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DETECTION OF *CRYPTOCOCCUS NEOFORMANS* BY SEMI-NESTED PCR IN CEREBROSPINAL FLUID

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ABSTRACT

Life-threatening infections caused by the encapsulated fungal pathogen *Cryptococcus neoformans* have been increasing steadily over the past 10 years. *Cryptococcus neoformans* is recognized as the most frequent fungal infection of the central nervous system (CNS) in immunocompetent as well as immunocompromised patients. We report the development of a semi-nested-PCR-based assay for the detection of C. *neoformans* in less than 100 yeast cells per ml of cerebrospinal fluid (CSF).
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INTRODUCTION

Cryptococcus neoformans is an encapsulated fungal organism that can cause disease in apparently immunocompetent, as well as immunocompromised, hosts. Most susceptible to infection are patients with T-cell deficiencies.[1,2] *C. neoformans* var. *neoformans* causes most cryptococcal infections in humans.

In the last decades, cryptococcosis has been assuming a prominent role at the public health level due to the growing number of AIDS individual cases.[3,4,5] It is an important opportunist systemic mycosis that involves mainly immunosuppressed individuals and starts when *C. neoformans* penetrates the organism, lodging primarily in the lungs and later presents a notable tropism for the central nervous system.[6,7] Molecular tests for detecting nucleic acids of infectious agents in biological samples have been developed for *C. neoformans.* These can be done in various clinical materials, such as blood, liquor, secretions, cutaneous scrapings, bronchial alveolar aspirate and urine.[8] For the diagnosis of neurocryptococcosis the application of more sensitive and specific laboratorial techniques are necessary in order to introduce early and specific antifungal therapy. PCR offers a good alternative.[9] It constitutes a method of choice for early alternative diagnosis to the conventional ones and contributes to supply important subsidies to the diagnosis of this pathology mainly when there is clinical suspicion of the disease.[10]

Confirmation of suspected clinical disease currently presents a challenge to the clinician, with difficulty in making a diagnosis, frequently delaying treatment.

Semi Nested PCR is, as the name suggests, a nested PCR with just one of the primers internal to the primers used in the first round. This method does not add as much specificity as having both primers internal to the primers in the first round, but for certain applications it will add enough specificity to get a specific PCR product.

The aim of the present work was to design a PCR for specific detection of *C. neoformans* directly in CSF specimens.

MATERIAL AND METHODS

Culture of the yeast cells

To optimize the amplification procedure, we used *Cryptococcus neoformans* cells, strain gatti-mating type α - molecular type VG1. Yeasts were cultured in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C with shaking to an OD600 of 1 (approximately 10^8 cells/ml). We diluted the cells to 10^6, 10^4, 10^2 and 10 cell per ml of CSF[1] (CSF sample was known to be negative for *C.neoformans*).

DNA Extraction

The yeasts of diluted samples were collected by centrifuging at 6000 RPM for 10 min. DNA extraction

[1]cerebrospinal fluid

was carried out by using silica powder and the specific lysis buffer (2% Triton X 100, 1% SDS, 0.1M NaCl, 0.01M Tris pH8, 10mM NaAc, 1mM EDTA, 2% 2-mercapto-ethanol).

After comparison of ITS rRNA sequences of *C. neoformans* with those of members of its most closely related taxa, three primers or probes were designed. The sequences (5`-3`) of these oligonucleotides, designated CN5, and CN6, and other primers used (ITS1) are as follows:

Primers, Two forward (ITS-1 + CN-5) and One Reverse (CN-6)

Round 1:
Forward: ITS-1 (3'-TCCGTAGGTGAACCTGCGG-5')
Reverse: CN-6 (3'-TTTAAGGCGAGCCGACGTCCTT-5')
Round 2:
Forward: CN-5 (3'-GAAGGGCATGCCTGTTTGAGAG-5')
Reverse: CN-6 (3'- TTTAAGGCGAGCCGACGTCCTT-5')

ITS primers are general fungal primers[12]; CN primers are *C. neoformans* specific.

PCR amplification: Semi nested-primers specific for internal transcribed spacer regions of ribosomal DNA of *C. neoformans*[12,13] were used. In the first amplification, we used the primers ITS-1 (3'-TCCGTAGGTGAACCTGCGG-5') and CN-6 (3'-TTTAAGGCGAGCCGACGTCCTT-5') which resulted in an amplicon of 250 bp. One microliter of DNA was amplified in a final volume of 25 µL, containing 10 mM Tris-HCl (pH 8.80), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µmol of each deoxyribonucleotide, 12.5 pmol of each primer, and 0.5 U of Taq DNA polymerase (Cinagen DNA Extraction Kit instance). PCR parameters used in the external reaction included an initial denaturation at 96°C for 3 min and 20 cycles of 96°C for 45 s, 50°C for 1 min, and 72°C for 1 min. Final extension was carried out at 72°C for 7 min. One microliter of the amplification product was then used as the template for the second reaction, carried out for 30 cycles under the same conditions as the first cycle but in the presence of the primers CN-5 (3'-GAAGGGCATGCCTGTTTGAGAG-5') and CN-6 (3'- TTTAAGGCGAGCCGACG TCCTT-5'), which produced an amplicon of 115 bp. PCRs were performed in a Mastercycler gradient thermal cycler (Eppendorf Scientific, Inc., Westbury, N.Y.).

Amplification products were electrophoresed through a 2% agarose gel and visualized with a UV transilluminator after ethidium bromide staining.

Controls

In order to monitor crossover contamination, sterile distilled water was included in the DNA extraction and was used as a negative control in the first and last samples in the nested PCR assay. Reaction mixtures without DNA were run in the first and nested PCRs to detect contamination.

Cloning and Sequencing

The PCR product was cloned into pGEM®-T Easy Vector (pGEM®-T Easy Vector Kit, Promega-USA). The ligation reaction was incubated overnight at 4°C. Transformation was performed with $CaCl_2$ procedure. The correct new plasmid was sent to sequencing (Cinnagen DNA Extraction Kit).

RESULTS

PCR amplification

The results of semi-nested PCR indicated that we can detect less than 100 yeast cells per mL of CSF (Fig. 1).

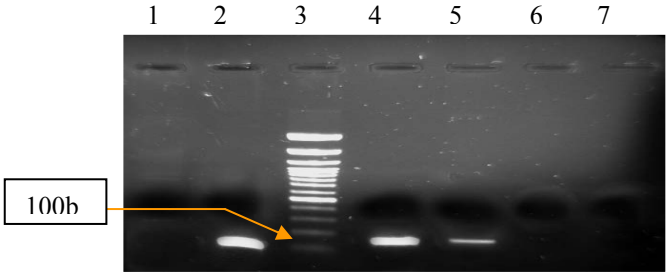

Fig 1. Lane 1- negative control, lane 2, 4, 5, 6- amplicons with cryptococcal cells numbering 106, 104, 102, 10 respectively, lane 3- DNA size marker 100bp plus (fermentas), lane 7- negative control.

Cloning and Sequencing

The result of sequencing and comparing of it with other references (Fig. 2) showed that our protocol amplified the 26 bp of 5.8S rRNA and 89bp of internal transcribed spacer 2 (ITS2) in round 2 of Semi-nested PCR.

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                    10        20        30        40        50
AJ876598  gaagggcatg cctgtttgag agtcatgaaa atctcaatcc ctcgggtttt
AJ876525  gaagggcatg cctgtttgag agtcatgaaa atctcaatcc ctcgggtttt
AJ876524  gaagggcatg cctgtttgag agtcatgaaa atctcaatcc ctcgggtttt
AJ876523  gaagggcatg cctgtttgag agtcatgaaa atctcaatcc ctcgggtttt
Sample    gaagggcatg cctgtttgag agtcatgaaa atctcaatcc ctcgggtttt

                ...|....| ....| ....| ....| .... | .... | .... | ....| ....|
                    60        70        80        90       100
AJ876598  attacctgtt ggacttggat ttgggtgttt gccgcgacct gcaaaggacg
AJ876525  attacctgtt ggacttggat ttgggtgttt gccgcgacct gcaaaggacg
AJ876524  attacctgtt ggacttggat ttgggtgttt gccgcgacct gcaaaggacg
AJ876523  attacctgtt ggacttggat ttgggtgttt gccgcgacct gcaaaggacg
Sample    attacctgtt ggacttggat ttgggtgttt gccgcgacct gcaaaggacg

                ....|....| ....|
                    110
AJ876598  tcggctcgcc ttaaa
AJ876525  tcggctcgcc ttaaa
AJ876524  tcggctcgcc ttaaa
AJ876523  tcggctcgcc ttaaa
Sample    tcggctcgcc ttaaa
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Fig 2. Comparison of our sample sequence and other reference sequences in Gene Bank with accession numbers AJ876598, AJ876525, AJ876524, AJ876523.

DISCUSSION

Identification of pathogenic fungi has changed dramatically over the past decade through direct examination of the tremendous variation present in DNA.[11]

The development of molecular diagnostic tests for mycotic infections requires information from an extensive sequence database.[12,13,14]

Laboratory diagnosis of (CSF) is traditionally based on microscopic examination of India ink preparations and on the detection of cryptococcal capsular polysaccharide antigen by a latex agglutination test[15]. Direct microscopic examination is a rapid but quite insensitive test and strongly depends on the operator's skills. The latex agglutination test is a more sensitive method but may still yield false-positive and false-negative results with either serum or CSF. [11,16,17,18] Moreover, the simple culture of CSF samples on Sabouraud agar is time-consuming; in fact, at least 4 days is necessary to detect positive cultures of *C. neoformans*[19] An enzyme-linked immunosorbent assay kit for the detection of capsular antigen is also available, with sensitivity comparable to those of agglutination tests.[20] PCR is a very powerful tool for clinical mycology. It allows the selective amplification of very small amounts of nucleic acids.[4,17] Nested PCR is able to monitor the presence of *C. neoformans* better than microscopic examination or the latex agglutination tests [21].These results demonstrate that the developed Semi-nested PCR is a promising method to be used in the analysis of CSF samples from patients suspected of having cryptococcal meningitis[22]. Moreover, since in the course of treatment of cryptococcosis the duration of therapy is still controversial, usually depending on the time needed for clearance of cryptococcal antigen from the CSF as demonstrated by the latex agglutination test[23, 24], the PCR may be a useful tool for the rapid diagnosis of acute cryptococcosis. It can be used for the diagnosis of neurocryptococcosis, therefore its use is advisable in the laboratorial routine[25].The nested PCR method is sensitive, specific, and reproducible and represents a promising method for analysis of CSF samples from patients with clinical suspicion of neurocryptococcosis.

In conclusion, we have successfully evaluated sensitive conventional nested PCR protocols for the detection of *C. neoformans* in cerebrospinal fluid samples. The true diagnostic value of these assays will be further evaluated in prospective studied on human cryptococcal disease.

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