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NEUROCRYPTOCOCCOSIS: DIAGNOSIS BY PCR METHOD

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SUMMARY

Cryptococcus neoformans detection was optimized using PCR technique with the objective of application in the clinical laboratory diagnosis. The amplification area was ITS and 5,6S which encodes the ribosomal RNA (rRNA). A total of 72 cerebrospinal fluid (CSF) samples were used, obtained from cases with and without AIDS. The patients had cryptococcal meningitis (n = 56) and meningitis caused by other agents (n = 16). The results demonstrated that PCR test had the highest sensitivity rates, superior to culture (85.7%) and to India ink test (76.8%). PCR was found to be sensitive in detecting 1 cell/mL and highly specific since it did not amplify other fungal DNA. The comparative analysis of the methods showed that PCR is more sensitive and specific and is applicable as an important laboratorial resource for neurocryptococcosis diagnosis.

KEYWORDS: *Cryptococcus neoformans;* PCR; Diagnostic methods; AIDS; Cryptococcosis

INTRODUCTION

Cryptococcosis is a cosmopolitan infection caused by *Cryptococcus neoformans*, an encapsulated yeast pathogenic to humans and animals[1,13,18].

In the last decades, cryptococcosis has been assuming a prominent role at public health level due to the growing number of AIDS individual cases[2,3,20]. 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[7,9,14]. 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[24]. 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[11]. 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[12]. The DNA of *C. neoformans* can be detected by initiators that encode the ribosomal RNA (rRNA) gene 16S-5S,8S-23S which is highly conserved[4,17].

In this work the single-step PCR technique on clinical samples was optimized by utilizing the initiator pairs CN-4/CN-5 that are specific for *C. neoformans* and do not amplify the DNA of other yeast[17]. This technology involves yeast acid nucleic isolation, amplification by PCR and identification by electrophoresis on polyacrylamide gel with silver salt staining.

MATERIALS AND METHODS

1. Biological samples: The CSF samples were obtained from individuals with and without neurocryptococcosis signals. The collection was carried out through lumbar and occipital puncture. The material was dripped directly into sterile tubes and divided into three groups for later analysis:

Group 1: Constituted of 43 individuals with or without clinical suspicion of meningitis and laboratorial diagnosis of meningitis, independent of age, color or sex with positive culture for *C. neoformans*.

Group 2: Constituted of 13 individuals with clinical picture and laboratorial diagnosis of meningitis, independent of age, color or sex, with negative culture and positive China ink test for *C. neoformans.*

Group 3: Constituted of 16 individuals with or without clinical suspicion of meningitis caused by other agents not related to *C. neoformans*, independent of age, color or sex, with negative culture and India Ink Test for *C. neoformans*.

Other methodologies were evaluated for DNA extraction from *C. neoformans* in culture and in CSF samples: from simple extraction with

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glass spheres[10,19,28], extraction of DNA with sodium iodide[15] and with guanidine thiocyanate[24] to more complex methods involving enzymes[22,25,26,27,29].

2. DNA extraction: The SANDHU extraction technique[24] was performed: 2 mL of clinical sample were placed in a sterile 15 mL polypropylene test tube and centrifuged at 5000 rpm during five min. The sediment was transferred to another sterile 1.5 mL microtube. Then 500 µL of the reagent GPT (6M guanidine thiocyanate) dissolved in 50 mM TRIS (pH 8.3) was added after mixing with an equal volume of phenol TRIS (pH 8.0). The resulting mixture was thoroughly stirred and immediately incubated by boiling water bath during 15 min. Next, 250 µL of chloroform: amyl alcohol (24:1 per volume) was added under vigorous stirring followed by centrifugation for five min at 12000 rpm. The aqueous phase was transferred to a new and sterile tube followed by addition of one mL 100% isopropanol. This set was freezer-stored for a minimum of one h and then centrifuged for 10 min at 12000 rpm. The supernatant was discharged. Next, 500 µL 70% ethanol was added to the sediment. Centrifugation during five min at 12000 rpm was performed and the sediment was resuspended in 50 µL buffer TE (10 mM TRIS, 1 mM EDTA, pH 8.0) and freezer-stored at -20 °C until use.

3. Primers: For the *C. neoformans* genome amplification we selected and tested primers "sense" CN-5 (3'GAA GGG CAT GCC TGT TTG AGA G 5') and "antisense" CN-4 (5'ATC ACC TTC CCA CTA ACA CAT T 3') as described by MITCHELL *et al.*[16] which are specific for *C. neoformans*.

4. PCR method: After the extraction, 5 µL DNA of each sample were added to the 200 µL Eppendorf type tubes. Next, buffer solution for PCR was pipetted [75 mM TRIS-HCl (pH 9.0), 50 mM KCl, 2 mM MgCl2, BIOTOOLS - B&M Labs, S.A]; 200 mM of each dNTP (dATP, dTTP, dGTP and dCTP, Pharmacia Biotech), 1.5 U/µL *Taq* DNA polymerase (BIOTOOLS - B&M Labs, S.A.); 10 ρmol/µl of each primer (Gibco-BRL Life Technologies NY-USES) for a reaction final volume of 50 µL. The amplification was realized in thermocycle GeneAmp 2400 (Perkin-Elmer, USA) and consisted of one cycle at 95 ºC for 7 min (hot start), 40 cycles at 95 ºC for 45 sec (denaturation), 60 ºC for one min. (annealing), 72 ºC for 1.5 min (extension) and 72 ºC for 7 min (final extension).

4.1. Analysis of the PCR products: The electrophoresis separation was accomplished on 8% polyacrylamide gel with 20 µL of the PCR product containing 10 µL of run buffer (6X concentrated). The system was submitted to 95 Volts during 6 h and later silver-stained through impregnation technique to permit observation of the DNA dark brown color bands (Fig. 1)

4.2. Specificity of the primers: The chosen primers CN-4 and CN-5 presented high specificity for *C. neoformans* serotypes A, B, C and D cultures for CSF samples where the yeast was present. The primer did not amplify other types of yeasts such as: *Candida albicans* ATCC 64548, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 750, *Candida glabrata* ATCC 90030, *Cryptococcus luteolus ICB*, *Cryptococcus terreus ICB*, *Cryptococcus diffluens* I*CB* 72 and *Rhodotorula glutinis* ICB 32 (Fig. 2).

4.3. Sensitivity of the PCR method: The technique was sensitive for the detection of up to 1 cell/ mL in CSF samples. The PCR amplification yielded a DNA fragment of 136 bp in all concentrations (Fig. 3). Each reaction was accompanied with control reagents and positive control with 6,000 cells/mL (data not showed).

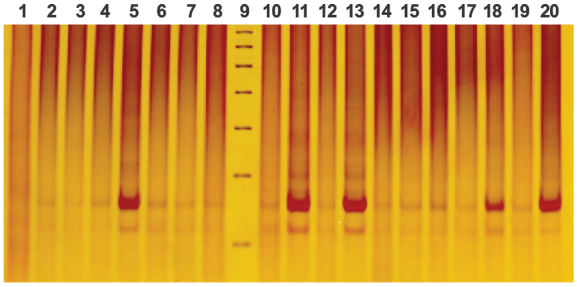

Fig. 1 - Electrophoretic separation on 8% polyacrylamide gel of the PCR products from *C. neoformans* in CSF sample extracted with guanidine thiocyanate. Line 1 reagent control; line 5 positive control; lines 2, 3, 4, 6-8, 10-20 samples of CSF; line 9: DNA ladder 100bp (standard);

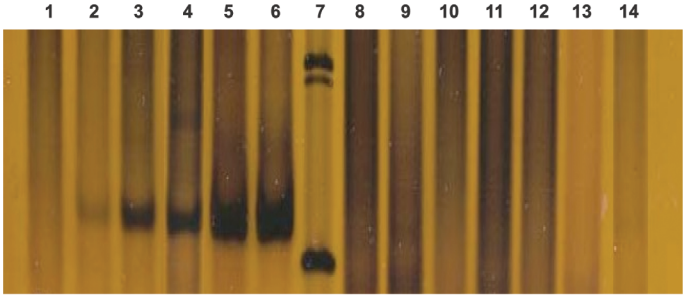

Fig. 2 - PCR Sensitivity. Line 1: reagent control; line 2: positive control; line 3,4,5 and 6: *C. neoformans* serotype A, B, C and D; line 7: DNA ladder 123 bp; lines 8, 9 and 10 *Cryptococcus terreus*, *Cryptococcus diffluens* and *Cryptococcus luteolus*; line 11: *Rhodotorula glutinis*, lines 12, 13 and 14: *Candida albicans*, *Candida tropicalis* and *Candida glabrata*.

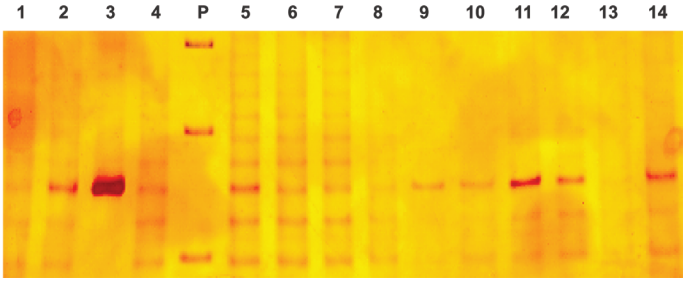

Fig. 3 - Electrophoresis on 8% polyacrylamide gel from clinical sample PCR product with different amounts of cells. Lines 1: 2 cells; 2: 11 cells; 3: positive control; 4: 70 cells; P: 100bp molecular weight DNA pattern; 5: 40 cells; 6: zero cell; 7: 1 cell; 8: zero cell; 9: 58 cells; 10: 1 cell; 11: 14 cells; 12: 65 cells; 13: 28 cells; 14: 110 cells.

RESULTS

In Table 1, the results obtained by culture, India Ink Test and PCR techniques were compared in the study of 72 CRL samples (groups 1, 2 and 3). It was found that all the techniques were 100% specific (75.9 - 100%) and that PCR technique amplifies only *C. neoformans* specie DNA[11,16].

Table 1
Comparison of results obtained by PCR method, culture and India ink test in 72 CSF samples from individuals with or without clinical diagnosis of cryptococcal meningitis

Samples n = 72		Cryptococcal meningitis n = 56	Other meningites n = 16	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	FP (%)	FN (%)
Culture	Pos	43	0	76.8	100	100	55.2	0	0
	Neg	13	16	(63.3-86.6)	(75.9-100)	(89.8-100)	(36.0-73.0)		
China Ink Test	Pos	48	0	85.7	100	100	66.7	0	33.3
	Neg	8	16	(73.2-93.2)	(75.9-100)	(90.8-100)	(44.7-83.6)		
PCR	Pos	52	0	92.9	100	100	80.0		
	Neg	4	16	(81.9-97.7)	(75.9-100)	(91.4-100)	(55.7-93.4)	0	20.0

Pos = positive; Neg = negative; PPV = positive predictive value; NPV = negative predictive value; FP = false positive; FN = false negative

The PCR method presented the greatest sensitivity with 92.9% (81.9 - 97.7%). The specificity was 100% (75.9 - 100%), with positive predictive value 100% (91.4 - 100%), there were no false positives; negative predictive value was 80.0% (55.7 - 93.4%) with 20% false negatives which refer to four samples of group 2 that presented positive India Ink Test. The India Ink Test sensitivity 85.7% (73.2 – 93.2), specificity 100% (75.9 - 100%), positive predictive value 100% (90.8 - 100%) and negative predictive value 66.7% (44.7 - 83.6%) generating a false negative of 33.3%, due to 8 samples of group one that presented positive culture but negative values with India Ink test. Nevertheless, the culture that was considered the gold standard of this study presented sensitivity 76.8% (63.3 - 86.6%), specificity 100% (75.9 - 100%), positive predictive value 100% (89.9 - 100%) and negative predictive value 55.2% (36.0 - 73.0%), generating false negatives of 44.8% due to 13 samples of group 2 that presented positive value with India ink test but negative culture.

DISCUSSION

In the present study, we optimized the method for the detection of *C. neoformans* by PCR technique in CSF samples with the objective of developing a new diagnosis resource that, associated with methods already used in laboratorial routine, can increase the diagnostic efficiency in cases where conventional techniques are inadequate[11].

At the standardization phase of DNA extraction method, the extraction efficiency, speed, simplicity of manipulation, ease of obtaining the material and low cost were taken into account; since these requirements are indispensable in order to ensure the method is applicable for routine laboratorial use. From simple DNA extraction with glass beads[10,19,28], DNA extraction with sodium iodine methods[16] and with guanidine thiocyanate[24] to more complex methods involving enzymes[21,22,25,26,27,29,30] all them were efficient for the extraction of DNA from cultures of *C. neoformans*. The extraction with guanidine thiocyanate is fast, presents easy execution, low cost and is much more efficient for CSF sample extraction than *C. neoformans* culture. This was demonstrated to be the only efficient technique for *C. neoformans* DNA extraction from clinical samples, working even with small amounts of yeast.

In order to evaluate the specificity of the primers, DNA of several ATCC and ICB yeast strains were tested and none of their DNA was amplified. Strains for the four serotypes of *C. neoformans* were also utilized as a standard. The validity of the methodology was studied through the evaluation of efficiency in the case of material obtained at the medical clinic. For that, CSF materials were studied as samples and were subdivided into three study groups:

Group 1, constituted of individuals with clinical diagnosis of neurocryptococcosis, with positive results for culture and India Ink test, PCR demonstrated high sensitivity (100%) and specificity (100%) without any false negatives; Group 2, constituted of neurocryptococcosis cases, with positive value to India Ink test and negative culture, PCR proved to be sensitive (69.2%) and specific (100%). In four individuals (30.8%) that presented negative PCR, two of the corresponding sample cells were not found; in the other two there were three and 46 cells, respectively. This was probably due to the capsule being thicker in clinical samples compared to those of cultures. Thus, the high thickness of the capsules may have considerably hindered the cellular lysis. Another hypothesis for this finding could be interferents in CSF, which may have inhibited the PCR. It is supposed that these interferents may be related to the great amount of antibiotics, anti-retroviral agents, proteases and other drugs administered to the AIDS patient, which may not have been eliminated at the DNA extraction. Occasionally, the presence of great capsular polysaccharide amounts can alter the activity of *Taq* polymerase and compromise the accuracy of PCR[5]. In group 3, constituted of individuals with other neurological diseases not related to *C. neoformans*, PCR was demonstrated to be specific (100%) and did not present fragment amplification in any of the samples and there were no false positive results.

Comparison of the three groups showed a predominance of 58 male individuals (80.6%) with a mean age between 36 to 39 years and a less expressive number of 14 female individuals (19.4%) with a mean age from 23 to 36 years.

PCR presented a higher sensitivity in relation to India ink test and culture due to the following factors that interfere in the *C. neoformans* growth and visualization: a) for the India ink test be positive it is necessary that there are more than 103 CFU/mL; b) *C. neoformans* shows different forms and sizes in CSF and sometimes does not present capsules, impeding its visualization; c) the culture medium composition has to offer some source of iron; d) some aberrant strains of *C. neoformans* are unable to grow in BHI (brain and heart infusion) medium, but grow well

on SDA (Sabouraud's dextrose agar); e) the growth can be significantly inhibited at temperatures between 39 - 40 ºC; f) some patients that received antifungal therapy can present negative culture, but positive result to India ink test at the end of therapy, irrespective of clinical improvement; and g) pH above 7.6 causes the death of the yeast[5].

India ink test presented 85.7% (73.2 - 93.2%) sensitivity; positive predictive value of 100% (90.8 - 100%) and did not present false negatives, but a negative predictive value of 66.7% (44.7 - 83.6%) in that 33.3% false negatives were found, amounting to eight samples from group one that were negative and under direct microscopy presented an absence of cells that probably harmed the color exam.

In relation to culture evaluation, a sensitivity of 76.8% (63.3 - 86.6%) was found due to 13 negative samples from group two. Of these, five samples were negative under direct microscopy, five samples presented low cellularity (3, 3, 28, 30 and 46 cells) and three high cellularity (110, 148 and 1080 cells). This can occur when antifungal therapy has already been initiated, thereby inhibiting cellular growth and negativation of the culture, but the presence of the yeast is detected by the India ink test and therefore in some cases is positive until the end of the therapy, irrespective of clinical improvement. It has yet to be clarified why these nonviable yeasts occasionally persist for months after the antifungal therapy[5].

RAPPELLI *et al.*[23] utilized the same protocol of DNA extraction of SANDHU *et al.*[24] and realized the identification of *C. neoformans* by "nested" PCR technique and observation of the DNA fragments on agarose gel stained with ethidium bromide. The demonstrated results, in accordance with SANDHU *et al.*[24] were significantly better for the following data:

In the protocol of RAPPELLI *et al.*[23] all CSF samples used had at least one cell per field in India ink test and presented latex agglutination titer 1:1024, data strongly indicative of neurocryptococcosis .

Some authors detected DNA from samples that did not present culture and India ink test, positive. These authors used "nested" PCR to increase the DNA detection sensitivity. This method is both time consuming and expensive besides the major inconvenience that there is a risk of causing contamination[6,8].

The primer pairs used in the "nested" PCR also amplify the saprophytic *Filobasidiella depauperata* and so can give a false positive result. The primers CN-4 and CN-5 used in this study do not amplify yeast other than *C. neoformans* because they are species-specific[17].

Hence, the strategy developed in this study allowed a reduction in the time for *C. neoformans* detection and identification since it entails just a single step for the amplification, which avoids contamination and offers satisfactory results with the same sensitivity as the method proposed by RAPPELLI *et al.*[23].

The results show that DNA extraction with guanidine thiocyanate was the only efficient method for *C. neoformans* DNA extraction in CSF samples, besides being fast and easy to perform. The PCR method has proven to be efficient in detecting up to one cell/mL in CRL samples, even in cases where antifungal therapy has been initiated. In the overall evaluation of the studied groups, this technique showed sensitivity of 92.9% and specificity of 100%. It can be used for the diagnosis of neurocryptococcosis, therefore its use is viable in the laboratorial routine.

The PCR method is sensitive, specific, and reproducible and represents a promising method for analysis of CSF samples from patients with clinical suspicion of neurocryptococcosis.

RESUMO

Neurocriptococose: diagnóstico por PCR

A detecção de *Cryptococcus neoformans* em líquor foi otimizada pela técnica de PCR. A amplificação foi realizada nas áreas ITS e 5,6S do RNA ribossomal (rRNA). Foram estudados 72 líquors obtidos de casos de pacientes com e sem AIDS. Os pacientes eram portadores de meningite criptocócica (n = 56) e meningite ocasionada por outros agentes (n = 16). Os resultados demonstraram que a técnica tem alta sensibilidade, superior a cultura (85,7%) e ao teste da tinta da china (76,8%). A técnica de PCR pode detectar 1 célula/mL de líquor e é altamente específica. A análise comparativa dos três métodos, tinta da china, cultura e PCR, demonstrou que o último é muito mais sensível e específico, podendo ser aplicável como importante recurso laboratorial no diagnóstico da neurocriptococose.

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