

ELISA for early diagnosis of histoplasmosis

Allan Jefferson Guimarães,¹ Claudia Vera Pizzini,¹
Herbert Leonel de Matos Guedes,¹ Priscila Costa Albuquerque,¹
José Mauro Peralta,² Andrew John Hamilton³
and Rosely Maria Zancopé-Oliveira¹

Correspondence

Rosely Maria Zancopé-Oliveira
zancope@ipecc.fiocruz.br

¹Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

²Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

³St John's Institute of Dermatology, Guy's Hospital, King's College, London, UK

An ELISA was developed and evaluated as a method for detecting antibodies against glycosylated and deglycosylated histoplasmin (HMIN). Sera from patients with histoplasmosis, paracoccidioidomycosis, sporotrichosis, coccidioidomycosis, aspergillosis, cryptococcosis and healthy donors were tested by ELISA against purified, deglycosylated histoplasmin (ptHMIN) and compared with purified, native (i.e. glycosylated) histoplasmin (pHMIN). Although cross-reactivity was not abolished when ptHMIN was used in the test, it was reduced (pHMIN ELISA 93 % versus ptHMIN ELISA 96 %). However, there were statistically significant differences between the sensitivities of these two methods for the detection of antibodies (pHMIN ELISA 57 % versus ptHMIN ELISA 92 %; $P < 0.001$) and between the efficiency of the methods (pHMIN ELISA 83 % versus ptHMIN ELISA 95 %; $P < 0.001$). These parameters compare better than previously published data relating to the use of treated HMIN in diagnostic ELISAs. Some of the reactivities of serum samples were compared by immunoblotting using deglycosylated HMIN and by immunodiffusion using the crude antigen. The results demonstrated that cross-reactions with heterologous sera in both ELISAs could also be observed in immunoblotting and arose from shared protein epitopes. These data suggest that ELISA using deglycosylated HMIN is a very sensitive diagnostic method and, by using commercially available antigen, it can be easily standardized and performed faster than previous Western blot-based tests using the same antigen. It provides a useful adjunct to existing methods of diagnosis that could be applied even in situations where laboratory facilities were relatively limited.

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INTRODUCTION

Histoplasmosis is a systemic fungal disease caused by *Histoplasma capsulatum*. The significance of histoplasmosis results from its worldwide distribution (Rios-Fabra *et al.*, 1994; Wheat, 2001), its ability to mimic other serious disease entities (Goodwin *et al.*, 1980; McKinsey *et al.*, 1997) and its propensity to cause serious disseminated infection in immunocompromised patients (Bradsher, 1996; Goodwin *et al.*, 1980; Wheat, 1996; Wheat & Kauffman, 2003).

Definitive diagnosis has typically relied either on direct visualization of the organism in tissue and/or the isolation of the causative organism, which is time-consuming and lacking in sensitivity (Bullock, 1995; Kwon-Chung & Bennet,

1992; Wheat, 2001). The detection of patients' antibody responses offers a more rapid alternative to microbiological means of diagnosis, and the detection of host anti-*H. capsulatum* antibodies by immunodiffusion (ID) and complement fixation (CF) tests is often used (Bullock, 1995; Kaufman *et al.*, 1997). Both the yeast and mycelial phases of the fungus produce a number of exoantigens in culture, the most important and characteristic being the H and M antigens. These two antigens are the primary immunoreactive constituents of histoplasmin (HMIN), the standard diagnostic reagent used in ID and CF for many years (Standard & Kaufman, 1976). However, it was appreciated at an early stage that HMIN in its native form was not an ideal serodiagnostic reagent, because of problems associated with the presence of cross-reactive carbohydrate components within it. This had a detrimental effect on the specificity of the CF test (Kaufman *et al.*, 1997) and resulted in the low sensitivity of ID, particularly in the early acute stage of disease (Pizzini *et al.*, 1999). An alternative approach to immuno-

Abbreviations: CF, complement fixation; HMIN, histoplasmin; ID, immunodiffusion; pHMIN, purified histoplasmin; ptHMIN, purified, treated histoplasmin.

diagnosis is to detect *Histoplasma* antigens in urine or serum, and this has proved particularly useful in cases of disseminated disease (Gomez *et al.*, 1997; Wheat *et al.*, 1991, 1997, 2002; Wheat, 2001; Wheat & Kauffman, 2003).

Since the 1980s, immunoassays with varying degrees of sensitivity and specificity have been developed for the detection of anti-*H. capsulatum* antibodies in clinical specimens (Brock *et al.*, 1983; Kumar *et al.*, 1985; Maiga & Marjolet, 1985; Raman *et al.*, 1990; Torres *et al.*, 1993; Zimmerman *et al.*, 1990). These variations have been attributed to the cross-reactive carbohydrate epitopes present on HMIN. One potential solution to this problem is the use of deglycosylated (periodate-treated) H and M antigens – indeed, immunoblotting using these reagents has been used successfully to detect specific responses (Pizzini *et al.*, 1999; Zancopé-Oliveira *et al.*, 1994a), although such methodology may lack sensitivity. Accordingly, in this paper, we describe the application of purified and chemically deglycosylated HMIN in an ELISA for the detection of antibody responses in patients with histoplasmosis.

METHODS

Antigen. HMIN was prepared from mycelial form cultures of *H. capsulatum* CDC6623 (ATCC 26320) according to Zancopé-Oliveira *et al.* (1993). HMIN was centrifuged at 1050 g for 10 min, filtered through a 0.45 µm membrane, concentrated 20-fold by pervaporation and dialysed against PBS (0.01 M, pH 7.2). The presence of the immunodominant H and M antigens in this antigenic complex was determined by ID. HMIN was purified by cation-exchange chromatography on CM Sepharose CL-6B columns. Antigens were eluted from the columns with a stepped salt gradient consisting of 0.1–1 M NaCl in 25 mM citrate buffer, pH 3.5. Fractions rich in H and M glycoproteins (pHMIN) were pooled and deglycosylated by mild sodium *m*-periodate (NaIO₄) oxidation to obtain purified and treated HMIN (ptHMIN) according to methodology previously described (Pizzini *et al.*, 1999; Zancopé-Oliveira *et al.*, 1994a). The protein concentration was measured by a dye-binding assay with respect to an albumin-globulin standard. The success of the deglycosylation procedure was evaluated by a comparison of the glycosylated (native antigen) and oxidized HMIN in immunoblots developed with a specific anti-HMIN polyclonal antibody and with anti-H and anti-M mAbs (Zancopé-Oliveira *et al.*, 1994a).

Serum samples. The criteria for the diagnosis of histoplasmosis in this population were based on positive culture and/or detection of the M-precipitin band, the H-precipitin band or both in the ID test, in persons with recent history of histoplasmosis. Fifty serum specimens were obtained from 34 patients with histoplasmosis; 21 of these patients provided a single serum sample and the remaining 13 patients provided a total of 29 serum samples. Among the 34 patients, 44% were culture-proven cases and all of them were serology (ID) positive. A total of 35 heterologous serum samples from patients with culture-proven mycotic diseases (seven with paracoccidioidomycosis, eight with aspergillosis, eight with sporotrichosis, eight with cryptococcosis and four with coccidioidomycosis), previously tested for the presence of precipitins against HMIN by ID, were also included in this study. ID was performed using whole HMIN as described previously (Kaufman *et al.*, 1997). All of the clinical samples were chosen randomly and were obtained from the Immunodiagnostic section serum bank, Mycology branch, DMIP/IPEC (FIOCRUZ, Rio de Janeiro, Brazil) as routine reference samples. In addition, 87 serum samples from healthy individuals previously tested

by ID against HMIN, and showing negative results, were included in this study as negative controls.

ELISA. Indirect ELISA was performed as described previously (Kostiala & Kostiala, 1981). Antigen (pHMIN or ptHMIN) was added (1 µg per well) to 96-well microtitre plates (Nunc-Immuno Starwell, MaxiSorp Surface) in 100 µl carbonate buffer (63 mM; pH 9.6) per well and incubated for 1 h at 37 °C and overnight at 4 °C. Plates were washed three times with washing buffer (10 mM PBS, 0.1% Tween 20, pH 7.3) and blocked with 200 µl of 5% (w/v) non-fat skimmed milk powder in washing buffer (blocking buffer) for 2 h at 37 °C. They were then washed three times with washing buffer and serum samples were added to each well at a dilution of 1:1000 in 100 µl incubation (blocking) buffer. The plates were then incubated again at 37 °C for 1 h. After three further washes with washing buffer, plates were incubated with goat anti-human IgG peroxidase conjugate (Jackson ImmunoResearch; peroxidase-conjugated affinipure goat anti-human IgG, Fc fragment specific) diluted 1:16 000 in 100 µl blocking buffer, at 37 °C for 1 h. After three subsequent washes, the reaction was developed with 100 µl per well of *o*-phenylenediamine dihydrochloride [OPD; 0.4 mg ml⁻¹ in 0.04% (w/v) H₂O₂] diluted in 0.01 M sodium citrate buffer, pH 5.5. The reaction was terminated by the addition of 50 µl 3 M HCl. Absorbances were measured on a microplate reader (Bio-Rad model 550) at 490 nm. The cut-off point for serum reactivity to the pHMIN and ptHMIN was set as the mean plus three standard deviations (SD) of log values of the healthy controls. Serum samples with absorbance values above the cut-off were considered positive.

Immunoblot analysis. Serum samples from patients infected with mycoses other than histoplasmosis that demonstrated absorbance values above the cut-off and those from histoplasmosis patients with absorbances below the cut-off point were tested by immunoblotting with deglycosylated HMIN (ptHMIN) as described previously (Pizzini *et al.*, 1999; Zancopé-Oliveira *et al.*, 1994a). This experiment was designed to ascertain whether any of the ELISA results were false-positives or false-negatives, respectively, since all of the homologous sera showed positive results and all of the heterologous serum samples presented negative results for anti-HMIN antibodies by ID.

Statistical analyses. Analyses were performed using SPSS (SPSS-Windows 8.0). Comparisons between the ELISA test using glycosylated and deglycosylated HMIN were made by chi-squared analysis. Comparisons of means were made by Student's non-paired *t* test. *P* ≤ 0.01 was considered statistically significant.

RESULTS AND DISCUSSION

The detection of antibodies in the sera of histoplasmosis patients directed against the H and M glycoproteins of HMIN by CF and ID has proved very useful in the diagnosis of this systemic mycosis. However, one important limitation of the use of these antigens in these tests has been the generation of false-positive results arising from antigenic cross-reactivity (Palmer *et al.*, 1977; Pizzini *et al.*, 1999). This cross-reactivity had been attributed to the glycosylated portion of the H and M antigens, and this hypothesis was confirmed after oxidation of the M antigen with sodium *m*-periodate, which cleaves hydroxyl groups from monosaccharides on glycoproteins. Mild periodate oxidation of the M antigen eliminated cross-reactivity in Western blots probed with sera from patients with aspergillosis, coccidioidomycosis, paracoccidioidomycosis and tuberculosis (Zancopé-Oliveira *et al.*, 1994a). This treatment did not affect the

integrity of the protein, as demonstrated by an analysis of its molecular mass and antigenicity (Zancopé-Oliveira *et al.*, 1994b).

Data from previous immunoassay studies, which incorporated deglycosylated antigens for antibody detection, confirmed that the use of such antigens improves test specificity. Thus, an EIA-inhibition assay that detected antibodies against the M antigen has been reported to be positive for 88 % of M-precipitin-positive patients (Brock *et al.*, 1983). However, serum specimens from patients with other fungal infections were not included in this study, and there have been no reports of prospective clinical evaluations with this test. More recently, we described the use of deglycosylated M and H antigens in a Western blot for the diagnosis of histoplasmosis (Pizzini *et al.*, 1999; Zancopé-Oliveira *et al.*, 1994a). This test showed high sensitivity and specificity, but is laborious and time-consuming when it is carried out in-house. Consequently, the format of this assay is not favourable for routine diagnostic use without the availability of commercially produced test strips.

The purity and characteristics of the pHMIN and ptHMIN used in the ELISAs, analysed by SDS-PAGE, are shown in Fig. 1. The untreated antigen contained glycoproteins of 94 (M antigen) and 116 (H antigen) kDa. These molecular masses decreased to 88 and 114 kDa, respectively, after periodate treatment.

The present study compared the use of pHMIN and ptHMIN in a rapid ELISA format and demonstrated that the assay was more sensitive and specific than ELISAs previously described when purified and/or deglycosylated HMIN were used (Brock *et al.*, 1983; Kumar *et al.*, 1985; Maiga & Marjolet, 1985; Raman *et al.*, 1990; Torres *et al.*, 1993; Zimmerman *et al.*, 1990). The chemical deglycosylation of HMIN by periodate increased the sensitivity and effectiveness of this ELISA.

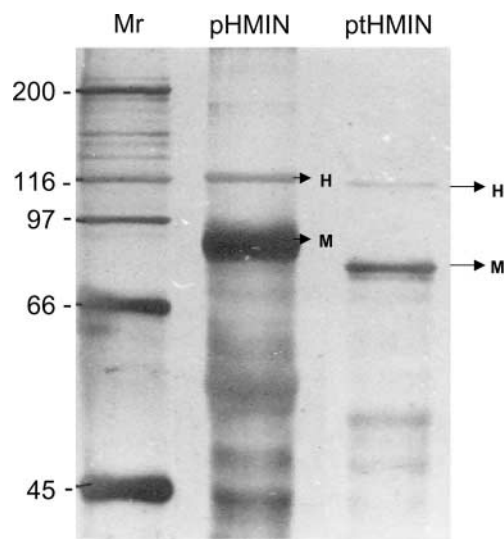


Fig. 1. SDS-PAGE of pHMIN and ptHMIN. Numbers on the left correspond to molecular mass markers in kDa.

Serum samples from 50 histoplasmosis and 122 non-histoplasmosis patients were tested for antibody reactivity, using pHMIN and ptHMIN in an ELISA, as shown in Fig. 2. The cut-off points of the ELISA were established as the mean absorbances plus three SD of the healthy controls. Accordingly, samples with absorbance values higher than 0.32 and 0.52 (when pHMIN and ptHMIN were used, respectively) were considered positive (Fig. 2a, b). Table 1 summarizes results obtained using both antigens. The number of sera giving positive ELISA results using ptHMIN to detect antibodies from patients with histoplasmosis was statistically higher when compared with ELISA results using pHMIN, showing an increased sensitivity for the treated antigen (92 % versus 57 %; $P < 0.001$). Two of the four ELISA-negative samples were from patients with AIDS and histoplasmosis; in such patients, the absence of a detectable antibody response may arise either from humoral immunosuppression or from the formation of immune complexes. Normal healthy controls did not show significant ELISA reactivity in either test (0 of 87 for ptHMIN and 1 of 87 for pHMIN).

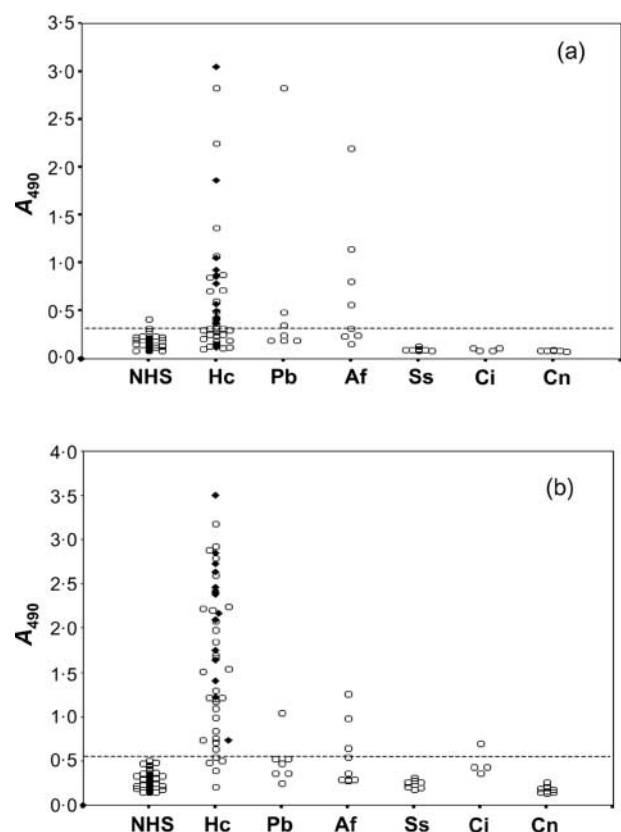


Fig. 2. Detection of antibody responses in sera from histoplasmosis patients, from patients with other proven mycotic diseases and from normal healthy controls by ELISA using glycosylated (a) and deglycosylated HMIN (b). \circ , Single serum samples. \blacklozenge , specimens from culture-proven histoplasmosis cases. The dashed horizontal line shows the cut-off point. Hc, Histoplasmosis; Pb, paracoccidioidomycosis; Af, aspergillosis; Ss, sporothricosis; Ci, coccidioidomycosis; Cn, cryptococcosis; NHS, normal healthy sera controls.

Table 1. Comparison of ELISAs using glycosylated and deglycosylated HMIN to detect antibodies in sera

Patient group (n)	Patients positive by ELISA/total number (%)	
	pHMIN	ptHMIN
Histoplasmosis (50)	28/50 (57)	46/50 (92)
Paracoccidioidomycosis (7)	3/35 (8.6)	1/35 (2.9)
Aspergillosis (8)	4/35 (11.4)	3/35 (8.6)
Sporothricosis (8)	0/35 (0)	0/35 (0)
Coccidioidomycosis (4)	0/35 (0)	1/35 (2.9)
Cryptococcosis (8)	0/35 (0)	0/35 (0)
Normal healthy controls (87)	1/87 (1.1)	0/87 (0)

To evaluate the specificity of the method, a total of 35 serum samples from patients with other proven mycotic diseases, plus 87 healthy controls, were included in this study. Cross-reactivity was seen in a small number of sera from patients with paracoccidioidomycosis (one serum in ptHMIN versus three sera in pHMIN), aspergillosis (three sera in ptHMIN versus four sera in pHMIN) and coccidioidomycosis (one serum in ptHMIN) (Table 1 and Fig. 2). Serum specimens from patients infected with *Sporothrix schenckii* and *Cryptococcus neoformans* did not show cross-reactivity for either antigen. Sera from normal healthy controls did not show any recognition of ptHMIN, and only one sample was reactive to pHMIN. Table 2 compares the overall specificity of the ELISA when using pHMIN and ptHMIN; the latter was not significantly different (93 % versus 96 %, respectively). Table 2 also summarizes the efficiency and negative and positive predictive value of the ELISA when using the two antigens. The efficiency of the ELISA using ptHMIN was statistically greater compared with the ELISA using pHMIN (95 % versus 83 %; $P < 0.001$).

The sensitivity for the ELISA assays was also evaluated using glycosylated and deglycosylated HMIN on the true-positive samples correctly identified (50 samples from histoplasmosis patients) and cross-reactivity with 35 true-negative

fungal control specimens. A specificity of 80 % was obtained for the ELISA using pHMIN (cross-reactivity was seen in specimens from patients infected with *Paracoccidioides brasiliensis* at 8.6 % and *Aspergillus fumigatus* at 11.4 %) excluding healthy controls. Although the cross-reactivity was not totally abolished ($P > 0.001$) after deglycosylation, the specificity of the ELISA when using ptHMIN increased to 86 % (with cross-reactions to *P. brasiliensis* of 2.9 %, *A. fumigatus* of 8.6 % and *Coccidioides immitis* of 2.9 %). However, the significant difference in the sensitivity, negative predictive value (NPV) and efficiency of the test between ptHMIN and pHMIN favours the use of the former in the ELISA (Table 2).

The reproducibility of the tests is shown in Fig. 3. The correlation coefficient (r^2) for pHMIN and ptHMIN were 0.981 and 0.988, showing uniformity throughout the results range of the ELISAs for both antigens. Comparisons of mean absorbances among the sera obtained from the 50 histoplasmosis cases and 87 serum samples from healthy individuals made by Student's non-paired *t* test showed statistically significant differences (pHMIN, histoplasmosis cases, mean 0.593 ± 0.655 versus healthy controls, mean 0.161 ± 0.054 , $P < 0.001$; ptHMIN, histoplasmosis cases, mean 1.673 ± 0.836 versus healthy controls, mean 0.264 ± 0.086 , $P < 0.001$). However, the same was not observed when absorbance values obtained from sera from patients with histoplasmosis ($n = 50$) were compared with fungal controls ($n = 35$) using pHMIN (mean 0.338 ± 0.592 , $P < 0.05$) and ptHMIN (mean 0.383 ± 0.265 , $P < 0.05$) by the same test. Inactivation of carbohydrate epitopes led to increased ELISA sensitivity, possibly because key protein epitopes became more exposed as a result of changes in the structure of HMIN.

The five false-positive results and the four confirmed histoplasmosis patient serum samples with absorbances lower than the cut-off value, together with negative and positive controls were used to probe blots of ptHMIN. The positive control recognized antigens at 88 and 114 kDa, consistent with the molecular mass of the M and H antigens, respectively. A 62 kDa species was also detected (Fig. 4). All

Table 2. Serological parameters for the ELISAs using glycosylated and deglycosylated HMIN

Sensitivity is the proportion of positive samples correctly identified by the test. Specificity is the proportion of negative samples correctly identified by the test. Efficiency is the sum of true positive and negative samples divided by the sum of all samples. PPV and NPV; positive and negative predictive value.

Parameter	pHMIN (%)	ptHMIN (%)	χ^2	<i>P</i>
Sensitivity	57 (43.1–70.9)	92 (84.4–100)	15.5	0.00008
Specificity	93 (88.4–97.6)	96 (92.5–99.5)	0.73	0.40
Efficiency	83 (77.3–88.7)	95 (91.7–98.3)	13.4	0.00025
PPV	78 (66.7–93.3)	90 (81.7–98.3)	0.97	0.32
NPV	84 (77.3–88.7)	95 (91.7–98.3)	1.37	0.24

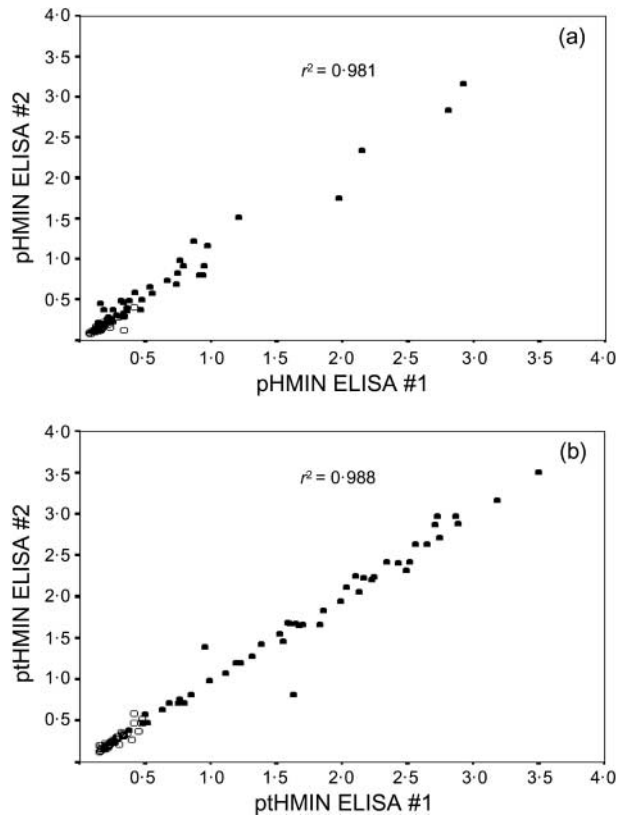


Fig. 3. Distribution and reproducibility of ELISA results using pHMIN (a) and ptHMIN (b). Closed data points, specimens from culture-proven histoplasmosis cases.

false-negative histoplasmosis patient serum samples reacted with the 88 kDa band (Fig. 4). Cross-reactivity was also observed in two false-positive serum samples; one serum from a patient with paracoccidioidomycosis and one from an aspergillosis patient weakly reacted with bands of 88 and 62 kDa. However, the possibility of concurrent and sub-clinical histoplasmosis cannot be excluded in these patients, since it is known that histoplasmosis may co-exist with other granulomatous diseases of the lung, including tuberculosis and other mycoses (Dijkstra, 1989; Wheat, 2001). Another possibility is that these antibodies arose from past exposure of these patients to *H. capsulatum*. Most cases of histoplasmosis (95%) are unapparent, subclinical or completely benign, and anti-M antibodies can remain elevated for several years following recovery. Such residual precipitins may lead to a misdiagnosis of histoplasmosis in patients with illnesses caused by other infectious agents (Wheat, 2001) or even in healthy residents of the area endemic for histoplasmosis (Brock *et al.*, 1983). Finally, the cloning, expression and characterization of the gene encoding the M glycoprotein (Zancopé-Oliveira *et al.*, 1999) has demonstrated that it has significant homology to catalases from *A. fumigatus*, *Aspergillus niger* and *Eimericella nidulans*. As such, there may be non-glycosylated epitopes on catalases from, for example, *A. fumigatus*, that contribute to cross-reactivity.

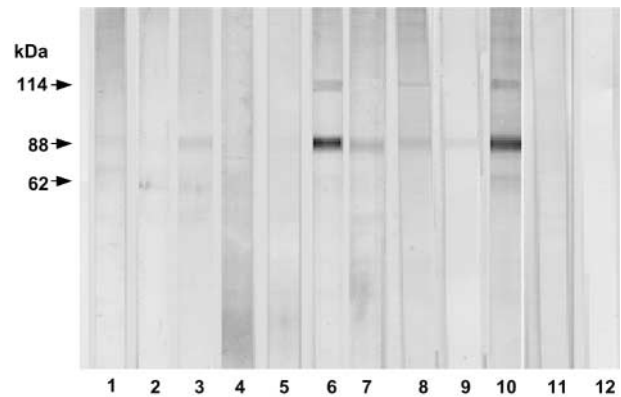


Fig. 4. Immunoblotting of ptHMIN probed with sera from the following patients. Lanes: 1, paracoccidioidomycosis; 2–4, aspergillosis; 5, coccidioidomycosis; 6–9, histoplasmosis (ELISA-negative); 10, histoplasmosis (positive control); 11, healthy individuals (negative control); 12, conjugate control. Black arrowheads correspond to the positions of deglycosylated H antigen (114 kDa), M antigen (88 kDa) and 62 kDa protein.

Polysaccharide antigen detection by radioimmune assay has also proved useful in the diagnosis of histoplasmosis, particularly in disseminated cases (Wheat *et al.*, 1991, 1997, 2002; Wheat, 2001; Wheat & Kauffman, 2003), although cross-reactivity may be a problem (Wheat *et al.*, 1997). In addition, the polysaccharide detection test has historically only been available at one centre in the United States, making it somewhat inaccessible to workers in developing countries. More recently, Gomez *et al.* (1997) developed a novel, though complex, test for the detection of antigenaemia in patients with histoplasmosis, which appears to be as sensitive as the RIA. In contrast to the radioimmune assay, the ELISA with purified and deglycosylated HMIN can easily be adapted for use in-house in regional centres, and it is clearly a sensitive and specific method for detecting antibodies in histoplasmosis. This assay is faster and more user-friendly than the equivalent Western blot-based test, and is highly sensitive; as such, it is valuable in the diagnosis of histoplasmosis even in situations where laboratory facilities are relatively limited.

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