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Role of western blot assay for the diagnosis of histoplasmosis in AIDS patients from a National Institute of Infectious Diseases in Rio de Janeiro, Brazil

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Running title: Western blot for histoplasmosis/AIDS patients

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ABSTRACT

Background: Histoplasmosis is a frequent fungal infection in HIV/AIDS patients, with high morbimortality rates when diagnosis and treatment are delayed. Antibody detection, which is faster than the gold standard culture test, hasten the laboratory investigation.

Objectives: To evaluate the role of WB for antibody detection in the diagnosis of histoplasmosis among HIV/AIDS patients.

Patients and Methods: Fifty patients with proven or probable histoplasmosis were included. Clinical, epidemiological and laboratory data were described in the same population after a review of their medical records. WB was performed using deglycosylated histoplasmin.

Results: 82% of patients were adult males and the mean age was 39.3 years. CD4+ T lymphocyte count less than 150 cells/mm^3 was observed in 62% patients. Antibodies against *Histoplasma capsulatum* M antigen were detected in 62% of patients, and against both M and H antigens in 28% of individuals. Sera from 10% of patients were nonreactive. Histoplasmosis was the first opportunistic infection in 38% of the cases. Disseminated and pulmonary histoplasmosis occurred in 84% and 16% of patients, respectively. The overall mortality was 16%.

Conclusion: WB could be useful for the histoplasmosis diagnosis in HIV/AIDS patients because of its easefulness and good sensitivity in a population where antibody production is hampered.

1. INTRODUCTION

Histoplasmosis is a systemic fungal disease which has a worldwide distribution. It is one of the most common respiratory mycosis in endemic regions of the United States and Latin America (1, 2). In Brazil, histoplasmosis has been described in several regions especially in the Midwest, Northeastern and Southeast regions of the country (3, 4).

The clinical spectrum of histoplasmosis ranges from asymptomatic, self-limited illness to a progressive disseminated disease. Although the clinical manifestations of histoplasmosis are well described, there is a significant overlap of clinical signs with other infectious diseases such as tuberculosis and *Pneumocystis* pneumonia, and the diagnosis cannot be achieved based solely on clinical information (5, 6).

Histoplasmosis is the most frequent opportunistic systemic fungal infection found in HIV-infected patients (7). It occurs in approximately 27% of patients with AIDS living in endemic areas and in 5% living in non-endemic areas (8). This mycosis could become progressive and spread rapidly from the lungs to other organs, presenting a high mortality in endemic areas if treatment is delayed (9, 10). The severe immunosuppression, characterized by count CD4+ T lymphocytes less than $150/mm^3$, is usually found in individuals that develop the disseminated form of mycosis (7, 11).

Access to highly active antiretroviral therapy (HAART) has resulted in a significant reduction in morbidity and mortality rates from AIDS in the world (12). However, even in the HAART era, disseminated histoplasmosis has been identified in a large

number of HIV-infected patients. Moreover, it can occur as the most common opportunistic infection associated to AIDS in some areas (13).

The definitive diagnosis of histoplasmosis requires the isolation of the *H. capsulatum* on specific culture media (6). However, *H. capsulatum* has a slow *in vitro* growth and can take up to 2–4 weeks to be identified by morphological characteristics, and require biosafety level 3 research facilities (14). Given these limitations, other laboratorial tests are used in the diagnosis of histoplasmosis, such as Giemsa preparations and histopathology using several staining methods, detection of anti-*Histoplasma* antibodies and specific antigens. More recently, molecular techniques for identification of *H. capsulatum* also have been applied (15).

Histopathology using stains for fungi is a definitive and rapid way for histoplasmosis diagnosis. However, expertise is mandatory to differentiate yeasts from other pathogens that resemble *H. capsulatum* yeast. As described by Assi et al. (16), assessing the turnaround times of the available methods for the histoplasmosis diagnosis, found that histopathologic and serologic results were released faster (2 and 3 days, respectively) than the other methods. In a review of cases in China, histologic evidence of yeast-like organisms resembling *H. capsulatum* in tissues provided the rapid diagnosis in 80.7% patients (17). Nonetheless, this screening requires an invasive procedure to obtain tissue. Previous studies from our group highlighted a western blot immunoassay (WB) using deglycosylated histoplasmin (ptHMIN) as an important method for diagnosis of histoplasmosis (18, 19). That way, the combination the histopathology and western blot tests could improve the diagnosis of histoplasmosis with fast results.

In this study, our main goal was the evaluation of the sensitivity of WB for the immunodiagnosis of histoplasmosis in HIV/AIDS patients. In addition, description of the clinical and epidemiological aspects of histoplasmosis in such patients at National Institute of Infectious Diseases in Rio de Janeiro – Brazil is provided.

2. MATERIALS AND METHODS

2.1 Design and population of study

A sectional and retrospective study was performed in HIV/AIDS patients diagnosed with histoplasmosis during hospitalization at the Evandro Chagas National Institute of Infectious Diseases (INI) – Fiocruz, during 2000 to 2015. INI is a public healthcare institution located in Rio de Janeiro State, southeast of Brazil, focused on clinical research, education programs, reference services, and multidisciplinary health assistance in infectious diseases.

Patients and clinical serum samples were identified by review of medical records and mycology laboratory´s registers. Inclusion criteria were a positive HIV serology according to the Brazilian Ministry of Health guidelines (20) and *H. capsulatum* growth in culture medium with mycelial-to-yeast conversion and/or detection of *H. capsulatum* yeast-like cells in histopathology, and/or a positive result in the double immunodiffusion (ID) test for histoplasmosis. Exclusion criteria was the unavailability of a serum sample collected at the time of histoplasmosis diagnosis stored (-20ºC) at the mycology laboratory. As controls, 22 serum samples of HIV-infected patients have been used: 14 without opportunistic infections and eight with other pulmonary infections (paracoccidioidomycosis, n= 2; aspergillosis, n= 2; cryptococcosis, n= 2 and tuberculosis, n= 2).

The classification of histoplasmosis cases was based on the consensus EORT/MSG (21). Proven histoplasmosis includes individuals with identification of *H. capsulatum* in cultures or histopathological tests. Probable histoplasmosis was considered for patients that have strong histoplasmosis clinical and epidemiological features, and a positive serological test (ID) for histoplasmosis.

Clinical and epidemiological data were obtained by a review of medical records. The variables analyzed included gender, age, time of HIV diagnosis, use of HAART, histoplasmosis as the first opportunistic infection, co-infection with tuberculosis, pulmonary radiologic aspects, site of *Histoplasma*-isolation, CD4+ T lymphocyte count, viral load, histoplasmosis clinical forms, and clinical outcome.

This study was approved by the Research Ethics Committee of the Evandro Chagas National Institute of Infectious Diseases - Fiocruz, accession number 19109913.0.0000.5262.

2.2 Western blot and double immunodiffusion

After the patients were included, their serum samples stored at -20ºC in our institution were recovered for antibody detection against *H. capsulatum* antigens by both WB and ID. WB was performed in stored serum samples from patients according to a previously established and validated protocol (18, 19). Results were obtained by visualization of band profiles on nitrocellulose membranes immunobilized by deglycosylated histoplasmin antigen (ptHMIN) (22, 23) corresponding to antibodies against the H and M antigens (molecular weight of 115

and 88 kDa, respectively). The ID test was performed following the methodology described (24) using the crude histoplasmin antigen (25).

2.3 Statistical analyses

Statistical analyses were performed with SPSS software, version 17.0. The epidemiological and laboratorial data were evaluated by bivariate analysis using Chi-square or Fisher exact test, when appropriate. For non-parametric data, we used the Mann-Whitney test. The median was used as a central tendency measure. The statistical significance was considered when the p-value was ≤0.05.

The diagnostic accuracy of the WB in the HIV-infected population was evaluated by sensitivity, specificity, and predictive values, with their respective 95% confidence intervals (CI).

3. RESULTS

3.1 Clinical, epidemiological and laboratory characteristics

In this study, we evaluated 50 HIV/AIDS patients with histoplasmosis from INI/Fiocruz, in a 16 year-period (2000-2015). Forty-one (82%) patients were male, and nine (18%) were female. The mean age was 39.3 years. Histoplasmosis was the first opportunistic infection in 38% of cases. Thirty-one (62%) patients had previous HIV-infection diagnosis, and the mean time of HIV-infection was 7.74 years. Among them, irregular adhesion to HAART was identified in 29 patients and two patients were HAART-naive.

Disseminated form of histoplasmosis occurred in 84% of patients, and pulmonary form in 16% of individuals. Detection of acid-fast bacilli in respiratory samples was performed in 44 patients and histoplasmosis co-infection with pulmonary tuberculosis was diagnosed in 17 individuals (38.6%). Another opportunistic co-infections identified in these patients were *Pneumocystis* pneumonia (n=5), cerebral toxoplasmosis (n=3), varicella-zoster virus (n=2), and syphilis (n=2). The CD4+ T lymphocytes counts were available for 47 patients, and the mean was 174,7cells/mm^3. Severe immunosuppression (CD4+ T lymphocytes < 150 cells/mm^3) was observed in 31 (66%) histoplasmosis patients. The viral load (VL) was available for 45 patients, and the mean VL was 217,877 copies/mm^3. Five individuals had undetectable VL and three patients had VL > 500,000 copies/mm^3 (Table 1).

Pulmonary radiologic changes were observed in 34 individuals. Micronodular interstitial infiltrate (n=23) was the main radiological profile identified, followed by condensation (n=2), pulmonary fibrosis (n=2), pleural fluid (n=1), and pulmonary abscess (n=1). Five patients had a mixture of radiographic profiles such as micronodular interstitial infiltrate with condensation (n=2), micronodular interstitial infiltrate with pleural fluid (n=2), and condensation with pleural fluid (n=1). Seven patients had normal chest X-rays, and nine individuals did not perform chest X-rays. The micronodular interstitial infiltrate occurred in 87% patients with disseminated form (20/23, *p*=0.079).

Fifty-seven biological samples for *H. capsulatum* culture were obtained from 46 patients. The isolation of *H. capsulatum* occurred in 84.7% of them (39/46). Positive specimens included 19 bone marrow samples (19/20; 95.0%), 11 skin or mucosa

biopsy samples (11/12; 91.7%), nine blood samples (9/11; 81.8%), six lymph node aspirate samples (6/6; 100%), and five respiratory samples such as sputum, tracheal aspirate and bronchoalveolar lavage (5/8; 62.5%). Some of the 39 patients that yield a positive *H. capsulatum* culture collected two different samples, and none of them collected two consecutive similar samples. Two patients had the histoplasmosis diagnosis by histopathological examination of smears stained by Grocott and PAS, which identified *H. capsulatum* as yeast-like structures and granuloma formation in skin biopsy and respiratory samples.

Forty-one (82%) patients had proven histoplasmosis and nine patients (18%) had probable histoplasmosis, according to the EORT/MSG criteria. The table 1 shows the main characteristics of histoplasmosis clinical forms. Briefly, disseminated histoplasmosis was the main clinical form observed in the group of proven histoplasmosis (n=40/42 – 95.2%), and pulmonary histoplasmosis was more frequently classified as probable histoplasmosis (n=7/8 – 87.5%). Two patients were transferred to other hospitals during histoplasmosis treatment, and they were excluded from the outcome evaluation. Death occurred in 8 (16.7%) patients, and discharge in 40 (83.3%) patients.

3.2 Serological aspects

The table 2 shows the WB performance for histoplasmosis diagnosis in different clinical forms in HIV/AIDS patients. Both H and M antigens were detected in 31% of patients, and detection of only M antigen in 69% of those patients that had a positive WB result (Fig. 1). All cases of pulmonary histoplasmosis had positive results by WB.

Positivity in the WB assay was not associated with severity of AIDS in the patients herein described (*p*=0.1504). Moreover, for individuals that had negative WB results (n=5), all with proven histoplasmosis by isolation and identification of *H. capsulatum* in culture, the median CD4+ T lymphocytes count was similar to patients with positive WB (24 vs. 109 cells/mm^3, respectively; *p* = 0.0628). Additionally, the median VL was similar in both groups of patients (4,027/mm^3 *vs.* 89,536/mm^3, respectively; *p* = 0.9856)

(Table 2).

False-positive results were observed in two samples due to cross-reactions in sera from patients with aspergillosis and cryptococcosis (n=1 each). The results of the WB diagnostic accuracy and their confidence intervals have shown a sensitivity of 90.0% (95% CI: 83.1 to 96.9%), specificity of 90.9% (95% CI: 84.3 to 97.6%), accuracy of 90.3% (95% CI: 83.4 to 97.1%), positive predictive value of 95.7% (95% CI: 91.1 to 100%), and negative predictive value of 80.0% (95% CI: 70.8 to 89.2%).

The ID test, performed simultaneously with the above mentioned WB revealed 82% sensitivity. Nine patients presented false negative results, all with the disseminated form of the disease. No cross-reactions were observed in the ID test.

4. DISCUSSION

Histoplasmosis can be considered one of the most important fungal infections related to immunosuppressed patients, especially HIV/AIDS patients, due to a significant prevalence (27%) of individuals with AIDS living in endemic areas of *H. capsulatum* (8, 26). The availability of HAART has resulted in a significant reduction in morbidity and mortality rates from AIDS in the world (12). However, a low

adherence to HAART and a late diagnosis of HIV are the main risk factors associated with the development of histoplasmosis in AIDS-patients (27, 28), as observed in the present study.

Definitive diagnosis of histoplasmosis is usually based on the isolation and identification of *H. capsulatum* in culture or by fungal identification in biologic samples with specific staining, mainly in the disseminated form (21). In this study, *H. capsulatum* was isolated in 87.7% of collected samples, with greater frequency in bone marrow aspirate, skin or mucosa lesions, and blood. However, it is important to highlight that culture is a time-consuming process and has limitations in sensitivity (29), especially in acute and disseminated histoplasmosis, which need to be quickly diagnosed for the immediate start of specific therapy (30).

As a solution for the time-consuming limitation of *H. capsulatum* isolation and identification in culture, immunological methods are alternately employed as important tools for the presumptive diagnosis of histoplasmosis, because they evaluate indirectly the presence of the pathogen in the host, by antibody and/or antigen detection.

Antigen detection based tests are non-invasive methods that can be performed in serum and urine samples for the diagnosis of histoplasmosis. Generally, they have a good sensitivity and specificity. However, it is available only in USA and Puerto Rico. *Histoplasma* galactomannan detection by radio immune assay was the first antigen detection test developed by MiraVista Diagnostics, which was subsequently modified as an EIA. A polyclonal EIA developed by CDC and a IMMY monoclonal

EIA also have demonstrated accuracies similar to the EIA MiraVista Diagnostics (31). A meta-analysis performed in 2016 to evaluate the diagnostic performance of these tests, demonstrated that the overall sensitivity for antigen detection in serum and urine was 81%, while specificity was 99% (32). In HIV patients from Latin America, the CDC test and IMMY EIA were evaluated with good performances. However, their commercialization is not available in the endemic areas of low and middle-income countries.

Antibody detection based tests are the main tools currently in use for the non-culture diagnosis of histoplasmosis given their general availability. However, a major limitation of these tests has been found in immunocompromised patients, whose ability to mount a humoral immune response is reduced. For instance,

an immunoelectrophoretic assay for *Histoplasma*-specific antibody detection was performed according to manufacturer's instruction (Beckman Paragon, France), has shown a sensitivity of 35.9% in HIV patients (33).

In a recent study, WB using a crude antigenic preparation showed an overall sensitivity of 50%, and when stratified, 66.7% and 25% of samples from histoplasmosis patients with or without HIV infection reacted with H and M fractions of *H. capsulatum* (34). In a previous validation study, WB using ptHMIN proved to be an efficient serological test for the diagnosis of histoplasmosis, with sensitivity of 94.9%, specificity of 94.1% and accuracy of 94.5% (19). In addition to its high sensitivity, this method is faster and safer than those one currently used in diagnostic

routine, such as culture examination, that can take up to four weeks to a final diagnosis and requires level three biosafety facilities (35).

It has been shown that detection of both anti-H and anti-M antibodies in serum samples is strongly suggestive of a conclusive histoplasmosis result (4). However, H and M bands were not detected by ID in any of the herein included patients, showing that this statement is not entirely correct in HIV/AIDS patients. Therefore, the association with the clinical assessment of the patient is mandatory. In this study, around 30% of patients, mainly those with disseminated histoplasmosis (13/14) had both anti-H and anti-M antibodies corroborating previous studies that indicate the presence of both bands are indicative of more severe forms of disease and more common in disseminated cases rather than in pulmonary cases (29, 36). The presence of only anti-M antibodies also can indicate a previous infection status (29).

In acute and chronic pulmonary histoplasmosis, the serological tests are very important due the significant difficulties to obtain suitable clinical samples for isolation of *H. capsulatum* in culture, besides the lack of sensitivity (4). Also, the sensitivity of serologic tests in acute histoplasmosis depends on the time of sample collection in relation to the infection. Tests performed in the first 4 weeks post infection are more often negative than positive. The sensitivity increases only if the test is done 6 weeks or more after infection (37).

Therefore, a previous study demonstrated that WB was an important tool for diagnosis the acute pulmonary histoplasmosis (18). Similar results were found out here since WB was positive in the eight patients suffering of acute and chronic

pulmonary histoplasmosis included in this study. However, the presence of H and M bands were detected in just one and anti-M antibodies (M band) in the other seven patients of this group. It has been demonstrated that the M antigen is more immunogenic than the H antigen, and the presence of anti-H antibodies occurs in less than 25% of patients (35). Double immunodiffusion demonstrates that anti-H antibodies were found only in 7.0% of sera from patients with acute pulmonary histoplasmosis (38). According to the greater sensitivity of western blot, those data could be underestimated.

Although WB has presented a high sensitivity, the specificity was not 100% since false negative results has been found in this work. Despite that the difference between CD4 cell counts among patients with positive and negative WB results was not statistically significant (p=0.06), probably due to the small sample size, there was a trend of lower CD4 cells in the false negative group. The decrease of CD4+ T cells as a consequence of HIV infection, reduces the IL-7 dependent activation of B lymphocytes that impairs the anti-*Histoplasma* antibody production (33, 39). This fact could explain the false negative results in some individuals with severe immunosuppression. Other methods based in *Histoplasma* antigen detection and molecular methods have shown higher sensitivity and specificity, and they can be used in individuals with histoplasmosis and lymphocytes CD4+ T counts less than 50 cells/mm^3. Unfortunately, these tests are not commercially available in Brazil or other developing countries where histoplasmosis is endemic (15).

In summary, histoplasmosis is a frequent neglected mycosis in Brazilian HIV/AIDS patients, with a life-threatening profile in these individuals. The application of faster and accurate diagnostic methods for this disease can help the early diagnosis and

treatment of this potentially fatal mycosis in this specific population. This study suggests that serologic tests to detect antibodies, such as western blot, using a high-quality antigen can be a useful laboratory tool for the diagnosis of histoplasmosis, even in immunocompromised patients living with HIV/AIDS. Although several publications have demonstrated very different results, our data has shown a very robust information in terms of specificity and sensitivity using these methods especially in HIV/AIDS patients. *Histoplasma* ptHMIN antigen sharing western blot immunoassay prompted us to introduce this method as a new tool for histoplasmosis diagnosis. Indeed, it will be a wise and useful choice in areas where molecular tests and antigen detection are not available, and in situations where laboratory facilities are relatively limited.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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Figure legend

Figure 1 – Demonstrative of serum reactivity of patients against ptHMIN antigen using western blot assay. Lanes: MW - Molecular weight; 1, 2, 3, 4, 6, 7, 8, 9 and 11 - positive samples (H and M bands); 5 and 10 - negative samples (absence of specific bands); 12 and 13 - positive samples (M band). Western blot was positive in 45 (90%) patients tested. Both H and M antigens were detected in 31% of patients, and detection of only M antigen in 69% of those patients that had a positive WB result.

Table 1 – Clinical, epidemiological and laboratory characteristics of histoplasmosis in HIV/AIDS patients from INI/Rio de Janeiro, 2000-2015 (n=50)

	Pulmonary form (n=8)	Disseminated form (n=42)	*p*-value[b]
Male (n=41)	6	35	0.623
Mean of age (years)	45.8	38.0	0.136
The first infection opportunistic (n=19)	4	15	0.459
Mean of time of HIV diagnosis (years)	5.75	8.07	0.519
Irregular use of HAART (n=29)	3	26	0.255
Co-infection with tuberculosis[a] (n=17)	2	15	0.206
Proven histoplasmosis (n=41)	1	40	**0.000**
Mean of lymphocyte T CD4+ (cells/mm^3) (n=47)	271	131	0.104
Mean of VL/mm^3 (n=45)	67,512	247,950	**0.017**
Isolation of *H. capsulatum* by culture (n=39)	0	39	**0.000**
Death (n=8)	1	7	1.000

[a]Diagnosis by isolation of Mycobacterium tuberculosis in sample of various clinical specimens; [b]p value < 0.05

Table 2 – Performance of western blot for histoplasmosis diagnosis in HIV/AIDS patients from INI/Rio de Janeiro, 2000-2015

	Pulmonary form (n=8)	Disseminated form (n=42)	*p*-value[a]
WB (n=50)			
Positive (n=45)	8	37	0.577
Negative (n=5)	0	5	
Antibodies detected (n=45)			
Anti-H and Anti-M (n=14)	1	13	0.402
Single Anti-M (n=31)	7	24	

[a]p value < 0.05