Original Article

Validation and clinical application of a molecular method for the identification of Cryptococcus neoformans/Cryptococcus gattii complex DNA in human clinical specimens

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The diagnosis of cryptococcosis is usually performed based on cultures of tissue or body fluids and isolation of the fungus, but this method may require several days. Direct microscopic examination, although rapid, is relatively insensitive. Biochemical and immunodiagnostic rapid tests are also used. However, all of these methods have limitations that may hinder final diagnosis. The increasing incidence of fungal infections has focused attention on tools for rapid and accurate diagnosis using molecular biological techniques. Currently, PCR-based methods, particularly nested, multiplex and real-time PCR, provide both high sensitivity and specificity.

In the present study, we evaluated a nested PCR targeting the gene encoding the ITS-1 and ITS-2 regions of rDNA in samples from a cohort of patients diagnosed with cryptococcosis. The results showed that in our hands, this Cryptococcus nested PCR assay has 100% specificity and 100% sensitivity and was able to detect until 2 femtograms of Cryptococcus DNA.

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Introduction

Cryptococcosis is a fungal infection caused by encapsulated yeasts of the phylum Basidiomycota, genus Cryptococcus. Although over 30 different species of Cryptococcus have been identified to date, just two closely related species – Cryptococcus neoformans and Cryptococcus gattii – cause the majority of human fungal infections.\(^1\) Based on specific polysaccharide capsule antigen analysis, subtyping data, and comparisons...
of the genomic sequences, pathogenic cryptococci have been
divided into five capsular serotypes: serotype A (C. neoformans
var. grubii), serotypes B and C (both C. gattii), serotype D (C. neoformans var. neoformans), and the hybrid diploid serotype
AD.4,5,6

C. neoformans is found worldwide, and it causes the
majority of cryptococcal infections in people with decreased
immunity (primarily AIDS patients, people undergoing immu-
nosuppressive therapies and those with lymphoproliferative
disorders), resulting in varying neurological complications.5
In contrast, C. gattii is primarily endemic to tropical and
subtropical regions, and it causes 70% to 80% of cryptococcal
infections in immunocompetent hosts.4,7,8

Although reporting fungal infections is not mandatory in
Colombia, South America, in 2012 Escandón et al. published
the results of a survey on cryptococcosis conducted in Colombi-
between 2006 and 2010. In this period, 526 reports with at
least one case of cryptococcosis were received. These cases
originated from 72% of the Colombian political divisions. The
most prevalent risk factor reported was HIV infection (83.5%),
with cryptococcosis defining AIDS in 23% of the cases. The
estimated mean annual incidence rate for cryptococcosis in the
general population was one in every 2.4 × 10^6 habitants,
while in AIDS patients this rate rose to one in 3.3 × 10^5. Neo-
cryptococcosis was recorded in 81.8% of the cases. Laboratory
diagnoses were based on direct examination, culture and latex
in 29.3% cases; of 413 Cryptococcus isolates analyzed, 95.6% were
identified as C. neoformans var. grubii, 1% C. neoformans
var. neoformans, and 3.4% C. gattii.9

Even though the majority of cryptococcosis cases reported
correspond to cryptococcal meningitis, the initial infection
is generally acquired by the inhalation of airborne fungal
propagules from an environmental source.10–12 Both C. neoform-
ans and C. gattii are capable of causing severe pulmonary and
central nervous system (CNS) infections in both immunocom-
potent and immunosuppressed individuals13–15; importantly,
up to 70% of these individuals will die within three months
of infection.16

The diagnosis of cryptococcosis infection is usually based
on isolation of the fungus from cultured tissue or body
fluids such as sputum, blood and cerebrospinal fluid, but
this method may require several days to detect and identify the
microorganisms. Although direct microscopic examination
is rapid, this method is relatively insensitive. Of rapid bio-
chemical and immunodiagnostic tests, which can be performed on
blood and/or cerebrospinal fluid,17,18 the detection of crypto-
coccal capsular antigen by latex agglutination is one of the
most helpful tests for fungi performed on a routine basis. Its
ease of use and sensitivity are better than other conventional
immunodiagnostic methods19–21; however, all of these meth-
ods have some limitations that may hinder final diagnosis.15

The diagnostic limitations and increasing incidence of
fungal infections have prompted the development of tools
for rapid and accurate diagnosis using molecular biologi-
cal techniques. Currently, molecular methods such as DNA
hybridization and PCR-based methods (particularly nested,
multiplex and real time PCR) provide both high sensitivity and
specificity. Improvements in PCR techniques have allowed the
detection of minimal amounts of DNA from the C. neoformans
species; in addition, PCR can be used in association with other
techniques, making it a valuable tool for molecular epidemi-
ology studies.20–24

Several target sequences have been utilized to identify
the C. neoformans complex, including URAS, CAP59, M13,
and ITS (18S, 5.8S, and 28S). The ITS region of rDNA has
been the most frequently used region for the detection of
fungal sequences because of its high degree of variation
compared to that of other ribosomal DNA regions facilitates
identification.25–27

Nested PCR stands out among the most-used PCR-based
techniques for detection and identification of C. neoformans
and C. gattii. In this technique, the DNA used in the reaction
is the product of a previous amplification, and it is very use-
ful when high sensitivity and specificity are desirable.28
The work of Rappelli et al. (1998)26 strongly influenced this area;
they developed a nested PCR protocol for the detection of C.
neoformans and C. gattii from samples obtained from patients
with neurocryptococcosis. The specificity and sensitivity of
this technique were tested using DNA from other microorgan-
isms, which were not amplified. Testing different dilutions of
fungal DNA samples resulted in the amplification of up to 10
fungal cells/ml.

Recently, Trilles et al. (2014) developed a hyperbranched
rolling circle amplification (HRCA) based on the PLB1 locus.
Used alone and in combination with a semi-nested PCR, this
technique was specific and highly sensitive. This new method
has great potential for use in direct diagnosis of cryptococcosis
from clinical specimens.29

For the treatment of cryptococcosis, amphotericin B,
fluconazole, and itraconazole are recommended as first-
line treatments for C. neoformans and C. gattii infections,
while voriconazole and posaconazole are used as secondary
therapies.30,31 However, worldwide, approximately 625,000
patients living with HIV/AIDS die from cryptococcal meningi-
itis each year (Centers for Disease Control and Prevention,
CDC, Atlanta, USA, http://www.cdc.gov/).

In the present study, we validated the nested PCR described
by Rappelli et al. (1998) in e ITS-1 and ITS-2 coding regions of
C. neoformans/C. gattii. The gold standard diagnostic technique
used in the validation was microorganism culture from clinical
specimens.26 We aimed at implementing this molecular assay
as an integral component of the diagnostic tests regularly used
in our laboratory.

Materials and methods

Clinical samples and isolates

Over the 17-month period from January 2011 to June 2012, 44
human clinical samples from 44 patients with confirmed cryp-
tococcosis were collected. The clinical specimens included:
bronchoalveolar lavage (BAL) (n = 10), bronchial lavage (BL)
(n = 6), biopsy (n = 4), and cerebrospinal fluid (CSF) (n = 24). All
of the specimens were collected at hospitals in Medellin, Colom-
bia, and sent to the Medical and Experimental Mycology Unit
of the Corporación para Investigaciones Biológicas (CIB), for
mycological diagnosis.

To assess the specificity of the nested PCR, 92 clinical
samples collected from patients with other respiratory
Infections (diagnosed by culture and/or specific stains) were analyzed. These clinical samples included specimens from patients diagnosed with: histoplasmosis (n = 10), paracoccidioidomycosis (n = 21), pneumocystis (n = 6), candidiasis (n = 16), aspergillosis (n = 19), and tuberculosis (n = 20). As negative controls (n = 51), we used 30 respiratory-negative samples (by culture and/or specific stains for cryptococcosis or other common respiratory infectious pathogens) as well as 21 peripheral blood samples from healthy individuals (Table 1). Additionally, the nested PCR specificity was also evaluated, using purified DNA isolated from cultures of different pathogen microorganisms (n = 35), previously identified by sequencing (Table 2).

**Processing of clinical samples**

Both respiratory tract specimens (BAL, BL, sputum) and body fluids (peritoneal, pleural and CSF) were collected in 50ml sterile Falcon tubes (Becton Dickinson) and centrifuged at 1550 x g for 30 min (Centra MP4R, IEC). Fresh tissues (biopsy) were manually homogenized in 3 ml of sterile saline solution. The pelleted samples and the homogenate obtained were used for culture and stain, and 0.6 ml of each sample was stored at −20°C for subsequent DNA extraction.

Processed specimens were cultured on Sabouraud Dextrose Agar and Mycosel (Becton Dickinson), incubated at room temperature (±18–22°C) for three weeks, and examined weekly for yeast colonies; the identification was performed using microscopic observation, phenotypic type enzyme (urease and phenoloxidase), and carbohydrate assimilation patterns (McTaggart et al., 2011). The biovarieties were determined by culturing the isolates on L-canavanine glycin bromothy- mol blue (CGB) selective medium (Klein et al., 2009). These procedures were carried out in a Biosafety Level 3 (BSL3) laboratory.

### Table 1 - Clinical samples used for the Cryptococcus neoformans/Cryptococcus gattii nested PCR validation.

<table>
<thead>
<tr>
<th>Clinical samples (n:187)</th>
<th>Negative controls (n:51)</th>
<th>Respiratory symptomatic (n:30)</th>
<th>Kind of sample</th>
<th>Type of sample</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL (n:25)</td>
<td>BL (n:4)</td>
<td>Sputum (n:1)</td>
<td>BAL (n:24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy individuals (n:21)</td>
<td>Whole blood (n:21)</td>
<td></td>
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</tr>
<tr>
<td>Positive controls (n:44)</td>
<td>Respiratory symptomatic (n:44)</td>
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<td></td>
<td></td>
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<tr>
<td>Biopsies (n:4)</td>
<td></td>
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<tr>
<td>Samples used to evaluate the specificity (n:92)</td>
<td>Respiratory symptomatic (n:92)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tracheal aspirates (n:9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biopsies (n:25)</td>
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<tr>
<td>Sputum (n:20)</td>
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<tr>
<td>LD (n:10)</td>
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<tr>
<td>BAL (n:24)</td>
<td></td>
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</tr>
</tbody>
</table>

*Note: Kind of patient: Respiratory symptomatic (n:30); Positive controls (n:44); Samples used to evaluate the specificity (n:92) *
DNA extraction

Table 2 – Purified DNA used to evaluate the Cryptococcus neoformans/Cryptococcus gattii nested PCR specificity.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Strain source</th>
<th>Nested PCR for the complex C. neoformans/C. gattii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus flavus</td>
<td>CIBb</td>
<td>–</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>CDC</td>
<td>–</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>CDC</td>
<td>–</td>
</tr>
<tr>
<td>Blastomyces dermatitidis</td>
<td>CDC 2008011573</td>
<td>–</td>
</tr>
<tr>
<td>Blastomyces dermatitidis</td>
<td>ATCC 26199</td>
<td>–</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida guilliermondii</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida dubliniensis</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida krusei</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida lusitanae</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida brucariensis</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida ohmeri</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida famata</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida orthopsilosis</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Candida metapsilosis</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Coccioidioides immitis</td>
<td>ATCC 28868</td>
<td>–</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>CIB</td>
<td>+</td>
</tr>
<tr>
<td>Cryptococcus gattii</td>
<td>CIB</td>
<td>+</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>CDC</td>
<td>–</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>G217B</td>
<td>–</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>G184B</td>
<td>–</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. capsulatum</td>
<td>CDC</td>
<td>–</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. duboisii</td>
<td>CDC/S822</td>
<td>–</td>
</tr>
<tr>
<td>Histoplasma capsulatum var. duboisii</td>
<td>CDC/S823</td>
<td>–</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Mycobacterium avium</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Paracoccidioides brasiliensis</td>
<td>ATCC 60855</td>
<td>–</td>
</tr>
<tr>
<td>Paracoccidioides brasiliensis</td>
<td>CBS/Pb339</td>
<td>–</td>
</tr>
<tr>
<td>Paracoccidioides brasiliensis</td>
<td>CBS/Pb18</td>
<td>–</td>
</tr>
<tr>
<td>Paracoccidioides lutzii</td>
<td>CIB</td>
<td>–</td>
</tr>
<tr>
<td>Schizopilomyces commune</td>
<td>CIB</td>
<td>–</td>
</tr>
</tbody>
</table>

a DNA was isolated from cultures of pathogenic microorganisms (≥35) previously identified by nucleic acid sequencing of genomic targets.
b CIB: Corporación para Investigaciones Biológicas, Medellin, Colombia.
c CDC: Centers for Disease Control and Prevention, Atlanta, USA.

DNA extraction

Two hundred microliters of each previously processed clinical sample or yeast suspension were used for DNA extraction and purification. The QIAamp® DNA Mini kit (Qiagen, Hildenberg, Germany) was used with some modifications: the initial incubation with lysis buffer was performed at 65°C for one hour, followed by AL buffer incubation at 90°C for 10 min and an additional incubation with recombinant lyticase (1U/ml) at 37°C for 45 min. For filamentous fungal isolates, DNA extraction was performed using the phenol-chloroform method or a commercial kit with Genomic G-100 columns (Qiagen Inc., CA) (Sambrook et al., 2001). DNA extraction from whole blood was performed using a protocol described by Einsele et al. (1997) with some modifications. The relative concentrations of DNA extracted were determined using a NanoDrop ND2000 (Thermo Scientific).

C. neoformans/C. gattii nested-PCR assay

C. neoformans/C. gattii specific primers that target the gene encoding the rDNA internal transcribed regions 1 (ITS-1) and 2 (ITS-2) were used in a nested PCR reaction as described by Rappelli et al. (1998), with some modifications. The master mix for the first PCR consisted of 10 μl of purified DNA in a total PCR volume of 50 μl with final concentrations of 2 mM MgCl₂ (Invitrogen), 0.2 mM of dNTPs Mix, 0.6 μM of each outer and inner primer (Invitrogen) and 0.02 units of Taq polymerase (Invitrogen). The mixture was incubated at 94°C for 5 min; 20 cycles of 94°C for 45 s, 55°C for 60 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. For the second (nested) PCR, the mix was similar to the first, except that 2 μl of the first PCR product was used as template DNA and the reaction mixture was incubated at 94°C for 5 min; for 30 cycles of 94°C for 45 s, 70°C for 60 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The final product of the nested PCR is an 116 bp fragment that indicates the presence of Cryptococcus DNA in the samples analyzed. As a positive control, 10 μl containing 10ng of purified C. neoformans DNA was used in all PCR assays. To detect any contamination, sterile water was included in the DNA extraction used as a negative control, and additional reaction mixtures without DNA were run during all procedures.

As a control to verify amplifiable DNA or to detect the presence of PCR inhibitors in the clinical samples, a PCR designed to amplify the human gene for β-globin was carried out as described by Bialek et al. (2005). All of the PCR reactions were run on a Peltier Thermal Cycler PT100 (MJ Research, USA). The PCR products were visualized by electrophoresis on 2% agarose gels (Sigma Chemical Co., St. Louis, MO, USA), using gel red and a UV transilluminator (Molecular Imager® Gel DocTMXLR+ BIORAD). All of the nested PCR products were sequenced to verify that the amplified DNA fragment corresponded to the C. neoformans/C. gattii target.

Detection limit

To establish the detection limit of the nested PCR assay, we extracted and quantified DNA from a C. neoformans yeast suspension and performed serial dilutions (1:2) ranging from 40.4 ng to 1 fg. Each of these dilutions was then used for a specific PCR, to determine the amount of DNA at the assay’s detection limit.

Data analysis

The sequences obtained were edited and aligned using Sequencer software (version 4.8), and homology searches of
all sequences were carried out using the BLASTn program from the National Center for Biotechnology Information, Washington, DC. The sequences were categorized according to E-values (error probability) as provided by BLASTn, using values lower than $1 \times 10^{-6}$.

Sensitivity and specificity for the *C. neoformans/C. gattii* nested PCR were calculated using the culture as the gold standard, according to the method of Galen and Gambino (1975).36

**Results**

**Cross-reaction assay**

None of the DNA isolated from cultures of related microorganisms previously identified by sequencing tested positive in the Cryptococcus nested PCR assay. By contrast, all purified DNA from the *Cryptococcus* yeasts, including *C. gattii*, tested positive in the nested PCR assay (Table 2).

**Detection of Cryptococcus DNA in clinical samples**

A total of 44 clinical samples from patients with cryptococcosis that was diagnosed by culture were analyzed through nested PCR. All of the BL, BAL, biopsies, and CSF samples were tested positive in the Cryptococcus nested PCR assay. Therefore, the ITS-1 and ITS-2 of rDNA nested PCR for the Cryptococcus *neoformans/Cryptococcus gattii* complex exhibited a sensitivity of 100% (Table 3A).

To assess the specificity of this nested PCR assay, 92 clinical samples collected from patients with other diagnosed respiratory infections by culture and/or specific stains and 51 negative controls (30 respiratory negative samples and 21 peripheral blood samples from healthy individuals) were analyzed. The ITS-1 and ITS-2 of rDNA nested PCR exhibited a specificity of 100% for the negative controls (0 positives/51 samples) as well as, for those samples (0/92) with other diagnosed respiratory infections (Table 3B).

The presence of PCR inhibitors was ruled out because all of the clinical samples with negative results in the Cryptococcus PCR assay allowed amplification of a specific fragment of the human β-globin gene.

**Detection limits**

DNA extracted from a *C. neoformans* yeast suspension was quantified and serial dilutions (1:2) were performed, ranging from 40.4 ng to 1 fg. The optimized nested PCR conditions in our laboratory allowed detection of 2 fg of Cryptococcus DNA (Fig. 1).

**Controls**

All of the DNA extraction controls tested negative in the nested PCR assays. Therefore, any possible cross-contamination during the extraction procedure was discarded.

**Discussion**

In our hands, the Cryptococcus nested PCR assay for rDNA ITS-1 and ITS-2 of has 100% sensitivity, as it tested positive for all 44 clinical samples (10 BALs, 6 BLs, 4 biopsies and 24 CSFs) from patients diagnosed with cryptococcosis. The specificity of this assay was also 100% as both the 51 negative controls (25 BALs, 4 BLs, 1 sputum, and 21 whole bloods) and the 92 clinical samples from patients diagnosed with infections other than cryptococcosis (10 histoplasmosis, 21 paracoccidioidomycosis, 6 pneumocystosis, 16 candidiasis, 19 aspergillosis, and 20 tuberculosis) gave negative results. However, in order to obtain negative and positive predictive values, a cohort of patients with syndromes that include *C. neoformans/C. gattii* as part of the possible causes will be evaluated in the future using both our PCR assays as the gold standard method until a final diagnosis is reached.

Just between the years 2006 and 2010 in our country, 526 reports of at least one case of cryptococcosis were obtained in
an epidemiological study conducted by Escandon et al. (2012), even knowing that the reporting of fungal infections was not mandatory in Colombia. In microbiology and mycology laboratories, the diagnosis of fungal infections is based on direct observation of macro/micro morphological characteristics, culture, or biochemical and serological tests that permit identification of the pathogen. Nevertheless, the aforementioned diagnostic strategies are not always sufficient for accurate cryptococcosis diagnosis. The implementation of molecular diagnostic tests with high sensitivity and specificity would be essential to mycology laboratories in our country. Early diagnosis that leads to adequate and prompt starting of antifungal therapy would be crucial in diminishing the severity of infection.

Accurate diagnosis of infectious fungal diseases is very difficult because nearly 70 thousand fungal species have been described. However, only approximately 270 species have been reported to result in infectious disease in humans, and the majority of them are not well adapted to growth at human body temperature (most of these fungi correspond to dermatophytic species causing superficial infections). Among the few species able to produce systemic fungal infections in humans are those included in the genus Cryptococcus, specifically C. neoformans and C. gattii.

In this context, the PCR technique has much strength compared to conventional methods: it is not laborious, can be used with a small sample, is able to detect a very low fungal load, and is a very rapid technique. Although other genetic targets, such as the CAP59 gene (involved in Cryptococcus capsule production) and alternative ribosomal DNA regions (SSU/LSU) have been used to identify different species of the C. neoformans complex, the high degree of variability of the ITS region of the rDNA makes it the most commonly used region for the detection and identification of several fungal sequences. Other studies using different molecular approaches for cryptococcosis diagnosis have also shown high sensitivity and specificity.

In our study, specific primers targeted to the ITS-1 and ITS-2 regions of rDNA were used according to the description of Rapelli et al. (1998). Additionally, in order to increase the disruption efficiency and liberate cryptococcal DNA from tissue homogenates, the DNA extraction protocol included an incubation period with recombinant lyticase (1 U/µl) at 37 °C for 45 min.

The current gold standard diagnostic test for cryptococcosis remains culture, despite its lack of sensitivity. Antigen tests are also regularly used to detect cryptococcal antigen (CrAg) by both enzyme immunoassay (EA) or latex agglutination (LA). These tests are sensitive and specific but require expertise, special storage, and a central reference laboratory. Additionally, cryptococcal antigen titers remain high even five months after effective therapy, which may lead to false positive test results. Recently, a lateral flow assay (LFA) with the ability to detect CrAg was developed, but although this rapid test exhibits good sensitivity and specificity, more validation studies are necessary.

In conclusion, in agreement with the results described by Rapelli et al. (1998), we found that Cryptococcus nested PCR is a sensitive, specific, and reproducible method to be used in the analysis of different clinical samples. We confirmed the high sensitivity with the ability to detect amounts down to 2 fg of Cryptococcus DNA. This nested PCR assay may be a useful tool not only for rapid diagnosis of acute cryptococcosis but also for monitoring patients during therapy and confirming clearance of the parasite in follow-up exams.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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