Validation and Clinical Application of a Molecular Method for Identification of *Histoplasma capsulatum* in Human Specimens in Colombia, South America

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Histoplasmosis is the most important mycosis endemic in the Americas and occurs by inhalation of the infectious propagules (microconidia) produced by the dimorphic fungus *Histoplasma capsulatum* (19, 32). It is amply distributed in most countries, being more prevalent in specific regions of United States, such as the Mississippi and Ohio River Valleys (14, 19). A high prevalence of histoplasmosis has also been observed in Central America (Mexico, Panama, Honduras, Guatemala, and Nicaragua), the Caribbean (Jamaica, Puerto Rico, Cuba, and Martinique), and South America (Venezuela, French Guyana, Colombia, Peru, Brazil, and Argentina) (16, 25).

The severity of histoplasmosis varies greatly depending on the intensity of the exposure to the fungus and on the immune status of the infected individual (18, 29). In patients with immunodeficiency disorders, and especially in those infected with HIV, histoplasmosis is considered an opportunistic infection (17, 20, 27); in addition, in a high proportion of cases, this fungal infection is manifested as a severe disseminated process which often leads to death if it is not treated promptly (17, 20, 27).

The diagnosis of histoplasmosis is usually accomplished by culture and microscopic examination of respiratory tract, biopsy, and body fluid specimens; nevertheless, these techniques yield positive results in only approximately 50% of the cases (9, 16, 18, 32). In addition, culturing of the fungus usually takes from 2 to 6 weeks, thus delaying the times to diagnosis and the initiation of therapy. Immunological tests that detect antibodies and/or antigens are also of value and may give results faster than culture. However, they show variable values of sensitivity and specificity and may often be negative for immunodeficient patients (18). The detection of antigen in serum and urine samples appears to be a sensitive and specific diagnostic tool, especially in HIV-infected patients (81 to 95% sensitivity with urine) (8, 12, 13, 26), although antigen detection shows cross-reactivity with the causative agents of other mycoses (12, 13, 16, 18, 30, 31).

In the last decade, several molecular approaches have been developed for the detection of *H. capsulatum* DNA in human clinical samples. Various studies have obtained high sensitivity and specificity values for PCR-based molecular tests, including a PCR (the Hc100 PCR) that detects a gene that codes for an *H. capsulatum* 100-kDa protein (Hc100), which is essential for the survival of *H. capsulatum* in human cells (3); a PCR that
determines 18S rRNA (2); a PCR that detects the internal transcribed spacer (ITS) region of the rRNA gene complex (21); and a PCR that detects the M and H antigens (4, 15). Some of these PCR assays have been tested with paraffin-embedded biopsy samples (3), blood specimens (22), infected mouse tissues (2), and samples from in vitro cultures; however, the DNA-based diagnosis of this fungal infection has not yet been established as a regular diagnostic tool, nor is a PCR assay commercially available (19).

In the present study, we evaluated over a 2-year period a cohort of patients with suspected or clinically diagnosed histoplasmosis, using a nested PCR targeting the gene coding for the 100-kDa protein previously described by Bialek et al. (3) and using fungal isolation in culture as the “gold standard” technique.

The results presented here are part of Cesar Muñoz’s master’s thesis for the Corporation of Biomedical Basic Sciences Master’s Program, Universidad de Antioquia, Medellín, Colombia.)

MATERIALS AND METHODS

Clinical specimens and cultures. In this prospective study performed between August 2005 and September 2007, 146 clinical samples were collected from 135 patients with suspected histoplasmosis at various hospitals in Medellín, Colombia, and were sent to the Medical and Experimental Mycology Unit at the Corporación para Investigaciones Biológicas (CIB) for mycological analysis and diagnosis of the infection. The respiratory tract specimens tested included bronchoalveolar lavage (BAL) fluid specimens (n = 40); bronchial lavage (BL) fluid specimens (n = 31); sputum samples (n = 8); biopsy specimens (n = 49); and body fluids (n = 18), including peritoneal fluid, pleural fluid, and cerebrospinal fluid (CSF), as well as whole blood. All specimens were initially cultured and then analyzed by a nested PCR assay (the Hc100 assay) for H. capsulatum.

To assess the specificity of the nested PCR assay, we further analyzed 60 clinical samples collected from patients diagnosed with respiratory infections different from histoplasmosis using culture and/or specific stains. These samples included 10 from patients with cryptococcosis, 10 from patients with paracoccidioidomycosis, 10 from patients with pneumocystosis, 10 from patients with candidiasis, 10 from patients with aspergillosis, and 10 from patients with tuberculosis. Negative controls (n = 49) consisted of 29 respiratory samples from patients with respiratory symptoms confirmed to be negative for H. capsulatum by culture as well as for the most common or most prevalent respiratory infectious pathogens. Additionally, we studied 20 peripheral blood samples from healthy individuals. In addition, 80 of the clinical samples or biopsy tissues were processed and stained with Wright and/or silver methenamine to identify intracellular structures compatible with H. capsulatum yeast cells.

The nested PCR was first evaluated by using 5 ng of purified DNA obtained from several related fungal pathogens maintained at the CIB or the Centers for Disease Control and Prevention (CDC; Atlanta, GA) collections (n = 22) (Table 1). Additionally, purified DNA from eight different H. capsulatum isolates from North America, Central and South America, and Africa were used as positive controls (Table 1).

**TABLE 1. PCR and DNA sequence analysis of different fungi and related microorganisms cultured in vitro**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Strain source</th>
<th>Molecular analysis</th>
<th>Fungi identified (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus fumigatus</td>
<td>CIB</td>
<td>–</td>
<td>A. fumigatus (99)</td>
</tr>
<tr>
<td>Aspergillus versicolor</td>
<td>CIB</td>
<td>–</td>
<td>A. versicolor (99)</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>CDC</td>
<td>–</td>
<td>A. flavus (100)</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>CDC</td>
<td>–</td>
<td>A. terreus (99)</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>CDC</td>
<td>–</td>
<td>A. niger (98)</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>CIB</td>
<td>–</td>
<td>C. neoformans (99)</td>
</tr>
<tr>
<td>Cryptococcus gattii</td>
<td>CDC 200814563</td>
<td>–</td>
<td>C. gattii (99)</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>CIB</td>
<td>–</td>
<td>C. albicans (100)</td>
</tr>
<tr>
<td>Candida guillermondii</td>
<td>CIB</td>
<td>–</td>
<td>C. guillermondii (99)</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>CIB</td>
<td>–</td>
<td>C. tropicalis (98)</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>CDC</td>
<td>–</td>
<td>C. parapsilosis (99)</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>CDC</td>
<td>–</td>
<td>C. glabrata (99)</td>
</tr>
<tr>
<td>Paracoccidioides brasilensis</td>
<td>ATCC 60855</td>
<td>–</td>
<td>P. brasiliensis (99)</td>
</tr>
<tr>
<td>Paracoccidioides brasiliensis</td>
<td>CIB/Pb339</td>
<td>–</td>
<td>P. brasiliensis (99)</td>
</tr>
<tr>
<td>Coccioides immitis</td>
<td>ATCC 28868</td>
<td>–</td>
<td>C. immitis (99)</td>
</tr>
<tr>
<td>Blastomyces dermatitidis</td>
<td>ATCC 200811573</td>
<td>–</td>
<td>B. dermatitidis (99)</td>
</tr>
<tr>
<td>Blastomyces dermatitidis</td>
<td>ATCC 26190</td>
<td>–</td>
<td>B. dermatitidis (99)</td>
</tr>
<tr>
<td>Chrysosporium keratinophilum</td>
<td>ATCC 14803</td>
<td>–</td>
<td>C. keratinophilum (99)</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>CIB 1980</td>
<td>+</td>
<td>H. capsulatum (99)</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>CDC/Thon</td>
<td>+</td>
<td>H. capsulatum (99)</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>G217B</td>
<td>+</td>
<td>H. capsulatum (100)</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>G184B</td>
<td>+</td>
<td>H. capsulatum (99)</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
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<td>+</td>
<td>H. capsulatum (98)</td>
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<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>CDC/2877</td>
<td>+</td>
<td>H. capsulatum (99)</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. dubsii</td>
<td>CDC/5822</td>
<td>+</td>
<td>H. capsulatum (99)</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. dubsii</td>
<td>CDC/5822</td>
<td>+</td>
<td>H. capsulatum (99)</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>CIB</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mycobacterium avium</td>
<td>CIB</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mycobacterium chelonae</td>
<td>CIB</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mycobacterium fortuitum</td>
<td>CIB</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**GenBank sequence**

<table>
<thead>
<tr>
<th>Nested Hc100/PCR</th>
<th>ITS 3 and ITS 4 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

**GenBank sequence**

Isolate Strain source Fungus identified

Aspergillus fumigatus CIB A. fumigatus (99)
Aspergillus versicolor CIB A. versicolor (99)
Aspergillus flavus CDC A. flavus (100)
Aspergillus terreus CDC A. terreus (99)
Aspergillus niger CDC A. niger (98)
Cryptococcus neoformans CIB C. neoformans (99)
Cryptococcus gattii CDC 200814563 C. gattii (99)
Candida albicans CIB C. albicans (100)
Candida guillermondii CIB C. guillermondii (99)
Candida tropicalis CIB C. tropicalis (98)
Candida parapsilosis CDC C. parapsilosis (99)
Candida glabrata CDC C. glabrata (99)
Paracoccidioides brasilensis ATCC 60855 P. brasiliensis (99)
Paracoccidioides brasilensis CIB/Pb339 P. brasiliensis (99)
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Blastomyces dermatitidis ATCC 200811573 B. dermatitidis (99)
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Histoplasma capsulatum var. capsulatum CIB 1980 H. capsulatum (99)
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Histoplasma capsulatum var. capsulatum G217B H. capsulatum (100)
Histoplasma capsulatum var. capsulatum G184B H. capsulatum (99)
Histoplasma capsulatum var. capsulatum CDC/3670 H. capsulatum (98)
Histoplasma capsulatum var. capsulatum CDC/2877 H. capsulatum (99)
Histoplasma capsulatum var. dubsii CDC/5822 H. capsulatum (99)
Histoplasma capsulatum var. dubsii CDC/5823 H. capsulatum (99)
Mycobacterium tuberculosis CIB – –
Mycobacterium avium CIB – –
Mycobacterium chelonae CIB – –
Mycobacterium fortuitum CIB – –

**GenBank sequence**
The clinical samples were cultured on Sabouraud dextrose agar and Mycosel (Becton Dickinson) by incubation at room temperature (±18 to 22°C) for 6 weeks and were microscopically examined weekly for the presence of mold colonies displaying tuberculeta macroceroma. Subsequent conversion to the yeast phase was demonstrated by culturing on brain heart infusion (BHI) agar (Becton Dickinson) containing 10% sheep blood at 37°C in a 5% CO₂ atmosphere.

**DNA extraction.** Two hundred microliters of each of the previously processed clinical samples (0.6 ml of pellet or homogenized tissue suspension stored at −20°C) or a suspension containing 5 × 10⁶ yeasts was used for DNA extraction and purification by using a DNA minikit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions.

Genomic DNA from the mold cultures was purified either by repeated phenol-chloroform extraction or by elution through Genomic G-100 columns (Qiagen Inc., Valencia, CA) with the recommended buffers and according to the instructions provided by the manufacturer. The relative concentrations of the genomic DNA extracts were determined with a NanoDrop ND1000 apparatus (Thermo Scientific, Wilmington, DE).

**Histoplasma capsulatum nested PCR assay.** *H. capsulatum*-specific primers which target the gene coding for the 100-kDa protein were used in a nested PCR, as described previously (3). The primers were synthesized by the DNA Chemistry Section, Biotechnology Core Facility, CDC. The inner primers amplify a 210-bp fragment from the first PCR product, and this amplification result is considered a positive result for *H. capsulatum*.

The PCR protocols were performed as described previously (3), with minor modifications. Briefly, the PCR consisted of 10 μl of purified DNA in a total PCR volume of 50 μl with final concentrations of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 1.5 U of *Taq* polymerase (Roche Diagnostic, Indianapolis, IN), 1 μM each outer primer, and 100 μM each deoxynucleoside triphosphate (Roche Diagnostics). The mix for the nested PCR was similar, except that 2 μl of the product from the first PCR, 50 μM each deoxynucleoside triphosphate, and 1 μM each inner primer set were used. The first PCR, the reaction for which contained the outer primer set, was cycled once at 94°C for 5 min; 35 times at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; and then once at 72°C for 5 min. For the second step, the reaction mixture was thermally cycled once at 94°C for 5 min, 30 times at 94°C for 30 s and 72°C for 1 min, and then once at 72°C for 5 min.

Ten microliters containing 5 ng of purified DNA from an *H. capsulatum* yeast culture was used as the positive control in all PCR assays. In order to detect any contamination, sterile water was included in the DNA extraction procedure as a negative control and was subsequently processed after every fifth sample in the nested PCR assay; in addition, reaction mixtures without DNA were run during all procedures.

To assess the clinical samples for the presence or the absence of amplifiable DNA, as well as the possible presence of PCR inhibitors, a nested PCR designed to amplify a human housekeeping gene, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (GenBank accession number J04038.1), was carried out as described previously (3).

**Panfungal PCR.** To confirm the identities of the different fungi cultured in vitro, we used primers ITS 3 and ITS 4, which target the ITS 2 region of the rRNA gene (34), followed by sequencing (Table 1). The products of the nested PCR assay were also sequenced. All PCR products were treated with the ExoSAP-IT reagent (USB Corporation, Cleveland, OH), according to the manufacturer’s instructions. DNA sequencing was performed at the Genomics Unit, Division of Food-Borne Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, with an ABI Prism 3730 genetic analyzer (Applied Biosystems).

**Detection limit.** In order to establish the detection limit of the nested PCR assay, DNA was extracted from serial dilutions made of a cell suspension from an *H. capsulatum* yeast culture containing 2.6 × 10⁶ cells/ml that was amplified by the nested PCR assay, as described above (2, 3).

**Data analysis.** The sequences obtained were edited and aligned by using Sequencer (version 4.8) software (Gene Codes Corp., Ann Arbor, MI). A search for the homology of our sequences with the sequences in the GenBank database was carried out by using the BLASTn program. The sensitivity, specificity, and predictive values for the *H. capsulatum* nested PCR were calculated as described by Galen and Gambino (11). We used culture to confirm the diagnosis. In addition, we calculated the positive and the negative likelihood ratios, as previously described by Dujardin et al. (10).

**RESULTS**

Detection of Hc100 in patients with either a clinical suspicion of or confirmed histoplasmosis. A total of 146 clinical samples from 135 patients with a clinical suspicion of *H. capsulatum* infection were analyzed. Sixty-seven samples (45.9%) were positive for histoplasma by culture; 20 of the 80 samples (25%) stained with Wright and/or silver methenamine demonstrated intracellular structures compatible with *H. capsulatum* yeast cells. PCR was positive for 76 samples (52.1%); 67 of these were also culture positive (Table 2). Nine samples in this group were positive by the nested PCR assay and negative by culture. The sequences of these nine amplification products showed identity values of 98% with the sequence of the gene coding for the *H. capsulatum*-specific 100-kDa protein. Three samples from patients with HIV infection were positive by staining but negative by both the Hc100 nested PCR and culture.

The presence of PCR inhibitors was ruled out, since all samples giving a negative result by the Hc100 nested PCR assay amplified the specific fragment of the human GAPDH gene (data not shown).

**Evaluation of control samples.** Sixty clinical samples from patients with infectious entities other than histoplasmosis were analyzed. Three of these patients, one of whom had been diagnosed with cryptococcosis and two of whom had been diagnosed with candidiasis (caused by *Candida albicans* and *Candida guilliermondii*, respectively) by culture, were positive by the Hc100 nested PCR (Table 3). Twenty-nine respiratory samples demonstrated to be negative by culture and/or staining for the most common respiratory fungal infections as well as for tuberculosis were used as controls (Table 3). Four BAL fluid samples were positive by the Hc100-specific nested PCR. Analysis of the sequences of
all of these samples showed that they had over 98% identity with the corresponding sequence for Hc100. The 20 peripheral blood samples from healthy individuals were negative by the Hc100 nested PCR.

All DNAs purified from the different *H. capsulatum* yeast cultures were positive by the nested PCR for Hc100, while none of the DNAs isolated from cultures of other microorganisms gave positive results by the Hc100 nested PCR (Table 1). The ITS sequences allowed us to identify the different fungal species studied.

No evident cross-contamination during the extraction procedure was observed. All DNA extraction controls were negative by the nested PCR assays.

The sensitivity and the specificity results for the nested PCR test were estimated on the basis of the findings for the 67 *H. capsulatum* patient isolates positive by both culture and PCR (Table 4). The Hc100 nested PCR exhibited a sensitivity of 100% and a specificity of 92.4% compared to the results for the clinical samples collected from patients with other respiratory infections diagnosed by culture and/or with specific stains. The corresponding positive and negative predictive values were 83% and 100%, respectively. In addition, we determined that the positive likelihood ratio was 25, while the negative likelihood ratio was 0.

**Detection limits.** The nested PCR optimized under our laboratory conditions allowed us to detect *H. capsulatum* DNA in a serially diluted suspension containing only 10 fungal cells. When the DNA extracted from 26,000 yeast cells was quantified and diluted, the nested PCR was still positive with the dilution containing 20 fg of DNA, which corresponds to approximately 8 genome equivalents or yeast cells (data not shown).

**DISCUSSION**

The diagnosis of histoplasmosis has historically been based on the findings of culture and microscopy, which lack sensitivity, require interpretive expertise, and in particular, fail to provide the timely diagnosis required for the institution of treatment of patients infected with both HIV and histoplasma (9). The advent of DNA-based diagnosis has resulted in the development of a number of molecular tests that employ different DNA targets (2, 4, 6, 15, 23). The choice of rRNA gene targets can contribute to an unacceptable rate of false-positive results, particularly when taxonomically close relatives cross-react with *Histoplasma* organisms (5). To circumvent this problem, Bialek et al. developed a nested PCR assay using the Hc100 gene, which they determined was specific for *H. capsulatum* (3). They recognized the need for this assay to be validated further with patient samples collected from a region where *Histoplasma* is endemic. This challenge was first addressed by Maubon et al. (22), who tested 40 samples from 27 patients in French Guiana, South America, and found that all of the cultures positive for *H. capsulatum* (*n* = 15) were also positive by the Hc100 PCR. The study we report here was designed to validate the Hc100 PCR assay for the diagnosis of histoplasmosis with samples from the largest study population evaluated to date: 146 clinical samples from 135 patients in Medellín, Colombia, suspected of having histoplasmosis. We obtained a sensitivity of 100% and a specificity 92.4% compared to the results for the negative controls and a specificity of 95.2% compared to the results for patients with other infectious diseases.

We also provide valuable information on the representativeness and reliability of the Hc100 gene as an indicator of *H. capsulatum* by verifying the presence of this gene in a series of *H. capsulatum* isolates from North, Central, and South America and from Africa, as well as by determining the absence of this target in the closely related fungi *Paracoccidioides brasilienis* and Blastomyces dermatitidis, as well as *Aspergillus, Cryptococcus, Candida, Coccioidoides,* and *Mycobacterium* species. These studies verified that clinical samples from patients with infections due to other respiratory pathogens would not be expected to display cross-reactivity with the Hc100 gene target.
We found that the Hc100 nested PCR assay was positive for 13 of the clinical samples from patients with respiratory symptoms who were negative for *H. capsulatum* by culture. Histoplasmosis is endemic in Colombia, and judging from the high rate of histoplasmin skin test positivity for healthy adults (22%), it can be speculated that such patients were infected with *H. capsulatum* (7, 24). If this value is reflected in the total number of inhabitants of the country, the number of infected persons would be close to 6 million. It is therefore possible to find subclinical histoplasmosis in both symptomatic patients and asymptomatic persons who remain healthy. A test as sensitive as the one that is being implemented could well detect these subclinical infections in patients who were not culture positive for *H. capsulatum*. These positive results may well occur for persons with latent histoplasmosis, in a manner similar to that for tuberculosis (17).

When we analyzed the 60 samples from patients with proven respiratory infections other than histoplasmosis, we found that one patient with established cryptococcosis and two patients with diagnoses of esophageal and pleural candidiasis, respectively, were positive by the Hc100 nested PCR. All amplified products were confirmed by sequence analysis, and these subclinical infections in patients who were not culture positive for *H. capsulatum* have been observed for persons with latent histoplasmosis, in a manner similar to that for tuberculosis (17).

When we analyzed the 60 samples from patients with proven respiratory infections other than histoplasmosis, we found that one patient with established cryptococcosis and two patients with diagnoses of esophageal and pleural candidiasis, respectively, were positive by the Hc100 nested PCR. All amplified products were confirmed by sequence analysis, and they showed over 98% identity with the gene coding for the *H. capsulatum*-specific 100-kDa protein. The findings suggest that the patients mentioned above may also have concomitant histoplasmosis that had not been previously detected by conventional tests. It is known that the identification of microorganisms by isolation in culture, especially if the microorganism is *H. capsulatum*, has its limitations. Culture methods can produce false-negative results for about 20% and 50% of patients with disseminated pulmonary histoplasmosis and chronic pulmonary histoplasmosis, respectively (28, 33). Concerning the cryptococcosis patient whose sample was positive by the Hc100 nested PCR, the clinical record showed that he had AIDS and a low CD4+ lymphocyte count (77 cells per microliter). Thus, in this group of patients, one may find non-apparent coinfections with AIDS-associated microorganisms (1). For the patients with candidiasis, we were, unfortunately, unable to access their medical records to obtain detailed data that could have revealed an explanation for their positive reactions.

In addition, we confirmed the detection limits of the test and showed that it is highly sensitive, detecting approximately either 10 yeast cells per reaction or *H. capsulatum* DNA at concentrations as low as 20 fg, suggesting that the Hc100 nested PCR reaches an analytical sensitivity similar to the analytical sensitivities reported in other studies (3, 4). Histoplasmosis is not a mandatory reportable disease; thus, its prevalence is difficult to calculate. We determined the positive and negative likelihood ratios and obtained values of 25 and 0, respectively. These values indicate that a positive result by the Hc100 nested PCR is about 25 times more probable for a patient with histoplasmosis than for an individual without histoplasmosis.

In conclusion, the Hc100 nested PCR is a promising diagnostic tool that can be implemented to detect *H. capsulatum* DNA in a variety of clinical samples. Thus, this molecular test appears to be much more sensitive than culture, with the latter being considered the gold standard. This method should be valuable in areas where *H. capsulatum* is endemic.

**ACKNOWLEDGMENTS**

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

**REFERENCES**


