

## ENVIRONMENTAL STRAINS OF *Cryptococcus neoformans* VARIETY *grubii* IN THE CITY OF SANTOS, SP, BRAZIL

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### SUMMARY

This study involved a total of 116 samples, 79 taken from pigeon droppings and 37 of atmospheric air taken close to accumulations of excrement. *Cryptococcus neoformans* var. *grubii* was isolated from 11 (13.9%) of these samples. Other species of *Cryptococcus* were also isolated from these samples, such as *C. albidus* (12.6%) and *C. laurentii* (8.9%). *C. neoformans* was not isolated from the air samples, though *C. albidus* (5.4%) was. All the strains of *C. neoformans* were found to belong to the A serotype (*C. neoformans* var. *grubii*). In regard to the studies with the antifungal agents 5-fluorocytosine, fluconazole, itraconazole, amphotericin B and voriconazole, by means of the microdilution method (EUCAST), we point out that one sample demonstrated resistance to fluconazole, this being especially significant because this is an environmental strain.

**KEYWORDS:** *Cryptococcus neoformans*; Pigeon droppings; Serotype; Antifungal agents.

### INTRODUCTION

*Cryptococcus neoformans* is an encapsulated yeast whose teleomorphic state is a basidiomycete - *Filobasidiella neoformans*. It is a cosmopolitan fungus frequently isolated from soil rich in organic material, particularly pigeon droppings<sup>4,9</sup>. Nowadays, in respect to its morphological, biochemical, immunological and epidemiological characteristics, *C. neoformans* comprises three varieties: *C. neoformans* var. *neoformans*, *C. neoformans* var. *gattii* and *C. neoformans* var. *grubii*<sup>5</sup>.

Currently, the varieties of *C. neoformans* may be considered as belonging to five serotypes: serotype A (var. *grubii*), serotype D and AD (var. *neoformans*) and serotypes B and C (var. *gattii* = *C. bacillisporus*)<sup>3,15,25</sup>.

It has been observed that this yeast may be present in a wide range of locations such as old buildings, church towers, stables, basements, sheds and various open-air public places, all of which could become infective foci<sup>2,22,27</sup>.

*C. neoformans* can occur in low-frequency in the human species, as part of the transitory microbiota<sup>18</sup>. Despite its saprophytic existence, *C. neoformans* is able to infect and cause disease in immunocompetent hosts (serotypes B and C) and, mainly, in immunocompromised patients (serotypes A, D and AD)<sup>28</sup>. It can also infect other mammals, insects and birds<sup>6</sup>.

In Brazil, *Cryptococcus* infection continues to be a lethal disease related to Aids. One possible hypothesis can be the existence of phenotypes that are resistant to antifungal therapy. New antifungal agents have become available, resulting in a greater number of therapeutic options and a demand for "in vitro" susceptibility testing<sup>12</sup>.

The Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) has developed a proposed standard broth microdilution procedure for the determination of antifungal MICs of some species of yeasts. This standard is based on the M27-A2 broth microdilution reference procedure of the National Committee for Clinical Laboratory Standards (NCCLS). It incorporates some modifications to allow the automation of the susceptibility method and to permit the reduction of the incubation period<sup>11</sup>.

It is generally held that the disease cryptococcosis is acquired from the environment through the inhalation of yeasts and/or dried basidiospores with incipient encapsulation, depending on the immunological state of the host<sup>6</sup>.

It should be noted that the place studied here, Santos, is a city with beaches attracting countless tourists, and has a significant number of residents with Aids. The aim of this study was to verify the occurrence of *C. neoformans* and its varieties in samples taken from the environment, pigeon droppings and atmospheric air, determining the prevailing serotype and the susceptibility to antifungal agents.

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## MATERIALS AND METHODS

**City studied:** The city of Santos is located on the coast of the state of São Paulo, 70 km from the city of São Paulo, and has a population of about 410,000. To the north it borders on the counties of Santo André, Mogi das Cruzes and Salesópolis; to the south, the Atlantic Ocean and Santo Amaro Island; to the east, the county of Bertioga; and to the west, the counties of Cubatão and São Vicente. The city comprises an island with an area of 39.4 km<sup>2</sup> and a mainland area of 231.6 km<sup>2</sup>. Its altitude is two meters, being predominantly flat but circled by high hills. Its climate is tropical. Together with other counties, it is part of the low coastland area denominated as the Baixada Santista. It has served as a port city for a long time (since 1543), and at its center there are historical constructions and monuments along with many churches. As a coastal city, it contains a large number of beaches and gardens. In all of these areas there can be found hundreds of pigeons, in constant contact with the residents and tourists.

**Environmental samples studied:** The environmental samples were collected from church towers, old buildings and dockside warehouses, as well as streets downtown and along the beaches.

A total of 79 samples of pigeon droppings were collected along with 37 samples of atmospheric air taken close to areas with accumulations of excrement.

The pigeon excrement was collected in sterilized plastic bags, using spatulas and brushes, from various sites within the urban area of the city.

Air contamination was verified at 37 locations by way of plates exposed for 15 minutes.

**Processing of the samples and yeast isolation:** The samples were processed less than 48 h after collection. Approximately 2 g of each pigeon dropping sample was suspended in a flask containing 50 mL of saline solution supplemented with chloramphenicol (0.1 g/L). After vigorous shaking for five min, the flask was allowed to rest for 30 min for phase separation.

A 2 mL volume of the supernatant was aspirated into test tubes for centrifuging. The sediment was serially diluted until 10<sup>-3</sup>, and the pure sample and its dilutions were cultured into Petri plates containing either Sabouraud dextrose agar (Difco) and niger agar (*Guizotia abyssinica*)<sup>14</sup>, both supplemented with chloramphenicol (0.1g/L). The dishes were placed into an incubator at 30 °C and observed daily for 15 days.

**Identification of isolated yeasts:** Selection was made of colonies with a creamy white or creamy beige aspect grown in the Sabouraud dextrose agar (Difco) or of dark-brown or black colonies grown in the niger agar. These were examined by microscope for morphological analysis, stained by a solution of cotton lactophenol blue and nigrosine and transferred to tubes containing Sabouraud dextrose agar for isolation. After growing, the colonies were cultured into tubes containing niger agar for verification of the phenoloxidase activity, and into Christensen's urea agar<sup>8</sup>. Positive samples were identified using a commercial yeast identification kit (API 20CAUX - bioMérieux). Culturing at 37 °C onto Sabouraud dextrose agar and in a "mycosel" agar (Oxoid) were also carried out<sup>19</sup>.

**Determination of the varieties isolated:** The varieties of *C. neoformans* isolated were determined by culturing in CGB medium (L-canavanine, glycine and bromotimol blue)<sup>17</sup>. The control consisted of standard strains of *C. neoformans* serotype A (ICB 163) and *C. neoformans* serotype B (ICB 162).

**Determination of serotypes:** Serotyping of the *C. neoformans* isolates was carried out using the Crypto Check kit (Iatron Laboratories, Tokyo, Japan) with specific antisera. The standard strains were also used<sup>18</sup>.

**Assessment of the samples in regard to antifungal agents:** Susceptibility tests were run using five antifungal agents (5-fluorocytosine - 5FC, fluconazole - FZ, itraconazole - IZ, amphotericin B - AB, and voriconazole - VZ). The minimum inhibitory concentration (MIC) was determined according to the standard technique established by document M27-A2<sup>24</sup>. These susceptibility tests included the standard sample of *C. neoformans* var. *neoformans* ATCC 90012.

The modifications include the use of RPMI 1640 supplemented with 2% glucose as the test medium, an inoculum size of 0.5 × 10<sup>5</sup> to 2.5 × 10<sup>5</sup> CFU/ml, flat-bottomed trays, and spectrophotometric reading with a 50% inhibition endpoint for azole agents and flucytosine and 90% to azoles. In addition, a previous evaluation showed that MICs obtained by the EUCAST broth microdilution method are in close agreement with those obtained using the NCCLS reference procedure<sup>11</sup>.

Stock solutions were prepared in dimethyl sulfoxide, except flucytosine, and fluconazole which was dissolved in sterile distilled water. The assay medium was RPMI 1640 without sodium bicarbonate and with L-glutamine buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid and supplemented with 18 g of glucose per liter to reach a final concentration of 2% (RPMI-2% glucose; Oxoid S.A., Madrid, Spain)<sup>10</sup>. Culture medium was prepared as a double-strength solution and sterilized by filtration. Sterile plastic microtitration plates containing flat-bottomed wells were utilized (Corning Costar Europe, Badhoevedorp, The Netherlands). The plates contained 100 µL of twofold serial dilutions of the antifungal drugs per well. Two drug-free medium wells for sterility and growth controls were employed. The final inoculum suspension contained between 0.5 × 10<sup>5</sup> and 2.5 × 10<sup>5</sup> CFU/mL, and an aliquot of 100 µL was added to each well of the microdilution plate. The MIC endpoints (to inhibit the growth at 50% to azoles and 5FC and 90% to AB) were determined after incubation at 30 °C for 72 h and analyzed according some breakpoints by NCCLS, 2002 or by some researches realized with drugs that have not these values standardized yet by this committee. We classified the strains in susceptible (S), susceptible-dose dependent (S-DD), intermediate (I) or resistant (R) without necessarily a correlation with clinical outcomes because for this are necessary studies of correlation "in vitro" and "in vivo". For FZ (S ≤ 8 µg/mL; 8 µg/mL > SDD < 16 µg/mL; R ≥ 16 µg/mL); IZ, VZ (S ≤ 0.125 µg/mL; 0.25 µg/mL ≥ SDD ≤ 0.5 µg/mL; R ≥ 1 µg/mL), 5FC (S ≤ 4 µg/mL; 8 µg/mL ≥ I ≤ 16 µg/mL; R ≥ 32 µg/mL); for AB (S < 2 µg/mL; R ≥ 2 µg/mL).

## RESULTS

Of the 79 samples of pigeon droppings analyzed (Table 1), 11 (13.9%) were positive for *C. neoformans*; 10 (12.6%) samples were

**Table 1**  
Yeast of the genus *Cryptococcus* isolated from environmental sources in the city of Santos, São Paulo state, Brazil

| Material        | Yeasts isolated      |      |       |                   |      |       |                     |      |       |
|-----------------|----------------------|------|-------|-------------------|------|-------|---------------------|------|-------|
|                 | <i>C. neoformans</i> |      |       | <i>C. albidus</i> |      |       | <i>C. laurentii</i> |      |       |
|                 | Pos.                 | Neg. | Total | Pos.              | Neg. | Total | Pos.                | Neg. | Total |
| Excrement       | 11                   | 68   | 79    | 10                | 69   | 79    | 7                   | 72   | 79    |
| Atmospheric air | 0                    | 37   | 37    | 2                 | 35   | 37    | 0                   | 37   | 7     |
| Total           | 11                   | 105  | 116   | 12                | 104  | 116   | 7                   | 109  | 116   |

positive for *C. albidus* and 7 (8.9%) samples were positive for *C. laurentii*.

Two samples of the total of 37 of atmospheric air were positive for *C. albidus* (5.4%) (Table 1).

Of the 11 isolates of *C. neoformans* from pigeon excrement, four were from church towers and seven from other places (Table 2).

All of the isolates of *C. neoformans* were found to belong to serotype A (*C. neoformans* var. *grubii*).

In regard to susceptibility to the antifungal agents, one sample (sample 2) presented resistance "in vitro" (MIC equal to 64 µg/mL) to

FZ. The results of the tests of susceptibility to antifungal agents are presented in Tables 3 and 4.

Fluconazole: The MIC values for FZ ranged from 0.12 to 64 µg/mL. Of the samples, 10 (91%) were susceptible and one (9%) was resistant.

Itraconazole: The MIC values for IZ ranged from 0.015 to 0.06 µg/mL. All the samples were susceptible to this drug.

**Table 2**

*Cryptococcus neoformans* var. *grubii* isolated from pigeon excrement collected in different locations, in the city of Santos, state of São Paulo, Brazil

| Location  | Sample number |            |
|---|---------------|------------|
|   | Collected     | Positive   |
| Church towers   | 17            | 04 (23.5%) |
| Old buildings   | 15            | 00 (0%)    |
| Health institutions   | 13            | 04 (30.7%) |
| Educational institutions  | 10            | 00 (0%)    |
| Dockside warehouses   | 07            | 01 (14.3%) |
| Open-air public places (downtown or beachside public squares/streets) | 17            | 02 (11.8%) |
| Total   | 79            | 11 (13.9%) |

**Table 3**

Determination of the minimum inhibitory concentration (MIC)\* (µg/mL) of *Cryptococcus neoformans* samples isolated from pigeon excrement in the city of Santos, state of São Paulo, Brazil

| Sample | 5FC | FZ   | IZ    | VZ    | AB   |
|--------|-----|------|-------|-------|------|
| 1      | 0.5 | 2    | 0.015 | 0.015 | 1    |
| 2      | 16  | 64*  | 0.06  | 0.06  | 1    |
| 3      | 8   | 2    | 0.015 | 0.06  | 0.5  |
| 4      | 4   | 0.25 | 0.015 | 0.03  | 0.5  |
| 5      | 2   | 2    | 0.03  | 0.12  | 1    |
| 6      | 4   | 1    | 0.015 | 0.015 | 0.5  |
| 7      | 1   | 8    | 0.03  | 0.25  | 0.25 |
| 8      | 2   | 0.12 | 0.015 | 0.03  | 0.5  |
| 9      | 2   | 0.5  | 0.015 | 0.03  | 0.5  |
| 10     | 8   | 0.5  | 0.015 | 0.06  | 0.5  |
| 11     | 2   | 0.25 | 0.015 | 0.03  | 0.25 |

\* resistant strain according to NCCLS, 2002; 5-FC = 5-flucytosine; FZ = fluconazole; IZ = itraconazole; VZ = voriconazole; AB = amphotericin B

**Table 4**

Evaluation of the range MIC and the susceptibility profile of the isolates

| Drug | Isolate number | Range MIC (µg/mL) | Susceptibility profile (%) |                  |                |                |
|------|----------------|-------------------|----------------------------|------------------|----------------|----------------|
|      |                |                   | S <sup>a</sup>             | SDD <sup>b</sup> | I <sup>c</sup> | R <sup>d</sup> |
| FZ   | 11             | 0.12-64           | 91                         | -                | -              | 9              |
| IZ   | 11             | 0.015-0.06        | 100                        | -                | -              | -              |
| VZ   | 11             | 0.015-0.25        | 91                         | 9                | -              | -              |
| 5 FC | 11             | 0.5-16            | 73                         | -                | 18             | 9              |
| AB   | 11             | 0.25-1            | 100                        | -                | -              | -              |

a: susceptible; b: susceptible-dose dependent; c:intermediate; d: resistant

Voriconazole: The MIC values for VZ ranged from 0.015 to 0.25 µg/mL. Ten (91%) of the samples were susceptible and one (9%) presented a susceptibility dependent on dose.

5-Flucytosine: The MIC values for 5FC ranged from 0.5 to 16 µg/mL. Eight (73%) of the samples were susceptible, two (18%) were intermediate and one (9%) presented a susceptibility dependent on dose.

Amphotericin B: The MIC values for AB ranged from 0.25 to 1.0 µg/mL. All of the samples were susceptible to this antifungal agent.

The results were interpreted according to document M 27 A2<sup>24</sup>.

## DISCUSSION

In a study carried out in 1994 in Mexico City, 251 samples of pigeon excrement were analyzed and *C. neoformans* was isolated in 52 (20.7%). The greatest frequency of the isolation of this yeast was from samples collected from public buildings (31.2%), followed by churches (22%) and residences (13.3%)<sup>7</sup>. In another evaluation, 522 samples of pigeon excrement collected from 14 cities in Puerto Rico were analyzed, of which 129 (24.7%) were positive for *C. neoformans*, all of the isolates belonging to the variety *neoformans*<sup>29</sup>.

Some researchers carried out a study on 634 samples of pigeon excrement collected in Turkey, in 1998, wherein *C. neoformans* was isolated from only 29 (4.6%) of the samples. Most of the isolates were obtained from samples collected from rooftops and pigeon nests<sup>32</sup>.

In the city of Rio de Janeiro, in 1993, 83 samples were studied from the soil and 13.2% of them were positive for *C. neoformans*<sup>21</sup>.

In a study carried out in the city of São Paulo, it was encountered positivity for this fungus in 21% of the places sampled from pigeon droppings<sup>23</sup>, while in the city of Rio de Janeiro, it was found a positivity of 31% in church towers with accumulation of pigeon excrement<sup>2</sup>.

The present study found positivity for *C. neoformans* in 13.9% of the samples of pigeon droppings from church towers, old buildings and open-air public places such as squares/parks and streets either downtown or at the beachside.

The method used in the present study was the same as that used by other authors<sup>2,23</sup> and was unlike that used by others<sup>21</sup> where samples were taken only from the soil with pigeon excrements.

It is likely that the low rate of isolation of the fungus in the current study is due to the low quantity of pigeon excrement available and the high temperatures in the city of Santos during the summer, as well as in other seasons. *Cryptococcus* does not grow at temperatures above 40 °C and the excrement is moreover subject to leaching as well as exposure to direct sunlight which can sterilize the sites contaminated by this yeast<sup>16</sup>. The saprophytic sources of *C. neoformans* have been researched in various parts of the world, but many questions concerning the ecology of the fungus and the epidemiology of cryptococcosis remain unresolved. In nature, excrement from pigeons and other birds, as well as from soils contaminated by these materials, represents the

most significant source of *C. neoformans* var. *neoformans*, while *C. neoformans* var. *gattii* has been isolated from vegetal fragments of trees in some cryptococcosis-endemic areas<sup>13,20,30</sup>. In Brazil, both varieties of the fungus are recognized as etiological agents of cryptococcosis<sup>28</sup>.

The incidence of cryptococcosis has shown marked increases over the last years. The number of reported cases of cryptococcosis has risen to relatively high levels, and is considered the most frequent systemic infection of opportunistic fungus in patients with Aids<sup>14</sup>. Official data indicate that cryptococcosis occurs in 7% to 8% of the patients with Aids in the United States, Europe and South America and in up to 35% of these patients in Africa. In Brazil it occurs in around 12% of these patients, with high rates of morbidity and mortality<sup>26</sup>.

It was reported a 54% isolation rate for *C. neoformans* from dust taken from houses occupied by Aids patients<sup>31</sup>.

One hundred and fifty four human residences were analyzed, collecting 824 samples of dust from inside the houses and from the soil and from bird excrement outside the houses. *C. neoformans* var. *grubii* was isolated from 20 (13%) of the residences; more specifically, from five of the 32 residences of Aids patients associated with cryptococcosis, four (8.9%) of the 45 residences of Aids patients not associated with cryptococcosis, and 11 (14.3%) of the 77 residences of apparently healthy individuals. The authors found that the main factor associated with household contamination by *C. neoformans* was the presence of birds either within the house or in its environments<sup>27</sup>.

In Brazil, serotype A (*C. neoformans* var. *grubii*) is the prevailing serotype found in environmental sources, such as pigeon excrement, vegetable remains and atmospheric air, followed by serotype B<sup>25</sup>.

In the current study the 11 isolates belonged to serotype A, in agreement with the literature review carried out by some authors concerning the Brazilian isolates of *C. neoformans*<sup>25</sup>. This same serotype A is also the most frequent in Brazilian clinical isolates (95%), followed by serotype B (18.2%) as reported in the above-mentioned review.

The samples were analyzed in respect to their susceptibility profile in regard to the five antifungal agents by way of the microdilution method. One strain was resistant to fluconazole, revealing a primary resistance to this drug; this finding is especially significant as this isolate was from an environmental sample. Other studies involving strains taken from the environment have warned about the resistance to this azolic derivative that is often used in the treatment of cryptococcal meningitis<sup>2</sup>. In respect to the other antifungal agents, all of the samples were either susceptible (IZ and AB) or presented dose-dependent susceptibility (VZ and 5FC).

So, this study has demonstrated that antifungal susceptibility results obtained by using the EUCAST proposed standard reference procedure are in close agreement with those achieved by the NCCLS M27A2 procedure. A great advantage of EUCAST standard is the reduction of the incubation time needed to determine the MIC (from 72h to 48h and 48 to 24 h ) and by this way, it may be more predictive of "in vivo" outcome for isolates that give unclear visual endpoints at a longer

incubation time due to trailing growth. It is apparent that the trailing phenomenon can be an important potential source of inaccuracy in MIC determination in these cases<sup>1</sup>.

The results obtained provide a susceptibility profile of the environmental isolates, nevertheless additional studies in relation to the appropriate conditions for the realization of the tests and the establishment of clinical correlations should be undertaken.

These findings reinforce the hypothesis that pigeons droppings are an important factor of yeast infection in the urban environment, underscoring the importance of our study for a city with a high Aids rate, as is the case in Santos.

## RESUMO

### Amostras ambientais de *Cryptococcus neoformans* var. *grubii* na cidade de Santos, SP, Brasil

Analisaram-se 116 amostras, sendo 79 de fezes de pombos e 37 de ar atmosférico de regiões com acúmulo de fezes. Isolou-se *Cryptococcus neoformans* var. *neoformans* de 11 (13.9%) destas amostras. Outras espécies de *Cryptococcus* também foram isoladas destas amostras tais como *C. laurentii* (8.9%) e *C. albidus* (12.6 %), o qual também foi isolado de amostras do ar (5.4 %). Todas as amostras de *C. neoformans* foram sorotipo A (*C. neoformans* var. *grubii*). Em relação à avaliação do perfil de sensibilidade às drogas antifúngicas (5-fluorocitosina, fluconazol, itraconazol, anfotericina B e voriconazol) pelo método da microdiluição (EUCAST, 2002), destacou-se a presença de uma amostra com valor de concentração inibitória mínima (CIM) elevado para fluconazol, sendo de grande significância, uma vez tratar-se de isolado ambiental.

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