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DNA fingerprinting pattern and susceptibility to antifungal drugs in *Cryptococcus neoformans* variety *grubii* isolates from Barcelona city and rural environmental samples

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Abstract

Cryptococcus neoformans var. *grubii* (serotype A) was isolated from 12 soil samples mixed with pigeon droppings (16.9%) from 71 soil samples in Barcelona and rural areas of Catalonia. *C. neoformans* was not isolated from indoor dust and *Eucalyptus* debris. PCR fingerprinting was performed in 22 representative isolates and all of them corresponded to the VNI pattern. Susceptibility testing for the 22 isolates of *C. neoformans* var. *grubii* showed that all of them were susceptible to amphotericin B. Three isolates presented MICs (Minimal Inhibitory Concentrations) ≥ 1 μg/ml to Itraconazole, five MICs ≥ 1 μg/ml to ketoconazole and four were fluconazole resistant, (MICs ≥ 64 μg/ml), while three of them were shown to have MICs ≥ 1 μg/ml to voriconazole. In spite that all isolates presented the same DNA fingerprinting pattern, the susceptibility to antifungals is very variable. The possibility of acquiring cryptococcosis infection with primarily resistant environment strains is feasible.

Key words: Barcelona, *Cryptococcus neoformans*, environmental isolates, *in vitro* susceptibility, PCR fingerprinting, triazoles, Barcelona

Introduction

Classically only one species, *Cryptococcus neoformans* with two varieties and four serotypes were described. Nowadays following the proposals of Franzot et al. [1] and Kwon Chung et al. [2] two different species and four different serotypes are accepted: *Cryptococcus neoformans* var. *neoformans* for serotype D, *C. neoformans* var. *grubii* for serotype A and *C. gattii* serotypes B and C. They constitute the main agents of human and animal cryptococcosis.

C. neoformans var. *neoformans* and variety *grubii* are cosmopolitan and cause cryptococcosis all over the world, pigeon (*Columba livia*) droppings are its major natural reservoir. In contrast, *C. gattii* (formerly *C. neoformans* var. *gattii*) has a more restricted geographical distribution, being prevalent in tropical and subtropical areas where it has been isolated from different species of *Eucalyptus* and other trees [3]. It has also been isolated in Spain from goats with cryptococcosis [4]. This yeast was also isolated from domestic dust in Rio de Janeiro [5]. Until now, there are no information about the presence and its prevalence in environment samples in Barcelona, a large Mediterranean city, where human cryptococcosis had been a high prevalence [6].

In 2003, a molecular epidemiological study by Meyer et al. [7] typed cryptococcal strains from

environmental and clinical origins with PCR fingerprinting. The isolates were classified into eight genotypes: VNI and VNII for A serotype, VNIII AD serotype, VNIV D serotype. The genotypes of *C. neoformans* var. *gattii* were VGI, VGII, VGIII for the B and VGIV for C serotypes. It was recommended that the *Cryptococcus*-positive isolates should be typed by PCR fingerprinting, by amplifying the repetitive and hypervariable regions of the genome, with a single primer specific to minisatellite or microsatellite DNA.

Cryptococcus neoformans susceptibility to current antifungal drugs in environment isolates is not well known [8] and there is scarce information on the susceptibility of non-clinical strains of *C. neoformans* to voriconazole [9].

The main goal of this work was to study the prevalence of *Cryptococcus* species and serotypes in natural habitats in Catalonia characterizing the isolates by PCR fingerprinting, and studying its susceptibility to current antifungal drugs, in order to provide a global view of the *Cryptococcus* ecology in this Mediterranean area.

Materials and methods

A total of 303 samples were collected from Barcelona city and rural areas from Catalonia in the northeast of the Iberian Peninsula during autumn and spring of the years 1998–2000. The sample distribution was as follows:

(1) Indoor dust samples (n = 100). Dust samples were taken from 100 different houses of Barcelona city, seven of those from AIDS patients hospitalized with cryptococcosis. Another 15 samples were from the houses of AIDS patient without cryptococcosis and 78 were from the houses of healthy persons. The samples were collected with a broom and a dustpan from the bedroom and living room. The dust from each abode was pooled and stored at 4 °C in individual dispensable plastic bags.

(2) Soil with pigeon droppings (n = 71). Seventy-one samples were collected from soil mixed with pigeon droppings from different streets and squares, from the outside of public buildings, around fountains, churches and monuments.

(3) Eucalyptus trees (n = 132). The Department of "*Agricultura Ramaderia i Pesca de la Generalitat de Catalunya*" provided information regarding the *Eucalyptus* species growing in Catalonia as well as their location. *Eucalyptus* samples were leaves, bark, soil with *Eucalyptus* debris, hollow tree debris, fruits and flowers. Fifty samples were from *E. camaldulensis*, and the remainder 82 from *E. globulus* and *E. dalrympleana*.

Samples were treated as described by Lazera et al. [5]. Then 0.1 ml of each supernatant was cultured on 10 Petri dishes containing *Guizotia abyssinica* agar seeds medium [10].

In five negative dust samples a suspension of 3×10^6 cells of *Cryptococcus neoformans* were added for growth control.

Eleven domestic dust samples were processed in duplicate at the Mycology Laboratory, Evandro Chagas Institute, in Brazil.

The cultures were incubated at 25 ± 1 °C in darkness and were checked daily for a week. All brown-pigmented colonies were subcultured in Sabouraud dextrose agar (bioMérieux S.A. 69280 Marcy l' Étoile-France) for identification.

Urease tests, susceptibility to cycloheximide at 0.01% and sugar assimilation (Auxacolor; Biorad, Marnes la Coquette, France) were performed with all suspicious colonies. The remaining yeasts were identified with the same sugar assimilation test. Once *Cryptococcus* was identified, isolates were cultured in CGB medium (L-canavanine–glycine–bromothymol blue) for differentiating *Cryptococcus gattii* from the *Cryptococcus neoformans*. The serotyping was performed by agglutination with a specific polyclonal antibody from the capsular polysaccharide using the Crypto-Check kit (Iatron Labs Inc., Tokyo, Japan).

PCR fingerprinting

For PCR typing and antifungal susceptibility, two colonies were randomly taken for each positive sample except in two cases in which only one colony was grown, then 22 representative isolates were studied.

DNA was extracted following the method of Lehman et al. [11].

Oligonucleotides of the minisatellite specific core sequence of the wildtype phage M13 (5′ GAGGGTGGCGGTTCT 3′) and of the simple repetitive sequence $(GACA)_4$ were used as single primers in the PCR. Amplification products were detected by staining 2% agarose gels with ethidi-

um bromide and photographing them under ultraviolet light.

Molecular types were assigned, according to the major bands in the patterns. All visible bands are included in the analysis, independent of their intensity [12].

For comparing, strain LA190 classified as VNI type by Meyers et al. [7], was used as reference.

The computer program Gel Doc 2000 software (Biorad Labs. Italy) was used to determine the genetic relatedness of the strains.

Antifungal susceptibility

The *in vitro* activity of amphotericin B (AMB), ketoconazole (KNZ), itraconazole (ITZ), fluconazole (FNZ) and voriconazole (VNZ) was tested against the same 22 representative strains by using the reference microdilution method M27-A (*National Committee for Clinical Laboratory Standard*) [13]. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as reference trains. Amphotericin B was provided by Squibb Labs (Esplugues de Ll; Spain), KNZ and ITZ from Janssen Research Foundation, (Beerse, Belgium), FNZ and VNZ from Pfizer Inc. (Central Research, Sandwich, UK).

FNZ was solubilized in sterile water and the rest in 100% dimethyl sulfoxide (DMSO).

Test was performed on sterile microdilution plates. RPMI 1640 medium (Sigma Chemical, St Louis, Missouri 63103, USA) with glutamine, but not bicarbonate, and 2% glucose with 0.165 M/l MOPS buffer (pH 7.2) were employed. The antifungal concentrations ranged from 0.03 to 16 μg/ml for AMB, KNZ, ITZ and VNZ and 0.125–64 μg/ml for FNZ.

The yeast inocula were adjusted to 0.5–2.5×10^3 CFU/ml. The plates were incubated at 35 °C and Minimal Inhibitory Concentrations (MICs) were read after 24 h for *C. parapsilosis* and *C. krusei*, and after 48 h for *C. neoformans*. The endpoints were determined visually and spectrophotometrically (A_{420}) after agitation with an automatic microplate reader (Multiskan, Labsystems, MS, Finland).

The MIC for AMB was the lowest drug concentration in which there was absence of turbidity. The MIC of azoles was the lowest drug concentration in a well that produced 80% reduction in turbidity compared with the growth control. Values of breakpoints proposed by Nguyen and Yu [14] were applied.

After the last reading was done, 10 μl from every well of the MIC and consecutives were seeded into Sabouraud dextrose agar for Minimal Fungicidal Concentration (MFC) determination. MFC was considered to be the minimum antifungal concentration that totally inhibited yeast growth.

Results and discussion

Cryptococcus neoformans was isolated from 12 soil mixed with pigeon droppings (16.9%), most of them from public areas of the old town of Barcelona, a city with a high population density of 316 inhabitants/hectare. Two other positive samples were obtained from small villages in Girona.

Eighty-five colonies of *Cryptococcus neoformans* were isolated from the positive samples, the yeast density was between 5×10^2 and 9×10^3 CFU/g . All of them corresponded to the variety *grubii* (serotype A). This variety has previously been isolated from environmental samples in other parts of Spain [15]. The prevalence of *C. neoformans* var. *grubii* in environmental specimens is in agreement with the results obtained for large cities in other European countries while the D serotype has also been found in France in pigeon droppings [16] and Belgium [17]. In Spanish clinical isolates, the prevalence of var. *grubii* was 62% although there were found to be significant regional differences [18].

Dust control samples inoculated with *C. neoformans* were always positive but *Cryptococcus neoformans* was not isolated from indoor dust samples, however, other yeasts were identified, including *Cryptococcus laurentii* ($n = 3$), *Cryptococcus albidus* ($n = 1$), *Candida famata* ($n = 2$), *C. zeylanoides* ($n = 4$), *C. lipolytica* ($n = 1$), other *Candida* sp. ($n = 23$), *Rhodotorula* sp. ($n = 8$) and *Aureobasidium* sp. ($n = 1$).

The 11 samples of indoor dust sent to Brazil and re-processed at the Mycology Laboratory were also found to be negative for *Cryptococcus neoformans*. These results suggest that indoor dust is not relevant as reservoir for *C. neoformans* in Barcelona.

Cryptococcus neoformans and *Cryptococcus gattii* were not isolated from *Eucalyptus* samples.

Recently, it has been described the first human autochthonous case of brain abscess by *Cryptococcus gattii* in Spain [19], but this species has not been isolated from environment; however, a larger number of samples and the inclusion of other materials, could allow their isolation as has been reported by others [20, 21].

The PCR fingerprinting of 22 selected isolates revealed a high level of homogeneity, with all isolates having the VNI pattern (7). Similar results were observed in most A serotype clinical strains isolated in other parts of Spain that were included in the Meyer's study [7].

Table 1 presents the MIC results for AMB, KNZ, ITZ, FNZ and VNZ using the 22 representative isolates. Results of MFC are presented in Table 2.

MICs of the *Candida* reference strains agreed with the NCCLS proposed values [13]. For AMB, all the *Cryptococcus neoformans* isolates were inhibited by MICs of 0.5 μg/ml. The MFC for the same antifungal showed a low GM of 0.31 μg/ml. With regards to KNZ, four isolates showed MICs of 8–16 μg/ml, three of these isolates presented MICs of 4, 8 and 16 μg/ml, to ITZ.

The highest MICs were seen for FNZ, four isolates presented MICs ≥ 64 μg/ml and another nine isolates MICs between 16 and 32 μg/ml. One of the strains with an MIC >64 μg/ml was inhibited with 0.25 of ITZ and 0.5 μg/ml of VNZ. The remaining three isolates with highest MICs to FNZ showed 8–16 μg/ml with VNZ.

Until now, there has been no reference method to determine MFCs for *Cryptococcus*, which could be useful to predict therapy failures. MFCs were highest for FNZ but six isolates presented values from 2 to 16 μg/ml against ITZ and KNZ. Five strains also showed MFCs from 2-to 16 μg/mL to VNZ.

Many studies refer to the *in vitro* antifungal susceptibility of clinical *C. neoformans* isolates [22]; however, data are scarce concerning environmental isolates. Franzot and Hamdan [8] did not find any difference between the susceptibilities of environmental and clinical isolates to amphotericin B, 5-flucytosine, ketoconazole, fluconazole and itraconazole, being all the 15 isolates susceptible to these azoles. Pfaller et al. [23] studied the *in vitro* activity of 566 clinical isolates of *C. neoformans* from Africa and USA. In this vast study, 90% of the strains were inhibited by 16 μg/ml of fluconazole while only three strains (0.5%), all from the USA, showed MICs ≥ 16 μg/ml. Two of the strains resistant to fluconazole showed MICs ≥ 1 μg/ml to voriconazole.

In our study, the coincidence of highest MICs and CFMs to the four azoles in most of resistant strains, suggest cross-resistances between them as it has been previously proposed [9].

Table 1. Minimal Inhibitory Concentrations (MIC) of 22 environmental isolates of *Cryptococcus neoformans* var. *grubii,* frequency MIC, range, Geometric mean (GM), MIC 90% and MIC 50% to amphotericin B, ketoconazole, itraconazole, fluconazole and voriconazole

MIC (μg/ml)	Antifungals				
	Amphotericin B	Ketoconazole	Itraconazole	Fluconazole	Voriconazole
<0.03	2	3	2	0	2
0.06	2	4	7	0	3
0.125	6	2	7	1	12
0.25	8	5	1	0	1
0.5	4	3	2	0	1
1	0	1	0	0	0
2	0	0	0	0	0
4	0	0	1	1	0
8	0	2	1	7	1
16	0	2	1	8	2
32	0	0	0	1	0
64	0	0	0	4	0
Range	<0.03–0.5	<0.03–16	<0.03–16	0.125–64	<0.03–16
GM	0.17	0.32	0.18	12.8	0.20
MIC90%	0.5	8	4	64	8
MIC50%	0.25	0.25	0.125	16	0.125

Table 2. Minimal Fungicidal Concentrations (CFM) of 22 environmental isolates of *Cryptococcus neoformans* var. *grubii,* frequency MIC, range, Geometric mean (GM), MIC 90% and MIC 50% to amphotericin B, ketoconazole, itraconazole, fluconazole and voriconazole

MFC (μg/ml)	Antifungals				
	Amphotericin B	Ketoconazole	Itraconazole	Fluconazole	Voriconazole
< 0.03	0	1	1	0	1
0.06	0	1	0	0	2
0.125	3	4	2	0	3
0.25	9	3	8	0	9
0.5	10	6	5	1	0
1	0	1	0	0	2
2	0	0	1	0	0
4	0	1	0	0	0
8	0	0	0	4	2
16	0	5	5	6	3
32	0	0	0	4	0
64	0	0	0	7	0
Range	0.125–0.5	< 0.03–16	0.125–16	0.5–64	< 0.03–16
GM	0.31	0.70	0.70	21.2	0.49
MFC90%	0.5	16	16	64	16
MFC50%	0.125	0.5	0.25	16	0.25

The results of this work can contribute to a better understanding of the ecology of *Cryptococcus neoformans* in the South of Europe and remarks the possibility of acquiring cryptococcosis infection with primarily resistant strains [24].

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