Evaluation of an enzyme-linked immunosorbent assay using purified, deglycosylated histoplasmin for different clinical manifestations of histoplasmosis

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Abstract

Diagnosis of invasive fungal diseases remains problematic, especially in undeveloped countries. We have developed an enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *Histoplasma capsulatum* using metaperiodate treated purified histoplasmin (ptHMIN). Our ELISA was validated comparing sera from patients with histoplasmosis, related mycoses, and healthy individuals. The overall test specificity was 96%, with sensitivities of 100% (8/8) in acute disease, 90% (9/10) in chronic disease, 89% (8/9) in disseminated infection in individuals without HIV infection, 86% (12/14) in disseminated disease in the setting of HIV infection and 100% (3/3) in mediastinal histoplasmosis. These parameters are superior to the use of untreated histoplasmin in diagnostic ELISAs. The high specificities, sensitivities, and simplicity of our ELISA support further development of a deglycosylated HMIN ELISA for clinical use and for monitoring the humoral immune response during therapy in patients with chronic and disseminated histoplasmosis.

Keywords

ELISA; antibody detection; histoplasmosis; serological diagnosis

Introduction

Histoplasmosis, a fungal infection caused by *Histoplasma capsulatum*, has a worldwide distribution and is one of the most common respiratory mycoses.– This fungus is endemic in certain areas of the United States, particularly in states bordering the Ohio River valley and the lower Mississipi River. In Latin America, histoplasmosis is prevalent in Venezuela, Ecuador, Brazil, Paraguay, Uruguay, and Argentina.,– In Brazil, positive histoplasmin skin tests occur in as many as 50% of the people living in diverse areas of the country where *H. capsulatum* is common. The clinical disease generally ranges from mild flu-like illnesses to progressive, disseminated disease, which manifests mostly in immunocompromised individuals– such as in patients with AIDS or receiving corticosteroids.

Immunocompromised patients with histoplasmosis frequently fail to mount an effective antibody response and specific antibodies to the disease cannot be routinely detected, which make its diagnosis difficult.,– A definitive diagnosis of histoplasmosis requires the isolation of the pathogen in culture, which is inefficient since its growth frequently takes more than three weeks on standard fungal media. Positive results commonly only occur with samples from patients with high fungal burdens, often in the setting of chronic or disseminated disease. Visualization of the organism in infected clinical specimens can also facilitate the diagnosis; however, this methodology has low sensitivity and structures from other microorganisms can be misidentified as *H. capsulatum*.,

In the absence of positive cultures, serological techniques such as immunodiffusion (ID),– complement fixation,, enzyme immunoassay– and radioimmunoassay– have been used to provide immunological evidence of *H. capsulatum* infection. These serological tests for the detection of either antibodies and/or antigen in clinical fluids specimens (such as serum, urine and liquor) offer a rapid alternative methodology for the diagnosis of histoplasmosis. Although the detection of *H. capsulatum* antigens in clinical samples shows high degrees of cross-reactivity and low sensitivity,, they are particularly useful when the detection of a patient's antibody is unlikely, such as in disseminated disease in an immunocompromised patient. Although the data for the MiraVista laboratory (MVista® Histoplasma Quantitative Antigen EIA) are excellent for certain presentations of histoplasmosis,, the assay is only performed in the United States of America and it is costly and impractical for use elsewhere. An ELISA has been recently described for the detection of progressive disseminated histoplasmosis antigenuria, demonstrating a sensitivity of 81% and a specificity of 95%. However, this assay relies on the use of polyclonal antibodies against *H. capsulatum* as both capture and detection reagents, and batch to batch variations may require intensive standardization procedures.

We have developed an ELISA assay for the detection of antibodies in sera from histoplasmosis patients using deglycosylated histoplasmin (ptHMIN). HMIN is a well characterized antigen obtained from *H. capsulatum* mycelia.,– The main components of HMIN to which antibodies can be detected are the C, M, and H antigens. The C antigen is a galactomannan responsible for the cross-reactivity observed with other fungal species and deglycosylation has been shown to increase specificity of the assay. The M antigen is a B catalase, and the H antigen is a β-glucosidase. Antibodies against the M and H antigen are

highly specific and their detection is particularly useful in diagnosis.– HMIN is stable at −20°C for long periods (> 48 months) and no significant batch to batch variations have been detected. We previously found that the ptHMIN ELISA detects antibodies in 92% of serum samples from patients with culture proven histoplasmosis and had a 96% specificity, versus 57% of sensitivity and 93% of specificity when only purified histoplasmin (pHMIN) was used.,

In the present study, we evaluated and validated the ELISA as a specific and sensitive method for the detection of antibodies in sera from patients with different clinical manifestations of histoplasmosis. Statistical analyses to characterize the reactivity profile and determine the reliability for detection of antibodies in each group were carried out. Also, we tried to establish a correlation between antibody titer and the clinical form of disease. This methodology was shown to be effective and further studies are being conducted to evaluate its use in the follow-up of clinical samples.

Materials and Methods

Antigen preparation

Histoplasmin (HMIN) was produced as previously described. Briefly, cells were centrifuged at 1,050 g for 10 min, and supernatant filtered through a 0.45 μm membrane, concentrated 20-fold and dialysed against PBS (0.01 M, pH 7.2). The presence of the immunodominant H and M antigens in the crude HMIN was determined by ID. HMIN was purified by cation-exchange chromatography on CM Sepharose CL-6B columns (Amersham Biosciences, NJ, USA). Elution was performed using a stepped salt gradient of 0.1–1 M NaCl in 25 mM citrate buffer, pH 3.5. Purified HMIN (pHMIN) was obtained after pooling the fractions rich in H and M glycoproteins and chemical oxidation of carbohydrate residues was achieved by sodium-*meta*-periodate (NaIO4) treatment to generate the purified and treated HMIN (ptHMIN) according to the methodology described previously.,, Antigen concentration was determined by a dye binding protein assay (Bio-Rad, NY, USA) with respect to a bovine albumin-globulin standard and aliquots of the final preparation were stored at −20°C.

Subjects

Patients with different clinical manifestations of histoplasmosis were included in this study. The diagnosis was based on the following criteria: i) biological material (including sputum, bronchoalveolar lavage, bone marrow aspiration, biopsies or cerebrospinal fluid) was submitted for direct examination with KOH 10% and culture on Sabouraud agar. Blood samples were cultured on brain heart infusion agar (BHI). Slants were incubated for 4–6 weeks at room temperature until the observation of white to brownish filamentous colonies. Microscopic examination of slide cultures revealing septate hyaline hyphae, with globose macroconidia, and microconidia presumptively identified the isolates as *H. capsulatum*. Conversion to oval yeast cells with single budding confirmed isolation of *H. capsulatum*; ii) tissue staining with H&E and GMS techniques, with histopathological findings revealing organisms consistent with *H. capsulatum* associated with clinical and radiological findings suggestive of histoplasmosis with or without positive epidemiological history; iii) seropositivity for anti-*H. capsulatum* antibodies (positive by either immunodiffusion (ID) or

Western blot (WB)) associated with clinical and/or radiological findings suggestive of histoplasmosis with or without a positive epidemiological history. From the number of cases selected, histoplasmosis patients were clinically classified into five clinical forms as described before depending on the severity and similarity of the symptoms. The main clinical features of each group are listed in Table 1. Additional criteria utilized to classify the patients in each clinical group included: travel history, fever, weight loss, and most importantly, the isolation of fungi from clinical samples. Approval for this study was granted by the Ethics Board, Instituto de Pesquisa Clinica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, in accordance with the ethical rules and regulations of our institution.

Sample size

Two approaches were used to determine the sample size requirements depending on the test performance. First, we used standard equations based on previously reported results of ELISA sensitivity and specificity. To determine the minimum number of histoplasmosis serum samples, we applied the following formula, using the sensitivity approach:

$$N_{sn} = \frac{(Z\alpha/_2)^2 \times (SN) \times (1 - SN)}{W^2}$$

A similar procedure was performed to calculate the minimum number of heterologous serum samples according to specificity using the following formula:

$$N_{sp} = \frac{(Z\alpha/_2)^2 \times (SP) \times (1 - SP)}{W^2}$$

Formulae specifications:

> N_{sn}: number of histoplasmosis patient serum samples based on the sensitivity values (homologous sera).
>
> N_{sp}: number of serum samples based on the specificity values (heterologous sera)
>
> $Z\alpha/_2$: the number of standard deviations in half of the two-tailed confidence interval (for the 95% confidence interval, $Z\alpha/_2 = 1.96SD$).
>
> SN: the estimated value for the performed sensitivity (92%).
>
> SP: the estimated value for the performed specificity (96%).
>
> W: maximum clinically acceptable width of the 95% of CI (half of the confidence interval).

Using this methodology and based on our recruited patients, a minimum of 29 histoplasmosis patients and 15 heterologous sera were required to evaluate the sensitivity and specificity of the ptHMIN ELISA, respectively.

A second approach used in this study was receiver operating curve (ROC) analysis. ROC analysis provides tools to select possibly optimal models and to discard suboptimal ones

independently from the cost context or the class distribution. To calculate the sample size, a 5% type I error rate was used. The sample sizes were determined using the same probability of 5% to reject or to accept the null hypothesis (type I and type II errors, respectively). Additionally, the accuracy (area of the ROC curve) of the test was set to be higher than 0.90, representing a high test accuracy and discriminatory power. The minimal required samples were calculated using the MedCalc (MedCalc, version 10.4.0.0). The required sample size was 23 subjects for each group in this case. For accuracy and ROC curve analysis, the test needed 23 normal subjects (negative group), 23 heterologous subjects (containing other mycosis than histoplasmosis) and 23 histoplasmosis-specific samples, giving a minimum of 69 required samples.

A total of 44 sera samples from homologous (histoplasmosis) and 35 sera samples from heterologous-infected (paraccocidioidomycosis [n=7], aspergillosis [n=8], sporotrichosis [n=8], coccidioidomycosis [n=4], cryptococcosis [n=8]) patients were tested by our ptHMIN ELISA. Additionally, 36 negative control sera (negative by ID testing) were collected from healthy donors at the Federal University of Rio de Janeiro and IPEC.

Serum samples were collected from patients (37 male and 7 female, age range 15 to 74 years) with different clinical forms of histoplasmosis (8 acute histoplasmosis, 10 chronic histoplasmosis, 9 disseminated histoplasmosis, 14 opportunistic histoplasmosis and 3 mediastinal histoplasmosis) (Table 2). A description of the isolation of *H. capsulatum* from the clinical samples of all the 44 histoplasmosis patients is shown in Table 3. Serum samples were obtained at a time-point closest to when individuals were diagnosed with histoplasmosis. The clinical samples were selected randomly from the Immunodiagnostic Section Serum Bank, Laboratory of Mycology IPEC (Fiocruz, Rio de Janeiro, Brazil). All specimens were stored at −20°C until tested and then at 4°C while the evaluations were performed.

Immunodiffusion

Serum samples were evaluated for the detection of antibodies by radial double ID as previously described. Briefly, the patient's sera were inactivated for 30 min at 56°C and stored at 4°C until the test was performed. ID slides were made by melting a 1% agarose solution in PBS and applying the solution to form a thin film on a commercially available glass slide (ThermoFischer Scientifics, USA). Wells were made in a hexagonal disposition, containing an extra well in the center. Serial two-fold dilutions were prepared to a final dilution of 1:1,024 in all confirmed positive samples. Ten microliters of each dilution were loaded onto individual wells. The crude histoplasmin antigen was diluted to previously standardized concentrations and used to load the central well. The slides were incubated at RT for 48 hours to allow formation of precipitins. Reaction was considered positive when the zone of equivalence lines gave a full identity and related to the presence of the M band only or both M and H antigens.

Evaluation of an enzyme linked immunosorbent assay

Indirect ELISA assay was performed as previously described. Briefly, ptHMIN was diluted in carbonate/bicarbonate buffer (63 mM; pH 9.6) to a concentration of 0.01 μg/μL and 100

μL were added to each well of 96-well microtiter plates (Nunc-Immuno Starwell, MaxiSorp Surface). The plates were incubated for one hour at 37°C followed by an overnight incubation 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 blocking buffer (5% [w/v] non-fat skimmed milk in washing buffer) for two hours at 37°C. After three washes, serum samples were added to each well in duplicate at a dilution of 1:1,000 in 100 μL of blocking buffer for one hour incubation at 37°C. Plates were washed and 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 of blocking buffer at 37°C for one hour. After washing, the reaction was developed using 100 μL per well of o-phenylenediamine dihydrochloride [OPD; 0.4 mg/mL in 0.04% (w/v) H_2O_2] diluted in 0.01 M sodium citrate buffer, pH 5.5. The reaction was stopped by the addition of 50 μL of 3 M HCl. Optical density (ODs) was measured on a microplate reader (Bio-Rad model 550) at 490 nm and the results from duplicate wells were averaged. This experiment was carried out twice on different dates under uniform laboratory conditions to avoid internal variations in order to ascertain the reproducibility of the assay. For each experiment, two controls were made: secondary antibody alone, to ensure that the reagent was not interacting with the antigen on the plate, and a blank control.

Receiving operation curves analysis

A receiver operator characteristic (ROC) curve was constructed with GraphPad Prism (version 5.01, GraphPad Software, Inc.) to assess variations in the sensitivity and specificity of antibody detection for the different clinical forms of histoplasmosis. A ROC plot was constructed using 1-specificity plotted against sensitivity values as a dependent variable and the area under curve was estimated by non-parametric integration. The optimum "cut-off" value was obtained from two-graph-ROC (TG-ROC) analysis., OD values of individual samples were considered as a cut-off and specificity/sensitivity values determined for each specific value, for a binary classification system as its discrimination threshold is varied. The cut-off value (or any specific value within an interval) showing the highest specificity/sensitivity parameter values, was defined as the definitive cut-off. Our optimal cut-off was also defined and confirmed by determining the efficiency and the Youden index to achieve maximum accuracy.

Statistical analysis

All data were subjected to statistical analysis with use of Prism 5.0 (GraphPad Software). P values were calculated by analysis of variance, Kruskall-Wallis or Pearson's correlation analysis. P values of <0.05 were considered to be statistically significant.

Results

Receiving operation curves and cut-off determination

The ROC curve analysis was used to evaluate the overall performance of the ptHMIN independent of the choice of cut-off value and a conventional ROC (Figure 1). The data used for analysis included patients with all forms of histoplasmosis. There were insufficient numbers for each manifestation to perform analyses individually. The area under the ROC

curve for ptHMIN representing the accuracy to predict antibody response in patients was 0.9491 ± 0.024 (95% CI, 0.9072 to 0.9910) (P<0.0001) (Figure 1A). Next, TG-ROC analysis allowed the calculation of optimal cut-off value when the sensitivity/specificity were plotted on the same graph against each sample value representative of each of the decision thresholds considered and assumed as the cut-off values, and the intersection point between the curves giving the highest values for both parameters was obtained (Figure 1B). For additional validation, we plotted efficiency versus OD and obtained a similar cut-off (Figure 1C). Hence, the established cut-off value was 0.480. The samples with OD values above the cut-off were classified as positive and those below were considered negative.

Evaluation of an enzyme linked immunosorbent assay evaluation

OD values for all samples were organized according to homologous and heterologous sera (Figure 2). ODs were significantly higher in sera from 44 patients with histoplasmosis (mean 1.646 ± 1.065, N=44) than in heterologous samples evaluated (mean 0.3539 ± 0.5179, N=71, P<0.0001) (Figure 2A). Differences in means (1.292±0.1483) and comparisons of parameters indicate that the test effectively discriminates between negative and positive samples. All the statistical evaluations and comparison among the groups are shown in Table 4.

We further examined whether test efficacy varied with the clinical form of disease. Mean OD values were determined for each group: acute (1.136±0.4903), chronic (2.306±1.119), disseminated (1.836±1.297), opportunistic (1.547±0.9911), and mediastinal (0.704± 0.05647) (Figure 2B). Statistical parameters such as maximum and minimum intragroup, median and standard deviation are shown in Table 5. The distribution of OD values varied in the clinical groups, but no statistical differences were found between them (P>0.05).

Importantly, correlation was found when the ELISA absorbance value of each sample was compared with immunodiffusion using a Pearson's correlation test based on the logarithmic function of antibodies titer (P=0.03, R squared = 0.2197) (Table 5). When the absorbance values of each single sample were plotted against the logarithmic value of its ID titer, a linear regression was produced between the average of absorbances and ID titers (Figure 3).

Antibodies could be detected in all the clinical forms of histoplasmosis. For acute (8 of 8) and mediastinal disease, the ptHMIN ELISA was positive for all patients. Also, 9 of 10 samples (90%) in chronic histoplasmosis, 8 of 9 samples (89%) in disseminated disease, and 12 of 14 samples (86%) in opportunistic histoplasmosis were positive. The overall sensitivity of the test was 91%. The serological parameters for each group analyzed are shown in Table 6. A higher mean of absorbances in the sera of chronic patients' samples was observed, indicating a higher overall amount of antibodies in this clinical group.

Discussion

Endemic mycoses can be challenging to diagnose and accurate interpretation of laboratory data is important to ensure the most appropriate treatment for patients. Although the definitive diagnosis of histoplasmosis requires the identification of *H. capsulatum* in infected

tissue, serological diagnosis can facilitate and provide a rapid identification of the fungus since recovery of the etiological agent is time consuming. We have developed an ELISA using metaperiodate treated purified histoplasmin (ptHMIN) that can improve and speed up histoplasmosis diagnosis in nations with limited resources.

We evaluated and validated this assay as a specific and sensitive method for the diagnosis of histoplasmosis. The statistical parameters of ptHMIN ELISA show that it is a powerful tool for the detection of antibodies in all the clinical forms of histoplasmosis. The ELISA data generated a ROC curve area of 0.944 for the prediction of a detectible antibody response. ROC analysis provides a description of the possibility of detecting the illness through a test that is independent of the incidence of the disease. In general, values higher than 0.9 reliably classify a test as acceptable. As could be seen, the curve was situated in the upper left-hand area of the ROC space, indicating a high accuracy, meaning that positive decisions are more probable when a case is actually positive than when it is actually negative. In terms of probability, this means that the probability of a test being positive when histoplasmosis is present is higher than the probability of the test being negative when histoplasmosis is absent. The overall sensitivity of the ptHMIN ELISA was 91% for all clinical forms of histoplasmosis. No statistical variation was observed when the test was repeated with the same samples. Scheel *et al.* recently published an ELISA intended for use in resource limited countries with an overall sensitivity of 81% and a specificity of 95% in detecting *H. capsulatum* antigen in urine from immunocompromised patients. However, this antigen-capture ELISA requires the production and careful characterization of polyclonal rabbit antibodies due to batch-to-batch variation. In contrast, ptHMIN is readily generated and simply stored.

Interestingly, detectable circulating anti-body levels varied for the different clinical forms of the disease, and the highest sensitivity was seen in the samples from patients with acute and mediastinal forms of histoplasmosis (100% in both cases). For these patients, isolation of *H. capsulatum* from clinical specimens lacks sensitivity, and antibody usually cannot be detected by the ID and CF. Notably, the efficacy of the urine radioimmunoassay is especially poor in acute histoplasmosis, making the ptHMIN ELISA useful even in such cases of this clinical form. Low sensitivity (86%) was exhibited by patients with histoplasmosis and concomitant HIV infection (opportunistic), which is most likely due to the lack of appropriate immune responses. The ptHMIN ELISA demonstrated a high specificity (96%) when testing sera from patients with other fungal infections usually exhibiting cross-reactivity in other immunoassays using crude HMIN, such as CF and WB., All the control sera (36 samples) were negative by ptHMIN ELISA, demonstrating a specificity of 100% for this group. The ELISA was highly discriminatory when heterologous sera were compared to sera from histoplasmosis patients (P<0.05).

In histoplasmosis, the association of severe disease, clinical form and antibody response may be relevant in monitoring patients and predicting prognosis. However, determinations of antibody titer over time using ID or CF have not previously been shown to correlate with clinical responses to infection. The amount of *Histoplasma* polysaccharide antigen detected in urine by radioimmunoassay, and in serum can be used to monitor a patient's response to

therapy, although most of the reported studies were carried out with AIDS patients,, and cross-reactivity may also be a problem.

We propose that a prospective study is warranted to determine the utility of the ptHIMN ELISA in monitoring response to therapy. In conclusion, the results of this study confirm the value of ptHMIN ELISA and demonstrate the utility of the test for the detection of antibodies in all the clinical forms of histoplasmosis. Future prospective studies will evaluate the usefulness of the ELISA in the follow-up of histoplasmosis patients during and after treatment.

Acknowledgments

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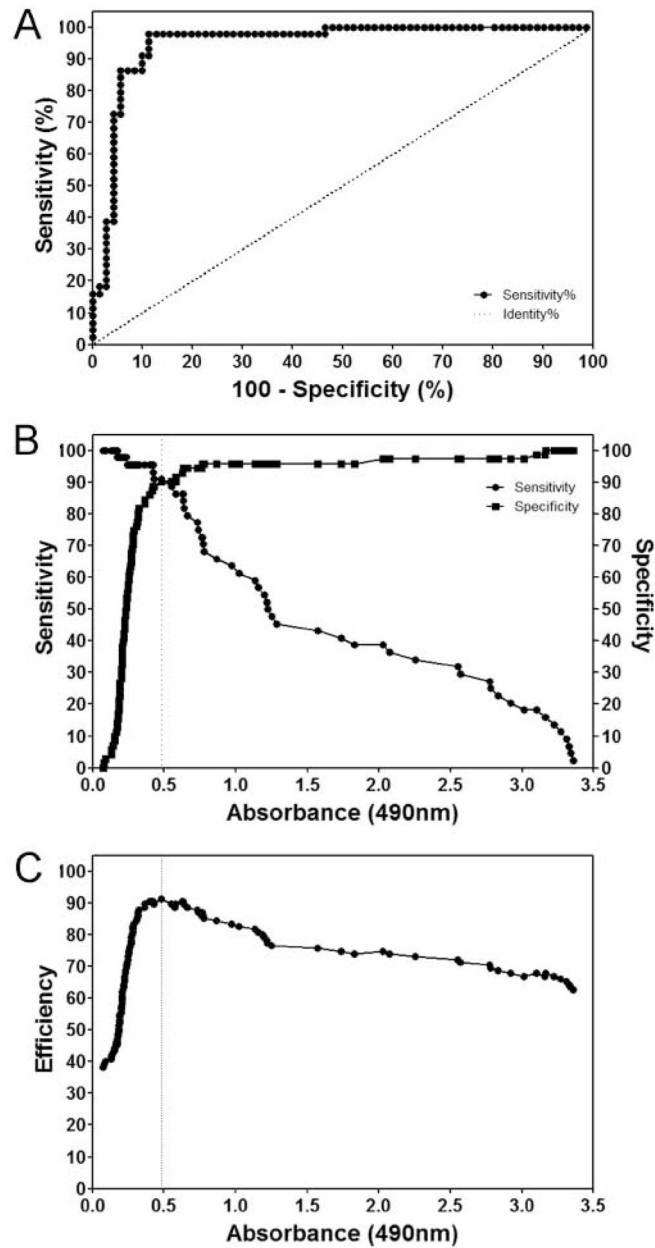
Figure 1.
(a) ROC plot of the ptHMIN specific antibody ELISA. (b) TG-ROC analysis of a ptHMIN specific antibody ELISA. The intersection point of the two graphs indicates the cut-off points at which the highest specificity and sensitivity can be achieved. (c) Youden index to achieve a maximum accuracy shows the efficiency as a function of the selected cut-off value, and confirms the best parameters with the chosen cutoff.
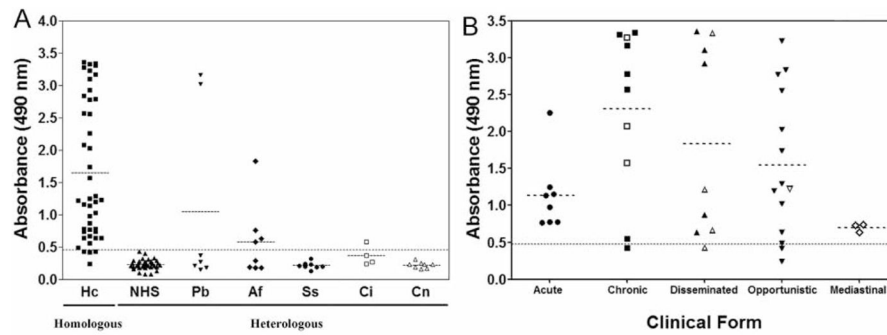
Figure 2.
(A) Detection of antibody responses in sera from histoplasmosis patients obtained before treatment, from patients with other proven mycotic diseases and from normal healthy controls by ptHMIN. Hc, Histoplasmosis; Pb, paracoccidioidomycosis; Af, aspergillosis; Ss, sporothricosis; Ci, coccidiodomycosis; Cn, cryptococcosis; NHS, normal healthy sera controls. Average for histoplasmosis group was 3.5 times higher than heterologous mycosis group and 7 times higher than normal human serum group (*P<0.001) (B) Detection of antibody responses in sera from histoplasmosis patients, with all clinical manifestations of the disease. All the samples are from patients with proven mycotic diseases. Solid symbols show samples from patients with paired fungal isolation at the moment the sample was obtained, whereas open symbols were negative, but fungal isolation previously reported. The dashed horizontal line shows the cut-off point corresponding to an OD of 0.480.
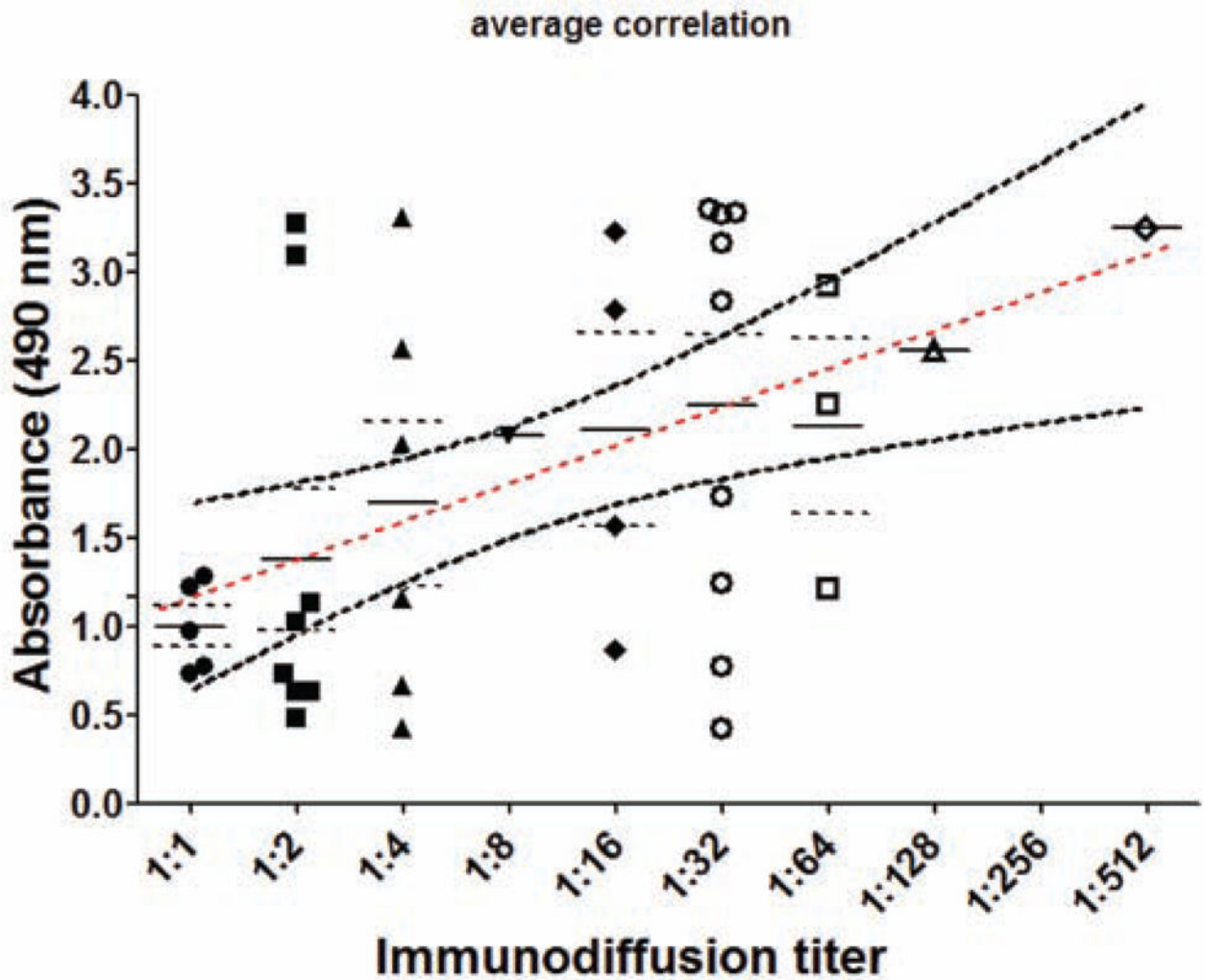
Figure 3.
Graph of the comparison of immunodiffusion and ELISA. The X axis shows the titer of the samples tested by immunodiffusion (logarithmic scale) and Y axis shows the absorbance. Dashed straight line represents the best fit line for linear regression and both curves above and below show the limits of the confidence intervals. Each symbol represents a single sample tested by both methods and the values were plotted on the graph. Correlation was found between the averages of ELISA absorbances from samples having the same ID titer.

Table 1

Classification of patients with histoplasmosis according to clinical-manifestation and diagnosis criteria.

Clinical forms	Diagnosis criteria and symptoms
Acute	Positive epidemiological history of recent exposure; abrupt onset, fever, headache, non-productive cough, aching or constricting substernal discomfort, pleuritic pain; scattered patchy pulmonary infiltrates and hilar adenopathy on chest radiograph.
Chronic	Malaise, fatigue, low-grade fever, mucoid sputum, chest pain, weight loss; reticulon odular and fibrotic lesions associated with cavitation on chest radiograph; and the presence of chronic obstructive pulmonary lung disease (not obligatory).
Disseminated	*Acute disseminated.* Preceded by the pulmonary acute form in half of the cases; fever; hepatosplenomegaly; anemia, leukopenia, thrombocytopenia; *Sub-acute disseminated.* Mild fever, weight loss, malaise; hepatosplenomegaly, focal lesions in various organs, most often in the gastrointestinal tract, adrenal gland, central nervous system, mucous membrane, skin; interstitial pneumonia; *Chronic disseminated.* Intermittent symptoms; low grade fever, fatigue, weight loss; one or more oropharyngeal, gastrointestinal, adrenal gland and/or CNS lesions; lungs are seldom involved.
Opportunistic (AIDS patients with disseminated disease)	Fever, cough; anemia, leukopenia, thrombocytopenia; hepatosplenomegaly; pulmonary involvement with diffuse interstitial infiltrates on chest radiograph; may progress to multi-organ involvement.
Mediastinal	Granulomatous mediastinitis – large caseous lymph nodes in the mediastinum.

Table 2

Demographic and clinical characteristic of patients with histoplasmosis evaluated in this study.

Variables	Results
Age range	15 to 74 years (median 36 years)
Gender	
Male	37 patients (84.1%)
Female	7 patients (15.9%)

Clinical forms (samples)	Diagnosis
Acute histoplasmosis	8 patients (18.2%)
Chronic histoplasmosis	10 patients (22.7%)
Disseminated histoplasmosis	9 patients (20.5%)
Opportunistic histoplasmosis	14 patients (31.8%)
Mediastinal histoplasmosis	3 patients (6.8%)

Table 3

Description of the isolation of *H. capsulatum* from the clinical samples of the 44 patients at the time of diagnosis. Single numbers identify samples isolated from a specific individual and in some cases, multiple samples showed positivity for growth of *H. capsulatum*.

Clinical samples	Clinical form				
	Acute (Patients 1–8)	Chronic (Patients 9–18)	Disseminated (Patients 19–27)	Opportunistic (Patients 28–41)	Mediastinal (Patients 42–44)
BAL	X	12, 15	27	X	X
Blood	X	10	X	28, 29, 31, 36, 41	X
Bone marrow	X	X	X	35	X
CSF	X	X	X	29	X
Lumbar puncture	X	X	19	X	X
Lymph node	X	X	24	30, 33, 38, 40	X
Nasal mucosa	X	X	21, 27	X	X
Oral lesion	X	X	22	39, 40	X
Skin	X	X	X	35, 38	X
Sputum	X	9, 11, 14, 15, 16, 17	21, 22, 27	39	X
Urine	X	X	X	32	X
Immunodiffusion titers (range)	1:1–1:64	1:2–1:32	1:2–1:64	1:1–1:512	1:1–1:2

BAL: bronchoalveolar lavage; CSF: cerebrospinal fluid.

Table 4

Differences in statistical parameters and descriptive measurement of the ELISA for detection of antibodies against ptHMIN.

	Homologous	Heterologous sera	
		Other mycoses	Normal human sera
Number of values	44	35	36
Minimum	0.2380	0.1295	0.0735
25% Percentile	0.7365	0.1820	0.1885
Median	1.220	0.2310	0.2288
75% Percentile	2.778	0.3640	0.2730
Maximum	3.358	3.159	0.4265
Mean (95%CI)	1.646 (1.323–1.970)	0.4811 (0.2347–0.7270)	0.2303 (0.2045–0.2560)
Std. Deviation	1.065	0.7166	0.07614

Homologous, Histoplasmosis sera; Heterologous, Sera from normal patients and from patients diagnosed with other mycoses included in the study.

Table 5

Statistical parameters and descriptive measurement of the ELISA for detection of antibodies against ptHMIN: comparison of the different clinical forms of the disease (P>0.05).

	Acute	Chronic	Disseminated	Opportunistic	Mediastinal	Total
Samples	8	10	9	14	3	44
Minimum	0.7700	0.4240	0.4240	0.2380	0.6390	0.2380
Maximum	2.254	3.339	3.358	3.222	0.7410	3.358
Median	1.054	2.675	1.215	1.257	0.7320	1.220
Mean (95%CI)	1.136 (0.726–1.546)	2.306 (1.506–3.106)	1.836 (0.838–2.833)	1.547 (0.966–2.113)	0.704 (0.564–0.844)	1.646 (1.323–1.970)
Std.	0.4903	1.119	1.297	0.9911	0.05647	1.065
Deviation Comparison to immunodiffusion (95%CI - one-tailed)	P=0.1721	P=0.6518	P=0.9119	P=0.0027	P=0.6159	P=0.0030
R squared correlation	0.3366	0.03074	0.002217	0.6505	0.3220	0.2197

Table 6

Serological parameters and descriptive measurements of the indirect ELISA for detection of antibodies in different clinical forms f histoplasmosis.

	Acute	Chronic	Disseminated	Opportunistic	Mediastinal	Total
Sensitivity	100%	90% (80.5–99.5)	89% (78.5–99.3)	86% (76.4–95.0)	100%	91% (86.6 – 95.3)
Efficiency	91% (80.9–100)	90% (80.5–99.5)	90% (80–100)	89% (80.6–97.4)	91% (74.5–100)	90% (85.5–94.5)
PPV	53% (35.4–70.6)	56% (40.6–72.0)	53% (36.4–70.0)	63% (50.0–75.9)	30% (3.5–56.5)	86% (79.6–90.4)
NPV	100% (93.6 – 100)	98% (93.3–100)	98% (92.4–100)	97%	100% (90.4–97.6)	94%

Sensitivity is the proportion of positive 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, respectively.