Cryptococcus neoformans var. *grubii* - Pathogenicity of environmental isolates correlated to virulence factors, susceptibility to fluconazole and molecular profile

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The pathogenicity of Cryptococcus neoformans *is heterogeneous and is associated with the expression of virulence factors. This study aimed to correlate the pathogenicity of* C. neoformans *var.* grubii *in BALB/c mice with in vitro virulence factors, fluconazole minimal inhibitory concentrations (MICs) and molecular profiles, before and after animal passage. Ten environmental isolates and one ATCC strain of* C. neoformans *var.* grubii *mating type α were evaluated. Most isolates (91%) killed 50% or more of the infected animals by day 24 postinfection and were recovered from the lungs and brains of surviving animals on days 7 and 14 postinfection. The burden of yeast in the lungs was more variable than that in the brain. The differences in the expression of virulence factors (growth at 37°C, presence and size of the capsule and production of melanin, urease, proteinase and phospholipase) by most isolates pre and postpassage in animals were not statistically significant. The fluconazole MICs in postpassaged lines differed by a one-dilution from the MIC of the corresponding prepassaged line for six isolates. Using molecular typing [polymerase chain reaction-fingerprinting with (GACA)$_4$ and M13], eight isolates were identified as VNI and three as VNII. We concluded that different isolates with the same molecular and phenotypic profiles, including isolates that are markedly hypervirulent, span a wide range of virulence and there were no changes in virulence factors in the postpassaged lines when compared with the corresponding nonpassaged lines.*

Key words: *Cryptococcus neoformans* - virulence factors - pathogenicity - molecular typing

Cryptococcus neoformans, an opportunistic fungus, is responsible for infections in immunocompromised hosts and three serotypes have been recognised: A (var. *grubii*), D (var. *neoformans*) and the hybrid AD. *C. neoformans* has well-defined mating types, constituted by one locus with two alternative alleles, mating type a and mating type α (Casadevall & Perfect 1998, Campbell & Carter 2006). Clinical and environmental isolates of *C. neoformans* are mostly of mating type α (Okabayashi et al. 2006).

Genetic differences between serotypes and among different isolates, both clinical and environmental, have been shown by phenotypic, immunological and, more recently, molecular typing methods. Techniques based on polymerase chain reaction (PCR) have been used to study the epidemiology of *C. neoformans*. PCR-based methods are more sensitive, specific and fast and are less likely to be influenced by external factors than traditional methods based on phenotypic characteristics (Meyer et al. 1993). By applying molecular techniques, it was possible to group *C. neoformans* into at least four molecular types: VNI and VNII (both var. *grubii*, serotype A), VNIII (serotype AD) and VNIV (var. *neoformans*, serotype D), according to Meyer et al. (2003). The molecular type VNI, followed by VNII, has predominated in clinical and environmental isolates in the central regions of Brazil, especially in the South, Southeast and Central Regions (Trilles et al. 2008).

C. neoformans pathogenicity is related to virulence factors including the presence and size of the polysaccharide capsule, the production of melanin by laccase, growth at 37ºC and secretion of the enzymes phospholipase, proteinase and urease (Kozel 1995, Casadevall & Perfect 1998). Studies on pathogenicity and on these virulence factors are fundamental to the understanding of the pathogenesis and biology of *C. neoformans*. Some of these virulence factors have been used in phenotypic typing (Kozel 1995, Idnurm et al. 2005).

It has been shown that the phenotypic characteristics of *C. neoformans* may be related to virulence and its susceptibility to antifungal drugs may change in cases of recurrent infection in immunocompromised patients, after experimental inoculation in animals and after long in vitro storage periods (Currie et al. 1995, Franzot et al. 1998, Blasi et al. 2001, McClelland et al. 2005, Clancy et al. 2006, Silva et al. 2006b). This phenomenon, called microevolution, has been reported by several authors (Franzot et al. 1998, Blasi et al. 2001, Almeida et al. 2007, Cavalcante et al. 2007).

C. neoformans can cause life-threatening fungal infections in immunocompromised individuals. The source of infection is the environment and there are some phenotypic and genotypic differences among isolates. Thus, this study aims to correlate pathogenicity and (i) virulence factors, (ii) the fluconazole minimal inhibitory concentration (MIC) and (iii) the molecular profiles obtained by PCR-fingerprinting in pre and postpassaged lines of environmental isolates of *C. neoformans* var. *grubii* from the city of Ribeirão Preto, state of São Paulo (SP), Brazil. In addition, we analyzed whether there were temporal differences in the parasite burden in the lungs and brains of animals at seven and 14 days after infection.

SUBJECTS, MATERIALS AND METHODS

Microorganisms - Ten environmental isolates of *C. neoformans* from bird excreta collected in Ribeirão Preto (Pedroso et al. 2009a) and the strain ATCC 90112 (var. *grubii*, mating type α) were analyzed in this study. Identification was conducted by classical methods based on urease production, the presence of a capsule, melanin production in L-dopa medium, the assimilation of nitrogen and carbon sources and the canavanine-glycine-bromothymol blue test. All identifications were confirmed using the ID32C system (bioMérieux AS, Marcy l'Etoile, France) and by DNA amplification using a species-specific forward primer CN-ITS1 (5'-TGGACTTTGGTCCATTTATCTAC-3') for the internal transcribed spacer (ITS) 1 and a universal reverse primer ITS4 (5'-TCCTCCGCTTATT-GATATGC-3'), according to Li et al. (2003). Isolates were incubated at 25-30ºC in Sabouraud dextrose agar (SDA) and subcultured bimonthly.

Determination of serotype and mating type - Genomic DNA was extracted as described by Bolano et al. (2001) with some modifications (Pedroso et al. 2009b) and the extracted DNA was maintained at -20ºC until use. Serotypes and mating types were determined using PCR with the primers described in Table I. Amplification reactions were performed in a final volume of 50 µL containing 60 ng genomic DNA, 1 x PCR buffer (Fermentas, Ontario, CA), 1.5 mM magnesium chloride, 0.2 mM each

dNTP, 21 pmol of each primer and 1 U Taq polymerase (Fermentas, Ontario, CA). Reaction conditions were as follows: initial denaturation at 94ºC for 10 min, 36 cycles at 94ºC for 1 min, annealing according to the temperature specified in Table I for 1 min, and extension at 72ºC for 1 min, and a 10-min final extension at 72ºC. Amplification products were maintained at 4ºC and then submitted to 1% agarose gel electrophoresis in 1 x tris-acetate (TAE)-ethylenediamine tetraacetic acid buffer at 75 V for 2 h. For each reaction, a known strain was used as a positive control. DNA fragments were observed under UV light in an ethidium bromide-stained gel, which was photographed using an image capturing system.

Experimental infection - Experiments were conducted according to the Ethical Principles in Animal Experimentation and were approved by the Committee on Ethics in Animal Use of the University of São Paulo, Ribeirão Preto Campus (protocol 05.1.327.53.7).

Female isogenic BALB/c mice, weighing 20 g, were utilized for the experimental infections. Microorganisms from recent cultures (72 h at 35ºC in SDA) were transferred to culture tubes containing 20 mL of brain heart infusion broth and incubated under constant agitation (200 rpm) at 35ºC for 48 h. Cells were separated by centrifugation (5,000 g for 5 min), washed three times with sterile physiological solution and adjusted to a concentration of 5×10^6 cells/mL. Mice were inoculated with 100 µL of cell suspension (5×10^5 cells) through the lateral vein in the tail. Each isolate preparation was used to inoculate nine animals (for a total of 11 groups of 9 animals each), of which five were observed daily for the development of infection and death. Two animals were euthanized by cervical dislocation on day seven and two on day 14. The brain and lungs were removed for determination of the number of colony forming units (CFUs). The removed organs were weighed and homogenized in a T18 basic Ultra Turrax Mixer (IKA, Wilmington, NC, USA) at 18,000 rpm for 30 s. Thereafter, serial dilutions to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were prepared and aliquots of 100 µL of each dilution were plated on SDA-chloramphenicol and incubated at 37ºC for 72 h. The results are expressed as mean of $\log_{10}$ CFU/g of each organ.

TABLE I

Nucleotide pairs and annealing temperatures employed in serotype and mating type determinations

Serotype and/or mating	Sequences (forward and reverse)	Annealing temperature (ºC)	Product (bp)	References
A *MAT* α	5'-GCTTGGCGTACGCTGTGG-3' 5'-GGCGTCGCTTGGTACGGGT-3'	60	1,150	Figueiredo (2007)
A *MAT* a	5'-TCCACTGGCAACCCTGCGGAG-3' 5'-ATCAGAGACAGAGAAGCAAGAC-3'	55	865	Okabayashi et al. (2006)
D *MAT* α	5'-GATTATCTCAGCAGCCACG-3' 5'-AAATCGGCTACGGCACGTC-3'	60	443	Okabayashi et al. (2006)
D *MAT* a	5'-GGTCCGCACTTGGGTAAGTG-3' 5'-GGCGTAGACGGACGAGCTC-3'	60	1,420	Figueiredo (2007)

bp: base pairs.

Evaluation of the virulence factors - Virulence factors were analyzed in pre and postpassaged lines in mice. Capsule production and thickness was determined according to the method of Pedroso et al. (2009b) using liquid Sabouraud medium containing 0.1% glucose. The results are expressed as scores: 0 for isolates without visible capsules, 1 for isolates with a small amount of visible capsule, 2 for isolates with a moderate amount of capsule (cell diameter plus capsule smaller than twice the cell diameter) and 3 for isolates with large capsules (cell diameter plus capsule equal to or larger than twice the cell diameter). Tests were scored by the same methods in both conditions.

Growth at 37ºC was measured according to the method of Pedroso et al. (2009b) and the results are expressed as scores: 0, no growth, 1, poor growth (few isolated colonies), 2, moderate growth (confluent colonies but smaller than the control colonies) and 3, growth equivalent to the control incubated at 30ºC.

Plates containing L-dopa medium (1.5% agar, 15 mM glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, 3.0 µM thiamine and 1.0 mM L-dopa) were used to measure the production of melanin after incubation at 30ºC for five days. Pigment intensity was recorded after grouping the isolates with equally coloured colonies according to the following scoring system: 0 for colonies without pigment, 1 for light brown colonies, 2 for brown colonies, 3 for dark brown colonies and 4 for black colonies.

Urease activity was determined by adding 100 µL of cell suspension (equivalent to McFarland scale 2) to 500 µL of urea liquid medium (Christensen urea). After 24 and 48 h of incubation, the activity was scored as follows: 0 for no colour (absence of activity), 1 for light pink colour, 2 for moderate pink colour and 3 for intense pink colour. Proteinase activity was detected on agar plates containing albumin that had been incubated at 30ºC for 14 days (Aoki et al. 1994). Plates containing egg-yolk agar were used to detect phospholipase activity after incubation at 30ºC for seven days (Echeverría et al. 2002). An opaque zone (phospholipase activity) or a dense white zone of hydrolysis (proteinase activity) around a yeast colony was expressed as Pz = cd/cdp, where cd is the colony diameter and cdp is the colony diameter plus the precipitation zone.

Susceptibility to fluconazole - The methodology followed recommendations of the M27-A2 document of CLSI/NCCLS (2002). Susceptibility was visually determined by comparing the turbidity of the fluconazole-treated cultures with the turbidity of a control culture. The MIC of fluconazole was defined as the concentration producing 50% inhibition. Strains of *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used controls.

TABLE II

Cumulative percentage of dead animals (n = 5) at the indicated days after inoculation of environmental samples of *Cryptococcus neoformans*

Time (days after infection)	Cumulative										
	CN13	CN17	CN18	CN19	CN20	CN22	CN23	CN24	CN25	CN26	90112
10	-	20	-	-	-	-	-	-	-	-	-
11	-	40	-	-	-	-	-	-	-	-	-
12	-	100	-	-	-	-	20	-	-	-	-
14	-	-	-	-	20	-	-	-	-	-	-
15	20	-	-	-	40	-	-	-	-	-	20
16	60	-	20	-	60	20	-	-	20	-	-
17	80	-	-	-	80	-	40	-	40	-	40
18	100	-	40	-	-	-	-	20	60	-	60
19	-	-	-	-	-	-	60	40	80	20	100
20	-	-	-	-	100	-	-	80	100	40	-
21	-	-	-	-	-	40	80	-	-	-	-
22	-	-	-	-	-	60	100	-	-	-	-
23	-	-	-	-	-	80	-	-	-	60	-
24	-	-	60	-	-	100	-	100	-	80	-
25	-	-	80	-	-	-	-	-	-	-	-
28	-	-	100	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	100	-
40	-	-	-	20	-	-	-	-	-	-	-
62	-	-	-	40	-	-	-	-	-	-	-
72	-	-	-	60	-	-	-	-	-	-	-
79	-	-	-	80	-	-	-	-	-	-	-
82	-	-	-	100	-	-	-	-	-	-	-

-: no animal dead in this time.

Molecular typing by PCR-fingerprinting - Molecular typing was completed according to the method of Meyer and Mitchell (1995) and Meyer et al. (2003) based on fragment amplification by PCR using only minisatellite sequences of the M13 phage (5′GAGGGTGGCGGTTCT-3′) and repetitive microsatellite sequences (GACA)$_4$ as probes. The final reaction volume was 50 μL and each reaction contained 200 ng DNA, 1 x enzyme buffer (Invitrogen, CA, USA), 60 pmol primer and 2.5 U Taq polymerase. The thermocycler conditions were as follows: 10 min at 94ºC, 36 cycles of 1 min at 94ºC, 1 min at 50ºC and 1 min at 72ºC and a final extension of 10 min at 72ºC. Five microlitres of the amplified DNA sample were submitted to 1.5% agarose gel electrophoresis in 1 x TAE containing 0.5 μg/mL ethidium bromide at 70 V for 3 h. The gel was visualized by UV light and photographed using an image capturing system. The molecular types were assigned by comparison to the reference strains of each of the molecular types, VNI, VNII, VNIII and VNIV. The DNA of the reference strains H99, C12, ZG287 and NIH33 (types VNI, VNII, VNIII and VNIV, respectively) were kindly provided by Dr Joseph Heitman of Duke University Medical Center, USA (Figueiredo 2007). The molecular profiles obtained by PCR-fingerprinting were analyzed based on the presence or absence of clear and well-defined bands in the digitized gel images.

Statistical analysis - The yeast burden in animal organs was measured at two times, seven and 14 days postinfection (time = factor), for each isolate (block); that is, we used an experimental structure consisting of completely randomized blocks (McPherson 1990) to test the effects of time and isolate on yeast burden in the lungs. Time (7 or 14 days) was considered one factor and the isolates were considered to be blocks. After significant effects of the isolates in the yeast burden were identified, the analysis was followed by the multiple comparison least significant difference test (LSD) to compare pairs of isolates (McPherson 1990). In all tests we used a significance level of 5%.

The same procedures described were used to analyze the time and the isolate effects on yeast burden in the brain.

RESULTS

All isolates were serotype A and mating type α according to PCR conducted with the probes listed in Table I.

In the infection experiment, all isolates were recovered from mouse brain and lungs on seven or 14 day postinfection, with exception of CN17, which killed all animals by day 12 postinfection. Most animals (> 50%) in each group died within 12-72 days and 91% of isolates killed 50% or more animals by day 24 postinfection (Table II).

The bar graphs in Fig. 1 show results for lungs and brains, in units of log$_{10}$ CFU/g, on days seven and 14 after inoculation with the *C. neoformans* isolates.

There was no significant difference between the burdens of microorganisms in the lungs on day seven and on day 14 (p = 0.086); however, there were significant differences (p = 0.008) in the microorganism burden among the isolates. Comparison of the isolates in pairs using the LSD test revealed the following differences: the burden of CN19 was different from those of CN13, CN20,

CN24, CN25, CN26 and 90112, the burden of CN18 was different from those of CN20, CN25, CN26 and 90112, the burden of CN20 was different from those of CN22 and CN23, the burden of CN22 was different from those of CN26 and 90112 and the burden of CN23 was different from those of CN25, CN26 and 90112.

When the same analysis was applied to brain data, the results showed a statistically significant difference in the burdens of yeast on days seven and 14 postinfection (p = 0.003) and among isolates (p = 0.002). The LSD test showed that the burden of CN19 was different from those of all other isolates and that the burden of CN18 was different from that of CN20. In both analyses, CN17 was not considered in the statistical tests because all animals died by day 12 after inoculation.

The results of the investigation of virulence factors and MICs for fluconazole are shown in Table III. All prepassaged lines grew at 37ºC and produced capsules, melanin and phospholipase; only CN24 did not show urease activity by 24 h. Most isolates (54.5%) had proteinase activity. The fluconazole MICs were 4 μg/mL (CN13, CN17, CN18, CN19 and CN24), 8 μg/mL (CN20, CN22, CN23, CN25 and CN26) and 2 μg /mL for 90112. The MIC values for the control strains were 2 μg/mL for *C. parapsilosis* ATCC 22019 (expected value, 1-4 μg/mL) and 32 μg/mL for *C. krusei* ATCC 6258 (expected value, 16-128 μg/mL).

Most of the isolates were not significantly different from the prepassaged lines with respect to any studied virulence factor in vitro. After 24 h, CN24, which was negative for urease activity in prepassaged line, became
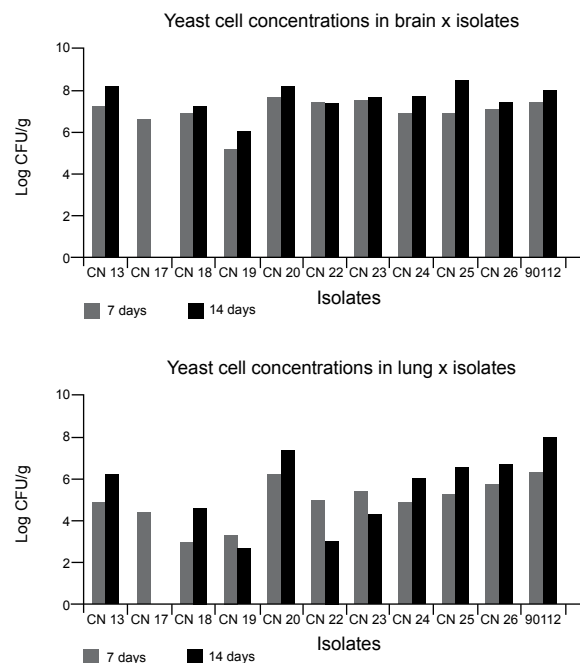

Fig. 1: concentration of viable yeast cells (log colony forming units/g) in lungs and brain of BALB/c mice, seven and 14 days after inoculation of a suspension containing 5 x 10^5 cells of different strains of *Cryptococcus neoformans* (CN). CFU: colony forming units.

TABLE III

Cryptococcus neoformans (CN) isolates characterized before and after inoculation in animals by virulence factors, fluconazole minimal inhibitory concentrations (MIC) and time of death of most animals

Isolate	Time of death[a] (days)	Growth at 37ºC (score)	Capsule (score)	Urease (24 h) (score)	Melanin production (score)	Proteinase (Pz)	Phospholipase (Pz)	MIC (fluconazol) µg/mL
CN13	16	3	2	3	3	0,91	0,74	4
CN17	12	3	1	3	4	1,00	0,83	4
CN18	24	3	3	3	4	1,00	0,59	4
CN19	72	3	2	3	4	0,85	0,69	4
CN20	16	3	2	3	3	0,90	0,89	8
CN22	22	3	2	3	3	0,83	0,79	8
CN23	19	3	2	1	4	1,00	0,60	8
CN24	20	3	3	0	4	0,87	0,67	4
CN25	18	2	1	2	3	1,00	0,71	8
CN26	23	3	2	3	3	0,83	0,67	8
90112	18	3	3	3	4	1,00	0,55	2

a: 60% of inoculated animals are dead; Pz = cd/cdp, where cd is the colony diameter and cdp is the colony diameter plus the precipitation zone.

positive but with a score 1. The fluconazole MIC changed by a one dilution for six isolates, CN24 (2 µg/mL), CN22, CN23, CN25, CN26 (4 µg/mL) and CN19 (8 µg/mL), when compared with the corresponding prepassaged lines.

PCR-fingerprinting allowed the isolates to be clustered into two molecular types, VNI (CN13, CN17, CN19 CN20, CN22, CN25 and CN26) and VNII (CN18, CN23 and CN24). Pre and postpassaged lines had identical electrophoretic band patterns, as shown in Figs 2, 3.

DISCUSSION

This study investigated the virulence of environmental isolates of *C. neoformans* var. *grubii*, mating type α, in immunocompetent BALB/c mice and analyzed the phenotype of the primary virulence factors, the fluconazole MICs and the molecular profiles by PCR-fingerprinting. The tissue yeast burden was evaluated seven and 14 days postinfection. Brain isolates obtained on day seven were compared to prepassaged lines.

The yeast burden in the brain showed considerable temporal variation (from 7-14 days), while there was no such variation in the lungs. Nine isolates from the brains and seven from the lungs had higher CFU counts after 14 days of infection. Only CN22 had a lower count on day 14 than on day seven in the brain. CN19 can be considered the least virulent isolate as it had the lowest number of CFU in the brain and lungs after both periods of incubation.

The duration of the animals' survival was also highly variable. The isolate CN17 was the most virulent, killing all mice between 10-12 days postinfection, while CN19 was the least virulent, with all mice dying between 40-82 days. This result showed the great variability in the virulence among the studied isolates. The statistical analysis identified the isolates with different behaviours when considering the tissue yeast burden and the survival duration after infection. Thus, the LSD test effectively

discriminated isolates that were different. These findings are important for future studies that analyze isolates with different characteristics and the relationship of these characteristics to the infectious process.
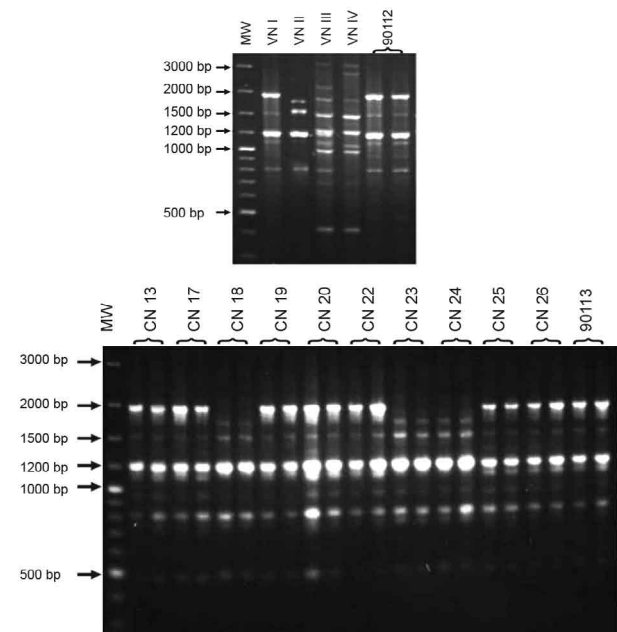

Fig. 2: agarose gel electrophoresis of amplified products by polymerase chain reaction-fingerprinting of *Cryptococcus neoformans* (CN) isolates using (GACA)$_4$ microsatellite regions as probe. Upper gel: VNI, VNII, VNIII, VNIV molecular type controls and ATCC *C. neoformans*; below: the first and second Lanes of each pair correspond to strains before and after inoculation into animals, respectively. Left Lane shows molecular weight (MW) markers (100 bp DNA Ladder Plus, Fermentas). bp: base pairs.

Of all isolates, 91% killed most experimental animals by day 24 postinfection. According to the data of Silva et al. (2006a), all strains studied that killed most animals in 24 days were considered highly virulent (69% of 62 strains). Other reports have suggested that *C. neoformans* var. *grubii* serotype A and mating type α isolates are more virulent than serotype D and mating type a isolates (Barchiesi et al. 2005). As expected, virulence was confirmed in the isolates in our study, but the variability in hypo and hypervirulence was intriguing and should be further investigated.

In vitro virulence factors, fluconazole MICs and molecular profiles were not strongly associated with the virulence in animals, suggesting the involvement of other factors. Several studies that have found an association between in vitro virulence and pathogenicity in *C. neoformans* emphasize that virulence is a multifactorial characteristic; that is, if an isolate lacks one virulence factor, this lack could be compensated by the presence of another virulence factor (Barchiesi et al. 2005, McClelland et al. 2005, Clancy et al. 2006). It has been agreed upon that the production and thickness of the capsule are important, although in some reports, thickness was the more important factor (Clancy et al. 2006) and, in others, it was not (McClelland et al. 2005). It is widely accepted that capsule thickness depends on growth con-

ditions and on the organ affected, with the capsule being thicker in the lungs (Zaragoza et al. 2009). Microevolution occurring during infection has been demonstrated to influence capsule size (Rivera et al. 1998). A recent review about this virulence factor sheds light on many obscure points, thus opening ways to new investigations in this field (Zaragoza et al. 2009).

It is also possible that limitations in the sensitivity and specificity of the detecting methods have precluded reliable quantification of virulence factors and discrimination of isolates. Some authors have suggested that rate of growth influences pathogenicity by increasing the number of microorganisms in the infected organ or the quantity of metabolites liberated (Clancy et al. 2006).

Regarding the molecular types, the isolates studied were VNI (7 isolates) and VNII (3 isolates). According to recent studies, the VNI is the type most common in clinical and environmental isolates in different regions of Brazil, with a prevalence ranging from 64-95.7% (Matsumoto et al. 2007, Trilles et al. 2008, Mora et al. 2010).

In this study, most isolates of *C. neoformans* recovered from animals did not show differences with respect to either phenotypic or molecular characteristics when compared to the prepassaged lines. Although this result demonstrated that animal passage did not change the features involved, it is possible that the time period (7 days) was insufficient for microevolution. According to several studies, microevolution occurred over longer periods of time and after several passages, as an adaptation to the host (Currie et al. 1995, Franzot et al. 1998, Blasi et al. 2001, McClelland et al. 2005, Clancy et al. 2006, Almeida et al. 2007, Cavalcante et al. 2007). One exception occurred with urease. Urease activity is an important virulence factor for dissemination and invasion and it was detected by 24 h only in the postpassaged line, demonstrating that a change in the environment led to changes in the expression of this virulence factor.

Regarding in vitro resistance to fluconazole, resistance was not detected in any of the isolates, but there were changes in the MIC of one dilution for six isolates; we do not consider this change to be substantial, according to literature data. Some authors have suggested that the MIC is related to virulence (Iwata et al. 1990, Clancy et al. 2006) and it changes after passage of the yeast in animals (Currie et al. 1995, Silva et al. 2006b). Currie et al. (1995) showed a four-fold MIC increase for amphotericin B and one to three-fold increase for fluconazole after passage of isolates in animals, although statistical analysis showed that only the amphotericin results were significant. On the other hand, Silva et al. (2006b) did not find differences in susceptibility to fluconazole or amphotericin after animal infection. Studies should be conducted with a larger number of isolates to clarify these contradicting results and to better understand the in vivo and in vitro susceptibilities of to *C. neoformans* antifungal drugs.

In the current study, it was not possible to determine if any one of the virulence factors studied had a stronger influence on pathogenicity or on tissue yeast burden than the other virulence factors. No genetic variation between pre and postpassaged lines was detected by the methodology applied. However, temporal differences
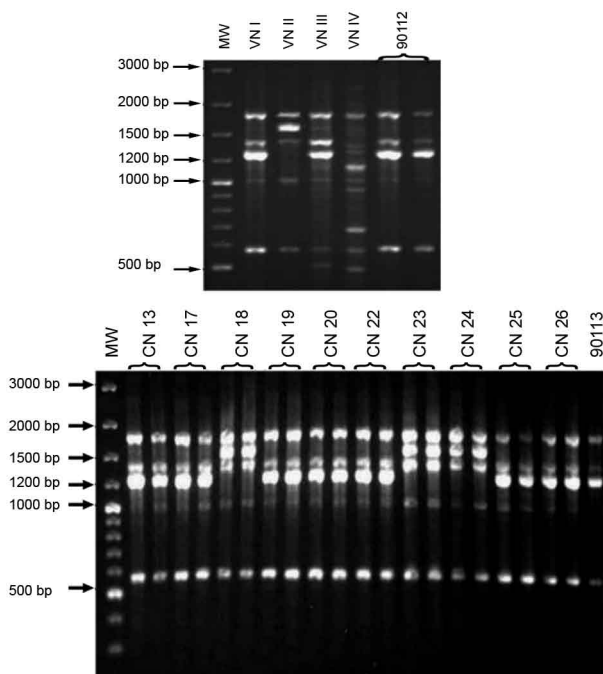

Fig. 3: agarose gel electrophoresis of amplified products by polymerase chain reaction-fingerprinting of *Cryptococcus neoformans* (CN) using M13 minisatellite regions as probe. Upper gel: VNI, VNII, VNIII, VNIV molecular type controls and ATCC *C. neoformans*; below: the first and second Lanes of each pair correspond to strains before and after inoculation into animals, respectively. Left Lane shows molecular weight (MW) markers (100 bp DNA Ladder Plus, Fermentas). bp: base pairs.

were demonstrated by the statistical tests with respect to the yeast burden in the brains of infected animals, but not in lungs. The isolates also showed large differences from one another, especially in lung yeast burden, as revealed by a multiple comparisons procedure. New studies of the factors related to *C. neoformans* virulence are necessary to develop new strategies to prevent and treat *C. neoformans* infections.

ACKNOWLEDGEMENTS

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