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## Original article

**Molecular diagnosis of cryptococcal meningitis in cerebrospinal fluid: comparison of primer sets for *Cryptococcus neoformans* and *Cryptococcus gattii* species complex**Marilena dos Anjos Martins<sup>a,b</sup>, Kate Bastos Santos Brighente<sup>a</sup>,  
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## ARTICLE INFO

## Article history:

Received 13 August 2014

Accepted 19 September 2014

Available online 16 December 2014

## Keywords:

Cryptococcal meningitis  
Acquired immunodeficiency  
syndrome  
Polymerase chain reaction  
Cerebrospinal fluid

## ABSTRACT

**Aim:** This study evaluated the use of polymerase chain reaction for cryptococcal meningitis diagnosis in clinical samples.**Materials and methods:** The sensitivity and specificity of the methodology were evaluated using eight *Cryptococcus neoformans*/*C. gattii* species complex reference strains and 165 cerebrospinal fluid samples from patients with neurological diseases divided into two groups: 96 patients with cryptococcal meningitis and AIDS; and 69 patients with other neurological opportunistic diseases (CRL/AIDS). Two primer sets were tested (CN4–CN5 and the multiplex CNa70S–CNa70A/CNb49S–CNb-49A that amplify a specific product for *C. neoformans* and another for *C. gattii*).**Results:** CN4–CN5 primer set was positive in all *Cryptococcus* standard strains and in 94.8% in DNA samples from cryptococcal meningitis and AIDS group. With the multiplex, no 448-bp product of *C. gattii* was observed in the clinical samples of either group. The 695 bp products of *C. neoformans* were observed only in 64.6% of the cryptococcal meningitis and AIDS group. This primer set was negative for two standard strains. The specificity based on the negative samples from the CTL/AIDS group was 98.5% in both primer sets.**Conclusions:** These data suggest that the CN4/CN5 primer set was highly sensitive for the identification of *C. neoformans*/*C. gattii* species complex in cerebrospinal fluid samples from patients with clinical suspicion of cryptococcal meningitis.© 2014 Elsevier Editora Ltda. Este é um artigo Open Access sob a licença de [CC BY-NC-ND](http://creativecommons.org/licenses/by-nc-nd/4.0/)

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## Introduction

Cryptococcal meningitis is one of the most serious systemic mycoses, mainly affecting immunocompromised patients and causing high morbidity and mortality. The course of infection is often a life-threatening disease that is typically observed in later stages of acquired immunodeficiency syndrome (AIDS).<sup>1-4</sup>

Cryptococcal meningitis affects approximately one million persons in the world each year and results in more than 400,000 deaths within three months after the onset of the disease.<sup>5</sup> Latin America, as a global region, has the third highest incidence of the disease, with around 54,400 cases annually.<sup>6</sup> Fortunately, in recent years, AIDS-associated cryptococcal meningitis has decreased dramatically in Brazil, which is a middle-income country with universal access to antiretroviral therapy.<sup>4,7</sup> However, until today, cryptococcal meningitis remains a common complication in Brazil and other Latin American countries as it represents the primary cause of opportunistic meningitis and the second-most frequent neurologic opportunistic infection in HIV-infected patients.<sup>6,7</sup>

Patients with cryptococcal meningitis usually present severe neurological manifestations, particularly secondary to intracranial hypertension.<sup>7-9</sup> A rapid laboratorial diagnosis is extremely important to identify *Cryptococcus* species.<sup>10</sup> Routinely, microscopic examination, cryptococcal polysaccharide antigen, and culture of cerebrospinal fluid (CSF) samples lead to the diagnosis.

Several studies have demonstrated the use of molecular methods for determining the genetic characteristics of *Cryptococcus* species.<sup>11-15</sup> Nevertheless, there is scarce information about the use of molecular diagnosis in clinical samples. Molecular methods such as polymerase chain reaction (PCR) potentially identify the fungus and the genotype of the strain.

Considering that no method is entirely effective in determining the presence of this pathogen, it is helpful to diagnose on the basis of results obtained from different methodologies to rule out false-negative or false-positive results. Hence, molecular methods such as PCR have proven to be useful for this purpose, since they can provide a definitive diagnosis as well as evaluation of specific treatment.

The aim of this study is to evaluate the use of PCR for molecular diagnosis of cryptococcal meningitis.

## Materials and methods

The sensitivity and specificity of the methodology were evaluated using *Cryptococcus* reference strains in the first part and CSF samples from patients with neurological diseases in the second part of the study.

### Patients and clinical samples

The assays were evaluated analyzing 165 CSF samples with commonly used, well-known laboratory and clinical diagnoses. The clinical samples were divided into two

groups. Group cryptococcal meningitis and AIDS (CM/AIDS) consisted of 96 CSF samples from patients with CM/AIDS. Cases of cryptococcal meningitis were confirmed using positive microscopic examination by India ink and latex agglutination of CSF samples.<sup>16</sup> All patients were admitted to the Instituto de Infectologia Emilio Ribas, Sao Paulo, SP, Brazil. The CRL/AIDS group (control) consisted of 69 CSF samples from patients with confirmed diagnosis (by clinical, radiological and laboratory features) of other neurological opportunistic infections including cerebral toxoplasmosis, progressive multifocal leukoencephalopathy, HIV-associated neurocognitive disorders, tuberculous meningitis, and neurosyphilis.

### Ethical considerations

All patients provided written informed consent. The institutional review board of both Instituto Adolfo Lutz and Instituto de Infectologia Emilio Ribas committees approved this study.

### Laboratory diagnosis

At the Instituto de Infectologia Emilio Ribas the CSF samples were collected in aseptic conditions, immediately transferred to sterile tubes, sent to laboratory and processed. Each sample was split in two tubes. One of them was used for microscopic examination by India ink and latex agglutination. The other was sent to the Instituto Adolfo Lutz for molecular diagnosis. Microscopic examinations by India ink was conducted as previously described.<sup>17,18</sup> CSF samples were centrifuged at  $3000 \times g$  for 10 min. One drop of the pellet was used for microscopic observation after India ink preparation. The polysaccharide capsule antigen was detected in CSF samples using a latex agglutination Kit (*Cryptococcus* Antigen Latex Agglutination Test-Immy) according to the manufacturer's instructions.

### Yeast preparation

The assays were also determined using DNA extracted from standard strains representing each molecular type including: WM 148 (serotype A, VNI), WM 626 (serotype A, VNII), WM 628 (serotype AD, VNIII), WM 629 (serotype D, VNIV), WM 179 (serotype B, VGI), WM 178 (serotype B, VGII), WM 175 (serotype B, VGIII), and WM 779 (serotype C, VGIV).<sup>12</sup> The strains were prepared as previously described.<sup>12</sup> Briefly, each isolate was plated on Sabouraud dextrose agar and incubated at 30 °C for 48 h. The yeast cells were transferred to a microcentrifuge tube containing 1 mL of 50 mM EDTA (Sigma Chemical), pH 8.0, mixed, and centrifuged at  $13,000 \times g$  for 15 min. The supernatant was removed, and the pellet was resuspended in 200  $\mu$ L of 50 mM EDTA, pH 8.0, containing 40  $\mu$ L of lysing enzyme from *Trichoderma harzianum* (10 mg/mL) (Sigma Chemical), and then incubated at 37 °C for 2 h. Subsequently, this mixture was centrifuged at  $13,000 \times g$  for 10 min, and the pellet was dissolved in lysis buffer containing 10 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 0.5% sodium dodecyl sulfate; 0.01% N-laurilsarcosyl; and 100 mg/mL proteinase K. The mixture was vortex mixed and incubated at 56 °C for 2 h.

### DNA purification and PCR

DNA molecules of protoplasts or CSF samples were extracted by QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. CSF samples were previously centrifuged for 15 min at 9000 × g. Packed cells were washed twice in PBS to prevent the action of any Taq polymerase inhibitor. Whole cells were incubated for 15 min at 100 °C in 200 µL of ultra-pure water containing four to five 1.5 mm glass beads. Next, the cells were processed in a TissueLyser disruptor (Qiagen) for two cycles of 3 min. After DNA extraction, the purity was determined by the ratio of O.D. at 260 and 280 nm in a NanoDrop ND100 (Thermo Fisher Scientific, Waltham, MA, US).

The amplifications were carried out with a kit purchased from Promega (Go Taq Green Master Mix). The PCR mix (12.5 µL) consisted of 1 unit of Green GoTaq® DNA polymerase, 10 mM Tris-HCl, pH 8.5; 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; and 200 mM of each dNTP. The reactions included the PCR mix, 10 µL of each DNA template and 25 pmol of each primer to a final volume of 25 µL. The amplifications were performed in an automated thermal cycler and each run contained one negative control (ultra-pure water) and one positive (DNA extracted from serotype A, VNI culture). After thermal cycles, PCR products were electrophoresed in 2% agarose gels in TBE buffer and stained with ethidium bromide. The size of fragment was based on a comparison with a 100-bp ladder.

### Primer set selection

The experiments were carried out by using two primer sets. The first set was the CN4–CN5 (5'ATCACCTTCCCACTAACACATT3' and 5'GAAGGGCATGCCTGTTTGAGAG3'), which amplified a 136-bp sequence from a specific gene coding region for rDNA of *C. neoformans* as target.<sup>15</sup> The amplifications were performed by one initial denaturation cycle for 5 min at 94 °C, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The procedure was completed by a final cycle extension for 10 min. The second was a multiplex PCR. The CNa70S–CNa70A (5'ATTGCGTCCACCAAGGAGCTC3' and 5'ATTGCGTCCATGTTACGTGGC3') amplified a 695-bp fragment of a region on *C. neoformans* chromosome 3 that includes the coding sequence of a putative aminotransferase gene. The CNb49S–CNb-49A (5'ATTGCGTCCATCCAAGGTGTTGTTG3' and

5'ATTGCGTCCATCCAACCGTTATC3') amplified a 448-bp fragment of a region on *C. gattii* chromosome 2, which includes the coding sequence of a putative polymerase.<sup>14,19</sup> The PCR products were amplified by one initial denaturation cycle for 5 min at 94 °C, 35 cycles of denaturation at 94 °C for 45 s, annealing at 65 °C for 60 s, and extension at 72 °C for 60 s. The procedure was completed by a final cycle extension for 10 min. To control the course of extraction and check for PCR inhibitors, all samples were assayed using the primer set β1–β2 (5'ACCACCAACTTCATCCACGTTACCC3' and 5'CTTCTGACACAACCTGTGTTCTACTAGC3'),<sup>20</sup> which amplified a 140-bp fragment of the human β-globulin gene. The reactions using the human primer set were run simultaneously with the same temperature protocol for the CN4–CN5 primer set and in the same PCR machine.

### Data analysis and quality assurance

Separate rooms were used for: (1) DNA extraction; (2) PCR mix and primer preparation; (3) adding DNA from clinical samples and positive control; and (4) post-PCR agarose gel electrophoresis analysis. DNA clinical samples were assayed in duplicate and, at least, twice. Clinical and laboratory (microscopic examination by India ink and latex agglutination) diagnoses were the gold-standard to establish sensitivity, specificity, positive (PPV) and negative (NPV) predictive values, which were calculated as: (i) sensitivity – ratio of true positives/(true positives + false negatives) × 100; (ii) specificity – ratio of true negatives/(true negatives + false positives) × 100; (iii) PPV – ratio of true positives/(true positives + false positives); and (iv) NPV – ratio of true negatives/(true negatives + false negatives).

## Results

Two PCR primer sets to determine *C. neoformans*/*C. gattii* species complex in clinical samples were evaluated since they amplified different genetic regions. Initially the primer sets were evaluated for sensitivity using the standard strains. CN4–CN5 primer set amplified the 136-bp product in all standard strains tested. The same DNA samples were also tested for CNa70S–CNa70A/CNb49S–CNb-49A multiplex. Four standard strains (serotypes A-VNI and VNII, serotype AD-VNIII, serotype D-VNIV) amplified the 695-bp fragment; and

**Table 1 – Evaluation of primer sets CN4–CN5 and CNa70S–CNa70A/CNb49S–CNb-49A in PCR for *Cryptococcus* standard strains.**

Standard strains	Primer sets	
	CN4–CN5	CNa70S–CNa70A/CNb49S–CNb-49A
WM 148 (serotype A, VNI)	Positive	Positive
WM 626 (serotype A, VNII)	Positive	Positive
WM 628 (serotype AD, VNIII)	Positive	Positive
WM 629 (serotype D, VNIV)	Positive	Positive
WM 179 (serotype B, VGI)	Positive	Negative
WM 178 (serotype B, VGII)	Positive	Positive
WM 175 (serotype B, VGIII)	Positive	Negative
WM 779 (serotype C, VGIV)	Positive	Positive

**Table 2 – Summary of the results using CN4–CN5 and CNa70S–CNa70A/CNb49S–CNb-49A primer sets in PCR using CSF samples from AIDS patients.**

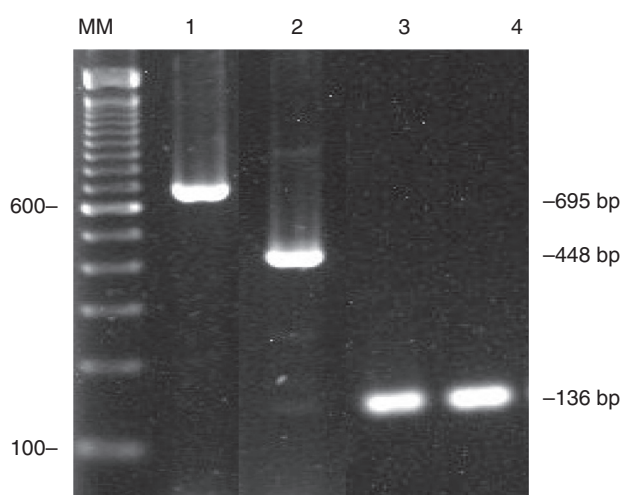
Primer sets	PPV <sup>a</sup>	NPV <sup>b</sup>	PCR results Number of samples (%)					
			Cryptococcal meningitis			Other neurological diseases		
			Positive <sup>c</sup>	Negative	Total	Positive	Negative <sup>d</sup>	Total
CN4–CN5	0.99	0.93	91 (94.8%)	5	96	1	68 (98.5%)	69
CNa70S–CNa70A	0.99	0.53	62 (64.6%)	34	96	1	68 (98.5%)	69

<sup>a</sup> Positive predictive value (PPV).

<sup>b</sup> Negative predictive value (NPV).

<sup>c</sup> Sensitivity (in percent) was calculated and determined considering the clinical and laboratory diagnosis described in Materials and methods section.

<sup>d</sup> Specificity (in percent) was calculated and determined considering the clinical and laboratory diagnosis described in Materials and methods section.



**Fig. 1 – Amplified PCR products of two different target regions of *Cryptococcus neoformans*/*C. gattii* species complex. CNa70S–CNa70A/CNb49S–CNb-49A multiplex amplified a 695-bp product of *C. neoformans* (line 1) and 448-bp of *C. gattii* (line 2). CN4–CN5 primer set, amplified a 136-bp product (lines 3 and 4). The DNA fragments were resolved in 2% agarose gels stained with ethidium bromide. Lane MM, 100-bp ladder.**

two (serotype B-VGII and serotype C-VGIV) amplified the 448-bp fragment. No amplified product was shown in two standard strains (serotypes B-VGI and VGIII) (Table 1). These experiments were conducted three times and, as an illustration, Fig. 1 shows the amplified PCR products of both primer sets used in this study. Both did not amplify other yeasts such as *Candida albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*.

The good quality of all 165 DNA samples was confirmed by the amplification of  $\beta$ 1– $\beta$ 2 primer set that amplified a PCR fragment of human  $\beta$ -globulin gene of 140-bp. Positive amplifications showed that no substance present in DNA samples inhibited the reactions.

Next, the sensitivity and specificity of the CN4/CN5 and the CNa70S–CNa70A/CNb49S–CNb-49A multiplex were determined in DNA samples extracted from CSF samples of both groups of patients. Table 2 shows the results of each group in

detail. The analysis of the clinical samples from the CM/AIDS group revealed that 94.8% of these samples were positive (91 out of 96) using the CN4/CN5 primer set. The specificity of the assay, based on the analysis of all 69 negative samples from CTL/AIDS group was 98.5%.

According to the multiplex CNa70S–CNa70A/CNb49S–CNb-49A, no 448-bp product of *C. gattii* (CNb49S–CNb-49A) was shown in the clinical samples of either group. The 695-bp products of *C. neoformans* were shown in only 64.6% (62 out of 96) of the CM/AIDS group. In CTL/AIDS, the specificity was also 98.5%. Only one sample out of 69 had a false-positive result. Although the specificity was good, the sensitivity was very low as well as the NPV (0.53).

## Discussion

Despite the observed decline of cryptococcal meningitis in AIDS patients, its occurrence still represents a determinant of poor prognosis in HIV-infected patients.<sup>6</sup> Additionally, this fungal infection in the central nervous system leads to severe neurological complications. In this way, its successful treatment depends on rapid and accurate identification of the causative agent.<sup>16</sup> Consequently, accurate and rapid diagnosis is critical. In recent years, great advances have been made in developing laboratory techniques to diagnose infectious diseases. Nevertheless, despite the well-established diagnosis of cryptococcal meningitis some aspects of the diagnosis remain challenging and there are still gaps that need to be addressed. For instance, a negative result from an *in vitro* culture might be due to the presence of nonviable or fastidious yeast in the sample. Thus, the use of PCR in CSF samples associated with methods already used at the laboratory could increase the diagnostic efficacy.

Several studies based on molecular methods have shown that *C. neoformans* isolates exhibit different degrees of genetic heterogeneity among clinical and environmental isolates. The primer sets used in these *Cryptococcus* studies were normally used for genotyping characterization.<sup>11,21–25</sup> However, there have been few studies evaluating the efficacy of such methodology in molecular diagnosis. Here, we chose two previously described primers' set widely used for *Cryptococcus* genotyping. Two primer sets were tested as they amplify different

genetic regions. The CN4–CN5 primer set amplified a 136-bp sequence from a specific region of gene coding for rDNA as target.<sup>15</sup>

The sensitivity, accuracy and specificity of a diagnostic test depend also on optimal working conditions. In order to improve the PCR sensitivity, the CSF samples should be processed rapidly within 48 h of collection to prevent Taq polymerase inhibition that could modify PCR results.<sup>26</sup> In addition, the DNA extraction and PCR inhibitors may be evaluated by a primer set that amplified human PCR fragments. In this study, positive amplifications showed that no substance present in DNA samples inhibited the reactions.

Firstly, the efficacy of both primer sets was analyzed using *Cryptococcus* standard strains and then the clinical samples. The CN4–CN5 primer set was highly sensitive since it amplified all *Cryptococcus* standard strains used in this study. These data were further confirmed using 96 clinical samples from the CM/AIDS group that revealed good sensitivity (94.8%) and negative predictive value of 0.93. Five cases of the CM/AIDS group were false negatives. The PCR results could be due to low or non-uniform distribution of specimens from which the DNA was extracted. Furthermore, the specificity was also good (98.5%) with high positive predictive value (0.99). Only one DNA sample from CLR/AIDS had a false-positive result. Similar results have been reported previously.<sup>27</sup>

The use in molecular diagnosis of a multiplex primer set could be an excellent alternative, since in the same reaction it could be possible to detect the organism and, at the same time, genotype the infecting strain. For this purpose, the CNa70S–CNa70A/CNb49S–CNb-49A multiplex was tested in this study. According to previous studies, 695-bp PCR products were shown in *C. neoformans* strains and a 448-bp fragment of *C. gattii*.<sup>14,19</sup> In this study, the PCR using the (CNa70S–CNa70A/CNb49S–CNb-49A) multiplex was unable to amplify two *Cryptococcus* standard strains (serotypes B-VGI and VGIII). In addition, no 448-bp product of *C. gattii* was shown in the clinical samples of either group. These data could suggest that the majority of the DNA samples from CM/AIDS had yeast belonging to *C. neoformans*. However, the 695-bp products of *C. neoformans* were shown only in 64.6% of the CM/AIDS group resulting in low sensitivity with very low NPV (0.53). In the CM/AIDS group, 34 DNA samples had false-negative results.

Collectively these data suggest that although the multiplex primer set could detect the organism and, at the same time, genotype the infecting strain, the sensitivity was too low. On the other hand, the primer set CN4/CN5 was highly sensitive for *C. neoformans/C. gattii* species complex determination in CSF samples from patients with clinical suspicion of cryptococcal meningitis or the treatment control.

### Conflicts of interest

The authors declare no conflicts of interest

### Acknowledgments

This study was supported by grants from: (i) FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil), Proc: 2011/13939-8, (ii) CNPq (Conselho Nacional de

Desenvolvimento Científico e Tecnológico, Brazil), Proc: 303489/2012-0. Jim Hesson of AcademicEnglishSolutions.com revised the English.

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