DEVELOPMENT OF A PCR MULTIPLEX FOR SEROTYPE AND MATING TYPE DETERMINATION IN *CRYPTOCOCCUS NEOFORMANS*

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Abstract- Multiplex Polymerase Chain Reaction (MPCR) is a molecular biology technique derived from PCR that is capable to amplify simultaneously two or more loci, thus being faster and more economic than conventional PCR. The aim of the present work was to establish the amplification conditions of a MPCR for the determination of serotype and mating type of *Cryptococcus neoformans*, the etiological agent of cryptococcosis. Standard isolates of *C. neoformans* were used for the development of the methodology. Genomic DNA was extracted with glass beads and the primers were obtained from the STE 20 gene sequence. The MPCR was successfully established, being considered reliable for the characterization of serotype and mating type of *C. neoformans*.
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Palavras-chave: *Cryptococcus neoformans*., serotype, mating type, PCR Multiplex
Área do Conhecimento: Ciências Biológicas

Introduction

Cryptococcus neoformans is a cosmopolite encapsulated yeast and etiological agent of the opportunistic mycosis cryptococosis. This disease manifests as meningoencephalitis in immunocompromised individuals such as AIDS patients [CASADEVAL and PERFECT, 1998].

Three varieties and five serotypes have been defined for *C. neoformans* based on genetic characteristics and serologic properties: *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *grubii* (serotype A), hybrid serotype AD, and *C. neoformans* var. *gattii* (serotypes B and C) [FRANZOT et al., 1999]. These serotypes differ in their ecological, molecular, and morphological characteristics as much as epidemiology, pathogenicity, physiology and geographic distribution [YAN et al., 2002]. Recently, the new species *C. gatii* was proposed based on differences between their teleomorphs and the biochemical differences between the anamorphs of the *C. neoformans* and *C. neoformans* var *gattii* [KWON-CHUNG et al., 2002].

Nowadays, PCR is the only tool available for determination of probable *C. neoformans* serotypes, because the “Crypto Check” kit (Iatron, Mitsubishi, Japan), that provided biochemical analyses of this feature, is not manufactured since 2005. The kit contained five serologic factors for specific antigenic determinants, allowing a quick identification of the serotypes of *C. neoformans* isolates.

Biotyping *Cryptococcus* isolates is an important epidemiological tool, since important characteristics are associated with different serotypes. In this context, the aim of the present work was to standardize the conditions for simultaneous amplification, with MPCR, for characterization of serotype and MAT of the varieties *grubii* and *neoformans* of *C. neoformans.*

Materials and methods

Samples: Standard isolates of *C. neoformans* (table 1) belonging to the fungal collection of the Mycology Laboratory of the Institute of Biomedical Sciences of the University of São Paulo, São paulo, Brazil (ICB-II/USP-SP) were maintained in Agar Sabouraud, at 25º C on the fungal collection of Federal University of Alfenas, Minas Gerais, Brazil (Unifal-MG).

DNA extraction: DNA extraction of the samples was made with glass beads (Sigma, St Louis, MO, USA) as proposed by Bolano et al (2001).

Establishment of the MPCR conditions: For the development of the MPCR, it is necessary that the primer set employed generates different sizes products, allowing the identification of the isolates. The establishment of the MPCR conditions for the characterization of serotype and MAT was performed with the primers described on table 2. Those primers were designed using the STE20 gene sequence.

Two PCR multiplex were performed. The first PCR multiplex (αAaD) was performed with the primers JOHE7264/7265 and JOHE7273/7275. The DNA of the standard samples ICB 110 and

ICD 170, respectively homozygotes for the alleles aD and αA and the DNA of the standard sample ICB 134, heterozygote αAaD were employed. The second PCR multiplex (aAαD) was made with the primers JOHE7270/7272 and JOHE7267/7268. The DNA of the standard samples ICB 163 and ICB aA, respectively homozygotes for the alleles αD e aA were used, as well as the mixture of the DNA of both isolates, obtaining the artificial heterozygote aAαD. According to Yan et al double heterozygotes aAαD are very rare.

The variable tested for the establishment of the methodology were primers annealing temperature, concentration of Taq DNA polymerase (Fermentas life Science) (1U or 1.5 U), concentration of $MgCl_2$ (1.5mM or 2mM), equimolar quantities of the two sets of primers (0.8 pmols or 10 pmols). Amplifications were realized in a thermocycler (Model 9700; Applied Biosystems).

Analysis of the MPCR products: Products were submitted to electrophoresis (80v for 60 min) in agarose gel (1.5%) and stained with ethidium bromide (1µg/ µL). The molecular weigh marker Ladder 250pb was applied. The gel was observed under UV light and the image was stored using the Kodak digital system EDAS-290.

Results

The best amplification conditions were obtained with the concentrations of 1U of Taq DNA polimerase, 1.5mM of $MgCl_2$ and 0.8 pmols of primers. Considering these results, the following protocol for MPCR was established in the present work: 50□L reactions containing 0.8 pmols of each primer, dNTP 0.2mM, buffer (10mM Tris-HCl, 50mM KCl, pH 8,8), $MgCl_2$ 1.5mM, Taq DNA polymerase 1U and DNA 60ng.

The PCR multiplex αAaD was produced in the following conditions: initial denaturing at 94ºC for 5 min followed by 30 cycles of 96°C for 60 seconds, annealing temperature of 61° C for 60s and extension at 72°C for 60 min. A final extension was performed at 72ºC for 10 min. The PCR multiplex aAαD followed the same conditions for initial denaturing and final extension, but consisted of 30 cycles of 96°C for 60 seconds, annealing temperature of 63° C for 60s and extension at 72°C for 60s.

The results obtained for MPCR αAaD can be observed on figure 1 and for MPCR aAαD on ficure 2. The primers JOHE 7264/7265 and JOHE 7267/7268 amplified 1200 pb length fragments and primers JOHE 7270/7272 and JOHE 7273/7275, 870 pb length fragments, as predicted by the nucleotide sequence of GenBank.

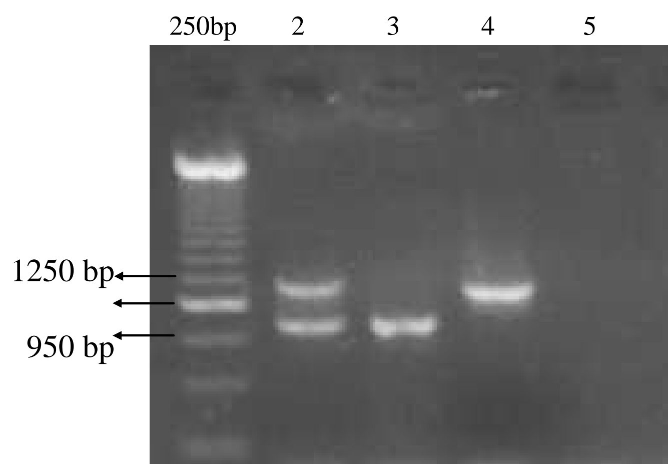

Figure 1 – Results obtained for MPCR αAaD of standard samples of *C. neoformans.* Lanes: 1 - 250 bp ladder, 2 - ICB 134, 3- ICB 110, 4 - ICB 170, 5 - blank.

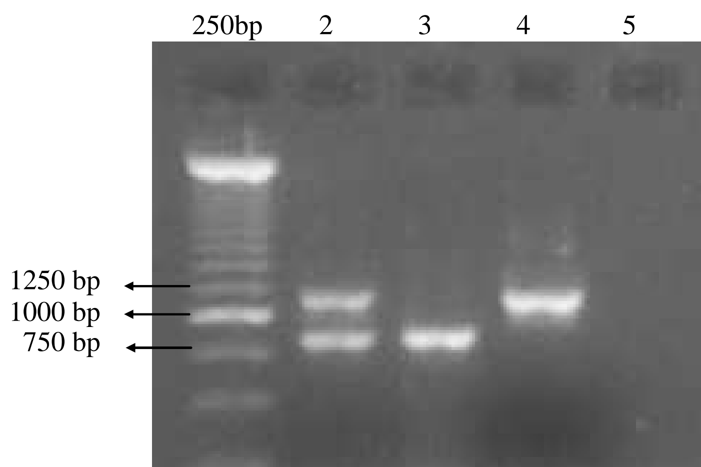

Figure 2 – Results obtained for MPCR aAαD of standard samples of *C. neoformans.* Lanes: 1 - 250 bp *ladder*. 2 - DNA artificial standard for two target sequences, 3 - ICB 163αD, 4-ICB aA, 5 – Blank.

Discussion

It can be observed on figures 1 and 2 that the intensity of all the bands amplified is similar, as recommended by Henegariu et al. [1997]. These authors developed a step by step protocol to achieve the ideal conditions of amplification, in the attempt to solve problems associated with MPCR, such as lack of amplification of some loci, uneven amplification and results with low reproducibility.

Those problems were not found in the present work, evidencing the success in standardizing the method.

As it can be observed on pictures 1 and 2 (spot 2), the detection of the hybrid serotype samples was possible by both MPCR proposed. Hybrids serotypes are not always detected when biochemical characterization is used.

New methods for determining MAT alleles of *C. neoformans* have been developed. One of such method uses MAT-specific primers in PCR conventional. The traditional crossing method was able to identify MAT of only a portion of the collection. In contrast, in the study made by Yan et al the conventional PCR method determined the MAT of all 358 strains.

The advantage of MPCR in comparison with conventional PCR is that the first has lower cost and quicker analysis of results. By simultaneously amplifying more than one locus in the same reaction, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory [HANEGARIU et al., 1997].

According to the experiments performed by Henegariu et al. [1997] a modification of 20 s to 2 min in the time of annealing does not modify the amplification significantly, so, this variable was not tested in the standardization of the MPCR. These authors emphasize that the annealing temperature is the most important variable to be tested in the standardization of MPCR. Many loci can be amplified individually in temperatures ranging from 56-66ºC. Our results show that the annealing temperature in MPCR was 1 or 2 degrees lower than the temperature used for individual amplification of the same loci. This fact was also observed by Henegariu et al. [1997] that reported a difference as great as 6 degrees.

There was no difference in the amplification of the samples when Taq DNA polimerase concentrations of 1U/50µL and 1.5U/50µL were tested. Henegariu et al [1997] performed tests with several Taq DNA polimerase concentrations and found that the ideal conditions were around 1-2U/25µL. The difference in the Taq DNA polymerase concentration obtained by Henegariu et al. [1997] and the concentration used in our work could be explained by the number of loci amplified. Those authors amplified seven loci simultaneously in contrast with two loci amplified in this study.

The MPCR performed with the concentration of 1.5 mM of $MgCL_2$ was more specific and showed bands with intensity comparable with the MPCR realized with 2 mm $MgCl_2$. The concentration of $MgCl_2$ affects the specificity of the reaction and is one of the crucial factors in PCR, determining the efficacy of the reaction. As a general rule, the concentration of $MgCl_2$ is greater for MPCR than for conventional PCR.

Amplification was obtained for primers concentrations tested, 0.8 pmols and 10 pmols. The last concentration caused an excess of primer in the reaction (data not shown), thus, the concentration of 0.8 pmols was used.

The MPCR methodology developed in the present work has been successfully employed in our group. Reimão et al. [2006] used the MPCR to characterize serotypes and mating types of 54 *C. neoformans* samples isolated from the hollows of living trees. As well, 22 samples of *C. neoformans* were characterized by this technique in the work developed by Carvalho et al. (2007).

In conclusion, it was possible to establish the MPCR. The reactions showed to be quick and effective for the characterization of serotype and MAT of *C. neoformans* of the varieties *grubii* and *neoformans,* proving to be a reliable alternative for this analysis.

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