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SEROTYPE AND MATING TYPE CHARACTERIZATION OF *Cryptococcus neoformans* BY MULTIPLEX PCR

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SUMMARY

Cryptococcus neoformans is an encapsulated yeast, etiological agent of cryptococcosis. The species is commonly associated with pigeon droppings and plant materials. The aim of the present work was to verify the presence of the yeast in pigeon droppings, and to identify the isolates obtained in serotypes and mating types (MAT). Ten samples of pigeon droppings were collected in the rural area of the city of Alfenas, Brazil. Samples were inoculated in agar Niger medium for fungal isolation and 22 isolates with characteristics of *C. neoformans* were obtained. The serotypes and MAT were determined by multiplex PCR using specific primers. Serotypes were also determined by using the Kit Crypto Check. Among the 22 samples evaluated, eight were identified as *C. neoformans* by classic identification tests. These samples were characterized as serotype A by the Kit Crypto check and as serotype A MAT α by the multiplex PCR. The present study reinforces the evidence that pigeon droppings are a reservoir for *C. neoformans* and confirms the prevalence of *C. neoformans* var. *grubii* (Aα) among environmental isolates. It also demonstrates that multiplex PCR is an acceptable alternative for serotype analysis because it reduces the costs for each reaction and analyses serotype and MAT simultaneously.

KEYWORDS: *Cryptococcus neoformans;* Serotype; Mating Type; PCR; Kit Crypto Check®.

INTRODUCTION

Cryptococcus neoformans is an encapsulated yeast, etiological agent of the opportunistic mycoses, cryptococcosis. This infection manifests mainly as meningoencephalitis in immunocompromised individuals. Cryptococcosis has been responsible for great morbidity and mortality rates among patients with AIDS[17].

Five distinct serotypes of *C. neoformans* are determined by specific antiserum: A, B, C, D and AD[1,24]. The occurrence is associated with ecological, morphological physiological and molecular features, epidemiology, pathogenicity and geographic distribution. The species is distributed in the varieties *neoformans* (serotypes A, D and A/D) and *gattii* (serotypes B and C). Recently, the variety *grubii* (serotype A) and the new species *C. gattii* were proposed[9]. The anamorphic stage of the yeast comprises two mating types (MAT), a and α, which can be crossed to produce the basidiomycetous *Filobasidiella neoformans*[5].

Cryptococcus neoformans var. *neoformans* is widely distributed in nature and has been isolated from several sources. The yeast is frequently associated with pigeon droppings but has also been isolated from soil, wood in decay, fruits and vegetables[4].

The aim of the present work was to identify samples of *C. neoformans* isolated from pigeon droppings in the rural area of the city of Alfenas, Brazil, to characterize the *C. neoformans* obtained in serotypes and MAT by PCR multiplex and in serotypes by the Kit Crypto Check® (Iatron, Tokyo, Japan). We also compared serotypes obtained by PCR multiples and by the Kit Crypto Check (Iatron).

MATERIAL AND METHODS

Ten samples of pigeon droppings were collected in nests from seven farms in the rural area of Alfenas city, MG, Brazil. The samples obtained were conditioned in clean and dry paper envelopes and processed immediately in the laboratory of Microbiology of the Federal University of Alfenas (Unifal-MG).

Sample processing: For the processing of the samples, we used the method proposed by SHIELDS & AJELLO (1966) modified. Four grams of each sample were solubilized under agitation for two min in 30 mL of sterile saline solution. After ten minutes, the supernatant was transferred for test tubes containing 2 mL of distilled water amended with 543 mg penicillin[6].

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The solution obtained was transferred on triplicate to plates containing agar Niger medium (*Guizotia abyssinica* - Niger seeds, 70 g; glucose, 10 g, chloramphenicol, 50 mg; biphenyl, 1 g, agar, 20 g; distilled water, 1000 mL). Samples were incubated at 28 °C and observed for five days.

Isolation and identification of *C. neoformans*: Yeast-like colonies showing a cream to brown pigmentation and mucoid characteristic were isolated in pure culture in Sabouraud Dextrose Agar (Difco, laboratories, Detroit, MI, USA) and observed microscopically with Indian Ink to investigate the presence of capsule[10], pigment production in agar Niger medium without biphenyl, urease production in modified Christensen's urea medium (peptone, 1 g; sodium chloride, 5 g; monopotassium phosphate, 2 g; glucose, 1 g; urea, phenol red 0.2%, 6 mL; distilled water, 1000 mL), growth at 37 °C[10], carbon source assimilation[23] and non assimilation of potassium nitrate[10].

Molecular characterization of *C. neoformans*: DNA extraction of the samples characterized as *C. neoformans* was made with glass beads (Sigma, St. Louis, MO, USA)[2]. Samples were submitted to a PCR with the aim to determine the serotype and MAT. Reference samples belong to the Culture Collection of the Biomedical Sciences Institute, University of São Paulo, São Paulo, Brazil.

The primers used for DNA amplification were based on the STE20 gene sequence[1]. Two PCR multiplex were performed to simultaneous amplification of serotype and MAT[22]. The first PCR multiplex (αAaD) was made with the primers JOHE7264/7265 and JOHE7273/7275 in the following conditions: 30 cycles of 96 °C for one min, 61 °C for 30s and 72 °C for one min. The sample ICB 134 was used as positive control C(+)1 and sample ICB 170, C(+)2. The second PCR multiplex (aAαD) was made with the primers JOHE7270/7272 and JOHE7267/7268 in the following conditions 30 cycles of 96 °C for one min, 63 °C for 30s and 72 °C for one min. The control for the simultaneous amplification of the bands generated by the primers 7270/7272 and 7267/7268 was obtained by the combination of the DNA of the samples ICB 163 and ICB Aa (data not shown). The primers are described on Table 1.

Serotype characterization by the commercial kit Crypto check® (Iatron): The isolates were cultivated in yeast extract-malt extract-agar (Difco) at 25 °C. After 48h of incubation the culture was suspended in sterile physiological saline solution at McFarland scale pattern 2 (about 6 x 10^8 CFU/mL). A drop of each seric factor (F1, F5, F6, F7, and F8) was placed in the corresponding cycle of the agglutination glass slides, and 50 µL of the *C. neoformans* suspension was added over each seric factor. The glass slides were homogenized with an agitator with rotational movement at 25 rpm for two min. The slides were read by direct observation of the small clots formed, as follows: serotype A, F1 and F7; serotype B, F1 and F5; serotype C, F1 and F6; serotype D, F1 and F8; and serotype AD, F1, F7, and F8.

RESULTS

Fifty-four yeast-like colonies were isolated from the ten analyzed samples of pigeon droppings. Among these, 22 were positive for the presence of capsule and showed cream to brown pigmentation in agar Niger without biphenyl, which are characteristics of *C. neoformans*. According to the carbon source assimilation test the following isolates were identified: two as *C. uniguttulatus*, one as *C. laurentii,* three as *C. albidus* and eight as *C. neoformans*. So, from the 22 analyzed samples, eight of them did not belong to the genus *Cryptococcus*.

The eight *C. neoformans* isolates were obtained from two farms, being six from dropping samples of five nests in the first farm and two dropping samples of two nests in the second farm.

All of the eight isolates evaluated agglutinated with the Kit Crypto Check (Iatron) sera and were classified as *C. neoformans* var. *grubii* (serotype A).

According to the multiplex PCR (Fig. 1), the C(+)1 control (ICB 134) was amplified resulting in one band of 1200 pb corresponding to the serotype A MATα alleles and another band of 870 pb related to the serotype D MAT a alleles. The amplification of C(+)2 control (ICB 170) resulting in a band of 1200 pb. The amplification of the eight *C. neoformans* isolates resulted in the presence of only one band, the 1200 pb one.

DISCUSSION

The isolation of *C. neoformans* from avian excreta has been reported by several authors[3,7,16,19,20]. STAIB & SCHULZ-DIETERICH (1984)

Table 1
Combination and sequences of the primers used for determination of serotype and mating type of *C. neoformans* by PCR multiplex αAaD and aAαD[2]

Gene or allele	*Primer*	Sequence 5'→3'	PCR product (pb)
MATα serotype A	JOHE 7264	AGCTGATGCTGTGGATTGAATAC	1200
	JOHE 7265	GTTCAATTAATCTCACTACCTGTAG	
MATa serotype D	JOHE 7273	GTTCATCAGATACAGAGGAGTGG	870
	JOHE 7275	CTCCACTGTCAAACCTACGGC	
MATa serotype A	JOHE7270	ATCAGAGACAGAGGAGGAGCAAGAC	870
	JOHE7272	TCCACTGGCAACCCTGCGAG	
MATα serotype D	JOHE7267	ATAGGCTGGTGCTGTGAATTAAG	1200
	JOHE7268	GTTCAAGTAATCTCACTACATGCG	

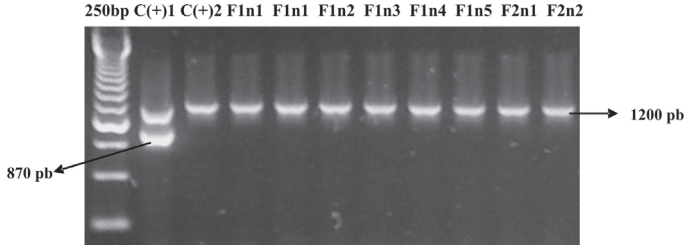

Fig. 1 - Electrophoretic analysis of the products obtained through the amplification by the multiplex PCR. Lane 1: 250 bp molecular size ladder (Invitrogen, Milano, Italy); Lane 2: C(+)1- reference strain ICB 134; lane 3: C(+)2- reference strain ICB 170; lanes 4 to 11: samples (F1 = Farm 1; F2 = Farm 2; n1-5 = nest 1 to 5).

concluded that the exposure to avian excreta explains at least partially the epidemiology of cryptococcosis.

REZENDE (2002) evaluated the presence of *C. neoformans* in pigeon and canary droppings in the urban area of the city of Alfenas and found the yeast in 11.11% of the samples analyzed. In the present work, *C. neoformans* could be isolated from 18.20% (eight in 44) of the samples analyzed. Similar data were obtained in other cities of Brazil and in other countries[11,14,25]. It has been speculated that plants in decay are the primary habitat of *C. neoformans*, which would explain the higher percentage of isolation in the rural area. On the other hand, this possibility does not explain the similarity with the percentage of isolation obtained in other works.

No non-specific bands were observed and no cross-reactions occurred. The produced fragments by the amplification of C(+)1 and C(+)2 controls correspond to the predicted sizes by the GeneBank (www.ncbi.nlm.nih.gov) nucleotide sequence.

The presence of 1200 pb band by the PCR multiplex allowed the identification of the variety *grubii* in the eight isolates.

Earlier studies had already demonstrated that MAT α was predominant among clinical and environmental isolates, despite the serotype[1,8,13,24]. In clinical samples, serotype A is more prevalent (77.95%) followed by serotype B (18.2%), AD (1.3%), D (0.4%) and C (0.2%)[15]. In the present study, serotype was determined by PCR multiplex and the Kit Crypto Check. Both methodologies showed to be easy to perform, reliable and demonstrated an acceptable concordance of results. The determination of the serotype by the Kit Crypto Check (Iatron) has also been performed by other authors[1,12]. BARRETO DE OLIVEIRA *et al.,* 2004, reported that PCR multiplex with specific primers could discriminate heterozygotes isolates (AD) identified as homozygotes (A) by the Kit. In the present study, heterozygotes isolates were not identified.

Kit Crypto Check allows the quick determination of serotypes in samples of *C. neoformans*, however, costs are elevated when compared to PCR, and the mating type is not identified. Besides, heterozygote isolates might not be discriminated by the Kit. The present study reinforces the evidence that pigeon droppings are a reservoir for *C. neoformans* and confirms the prevalence of *C. neoformans* var. *grubii* (Aα) among environmental isolates. It also demonstrates that PCR multiplex is an acceptable alternative for serotype analysis because it reduces the costs for each reaction and analyses the mating type and serotype simultaneously. It represents a rapid, simple and relatively economical tool for epidemiological and virulence studies.

RESUMO

Caracterização de sorotipo e "mating type" de *Cryptococcus neoformans* por PCR multiplex

Cryptococcus neoformans é levedura encapsulada, agente etiológico da criptococose. As espécies são comumente associadas com fezes de pombos e material vegetal. O objetivo do presente trabalho foi verificar a presença de leveduras em fezes de pombos e identificar os isolados em relação aos sorotipos e "mating types". Dez amostras de fezes de pombos foram coletadas na zona rural da cidade de Alfenas, Brasil. As amostras foram inoculadas em agar Niger e 22 isolados com características de *C. neoformans* foram obtidos. Os sorotipos e "mating types" foram determinados pela PCR multiplex e os sorotipos foram identificados também pelo Kit Crypto Check. Dentre as 22 amostras avaliadas, oito foram identificadas como *C. neoformans* através dos testes clássicos. Estas amostras foram caracterizadas como sorotipo A pelo Kit Crypto check e como sorotipo A MATα pela PCR multiplex. O presente estudo reforça a evidência de que as fezes de pombos constituem reservatório para *C. neoformans* e confirma a prevalência de *C. neoformans* var. *grubii* (Aα) nos isolados ambientais. PCR multiplex é uma alternativa aceitável para análise do sorotipo porque reduz os custos de cada reação e analisa simultaneamente os sorotipos e "mating type".

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