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## ***Cryptococcus neoformans* STRAINS ISOLATED FROM CHURCH TOWERS IN RIO DE JANEIRO CITY, RJ, BRAZIL**

Francisco de Assis BARONI(1,2), Claudete Rodrigues PAULA(2), Ériques Gonçalves da SILVA(2), Flávio Cesar VIANI(2), Irma N.G. RIVERA(2), Maria Tereza Barreto de OLIVEIRA(2) & Walderez GAMBALE(2)

### SUMMARY

Cryptococcosis has been a significant cause of morbidity and mortality in patients with Aids. Many reservoirs of the agent *Cryptococcus neoformans* have been reported, but the ecology of this yeast must be elucidated in order to establish surveillance programs and to prevent infections. The objective of this study was to evaluate the presence of *C. neoformans* in Rio de Janeiro City, RJ, Brazil. Ten churches were selected for sampling and detection of the yeast collecting pigeon dropping, air samples from church towers and neighboring areas during one year. The data demonstrated that *C. neoformans* has been present in every church selected and was present in 37.8% of 219 pigeon dropping samples. As well as, the yeast was isolated from soil, insects, eggs, pigeon nests and feathers. Fifteen air samples (4.9%) were positive. The growth on C.G.B. medium showed that all strains belonged to *C. neoformans* var. *neoformans*, with 98.8% of the strains belonging to serotype A.

**KEYWORDS:** *Cryptococcus neoformans*; Serotypes; Pigeon dropping; Ecology.

### INTRODUCTION

The yeast *Cryptococcus neoformans* has currently been classified according to its perfect state, *Filobasidiella*<sup>13,14</sup>. Single species has been described, composed by two varieties and five serotypes: *Filobasidiella neoformans* variety *neoformans* (serotypes A, D, and A/D) and *F. neoformans* variety *bacillispora* (serotypes B and C). The varieties *neoformans* (serotypes A, D, A/D) and *gattii* (serotypes B and C), which belong to teleomorph *F. neoformans*, have been correlated (*Cryptococcus neoformans*). More recently, the variety *grubii* (serotype A) and the new specie, *C. bacillispora* (var *gattii*), have been proposed<sup>1,2,6,8</sup>.

Cryptococcosis has been responsible for great morbidity and mortality rates among patients with Aids<sup>7,27,28</sup>, considered the fourth most common infection in the immunocompromised individuals<sup>11</sup>. The majority of *C. neoformans* isolates recovered from HIV-positive patients have been serotype A and a similar prevalence has been found among patients that have developed the acquired immunodeficiency syndrome<sup>3,19,26,27,31</sup>. According to LAZERA *et al.*, 1993<sup>15</sup> some sources of *C. neoformans* around the urban perimeter of Rio de Janeiro city, RJ, Brazil, have been verified. One strain of *C. neoformans* var. *neoformans* was isolated from an abandoned house and another strain of var. *gattii* was isolated from guano in an old house. According to PASSONI *et al.*, 1998<sup>24</sup>, the distribution of *C. neoformans* var

*neoformans* in Rio de Janeiro city has not restricted to the biotype because they verified the agent at patient's house with cryptococcosis, associated to Aids, at a house of Aids patient, without association with cryptococcosis and even in houses with healthy individuals. Also in Brazil, MONTENEGRO & PAULA, 2000<sup>20</sup>, demonstrated the presence of *C. neoformans* var. *neoformans* and var. *gattii* in São Paulo city and in urban areas. Brazilian studies have shown the prevalence of serotype A (78%) on clinical isolates. However, serotype B has most frequently been isolated from the northeastern region<sup>22</sup>.

In many situations, reports of cryptococcosis have been associated to pigeon droppings as the source of infection; however, an epidemiological analysis showed that patients with pigeons contact had a high exposure risk<sup>16</sup>. The major problem is that *C. neoformans* remains viable on dry pigeon droppings, for many years, being a reservoir of inhaled infecting particle, persisting with small capsule which are compatible with alveolar deposition.

In Rio de Janeiro, a very attractive international tourism city, there are pigeons as "permanent members of its postcard". Several church towers and old buildings have been densely populated by pigeons that can be daily seen around the people.

In this study, we aimed to verify the presence of *C. neoformans* and respective serotypes in church towers (pigeon droppings and air

(1) Universidade Federal Rural do Rio de Janeiro, UFRRJ, Rio de Janeiro, Brazil.

(2) Institute of Biomedical Sciences, University of São Paulo, USP, São Paulo, Brazil.

**Correspondence to:** Francisco de Assis Baroni, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Instituto de Veterinária, Departamento de Microbiologia e Imunologia. km 47 antiga Rio/São Paulo, Caixa postal 74585, Seropédica, RJ, Brazil. Phone/Fax: +55-21-26827075. E-mail: baroni@ufrj.br

samples), at Rio de Janeiro city. In addition, we also studied related material as soil, insects, pigeon eggs and nests.

## MATERIAL AND METHODS

**1. Sampling sites:** Ten churches in Rio de Janeiro city were selected for sampling mainly pigeon droppings, however air samples and other materials (soil, nests, eggs and insects) were also collected at the selected areas (Table 1). Sometimes, when it was observed the presence of great quantity of excreta in the area, more than a single sample was collected. Ten churches were sampled in ten distinct occasions and a total of 219 excreta samples were collected from January to December 1998. The number of samples per church varied according to the sampled area (towers, or towers and stairs, and area over the church's nave).

**2. Pigeon dropping sampling:** Pigeon excreta samples were collected using sterile spatulas, transferred to sterile plastic bags (18.5 x 23.5 cm) and properly labeled according to site and date. The average sample weight was around 500 g. Latter, soil, insects, pigeon nests, and eggs were also collected. Samples were carried on to the laboratory under refrigeration and stored until used.

**3. Air sampling:** The presence of *C. neoformans* in atmospheric air at church towers was investigated on 304 samples. Petri dishes containing DOPA medium<sup>14</sup> and Sabouraud dextrose agar medium (SDA-Difco) were directly exposed to air for 15 minutes. Four to seven dishes per sample were exposed, depending on the area dimensions (approximately one Petri dish per m<sup>2</sup>). The plates were incubated at 32 °C from three days to two weeks.

### 4. Procedure

**4.1. Pigeon droppings:** Portion of excreta (approximately 20-30 g) from each sterile plastic bag, was aseptically removed, weighted, and transferred to Erlenmeyer flasks containing a saline solution (0.9%) with chloramphenicol (200 mg/L), achieving 1:10 dilution (w/v). The material was homogenized by shaking and allowed to stand for 30 minutes. Aliquots of 0.5 mL supernatant were streaked on DOPA agar and Sabouraud dextrose agar medium - SDA - (Difco) with chloramphenicol (200 mg/L), using a Drigalski loop. Cultured samples were incubated at 32 °C and observed daily for 15 days.

**4.2. Additional material collected:** *Soil (8 samples):* it was followed the same procedure described above (item 4.1). *Pigeon nests:* 15 nests were crushed, added to a saline solution (0.9%) containing chloramphenicol (200 mg/L) and homogenized very well. The procedure for analysis was identical to that one used for pigeon excreta, but the nests were not weighted. The pieces were broken on smaller parts, around 1-2 cm. *Bird eggs:* 11 eggs were first immersed on iodine solution (0.8%) for 10 minutes, and after left in sterile saline solution. Each egg was placed aseptically on an Erlenmeyer flask containing 150 mL of saline solution, in which chloramphenicol had been added, and the flasks were placed on shaker until breaking the egg and then, material was homogenized. The mixture was allowed to stand for 30 minutes, after which 1.0 mL supernatant streaked onto DOPA and SDA media. The plates were incubated at 32 °C. *Insects (Coleoptera spp.):* 19 insects were immersed on iodine solution and dipped on at five consecutive times in sterile saline solution containing chloramphenicol. The insects were then dried on filter paper and ground up. The fragments

were streaked onto DOPA and SDA plates. Insects not crushed were streaked onto DOPA medium.

**5. Isolation and identification of *Cryptococcus neoformans* strains:** Seventy-two hours after inoculation, colonies with a mucous appearance on SDA medium and brown colonies on DOPA medium were selected. Each colony was suspended on sterile saline, observed under an optical microscope, and re-cultured on DOPA medium to confirm the production of phenoloxidase. From each Petri dish streaked, one colony was randomly selected and transferred to SDA medium, both for maintenance and identification<sup>13</sup>. The identification was accomplished by negative growth on medium containing cycloheximide, urease production on Christensen medium, phenoloxidase production on DOPA medium and carbon and nitrogen assimilation tests<sup>13</sup>.

**6. Serogrouping and serotyping:** *Cryptococcus neoformans* strains were biochemically typed, using C.G.B. medium (canavanine-glycine-bromotimol blue agar medium)<sup>12,13</sup>. Isolates grew on SDA medium (Difco) for 48 hours, were streaked onto C.G.B. medium and incubated at 32 °C. Tests were performed on duplicate and cultures were daily observed for 10 days. Controls for A, B, C, D and A/D serotypes were: *C. neoformans* - ATCC 34872 (Serotype A), ATCC 32269 (Serotype B), ATCC 24066 (Serotype C), ATCC 28958 (Serotype D) and ATCC 48184 (Serotype A/D). After the serogrouping, strains were serotyped by slide agglutination with specific antiserum (Crypto Check Iatron, RM 304-K Laboratories Inc., Tokyo, Japan).

## RESULTS

*C. neoformans* was isolated from the total of the churches and from pigeon dropping samples collected on nine from the ten church buildings selected. This yeast was isolated from air samples at six church buildings (Table 1).

The largest number of colony former unit (CFU) occurred on the samples from churches with larger excreta concentration. *Acanthamoeba* spp. was found on fecal material collected from church 4.

From a total of 219 excreta samples, 83 (37.8%) were positive for *C. neoformans*. From 11 processed pigeon eggs, two isolates were obtained (18.18%). From 15 processed pigeon nests samples, three were positive (20.0%). From 19 samples of insects, only one isolate (5.26%) was obtained. From eight soil samples, two strains were obtained (25%). Fifteen (4.93%) were recovered from 304 air samples. The number of colonies on Petri dishes exposed to the air was smaller (< 50 CFU), in comparison, to the number of colonies obtained from diluted excreta on saline solution (> 50 CFU).

Considering only excreta, church 3 provided the largest number (14/6.4%), followed by church 9 (11/5.0%) and church 5 (9/4.1%) (Table 1).

Serogrouping using C.G.B. medium, showed that all strains were susceptible to canavanine and, therefore, have belonged to *C. neoformans* var. *neoformans*.

The serotyping revealed that 98.8% of the strains were serotype A (var. *grubii*) and just 1.2% serotype AD (var. *neoformans*).

**Table 1**  
*C. neoformans* isolated from church towers in Rio de Janeiro city, Brazil

Church number	Positive sample	Months									
		Jan.	Feb.	Apr.	May	Jun.	Jul.	Aug.	Oct.	Nov.	Dec.
1	5	-	PD	-	PD*	PD	PD	-	-	-	-
2	9	-	-	PD	PD	-	-	PD	PD	PD	PD
3	14	PD	-	-	-	-	PD	PD	PS	PD	PD
4	2	-	-	-	-	-	-	-	-	PN	-
5	13	-	-	-	-	-	-	PD	A	PD	PD
6	5	-	PD	-	-	PD	-	PD	-	A	PD
7	9	-	-	-	-	PD	-	PD	A	A	PD
8	1	-	-	-	-	-	-	-	-	PD	-
9	20	-	-	PD	-	PD	PD	PE	PE	S	PD
10	05	-	-	PD	-	S	S	-	S	-	-
Total	83	1	2	3	2	8	6	12	17	22	10

A = air; I = *Coleoptera* spp., PD = pigeon dropping, PE = pigeon eggs, PN = pigeon nest, S = soil, \* A/D serotype; (-) negative.

## DISCUSSION

The occurrence of the aetiologic agent of cryptococcosis in church towers and adjacent areas found in this study could be due to the environmental conditions favoring growth of *C. neoformans*, as mild air temperature, large amounts of pigeon excreta, a sheltered environment from direct solar radiation and a proper pH. Overall, the number of colonies on Petri dishes exposed to ambient air was small (< 50 CFU). In a previous study where similar methodology had been used for testing the presence of *C. neoformans* in the air, BULMER<sup>4</sup> showed that the recovery depends, among other factors, of wind directions. At church 10, where there was a little air circulation, the propagules on suspension were less expected to be found and the recovery of *C. neoformans* by direct exposure to Petri dishes was not successful (Table 1).

Based on our results, the highest isolation rate was recorded in November, similarly to that one occurred in São Paulo city, pointing out that is spring in Brazil<sup>20</sup>. The temperature influences the proliferation and survival of this yeast-like fungus<sup>16</sup>. Very high summer temperature in Rio de Janeiro could inhibit *C. neoformans* growth or

even could cause cell inactivation, perhaps explaining low rates of isolation during this season.

Recovery of *C. neoformans* from beetles was not surprising, since these insects are coprophagous. Because beetles widely spread, they are able to carry on *C. neoformans* over huge distances. Thus, these insects could be a hazard for public health care.

Just a few reports of *C. neoformans* isolation from pigeon nests and related material have been available. Some studies carried out in Brazil reported the presence of this fungus in nests<sup>17,31</sup>. Although pigeons can carry *C. neoformans* on their beaks, feet and feathers, it remains to be showed that these ones provide appropriate nutrients for *C. neoformans* growth<sup>10</sup>. This yeast has been associated to other yeasts during delignification of wood<sup>9</sup>. It has also been proposed that the variety *gattii* has been better growing on trees with high lignin concentration.

Isolation of *C. neoformans* from soil at the church towers most likely reflects contamination by pigeon excreta deposits. Interestingly, amoebae (*Acanthamoeba* spp.) was detected on the pigeon dropping

samples collected at church 4, where *C. neoformans* was isolated, only from a nest and air samples (data not showed). Trophozoites of *Acanthamoeba polyphaga*<sup>5,22</sup> were reported to phagocytize *C. neoformans* in larger numbers, as *Acanthamoeba palestinensis* does<sup>29</sup>. MALLIARIS *et al.*, 2004<sup>18</sup> showed that the interaction between *C. neoformans* and *Acanthamoeba castellanii* was primarily extracellular with a paucity of phagocytic events. They have suggested that the difference on the capsule structure may be responsible for the different interaction between cells of varieties *neoformans*, *grubii* and *gattii* with amoebae.

Obviously, the *C. neoformans* isolation from pigeon droppings depends on several environmental factors including the material amount. In church 8, *C. neoformans* was isolated once and *Acanthamoeba* spp. was not observed, in spite that the material amount was scarce.

Biochemical results showed that all environmental isolates studied were variety *neoformans* and, from these ones, 98.8% were serotype A. In Brazil, NISHIKAWA *et al.*, 2003<sup>22</sup> reported the greater frequency of serotype A on pigeon samples and serotype B on tree samples. Additionally, they reported serotype A (77.95%), B (18.2%), AD (1.3%), D (0.4%) and C (0.2%) on clinical samples. It has been reported worldwide the association of *C. neoformans* serotype A with cryptococcosis, among HIV- positive individuals<sup>3,26,27,31</sup>. However, this fact must be better studied using molecular epidemiology methods in order to establish the direct relationship between the disease and the serotype.

The cryptococcosis is more frequently found in patients with Aids (48%) and other diseases associated to cellular deficiency (33%)<sup>25,30</sup>.

Meningoencephalitis has been the most common form of cryptococcosis infection. The disease is more frequent in adult people, in spite that in Brazil the incidence in childhood population increased to 24%<sup>25</sup>.

A review of cryptococcosis Brazilian experience was published in 2003 by PAPPALARDO & MELHEM<sup>23</sup>, showing the great importance of this disease for morbidity and mortality rates.

This study is the first report of *C. neoformans* presence from church towers in Rio de Janeiro city. The towers have sheltered pigeons as well as large deposits of the bird excreta, facilitating this microorganism spreading by airborne propagules and, very likely, migratory patterns of insects (beetles). It needs to be reminded that according to BULMER (1990)<sup>4</sup>, the problem is that *C. neoformans* remains viable on dry excreta for two years<sup>16</sup>. Old buildings and churches of historical interest have been abundant in Rio de Janeiro and the significance of these spaces as ecological niches for *C. neoformans* must be seriously considered by public health authorities.

## RESUMO

### *Cryptococcus neoformans* em torres de igrejas da cidade do Rio de Janeiro, RJ, Brasil

*Cryptococcus neoformans* é um fungo que ocasiona micose de alta morbidade e mortalidade, especialmente em pacientes com Aids.

Muitos reservatórios de *C. neoformans* têm sido relatados, mas a ecologia desta levedura deve ser ainda elucidada para se estabelecer programas de vigilância e prevenção desta infecção. O objetivo deste estudo foi o de avaliar a presença de *C. neoformans* no Rio de Janeiro, RJ, Brasil. Dez igrejas foram selecionadas para este estudo, coletando-se fezes de pombo, amostras de ar, das torres das igrejas e de áreas vizinhas, durante um ano. Os resultados revelaram que *C. neoformans* estava presente em todas as igrejas e em 37,8% das 219 amostras das excretas das aves.

Ao mesmo tempo, o fungo foi isolado do solo, insetos, ovos e ninhos de pombos. Quinze (4,9%) do total das amostras de ar foram positivas. O crescimento no meio de CGB revelou que todas as amostras pertenciam a *C. neoformans* var. *neoformans*, e 98,8% destas amostras pertenciam ao sorotipo A.

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