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# Use of Polymerase chain Reaction for *Cryptococcus neoformans* Genome Detection in Cerebrospinal Fluid for Neurocryptococcosis Diagnosis

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## Summary

Cryptococcosis is a systemic infection caused by encapsulated yeast of *Cryptococcus* genus, has a worldwide distribution, affecting about 1/3 of human immunodeficiency virus (HIV) carriers and thus, is considered an opportunistic infection. Rapid and specific diagnostic tests for fungal infections are extremely important to effective treatment of infected patients. With the progress of molecular biology has been possible to develop new techniques for diagnosis and *Cryptococcus* spp identification. This study aimed standardizes a *Cryptococcus* spp DNA extraction method; determine the detection limit of fungus genome by PCR (Polymerase Chain Reaction) technique and determine the sensitivity and specificity of this technique in biological samples from infected patients. To standardize the DNA extraction were compared two ways for breaking fungus cell wall: the first one, using only glass beads, and the second one, using CTAB reagent. For standardization of conventional PCR and real time PCR (q-PCR), primers previously described were used, specific for *Cryptococcus* genus (CN4-CN5). A culture of *C. neoformans* (ATCC 24067) was used as a positive control. For q-PCR standardization for *Cryptococcus* spp genome detection and quantification in cerebrospinal fluid (CSF) were used 56 samples collected from positive neurocryptococcosis patients, which diagnostic was confirmed by other tests such as India ink, culture, latex and clinical findings. As negative control were used 44 CSF samples collected from negative neurocryptococcosis patients, according the same tests mentioned above. To confirm the specificity of the primers, q-PCR was tested in genomes of other fungal species. According to the results, no significant difference was observed between two DNA extraction methods, suggesting that for DNA extraction in CSF samples it is not necessary to use CTAB to lyse the fungus cell wall. The CTAB residues may inhibit the q-PCR, which is highly sensitive and requires a high degree of purity of the DNA. With respect to the detection limit, it was observed that the lowest DNA concentration detected by q-PCR was 47.0 femtograms (10-5) DNA/µL. The specificity test showed that primers were 100% specific, because it was not aligned in the other fungi species genomes tested. All 56 CSF samples of confirmed neurocryptococcosis patients were positive in q-PCR, while 18 (18.8%) CSF samples among negative neurocryptococcosis patients group were positive in q-PCR. This divergence of results can be explained by the fact that q-PCR has higher sensitivity than other tests used in cryptococcal meningitis diagnosis. Therefore, the q-PCR technique proved to be feasible for neurocryptococcosis diagnosis support, after it is well standardized, as in this study.

**Keywords:** Neurocryptococcosis; HIV; *Cryptococcus* spp; Polimerase chain reaction; Molecular diagnosis; q-PCR

## Introduction

The neurocryptococcosis is a systemic fungal infection caused by inhaling basidiospores and yeast cells of the fungus *Cryptococcus neoformans*, which after spending some time in the lungs, is spread through the blood and lodged primarily in the brain and meninges [1,2]. The disease affects about one million people worldwide each year and 400,000 deaths are computed from patients who die within three months after the conception of the disease [3].

The neurocryptococcosis primarily affects patients with immunosuppressive diseases such as acquired immunodeficiency syndrome (AIDS) or lympho proliferative neoplasias, sarcomas, or even those patients that received transplants and are at the use of immunosuppressive therapy [4,5]. The disease emerged in the 80s with the AIDS pandemic, affecting currently, about one-third of individuals carrying the human immunodeficiency virus (HIV), making it the fourth most important cause of death in these patients [6,7].

The fungus tropism for the central nervous system (CNS) is due to the high concentration in the cerebrospinal fluid of important nutrients for fungal growth as thiamine, glutamic

acid, glutamine, carbohydrates and minerals [8]. Moreover, the lack of activity of the complement system and weak or absent in brain tissue inflammatory response, enable persistence of fungal cells in the meninges [9,10].

*Cryptococcus* (*Filobasidiella*) *neoformans* is an encapsulated basidiomycete, ubiquitous in the environment and can be found in soil, trees and bird droppings, because its cells are highly resistant to desiccation [11,12]. The species was divided into two varieties: *Cryptococcus neoformans VAR. neoformans*, with serotypes A, D and AD; *Cryptococcus neoformans var gatii*, with serotypes B and C [13]. A new variety, *Cryptococcus neoformans* VAR. *grubii*, was proposed by Franzot and colleagues [14], with the reclassification of serotype due to molecular and epidemiological differences between serotypes A and D.

The variety neoformans has a worldwide distribution associated with soil contamination by bird droppings and generally is responsible for cryptococcosis in immunocompromised patients, particularly in individuals infected with HIV [11,15-17]. *Cryptococcus neoformans* VAR. *gattii* is geographically restricted to the tropical and subtropical climate, commonly found in association with trees species.

*Eucalyptos camaldulensis* and *E. tereticornis*, being associated with cryptococcosis in immunocompetent individuals [18-20].

## Neurocryptococcosis diagnosis

Laboratory diagnosis of neurocryptococcosis is based on three fundamental methodologies: fresh examination, which consists of yeast observation in clinical material stained with India ink; fungus isolation in artificial culture medium and biochemical tests for identification and search for circulating antigens [13]. Fungus detection is performed in biological samples such as cerebrospinal fluid (CSF), bronchial lavage, secretion of mucocutaneous injuries, urine, macerated tissue obtained by biopsy and bone marrow aspiration [5].

The India ink applied to direct testing of *C. neoformans* in clinical material provides a differential, faster and cheaper diagnosis, and the analytical sensitivity of the method range from 1000 to 10,000 cells per mL. In cases of HIV patients, which are observed a high burden of yeast, test sensitivity reaches 80% of positive cases of neurocryptococcosis. On the other hand, in immunocompetent patients this percentage can drops to 30-50% [11]. In addiction, this method relies heavily on good technical operator training [21].

Various artificial culture media may be used for *C. neoformans* growing, such as Agar Sabouraud, Agar niger, Agar L-dopa or dopamine [22]. A positive urine culture may be indicative of disseminated cryptococcosis, even in the absence of clinical signs involving the urinary tract, especially in patients with severe immunodeficiency [23,24]. The cultivation of CSF samples on artificial medium is time consuming, requiring at least 4 days to detect *C. neoformans* positive cultures and, in some instances, the liquor may result in negative cases due to the small load of viable micro-organisms, making it a problem in the monitoring of chronic patients [25].

Serological tests can be used to aid in the diagnosis of cryptococcosis, such as agglutination tests with latex particles, to search polysaccharide antigen of this fungus [26]. ELISA (Enzyme Linked Immunosorbent Assay) for detection of *C. neoformans* antigens has been described by Casadevall et al. [27] and Mukjerjee & Casadevall [28]. This test has many advantages, including a clear discrimination between positive and negative results, quantitative and highly sensitive information [29]. The biological materials used for testing are cerebrospinal fluid, serum or urine [11].

High antigen securities generally correlate with disease severity and likewise a decrease corresponds to a good prognosis. False-positive reactions may occur related to rheumatoid factor, diseases caused by *Trichosporon beigelii* and Gram-negative bacilli [30-34].

The molecular methods such as polymerase chain reaction (PCR) represent an excellent alternative for early cryptococcosis diagnosis in comparison with the conventional methods, since it is able to detect lower fungal burden and can be used in small quantities of biological samples without cultivation [35-41].

Ribosomal subunit genes (rDNA) have been exploited for fungal molecular taxonomy and identification, due to the high degree of variability of its nucleotide sequences, which allow distinguishing between genus, species and varieties of fungi [42]. Thus, this study was conducted in order to test the PCR technique application to detect *Cryptococcus* spp. regions of rDNA in CSF samples and, therefore applied to the diagnosis of cryptococcal meningitis. Once developed, this technique can be used routinely in clinical laboratories, aiding in the diagnosis and cure of disease monitoring.

# Materials and Methods

## Extraction of genomic DNA

In order to evaluate the efficacy of fungal DNA extraction in cerebrospinal fluid (CSF) samples were compared two different methods of DNA extraction. For both methods, were used 10 CSF samples from positive neurocryptococcosis patients, confirmed by fresh examination stained with India ink and/or by culture findings and/or by Latex test. The patients were examined in Eduardo de Menezes Hospital, Belo Horizonte, Brazil and the CSF samples were kindly provided by the laboratory CSF collection.

The first DNA extraction protocol used glass beads to break the fungus cell wall. For this method, were added 100 mg of sterile glass beads in a sterile 2.0 mL microtube containing 500 µL of Lysis I sterile buffer (3M MW=342.3 Sucrose, 10 mM Tris-HCl $p^H$ 7.5, 5 mM $MgCl_2$, Triton X-100 1%) and 500 µL of each CSF sample. The suspension was stirred vigorously on magnetic stirrer (3 pulses of 30 seconds). Was added 10 µL of Proteinase K 20 mg/mL, gently homogenizing the tube and incubating for 30 minutes at 65°C. After incubation, was added

to 250 µL of sterile Lysis II buffer (0.075 M NaCl, 0.024 M Na-EDTA pH 8.0 and sterile milli-Q water qs 100 ml) and 5µL of sodium dodecyl sulfate (SDS) at 20%, stirring in vortex for 30 seconds. After that, was added 15 mL of 6 M Sodium Chloride (NaCl), stirring again in vortex for 30 seconds and centrifuged for 15 minutes 5,000 g. The resulting supernatant was recovered into a new sterile 1.5 mL microtube and added to an equal volume of cold absolute isopropanol, homogenized and waited for 15 minutes until the total precipitation of DNA. After precipitation of the DNA, the microtube was centrifuged for 5 minutes at 14,000 g and the supernatant was discarded. The precipitated DNA was washed three times with 1 ml of 75% Ethanol solution. After each wash the microtube was centrifuged at 14,000 g for 1 minute. The final supernatant was discarded, leaving the microtube inverted on paper filter for dry at room temperature until total evaporation of organic residues. After, was added 100 µL of sterile milli-Q water and this was incubated for 10 minutes at 65°C thermo block for hydrating DNA.

The second DNA extraction protocol was used the bromide reagent cetyltrimethylammonium (CTAB) [43], without using glass beads. The DNA extraction using organic solvent CTAB is very applied in plant tissues, biological materials, blood, body tissue, muscle tissues, fungi and bacteria, being an inexpensive method and easy to apply in laboratories [44,45]. In this protocol were used the same procedures as described in the first protocol; however, were not added glass beads in Lysis I sterile buffer and added 700 uL CTAB (20 ml Tris-ClPh 7.5, 20 mL 41% NaCl, 20 ml 0,5M EDTA; 20 mL 10% CTAB and sterile milli-Q water qs 100 mL) to each CSF sample.

Following the procedures, DNA samples was measured by absorbance at 260/280 nm, using the equipment NanoVue Plus–GE (USA).

## Conventional PCR

To standardize the PCR reactions a culture of *C. neoformans* (ATCC 24067) was used as Positive control. The pair of primers CN4 Forward (5'-ATC ACC CTA CCA TTC ACA CATT-3 ') and CN5 Reverse (5'-GAA GGG CAT GCC AGA TGT TTG-3') was chosen [46]. It was used Blastn program (blast.ncbi.hlm.nih.gov/blast.cgi) to assess the specificity in silico of these primers to *Cryptococcus* genus.

The PCR reaction was performed in final volume of 15 µL, containing sterile milli-Q water qs, 3.0 mM $MgCl_2$ (Ludwig BIOTEC, Brazil), 0.2 mM dNTPs (Ludwig BIOTEC, Brazil), 0.25 mM of each CN4-CN5 initiator, 1 U *Taq* DNA polymerase and PCR reaction buffer (Ludwig BIOTEC, Brazil, 47.0 ng/µL DNA template. PCR reactions were performed in Thermal Cycler thermocycler Verite - Applied Biosystems (USA), by means of the initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds for denaturation, 62°C for 30 seconds for annealing, 72°C for 30 seconds for extension. It also included a final extension cycle at 72°C for seven minutes. The amplifications products were visualized on a 7% polyacrylamide gel, stained by Silver Nitrate 0.2%.

## Real-time PCR (q-PCR)

Real time PCR was standardized using the Power Sybr Green Mastermix (Applied Biosystems, Brazil). The reactions were performed in the equipment ECO Real-Time PCR-ILLUMINA (USA).

Standardized q-PCR was performed following the steps recommended in the (MIQE) Johnson et al. [47] as described below:

I- Array of primers was performed by varying the concentration of CN4 and CN5 primers of 0,3 µM, 0,15 µM and 0.25 uM. We used the DNA sample *C. neoformans* ATCC 24067 (47.0 ng/uL); 1X mastermix Power Sybr Green and sterile ultrapure water (qs 10µL). All concentrations were tested in triplicate. The run was performed as absolute quantification, following the pattern of cycling: 10 minutes at 95°C; 40 cycles of 95°C-10 seconds and 62°C-30 seconds; followed by Melt curve: 95°C-15 seconds, 55°C-15 seconds and 95°C-15 seconds. At the end, the reaction data were analyzed in the equipment program-Eco-software version 4.0. The parameters to determine the best primer concentrations were higher ΔRN by considering the final cycle of amplification in a smaller Cq with a lower standard deviation of the triplicates.

II- q-PCR reaction used 1X Sybr Green Mastermix Power, 0.15 mM each primer and sterile ultrapure water. The cycling conditions were 95°C-10 minutes, 40 cycles of 95°C-10 seconds and 62°C-30 seconds, followed by Melt curve: 95°C-15 seconds, 55°C-15 seconds and 95°C-15 seconds. To confirm the amplification of the expected 136 base pairs fragment, the final reaction product was visualized on a 7% polyacrylamide gel, stained by Silver Nitrate 2%.

## Determination of detection limit and PCR efficiency curve

To study the detection limit of PCR were used genomic DNA extracted from *C. neoformans* ATCC-24067 using Protocol 1, which did not use CTAB. From initial concentration of 47.0 ng/µL, a serial dilution was made starting at 1:10 in a final volume 50 µL (5 DNA in 45 µL sterile ultrapure water). From the first 1:10 dilution were carried out serial dilutions (10 -2, 10 -3, 10 -4, 10 -5, 10 -6, 10-7 ng/µL). Efficiency curve was analyzed following MIQE parameters, which evaluates the slope, the R2 and efficiency parameters. Ideal slope was between -3.2 and -3.5, R2>0.98 and efficiency of 90 to 105%. The detection limit is the lowest detectable DNA mass.

## Specificity *in vitro* test

To confirm *in vitro* specificity of primers, the PCR reaction was performed using genomes extracted from another pathogenic fungi: *Candida albicans*-ATCC 52A and ATCC 18804, *Candida krusei*- ATCC 6258, *Candida glabrata*- ATCC Y59, *Paracoccidioides brasiliensis*- Pb18 and *P. lutzii*- Pb01. Samples of *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Sporothrix* sp. and *Histoplasma capsulatum*, belong to the collection of Mycology Laboratory of IEP/Santa Casa de Belo Horizonte Hospital. For this reaction primer concentrations and cycling conditions

were the same as in previous tests. The analysis was based on samples amplification or not, in any cycle, even in the final. The analysis method was recommended by MIQE in assays for pathogen detection - End Point analysis or presence/absence [47].

### Confirming q-PCR amplifications in CSF samples

After standardization, q-PCR was tested in 100 CSF samples, also provided by laboratory CSF collection from Eduardo de Menezes Hospital, Belo Horizonte, Brazil. Among these samples, 56 were from positive neurocryptococcosis patients, confirmed by fresh examination stained with India ink and/or by culture findings and/or by Latex test. The remaining 44 samples were from negative neurocryptococcosis patients, according to above mentioned tests.

## Results and Discussion

### Comparison of different DNA extraction methods in CSF samples

In the first DNA extraction in CSF samples protocol, which was used glass beads to break the fungi wall, the average of 10 DNA extracted measurements was 11.3 ng/µL and in the second protocol, which was used CTAB to break the fungi wall, the average of 10 DNA extracted measurements was 9.9 ng/µL. There was no significant difference between extractions methods, being P=0.545 (P>0.05), calculation was performed by Mann Whitney nonparametric method in the program Graphpad Prism 5 (Figure 1).

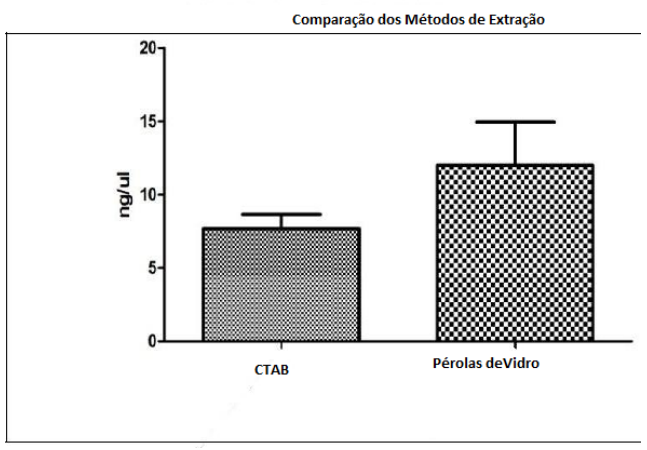



**Figure 1:** Average of DNA extracted measurements using glass beads and using only CTAB to lyse cell wall of the fungus

The results suggest that for DNA extraction in CSF samples it is not necessary to use CTAB to lyse cell wall of the fungus. The CTAB is a detergent commonly used for cell wall lysis, but their residues may inhibit the q-PCR, which is highly sensitive and requires a high degree of purity of DNA [45,48]. As demonstrated by Tanaka et al. [38], glass beads promote mechanical destruction of *C. neoformans* capsule, being a suitable DNA extraction process and furthermore, not generate chemical waste, being a safer alternative to working with sensitive techniques such as q-PCR.

### Primers selection and research *in silico* and *in vitro*

The selected pair of primers showed no complementarity with human genome and other pathogenic fungi, according to *in silico* analysis performed in Blastn software.

Primers were aligned with genomic sequences of the *Cryptococcus* species. The primers CN4-CN5 flanking ITSrDNA region, generating a fragment of 136 bp, binding in genoma of the three varieties *Cryptococcus neoformans* VAR. *grubii*, *Cryptococcus neoformans* VAR. gatti and VAR. neoformans (data not show). Despite this primer pair does not differentiate between *Cryptococcus* varieties, it can be an important tool for rapid diagnosis, enabling the planning of treatment or cure control, already suggested by other studies [49].

According to *in vitro* results for q-PCR, CN4-CN5 primers showed 100% specificity since there was no amplification of any genome of tested species, demonstrating that it can be used in *Cryptococcus* genomic DNA detection. These data are confirmed by the research of other authors [35,46,50].

### Detection limit of the PCR techniques

According to the results obtained from this study, conventional PCR showed a detection limit of 0.0047 ng/µL, whereas the q-PCR presented a detection limit of 0,000047 ng/µL (Figure 2). Thus, q-PCR detection limit is 100 times less than that of conventional PCR, demonstrating the high sensitivity of q-PCR. This result indicates that q-PCR can be very useful to control cure once fungal load tends to decrease with treatment of the patient and also, can be used in diagnostic in immunocompetent patients, who typically have low fungal load, according describe by Casadevall & Perfect [11].

Moreover, the technique has a high sensitivity, since the 56 samples confirmed positive by routine laboratory tests were all positive in q-PCR (Figure 3 & 4). Among 44 negative samples, 8 (18.18%) were positive on q-PCR. Several factors must be considered when analyzing samples that were positive on q-PCR and negative in routine of Hospital laboratory, among them the low fungal burden in the CSF, which can hamper the growth of fungus in culture or its viewing in fresh examinations [11]. It may also be that DNA is detected from fungal cells which are not viable and remained in liquor after treatment, since these samples were from patients who were under treatment. We have no treatment date about these neurocryptococcosis negative patients.

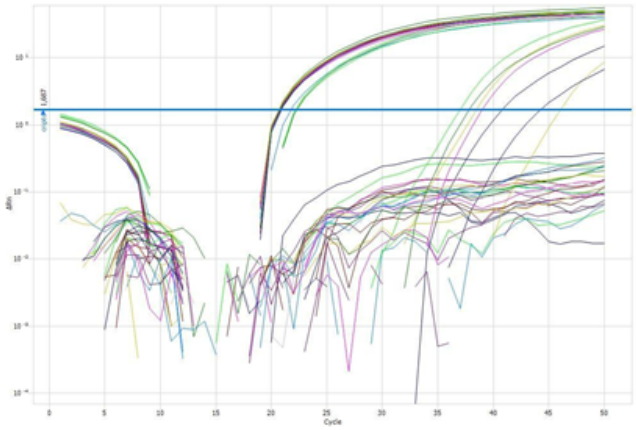
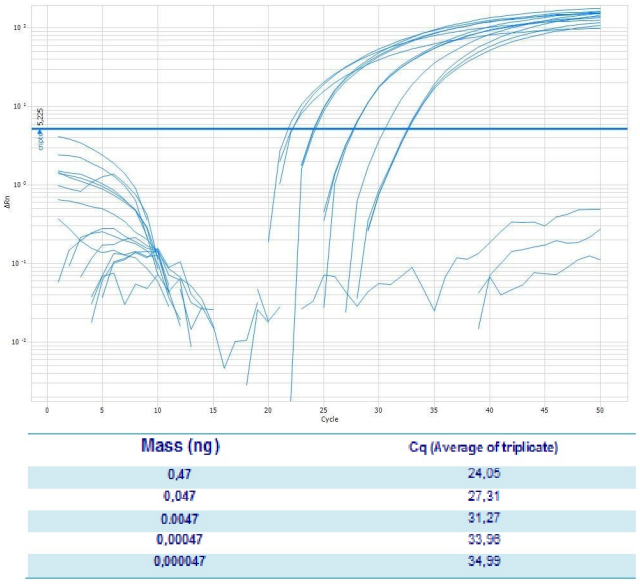


**Figure 3:** Amplification graph curve above cutting line are positive CSF samples and below cutting line negative samples. In all q-PCR reactions were used positive controls ATCC *Cryptococcus neoformans* and negative control NTC (No template control).



| Mass (ng) | Cq (Average of triplicate) |
|---|---|
| 0,47 | 24,05 |
| 0,047 | 27,31 |
| 0,0047 | 31,27 |
| 0,00047 | 33,98 |
| 0,000047 | 34,99 |

**Figure 2:** Efficiency curve of q-PCR in accordance with different dilutions of DNA extracted from CSF samples.

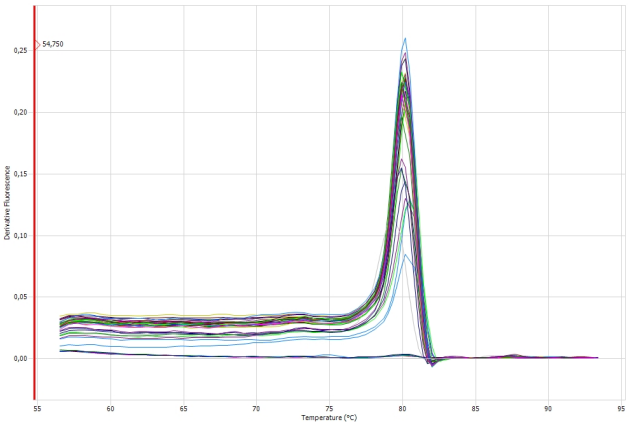


**Figure 4:** Melting curve of CSF samples, showing peak of denaturation at 80 ° C and negative samples without linear peak at point 0.

These results open perspectives for q-PCR use in neurocryptococcosis´s diagnosis. Some studies have demonstrated the q-PCR sensitivity and specificity for genome of fungi detection in biological samples as cerebrospinal fluid, serum and urine [21,36,50]. PCR is an important tool to be used in cryptococcosis diagnosis as it has greater sensitivity than culture, and fresh examination stained with India ink.

In a study conducted by Paschoal et al. [35], PCR was compared with two other *Cryptococcus* detection techniques, and was shown that although the fresh examination is a faster technique, the sensitivity is only 85.7%. The culture had a sensitivity of 70.8%, while the PCR showed 92.9% sensitivity. The specificity was 100% for all three techniques. In another important study, Leal et al. [49], have standardized a method of multiplex PCR that allows differentiation between *C. neoformans* var. *neoformans* and var. *Gatti*, without samples cultivation, making diagnosis more secure and agile.

In addition, PCR is an improvement on the serology once it detects the pathogen genome in cerebrospinal fluid where it is not possible to detect circulating antibodies, becoming an important tool for diagnosis of infectious diseases on CNS such as neurocryptococcosis.

Rapid and specific diagnostic tests for fungal infections enable more effective treatment and, therefore decrease the morbidity and mortality of diseases [10]. With the advancement of molecular biology has been possible to develop new techniques for diagnosis and serotype identification of *Cryptococcus* sp, enabling the identification of serotypes from biological materials without necessarily cultivating fungus [50]. In addition to the speed on detection of fungos´s genome, PCR have shown reduced costs of diagnoses each year, making it one of the most cost tools / benefit [21,35,36].

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