total	21	13	34	31	1	32	52	13	65
KAPPA	0.04			0.65			0.37		

DI - double immunodiffusion; IB - immunoblotting; R - reactive, NR - no reactive.

^a negative control sample included to perform the calculation.

ies presence detected by serological methods, combined with clinical and / or radiological evidence of infection can be considered as a criterion for diagnosis of histoplasmosis in cases where the isolation of the etiologic agent was not possible (Leimann *et al.*, 2005). In this reported outbreak, four individuals exposed to the same environmental conditions got ill and all of them showed high fever, followed by holocraniad headache, cervical lymphadenomegaly and myalgia complaint (Zöllner *et al.*, 2010). The clinical and epidemiological resemblances between the patients needed of laboratorial confirmation as soon as possible. This study shows the potential role of immunological assays for diagnosis of acute histoplasmosis, including asymptomatic form; in that the culture is a slow and low sensibility method for this clinical form (~15%) (Guimarães *et al.*, 2006). Importantly, in this study only four individuals presenting exacerbated clinical symptoms were assessed mycologically. In these four patients the Grocott-Gomori methenamine silver stain revealed intracellular yeast forms and cultures of sputum samples and brochoalveolar lavage revealed the presence of *H. capsulatum* (Wheat and Kauffman, 2003, Wheat, 2003). The two serological methods routinely employed for immunodiagnosis of histoplasmosis are complement fixation (CF) and double immunodiffusion (DI). Although in past CF testing had been used among many laboratories, in Brazil, it was replaced by DI assay, because to its simplicity, reliability, and potential cost effectiveness. DI is highly specific for the detection of anti-M and anti-H antibodies, but shows variable sensitivity (70-100%) (Guimarães *et al.*, 2006). The IB is a immunoenzymatic methodology and its use has the advantage to be the first evidence of *H. capsulatum* infection in some cases. This happened due to the possibility of earlier detection of seroconversion by DI (Fava Netto *et al.*, 1967) as demonstrated in this study. Of 34 confirmed cases serologically, 22 (61.8%) samples had reactivity to *H. capsulatum* by IB on the first lot. The k concordance index has been described as a way to comparatively analysis the agreement between two diagnostic assays with qualitative results (reagent or non-reagent). In this case, the degree of concordance in the second batch of samples was good ($k = 0.65$) and corroborates to the phenomenon of seroconversion. Due to the DI semiquantitative property, the

disease evolution could be laboratory followed by decrease of antibodies titers. Therefore, the concomitant use of two immunoassays was more efficient than isolated methodologies.

In summary, this report demonstrated as well as proven the potential usefulness of two different serological assays as complementary for elucidation of cases, especially when the mycological tests were not requested and or available. The IB assay showed more sensitivity, in turn, the DI is more suitable to monitor disease prognosis. Furthermore the use of a more sensitive method such as IB has allowed us to provide the Vigilância Epidemiológica de Areias a rapid and reliable response allowing appropriate action to be taken.

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Declaration of interest

All authors no reported conflicts.

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