SEROTYPE AND MATING TYPE CHARACTERIZATION OF *CRYPTOCOCCUS NEOFORMANS* ISOLATED FROM PIGEON DROPPINGS COLLECTED IN THE RURAL AREA OF THE CITY OF ALFENAS, MG

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Abstract- *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 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 PCR multiplex. The present study reinforces the evidence that pigeon dropping 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 serotype and MAT simultaneously.
Key words: *Cryptococcus neoformans;* serotype; *mating type*; PCR; Kit Crypto Check®.
Área of Knowlegment: Biology

Introduction

Cryptococcus neoformans is an encapsulated yeast, etiological agent of the opportunistic mycoses, criptococosis. This infection manifests mainly as maningoencephalitis in immunocompromised individuals. Criptococosis 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 e AD [24, 1]. 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. bacillispora* (var. *gattii*) were proposed. 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, Brasil, 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 nest found in farms localized in the rural area of the city of Alfenas, MG, Brazil. The samples obtained were conditioned in clean and dry paper envelopes and processed immediately in the laboratory of Microbiology of the University of Alfenas –MG.

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

The solution obtained was transferred on triplicate to plates containing agar Niger medium (*Guizotia abyssinica* _ Niger seeds, 70g; glucose, 10g, chloramphenicol, 50mg; biphenyl, 1g, agar,

20g; distilled water, 1000ml). Samples were incubated at 28°C and observed for five days.

2) Isolation and identification of *C. neoformans* isolates: Yeast-like colonies showing a cream to brown pigmentation and mucoid characteristic were isolates in pure culture in Sabouraud Dextrose Agar (Difco, laboratories, Detroit, MI, USA) and observed microscopically with Indian Ink to investigate the presence of capsule [9], pigment production in agar Niger medium without biphenyl, urease production in modified Christensen's urea medium (peptone, 1g; sodium chloride, 5g; monopotassium phosphate, 2g; glucose, 1g; urea, phenol red 0,2%, 6ml; distilled water, 1000 ml), growth at 37°C [9], carbon source assimilation [23] and non assimilation of potassium nitrate [9].

3) Molecular characterization of *C. neoformans* isolates:

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 (Table 1).

The primers used for DNA amplification were based on the STE20 gene sequence [13]. 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 1 min, 61° C for 30s and 72°C for 1 min. The sample ICB 134 was used as positive control. 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 1 min, 63°C for 30s and 72°C for 1 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. The primers are described on table 2.

Samples were analyzed and compared with the reference samples for identification of the serotype and MAT.

4) Serotype characterization by the commercial kit Crypto check® (Iatron):

The isolates were cultivated in yeast extract-malt extract-agar (Difco) at 25℃. After 48 h 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 2 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 samples analyzed. 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*. The carbon source assimilation test identified eight isolates as *C. neoformans*, two as *C. uniguttulatus*, one as *C. laurentii* and three as *C. albidus*. Eight samples were not identified as *Cryptococcus* spp.

All of the eight isolates evaluated agglutinated rapidly with the Kit Crypto Check (Iatron) sera and were classified as *C. neoformans* var *grubii* (serotype A). According to the PCR, the eight isolates evaluated were identified as serotype A and MAT α, allowing the identification of the variety *grubii*. Thus, the samples could be characterized as *C. neoformans* var. *grubii*.

Discussion

The isolation of *C. neoformans* from avian excreta has been reported by several authors [3, 16, 20, 7, 19]. Staib (1984) concluded that the exposure to avian excreta explains at least partially the epidemiology of criptococosis.

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 30% of the samples analyzed. Similar data were obtained in other cities of Brazil and in other countries [25, 10, 14]. 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.

The PCR multiplex showed that all the isolates obtained in the present work were serotype A MAT α, therefore *C. neoformans* var *grubii*. Earlier studies had already demonstrated that MAT α was predominant among clinical and environmental isolates, despite the serotype [8, 12, 24,

[13].In clinical samples, serotype A is more prevalent (77.95%) followed by serotype B (18.2%), AD (1.3%), D (0.4%) e C (0.2%) [15]. In the present study, serotype was determined by PCR multiplex and the Kit Crypto Check. Both methodologies shoed 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 [11, 13]. 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 dropping 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.

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