Molecular typing and antifungal susceptibility of clinical sequential isolates of *Cryptococcus neoformans* from Sao Paulo State, Brazil

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Abstract

The antifungal susceptibility profiles and the genetic variability of 83 sequential clinical isolates of *Cryptococcus neoformans*, including four *Cryptococcus gattii* isolates, obtained from 38 Sao Paulo AIDS patients with cryptococcal meningitis were assessed by electrophoretic karyotyping and random amplified polymorphic DNA (RAPD) analysis. The majority of the *Cryptococcus neoformans* isolates were highly susceptible to amphotericin B and fluconazole. Twenty percent of the minimum inhibitory concentration values for amphotericin B varied from 0.5 to 1 $\mu g\,mL^{-1}$. For fluconazole, 22% occurred in the range 8–16 $\mu g\,mL^{-1}$. Sequential isolates from nine patients showed a trend towards lower susceptibility to fluconazole, flucytosine, itraconazole and amphotericin B. The results of molecular typing by electrophoretic karyotyping and RAPD analysis showed the presence of 22 electrophoretic karyotypes (EK) and 15 RAPD profiles that were highly correlated. Our results provided evidence for the occurrence of genetic changes in some strains associated with microevolution during the course of infection. We also observed both microevolution and simultaneous coinfection with two distinct *Cryptococcus neoformans* strains in one patient. In some patients, we found changed EK- and RAPD patterns in association with increased MIC values.

Introduction

Cryptococcus neoformans is an ubiquitous and opportunistic yeast causing life-threatening meningoencephalitis in immunocompromised patients, who show a high tendency to relapse despite effective antifungal therapy. The fungus is commonly associated with pigeon droppings, decaying leaves and wood debris (Lazera *et al.*, 2000). The incidence of cryptococcosis in immunocompromised patients varies according to the population, the region and the period studied. From 1980 to 2002, 215 810 cases of AIDS were registered in Brazil. Six percent of these patients had cryptococcosis at the time of diagnosis, and Sao Paulo was the state with the highest incidence (Ministério da Saúde do Brasil, 2002).

Several molecular typing methods have been used in epidemiological analyses of clinical and/or environmental isolates of *Cryptococcus neoformans*, including electrophoretic karyotyping, PCR fingerprinting, random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism analysis and amplified fragment length polymorphism (AFLP) analysis (Brandt *et al.*, 1996a, b; Fries *et al.*, 1996; Franzot *et al.*, 1997; Almeida, 2000; Calvo *et al.*, 2001; Horta *et al.*, 2002; Casali *et al.*, 2003; Meyer *et al.*, 2003; Barreto de Oliveira *et al.*, 2004; Igreja *et al.*, 2004).

A South American study performed with strains from nine countries showed low genetic variability and demonstrated the presence of only three molecular types in Brazilian isolates (Meyer *et al.*, 2003). Franzot *et al.* (1997) also suggested limited genetic diversity, as did Casali *et al.* (2003). In contrast, Horta *et al.* (2002) revealed considerable genetic differences using the same PCR method, but

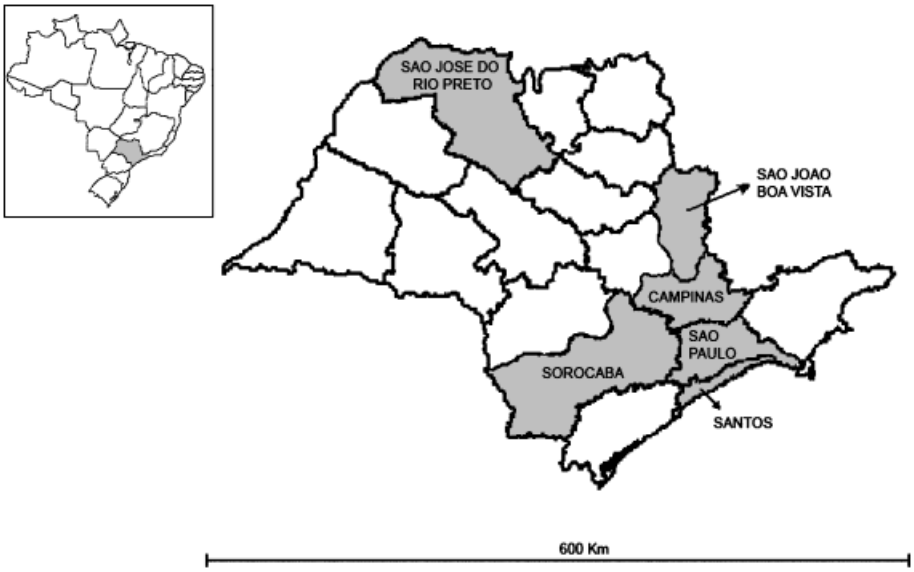

Fig. 1. Map of São Paulo State showing the location of the districts where the *Cryptococcus neoformans* and *Cryptococcus gattii* isolates were collected.

applying other primers. In another study, Calvo *et al.* (2001) found nine electrophoretic karyotype (EK) profiles among 25 Brazilian isolates of *Cryptococcus neoformans* var. *neoformans*. Few studies have analyzed sequential isolates from cryptococcosis patients. Igreja *et al.* (2004) and Rezende (2002) demonstrated the persistence of the same strain through the entire course of the disease in an individual, even under pressure of antifungal therapy. In fact, recurrent infections may be due to the persistence of the original infecting strain (Fries *et al.*, 1996; Branchini & Papaiordanou, 2000; Meyer *et al.*, 2003).

In AIDS patients, cryptococcosis is considered incurable and requires lifelong antifungal therapy (Mitchell & Perfect, 1995). Current regimens for treatment of the disease remain focused on amphotericin B, with or without flucytosine, for induction treatment, whereas fluconazole remains the agent of choice for long-term maintenance treatment (Mitchell & Perfect, 1995; Powderly *et al.*, 1995; Brandt *et al.*, 2001). Previous studies have demonstrated that most recurrences of cryptococcal meningitis during maintenance therapy were due to the persistence of the original infecting strains rather than reinfection with a new cryptococcal strain (Brandt *et al.*, 1996a, b; Casadevall & Perfect, 1998; Pfaller *et al.*, 1998) or a decrease in drug susceptibility (Currie *et al.*, 1995; Rodero *et al.*, 2003).

There is accumulating evidence that cryptococcal strains can undergo genetic changes *in vitro* and *in vivo* (Franzot *et al.*, 1997; Fries & Casadevall, 1998). These changes have been demonstrated to occur in the EK profiles after passage through mice (Kwon-Chung *et al.*, 1992) and in serial isolates obtained from individual patients (Brandt *et al.*, 1996a, b; Almeida, 2000). This phenomenon, termed microevolution, may contribute to survival in the host by providing a means to evade host defenses (Fries & Casadevall, 1998).

Even though *Cryptococcus neoformans* is a leading cause of life-threatening fungal infection among HIV-infected patients in Brazil, little is known about its susceptibility to antifungal drugs and the molecular epidemiology, especially with respect to clinical serial isolates. Hence, the aims of this study were to compare sequential clinical isolates of *Cryptococcus neoformans* from several São Paulo districts by determining their susceptibility profiles with the microdilution method and to assess their genetic variability by EK and RAPD analysis.

Materials and methods

Fungal isolates

Eighty-three isolates of *Cryptococcus neoformans* were collected at different time intervals from 38 AIDS patients from various geographic areas of Sao Paulo State (Fig. 1), before the introduction of highly active antiretroviral therapy (HAART). These isolates were maintained in the Culture Collection of the Instituto Adolfo Lutz Laboratory, a Public Health National Reference Center, located in six different cities in São Paulo State, Brazil. Twenty-two isolates originated from São Paulo, 24 from Campinas, 16 from São José do Rio Preto, 13 from Santos, six from Sorocaba and two from Sao Joao da Boa Vista.

The *Cryptococcus neoformans* cultures were grown at 30 °C on Sabouraud dextrose agar with chloramphenicol. All isolates were identified by morphologic, physiologic and biochemical characteristics (Klepser & Pfaller, 1998).

Antifungal susceptibility testing

The individual minimum inhibitory concentrations (MICs) were determined following the microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (Sanati *et al.*, 1996; NCCLS M27-A, 1997) with modifications (Rodriguez-Tudela *et al.* 1996). The modifications included the use of Roswell Park Memorial Institute (RPMI) 1640 (Sigma Aldrich Quimica SA, St Louis, MO) with L-glutamine buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid and 1 M NaOH supplemented with 18 g of glucose per liter (RPMI/2% glucose), and spectrophotometric adjustment of the inoculum preparation to match the turbidity of a 0.5 McFarland standard. The antifungal agents utilized were amphotericin B (Sigma Aldrich Quimica, S.A.), 5-fluorocytosine (Sigma Aldrich Quimica S.A.), fluconazole (Pfizer S.A., New York, NY), and itraconazole (Janssen S.A., Titusville, NJ). Amphotericin B and itraconazole were dissolved in dimethyl sulfoxide (Sigma Aldrich Quimica S.A.), and 5-fluorocytosine and fluconazole were dissolved in sterile distilled water. All drugs were then diluted in the test medium and dispensed into 96-well flat-bottomed microdilution trays and frozen at -20 or $-70\,^{\circ}C$ until use. The final concentrations ranged from 0.125 to 128 $\mu g\,mL^{-1}$ for fluconazole and 5-fluorocytosine, and from 0.0313 to 16 $\mu g\,mL^{-1}$ for amphotericin B and itraconazole. The microdilution trays containing 100 µL of the twofold serial dilutions of the antifungal drugs in RPMI 2% glucose medium were inoculated with 100 µL of inoculum to a final concentration of 2.0×10^3 CFU mL^{-1}. Two drug-free medium wells for sterility and growth controls were used. The MICs were determined after 48 h of incubation, and the optical density of each well was read with a microplate reader (Bio-Rad) at 490 nm. For amphotericin B, the MIC breakpoint was defined as the lowest drug concentration causing a reduction in growth of 90% or more, compared with growth in the control well. For 5-fluorocytosine and the azole drugs, the MIC breakpoint was defined as the concentration producing 50% inhibition. *Candida parapsilosis* ATCC 22019 and *Cryptococcus neoformans* ATCC 90012 were included in each test as quality and reference control organisms, respectively. The reference values of MICs of all drugs were obtained in accordance with the guidelines of CLSI document M27-A2 (NCCLS M27-A2, 2002).

Electrophoretic karyotyping

Karyotype analysis was performed with contour-clamped homogeneous electrophoretic field (CHEF) technology. Briefly, *Cryptococcus neoformans* isolates were grown on Sabouraud's dextrose agar at 30 °C for 48 h and then inoculated into 20 mL of YEPD medium (1% yeast extract, 2% peptone and 2% dextrose, supplemented with 2.9% NaCl) and incubated at 30 °C overnight. The cells were harvested by centrifugation, washed twice in SCE (20 mM sodium citrate, pH 5.6, 50 mM EDTA, pH 8.0, 0.9 M sorbitol), resuspended in SCE containing Novozym (10 mg mL^{-1} Novozym 234; NovoNordisk, from *Trichoderma harzianum*, Denmark) and incubated at 37 °C for 2 h. Spheroplasts were pelleted and washed twice in 1 mL of SCE. Chromosomal DNA plugs of *Cryptococcus neoformans* were prepared by mixing a protoplast suspension with 1.4% low-melting-point agarose solution (Bio-Rad, Philadelphia, PA) to yield a final agarose concentration of 0.7%. The mixture was dispensed into molds to form agarose plugs. After solidification, the plugs were incubated for 2 h at 56 °C in a solution of 0.25 M EDTA and 1% sodium dodecyl sulfate (SDS), and subsequently in NET-Prok buffer (0.01 M Tris, 0.45 M EDTA, 1% sarcosine, 1 mg mL^{-1} proteinase K) for 24 h. Agarose inserts were then washed with the same buffer and incubated a second time. Subsequently, the plugs were washed four times with 3 mL of 0.05 M EDTA, pH 7.8, and stored at 5 °C until use. Electrophoresis was performed in 1.0% agarose (Sigma Aldrich Quimica S.A) and 0.5 × TBE buffer (0.5 M Tris, 0.5 M sodium borate, 0.005 M EDTA) at 10 °C and 6.0 V cm^{-1}. Gels were run for 30 h with a switch time of 60–120 s (Barchiesi *et al.*, 1995). *Saccharomyces cerevisiae* chromosome DNA molecular weight markers (Bio-Rad) were included in each gel as standards. After electrophoresis, gels were stained with ethidium bromide (final concentration 0.5 $\mu g\,mL^{-1}$) and photographed under UV light. Banding patterns were evaluated by visual inspection and analyzed using the ImageMaster VDS system (Amersham Pharmacia Biotech, Piscataway, NJ). Isolates were considered to be identical only if all bands matched exactly. Isolates with EK profiles differing only by the position of bands or by the presence/absence of one band were considered to be microevolutionary variants of each other. Isolates with EK profiles differing by two or more bands were considered to be different strains. The computer program GelComparII, version 2.0 (Applied Maths, Belgium), was used to determine the genetic relationship of the isolates. Similarity coefficients were calculated using the Dice algorithm, and cluster analysis was performed by the unweighted pair group method with arithmetic mean (UPGMA). Isolates with EK profiles that grouped in clusters with similarity values above 0.8 were considered to belong to the same strain as a result of microevolution (Soll, 2000).

RAPD analysis

Genomic DNA was extracted and purified by the method of Lasker *et al.* (1992) with a slight modification. The spheroplasts were prepared as cited above and thereafter lysed in TEN buffer with 1% SDS (1 M Tris-base, 0.5 M EDTA, 1 M NaCl, 1% SDS). Then 750 µL of chloroform/isoamyl alcohol

(24 : 1) was added, the suspension was shaken and centrifuged for 13 min at 15 ***g***, and the aqueous phase was transferred to a new tube. To precipitate the DNA, 750 μL of isopropanol was added and the mixture was incubated at − 20 °C for 30 min. The DNA pellet was washed with 70% ethanol, centrifuged and air-dried. Finally, the DNA was resuspended in 80 μL of TE (40 mM Tris-base, 2 mM EDTA). The RAPD analysis was carried out with primer 6 (5′-d[CCCGTCAGCA]-3′) from the Ready-to-Go kit (Amersham Pharmacia Biotech) using a total volume of 25 μL, and in accordance with the manufacturer's instructions. The following PCR cycle conditions were used: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min and amplification at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplification products were separated by electrophoresis on 2% agarose gels in 1× TBE buffer at 150 V for 2.5 h, stained with 0.5 μg mL^{-1} ethidium bromide and visualized under UV light. Banding patterns were analyzed both by visual comparison and by the ImageMaster VDS Software (Amersham Pharmacia Biotech). The computer program GelCompar II version 2.0 was used to determine the genetic relationship of the isolates. Gels were normalized using *S. cerevisiae* chromosomal DNA standards as a reference. RAPD profiles were analyzed by GelCompar software Version 2.0 (Applied Maths). The similarity coefficient (S_{AB}) between patterns for every pair of isolates A and B was computed with the formula $S_{AB} = 2_E/(2_E + a + b)$, where E is the number of common bands in the patterns of A and B, a is the number of bands in pattern A with no correlates in pattern B, and b is the number of bands in pattern B with no correlates in pattern A. Dendrograms based on S_{AB} values were generated by UPGMA, implemented in the GelCompar software. An S_{AB} value of 1.00 indicates that the banding patterns for strain A are identical with that of strain B; S_{AB} values of 0.80–0.99 represent highly similar, but nonidentical, strains, and may suggest the occurrence of microevolution in a single strain; and S_{AB} values below 0.80 represent unrelated strains (Soll, 2000; Pfaller *et al.*, 2005).

Results

From a total of 83 cryptococcal isolates, 78 were recovered from 37 patients and identified as *Cryptococcus neoformans* var. *neoformans*. Four sequential isolates of *Cryptococcus gattii* were obtained from one patient, and from another patient both *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus gattii* were isolated. The MIC values obtained for the 83 isolates varied from 0.5 to 16 μg mL^{-1} for fluconazole, from 0.03 to 1 μg mL^{-1} for itraconazole, from 2 to 16 μg mL^{-1} for 5-fluorocytosine and from 0.03 to 1.0 μg mL^{-1} for amphotericin B. In this study, 35% of isolates were considered susceptible to itraconazole (0.25–0.5 μg mL^{-1}) and 8.4% (≥ 1 μg mL^{-1}) were resistant. On the other hand, 67.5% were intermediately susceptible to 5-fluorocytosine (8–16 μg mL^{-1}). The majority of the *Cryptococcus neoformans* isolates were highly susceptible to amphotericin B and fluconazole, but 20% had MIC values for amphotericin B in the range 0.5–1 μg mL^{-1}, and 22% had MIC values in the range 8–16 μg mL^{-1} for fluconazole.

Remarkably, the MIC_{90} for itraconazole was 1 μg mL^{-1} in Campinas, the highest value observed in all the cities, followed by 0.5 μg mL^{-1} in São Paulo. Isolates from Santos, followed by those from São José do Rio Preto, were more susceptible to all drugs investigated than those from the São Paulo and Campinas regions. Amphotericin B in all regions (MIC_{90}, 0.25–0.5 μg mL^{-1}) and itraconazole (MIC_{90}, 0.125–0.5 μg mL^{-1}) in São Paulo city, Santos and São José do Rio Preto were considerably more potent than fluconazole (MIC_{90}, 4.0–8.0 μg mL^{-1}) and 5-fluorocytosine (MIC_{90}, 8.0–16.0 μg mL^{-1}) against isolates from all four geographic areas (Table 1 and Fig. 1).

Interestingly, sequential isolates from nine patients demonstrated reduced susceptibility to all the drugs investigated. Isolates from two patients (20 and 32) showed an increase in MIC values of two or three dilutions for 5-fluorocytosine, five patients (2, 4, 11, 20 and 29) for fluconazole, two patients (24 and 28) for itraconazole and one (21) for amphotericin B. Isolates from one patient (20) showed reduced susceptibility to two drugs, namely 5-fluorocytosine and fluconazole (Table 2).

All the isolates grouped into 22 EK profiles (Fig. 2), and the dendrogram showed a high similarity among 15 EKs (68.2%) from São Paulo state (Fig. 3). The average S_{AB} for the entire collection of 83 *Cryptococcus neoformans* isolates was 0.71 ± 0.17. Seventy-one isolates were distributed in 14 EKs, each with a similarity above 0.90. However, the similarity coefficients between the different EKs varied from 0.65 to 1.0. The remaining isolates grouped in other minor clusters with a lower degree of similarity (Fig. 3). Three main clusters were defined by coefficients of similarity above 80%. One of these (I) contained 10 isolates from six patients, with two subclusters (labeled Ia and Ib) showing 83–100% and 92–100% similarity within the subclusters, respectively. Another large group (II) with 68 isolates (82%) from 34 patients showed high homogeneity among the isolates, with similarity coefficients of *c.* 0.80. The two subclusters IIa and IIb had *c.* 90–100% similarity. One of them (IIa) contained isolates from São Paulo, Campinas, Santos and São José do Rio Preto, with a predominance of the EKs A, B, C and D (90% similarity), and included 55% of the strains.

A third cluster (III) of four isolates with a similarity coefficient below 0.70 comprised all *Cryptococcus gattii*

Table 1. MIC_{50} values of fluconazole (FLC), itraconazole (ITC) and 5-fluorocytosine (5FC), and MIC_{90} values for amphotericin B (AMB) of 83 samples of *Cryptococcus neoformans* isolated from 38 patients with HIV-related cryptococcosis from different regions of São Paulo State, Brazil

		Number of isolates inhibited at MIC ($\mu g\,mL^{-1}$) of									
Antifungal agents	Region (no. of isolates)	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16
FLC	São Paulo (22)						1	7	8	6	
	Campinas (24)					2		4	12	5	1
	S. J. Rio Preto (16)							2	9	5	
	Santos (13)							4	9		
	Sorocaba (6)							1	3	2	
	S. J. Boa Vista (2)								2		
AMB	São Paulo (22)		3	4	9	6					
	Campinas (24)	1	5	4	8	4	2				
	S. J. Rio Preto (16)		4	5	5	2					
	Santos (13)	1	6	1	4	1					
	Sorocaba (6)			4	1	1					
	S. J. Boa Vista (2)		2								
5FC	São Paulo (22)								2	14	6
	Campinas (24)								8	9	7
	S. J. Rio Preto (16)								6	4	6
	Santos (13)							2	3	6	2
	Sorocaba (6)							1		3	2
	S. J. Boa Vista (2)								2		
ITC	São Paulo (22)	2	2	6	6	5	1				
	Campinas (24)	3	5	9	2	3	2				
	S. J. Rio Preto (16)		8	7		1					
	Santos (13)		3	2	7	1					
	Sorocaba (6)				2		4				
	S. J. Boa Vista (2)		2								

MIC, minimal inhibitory concentration.

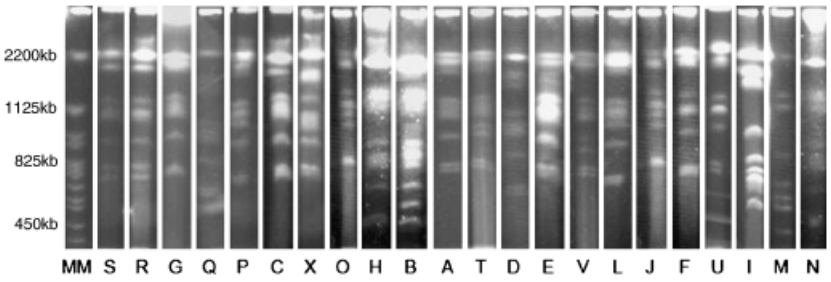

Fig. 2. EK patterns of *Cryptococcus neoformans* strains from São Paulo State. The letters correspond to the different types of EKs. Lane MM represents the *Saccharomyces cerevisiae* size standard.

isolates. The only isolate of cluster IV showed the most dissimilar EK if compared to the other isolates.

Thirty-six patients had sequential isolates, and nine of these showed increasing MICs over time (Table 2). MICs of fluconazole, 5-fluorocytosine, itraconazole and amphotericin B increased twofold or threefold from those of isolates of six patients (2, 4, 21, 24, 28 and 32), and all these isolates possessed identical EK patterns. However, isolates from three patients (11, 20 and 29) had increased MIC values for fluconazole, and showed changing EKs. One of these (20) also showed an increase in MIC for 5-fluorocytosine (Table 2). All these strains had EKs showing small changes in the position or number of bands and showed $\geq 80\%$ similarity (Fig. 3). The five sequential isolates of patient 20 showed a rise in MIC from 2 to $16\,\mu g\,mL^{-1}$ for both fluconazole and 5-fluorocytosine and belonged to three different EK profiles (sample 163 with EK V, sample 164 with EK A, sample 165 with EK T, and samples 199 and 204 with EK A; Fig. 4). The first isolate (163), with an MIC of $2\,\mu g\,mL^{-1}$, showed a coefficient of similarity of 90% with the other isolates with MICs $> 8\,\mu g\,mL^{-1}$. Furthermore, the last of these isolates had an MIC of $16\,\mu g\,mL^{-1}$ and showed chromosomal variation in two band positions (Fig. 4). Patient 29 had two isolates with different EK profiles of high similarity (90%).

Table 2. Representative results showing increase of antifungal MIC values, EKs and RAPD profiles of sequential isolates of *Cryptococcus neoformans* from individual patients

		Change in MIC values				Genotypic profiles	
Patient	Isolates	FLC ($\mu g\,mL^{-1}$)	AMB ($\mu g\,mL^{-1}$)	5FC ($\mu g\,mL^{-1}$)	ITC ($\mu g\,mL^{-1}$)	EK	RAPD
2	379	2				B	Ia
	381	8				B	Ia
4	426	1				B	Ia
	578	4				B	Ia
11	13	2				G	Ib
	49	8				S	Ia
20	163	2		4		V	Ia
	164	8		8		A	IIa
	165	8		8		T	IIa
	199	16		16		A	IIa
	204	16		16		A	IIa
21	192		0.125			J	Iia
	196		0.5			J	Ib
24	222				0.125	F	Ia
	65				0.5	F	Ia
28	78				0.0313	E	IIIa
	101				0.125	E	Ia
29	60	2				L	Ia
	68	8				C	Ia
32	147			4		D	Ia
	159			16		D	Ia

MIC, minimal inhibitory concentration; FLC, fluconazole; ITC, itraconazole; 5FC, 5-fluorocytosine; AMB, amphotericin B; EK, electrophoretic karyotype; RAPD, random amplified polymorphic DNA.

One isolate from the patient with four sequential isolates of *Cryptococcus gattii* showed EK variation, with a similarity value of 87% to the other three isolates. All these isolates were susceptible to drugs, and no increase of MIC values for any of the drugs was observed.

All the isolates grouped into 15 RAPD profiles in four main clusters (Fig. 5). The average S_{AB} for the entire collection of 83 *Cryptococcus neoformans* isolates was 0.81 ± 0.17. Three clusters had a coefficient of similarity above 70%, one containing 56 isolates from 30 patients (cluster I), which comprised two subclusters. Cluster Ia contained two subgroups, namely one with 46 isolates from 25 patients and a second with three isolates from three patients. Subcluster comprised two subgroups, the largest of which contained five isolates from three patients. Cluster II with 12 isolates from eight patients showed two subclusters IIa and IIb. Subcluster IIa grouped nine isolates from six patients. Cluster III, containing eight isolates within two subclusters, IIIa and IIIb, showed a low degree of similarity ($< 75\%$) with the other groups. A minor group (IV) included the *Cryptococcus gattii* isolates, with a similarity of 0.90, and comprised two subgroups. One patient had a mixed infection with *Cryptococcus gattii* and *Cryptococcus neoformans* var. *neoformans*.

Both molecular typing methods confirmed the presence of closely related groups of isolates with a similarity above 80%. Small variations occurred in some strains associated with an increase in observed MIC values. The great majority of the serial samples (88.5%) showed a similar degree of genotypic relatedness using both methodologies. The isolates from patients 11 and 29 had modifications in both their EK and RAPD profiles (Figs 3–6 and Table 2). The first isolate from patient 11 in the subcluster Ia had a changed MIC for fluconazole, and the second isolate from this patient occurred in subcluster Ib, showing *c.* 80% similarity in the RAPD and EK patterns. The two isolates from patient 29, showing an increase of MIC for fluconazole, occurred in this larger RAPD cluster. Interestingly, all the samples of the RAPD IIa cluster (Fig. 5) were intermediately susceptible or resistant to 5-fluorocytosine, and the five isolates from patient 20 had increased MICs for fluconazole and 5-fluorocytosine (from 2 to $16\,\mu g\,mL^{-1}$ and from 4 to $16\,\mu g\,mL^{-1}$, respectively). The first of these isolates, with MICs of $2\,\mu g\,mL^{-1}$ for fluconazole and $4\,\mu g\,mL^{-1}$ for 5-fluorocytosine, had a different EK profile with 84% similarity. The other four isolates, with MICs ranging from 8 to $16\,\mu g\,mL^{-1}$ for fluconazole and 5-fluorocytosine, had higher than 90% similarity using both typing methods, thus suggesting that the first isolate is a different strain from the others (Figs 3 and 5 and Table 2).

The two isolates from patient 21 had increased MIC values for amphotericin B (Table 2). The first isolate

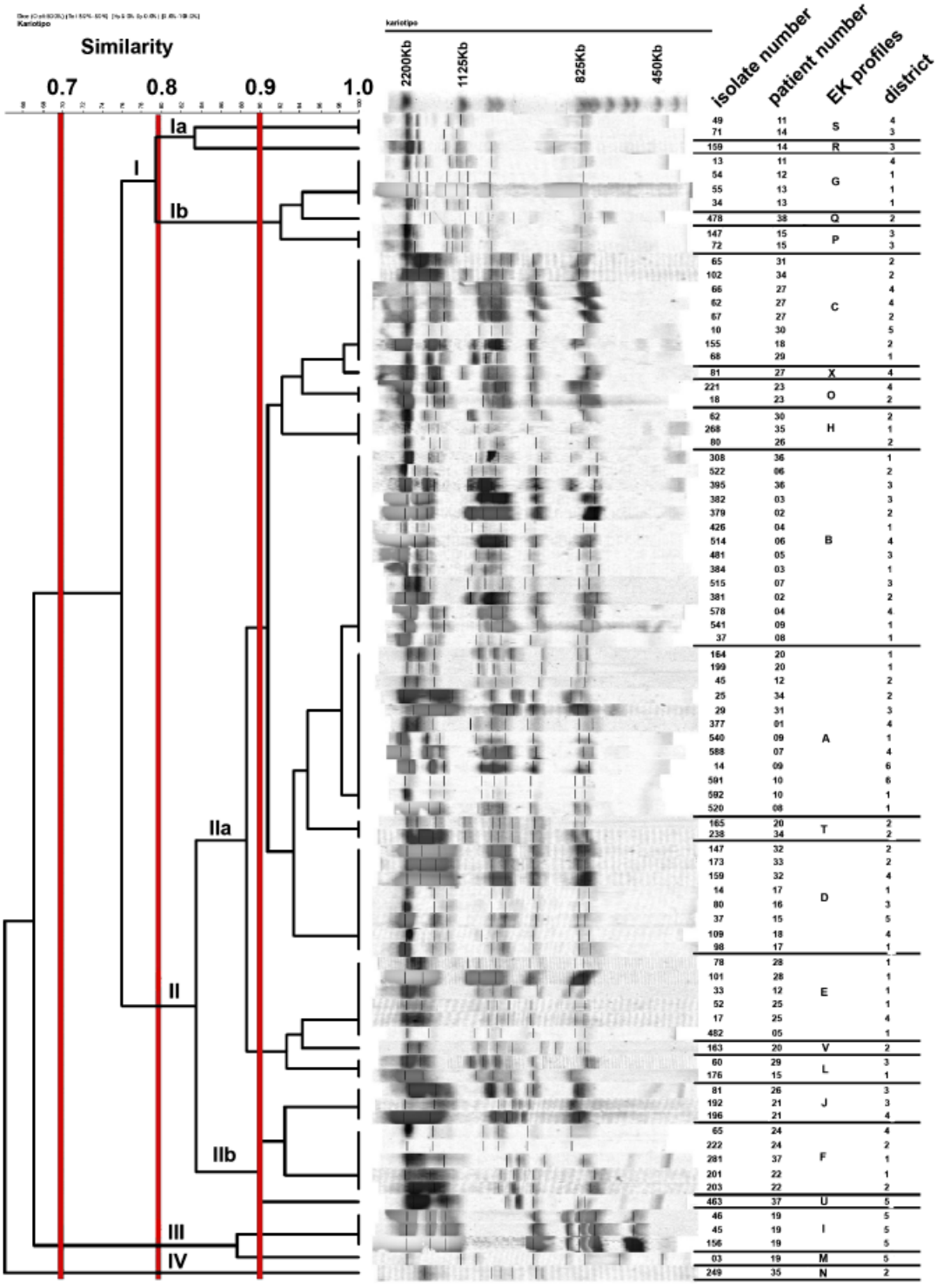

Fig. 3. Dendrogram of the EK patterns obtained from *Cryptococcus neoformans* and *Cryptococcus gattii* sequential isolates. The district numbers are: 1, Campinas; 2, São Paulo; 3, Santos; 4, São José do Rio Preto; 5, Sorocaba; 6, São João da Boa Vista (all isolates from patient 19 are *Cryptococcus gattii*).

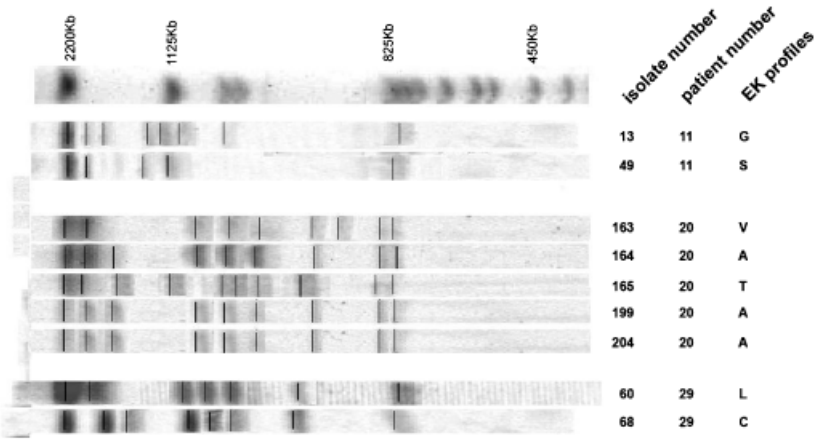

Fig. 4. EK profiles of sequential isolates of *Cryptococcus neoformans* from patients who showed increased MIC values and changes in the EK profiles. The letters correspond to the different EKs.

belonged to RAPD cluster IIa and the second to subgroup Ib (76%), but they had the same EK type.

Discussion

Cryptococcal infections of immunocompromised individuals remain a significant concern. The available data suggest that the main cause of therapy failure in AIDS-associated central nervous system cryptococcosis is low immunity of the host. Even though the role of factors in the follow-up of AIDS-associated cryptococcosis is well known in Brazil, and despite the administration of HAART and other advanced therapies that decreased the disease rates, the frequency has not diminished so markedly as in the USA and western Europe (Pappalardo & Melhem, 2003).

Most studies in Latin America have been limited to clinical and epidemiologic aspects (Rozenbaum & Goncalves, 1994), and there have been few studies on the molecular epidemiology of clinical isolates from this area (Franzot *et al.*, 1997; Calvo *et al.*, 2001; Horta *et al.*, 2002; Pappalardo, 2002; Casali *et al.*, 2003). In Brazil, little is known about the antifungal susceptibility and genetic diversity of sequential isolates of *Cryptococcus neoformans* obtained from single patients in different localities to explain the sustained incidence and recurrence rates (Pappalardo, 2002; Igreja *et al.*, 2004). We studied clinical strains of *Cryptococcus neoformans* from 38 Brazilian AIDS patients with cryptococcal meningitis from six regions of São Paulo State isolated during the pre-HAART period by analyzing their molecular and antifungal susceptibility profiles. *Cryptococcus neoformans* var. *neoformans* was predominant among the 83 isolates observed in our survey, and there was a low incidence of *Cryptococcus gattii*. *Cryptococcus neoformans* is prevalent among immunosuppressed patients, who are mainly HIV-infected patients, not only in Brazil, but also worldwide (Franzot *et al.*, 1997; Calvo *et al.*, 2001; Pappalardo, 2002; Casali *et al.*, 2003; Meyer *et al.*, 2003; Igreja *et al.*, 2004; Delgado *et al.*, 2005).

In our study, most of the strains were considered susceptible to fluconazole, intermediately susceptible to 5-fluorocytosine and dose-dependent susceptible to itraconazole, according to the CLSI guidelines (NCCLS M27-A2, 2002). All strains were susceptible to amphotericin B, although interpretative breakpoints for amphotericin B do not exist, due to a lack of clinical correlation between *in vitro* and *in vivo* results (Cuenca-Estrella & Rodriguez-Tudela, 2002). Current data suggest that the microdilution test does not permit reliable detection of amphotericin B-resistant isolates. For *Cryptococcus neoformans*, in contrast to *Candida albicans* and a few other fungal species, there is only limited experience of susceptibility testing and the respective breakpoints. In this study, we observed that MIC values for *Cryptococcus neoformans* remained relatively stable, with a few exceptions. Furthermore, it appeared that *c.* 90% of the infections resulted from infection with a single cryptococcal strain. However, the MICs of fluconazole, 5-fluorocytosine and itraconazole were observed to increase in serial isolates, ranging from 2 to 16 $\mu g\,mL^{-1}$, from 4 to 16 $\mu g\,mL^{-1}$ and from 0.0133 to 0.5 $\mu g\,mL^{-1}$, respectively. These values are higher than those described previously (Barchiesi *et al.*, 1995; Franzot & Hamdan, 1996; Klepser & Pfaller, 1998; Calvo *et al.*, 2001). Resistance in *Cryptococcus neoformans* clinical isolates remains uncommon and has not increased in the last decade in the USA and the UK (Pfaller *et al.*, 2005), but local occurrence of resistance has been reported from other areas (Sar *et al.*, 2004; Perkins *et al.*, 2005). The reasons for this tendency are not clear, but may be related to the less appropriate use and dosage of fluconazole and 5-fluorocytosine over the years, the presence of mixed strains in a single infection course, or even the acquisition of a new strain with lower antifungal susceptibility. On the other hand, patients suffering from these infections are difficult to treat, and the majority could have relapses

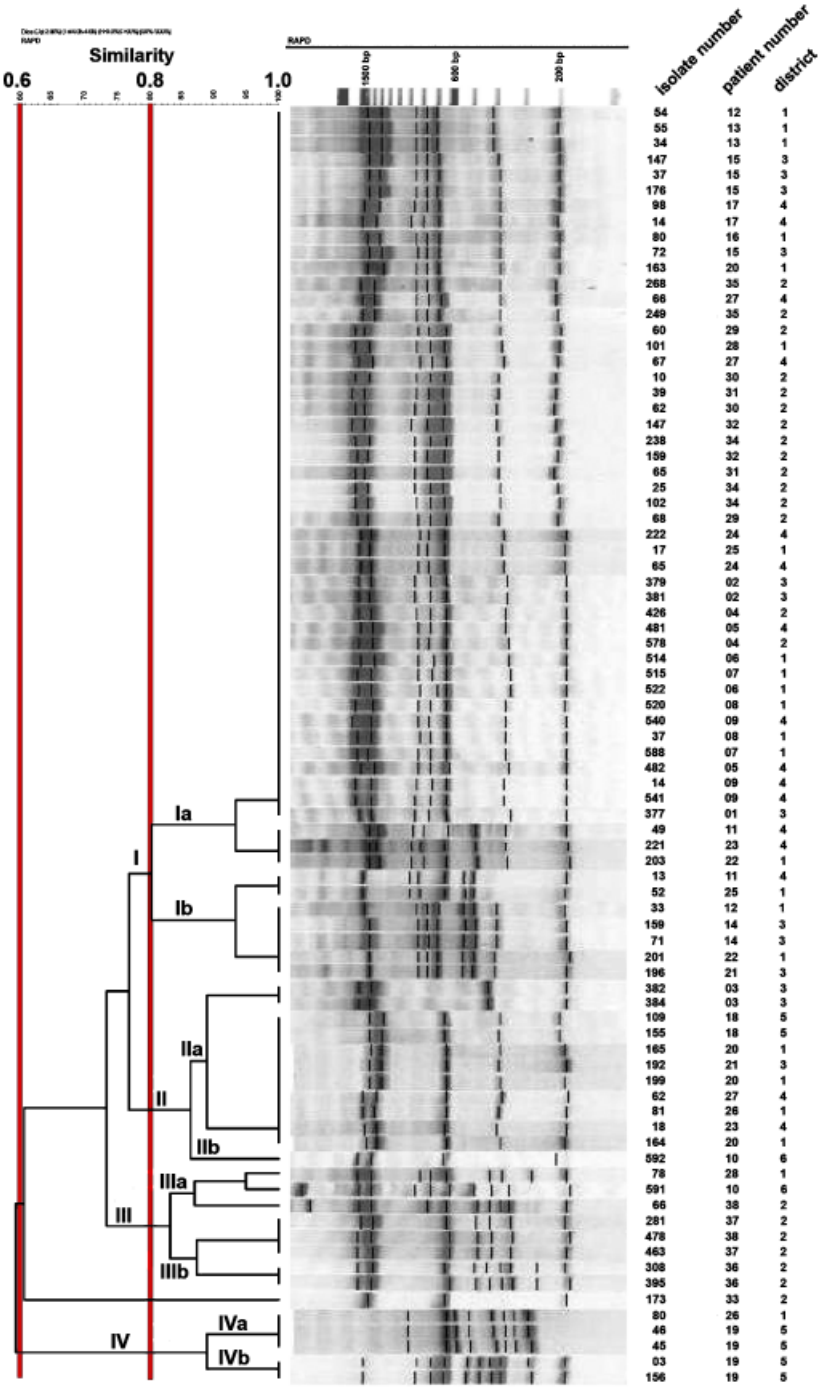

Fig. 5. Dendrogram of the RAPD profiles obtained from *Cryptococcus neoformans* and *Cryptococcus gattii* sequential isolates with random primer 6. The district numbers are: 1, Campinas; 2, São Paulo; 3, Santos; 4, São José do Rio Preto; 5, Sorocaba; 6, São João da Boa Vista. All isolates from patient number 19 are *Cryptococcus gattii*.

(Pappalardo & Melhem, 2003). This study revealed increased MIC values, mainly for fluconazole (three patients), that are associated with changes in the EK and RAPD patterns. The MIC values for sequential isolates increased significantly in nine patients (21%), thus supporting the idea that drug resistance may emerge during therapy, which is in agreement with data from Pappalardo (2002), who found resistance in 12% of 168 serial samples collected from 35 patients. Long-term exposure to fluconazole is one factor that may lead to the selection of azole-resistant strains (Barchiesi *et al*., 1995; Colombo *et al*., 1995; Currie *et al*., 1995; Brandt *et al*., 1996a, b; Nguyen & Yu, 1998; Pfaller *et al*., 2005), and our results suggest the presence of the fluconazole-heteroresistant phenotype of *Cryptococcus*

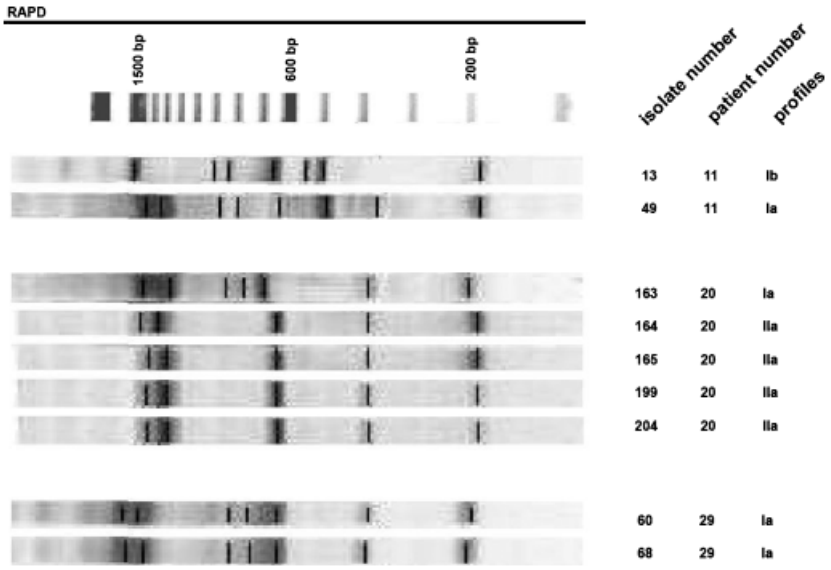

Fig. 6. RAPD profiles of sequential isolates of *Cryptococcus neoformans* from patients who showed increased MIC values and changes in the EK profiles.

neoformans in some sequential clinical isolates. Mondon *et al.* (1999) investigated serial isolates from two infected patients and showed that each isolate produced cultures with heterogeneous fluconazole susceptibility and that the proportion of subpopulations resistant to fluconazole (MIC = 64 μg mL^{-1}) increased steadily over time. Whether the heteroresistant phenotype in *Cryptococcus neoformans* accounts for the clinical failures of fluconazole therapy is currently unknown. These observations suggest a clinical significance for the heteroresistant phenotype as a potential cause of failure of fluconazole treatment in immunosuppressed patients. Acquired resistance is more frequently seen in *Candida* spp. than in *Cryptococcus neoformans* (Maenza *et al.*, 1997; Berg *et al.*, 1998; Perfect & Cox, 1999). Friese *et al.* (2001) reported a case of cryptococcal meningitis in which a fluconazole-resistant strain emerged (MIC 64 μg mL^{-1}), but some isolates with increased MIC values remained susceptible to another triazole (itraconazole) agent. Rodero *et al.* (2003) documented the development of fluconazole resistance after three episodes of meningitis, and concluded that it was probably caused by the fluconazole maintenance therapy.

In this research, we observed a high correlation between distinct genetic profiles in serial samples identified by two molecular typing methods and the tendency to become resistant to antifungal drugs. Barchiesi *et al.* (1995), Brandt *et al.* (1996a, b) and Calvo *et al.* (2001) described similar results. In contrast, Rodero *et al.* (2003) analyzed five sequential *Cryptococcus neoformans* isolates from an AIDS patient with recurrent meningitis, and all belonged to the same genetic profile.

Electrophoretic karyotyping has been found to be a useful technique to distinguish between *Cryptococcus neoformans* isolates (Boekhout *et al.*, 1997; Klepser & Pfaller, 1998). The number of EK patterns among our isolates was comparable to the number observed in previous Brazilian studies (Franzot *et al.*, 1997; Calvo *et al.*, 2001), and higher than in other studies (Fries & Casadevall, 1998; Jain *et al.*, 2005). In our study, 22 EK patterns were found among 83 isolates. The high frequency of chromosomal differences occurring among strains has made electrophoretic karyotyping a highly discriminatory technique to distinguish among isolates (Barchiesi *et al.*, 1995; Sukroongreung *et al.*, 2001). In São Paulo State, Campinas is the region with the highest strain diversity, followed by São Paulo, São José Rio Preto and Santos. However, some highly similar karyotypes were observed predominantly, thus suggesting the occurrence of clonal spread of this EK in São Paulo State. Among sequential isolates recovered from the same patients, limited karyotype variation exists.

Our results show evidence of changed EKs occurring in 50% of the strains during infection. The majority of these alterations did not involve significant modifications of the chromosomes, since the majority of strains presented 80% similarity of the EKs. These genetic changes were markedly associated with an increase of the MIC values. This was particularly evident in isolates obtained from three patients; two of them exhibited EKs modified by the acquisition of a new chromosomal profile, thus suggesting the occurrence of microevolution (Fries & Casadevall, 1998). It should be noted that chromosomal rearrangements are common in *Cryptococcus neoformans*, and karyotype instability could explain the apparent emergence of new EKs within a given strain. Variation in EKs due to karyotype instability is usually indicated by change of a single chromosomal band, whereas differences of two or more bands suggest the

presence of different strains. Chromosomal rearrangement may occur as an adaptation of the isolate in response to suboptimal levels of antifungal drugs (Brandt *et al.*, 1996a, b). In some patients, two strains infecting simultaneously during the same episode of infection may be present (Klepser & Pfaller, 1998; Jain *et al.*, 2005). One patient (20) in our analysis presented three different EK profiles (namely, EK V, A and T) during five episodes of infection, thus suggesting infection with closely related strains (Table 2 and Figs 3 and 4). This differentiation in EK types was associated with increasing MIC values from the first to the fifth isolate from 2 to 16 $\mu g\,mL^{-1}$ for fluconazole, and from 4 to 16 $\mu g\,mL^{-1}$ for 5-fluorocytosine. Three profiles, namely EK V (sample 163), T (sample 165) and A (samples 164, 199 and 204), of the serial isolates of this patient accompanied a threefold increase of MIC, as has been reported previously (Brandt *et al.*, 1996a, b). Our study suggests that *Cryptococcus neoformans* strains may undergo significant diversification *in vivo* as a result of microevolution associated with the heteroresistant phenotype. Cryptococcosis is a chronic disease, and patients are frequently infected for long periods (i.e. weeks or months), during which the fungus can undergo microevolution. Interestingly, the *Cryptococcus neoformans* genome is rich in transposons, which may play a role in microevolution, as suggested by Jain *et al.* (2005).

The development and application of techniques designed to differentiate between individual isolates are of particular relevance because of the frequent recurrence of cryptococcal infections in HIV-infected patients once antifungal drug therapy has ceased. RAPD analysis has been used to demonstrate intraspecific diversity in *Cryptococcus neoformans* (Franzot *et al.*, 1998; Horta *et al.*, 2002). In our study, the analysis with random primer 6 showed a high similarity among 67.5% of the isolates from São Paulo State, yielding four RAPD profiles. These results were in accordance with an Ibero-American study that showed that the molecular type VNI is the most common type among three molecular types present in Brazil (Meyer *et al.*, 2003). Casali *et al.* (2003) showed a predominance of *Cryptococcus neoformans* var. *grubii* molecular type VNI in the southern region of Brazil, and Horta *et al.* (2002) found a predominance of RAPD profile F in clinical isolates. In addition, the studies of Franzot *et al.* (1997) suggested the presence of limited genetic diversity as well. In contrast, our results show low genetic similarity among 31% of the strains, which may suggest that genetically distinct strains colonize São Paulo State. This seems to be in accordance with an AFLP study published by Barreto de Oliveira *et al.* (2004).

Our results provide evidence for the occurrence of genetic changes in some strains during the course of infection by both genotyping methods. In some patients, we found changed karyotype-and RAPD patterns in association with increased MIC values. The mechanism causing the genetic diversity among serial isolates is still unclear, but it is possible that the fungal population changes and adapts in order to escape pressure by antifungal drugs or eradication by the immune system. *Cryptococcus neoformans* is capable of microevolution *in vivo*, and new genotypic variants may emerge, allowing the organism to persist. Furthermore, we provide evidence that *Cryptococcus neoformans* may undergo phenotypic and genotypic changes during the early stages of infection in humans, prior to or during antifungal therapy.

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References

Almeida AMF (2000) Análise do cariótipo eletroforético de diferentes amostras de *Cryptococcus neoformans* e correlação com suscetibilidade a drogas antifúngicas. MSc. Thesis, Universidade Estadual Paulista, Araraquara, SP, Brasil.

Barchiesi F, Hollis RJ, Messer SA, Scalise G, Rinaldi MG & Pfaller MA (1995) Electrophoretic karyotype and in vitro antifungal susceptibility of *Cryptococcus neoformans* isolates from AIDS patients. *Diagn Microbiol Infect Dis* **23**: 99–103.

Barreto de Oliveira MT, Boekhout T, Theelen B, Hagen F, Baroni FA, Lazera MS, Lengeler KB, Heitman J, Rivera IN & Paula CR (2004) *Cryptococcus neoformans* shows a remarkable genotypic diversity in Brazil. *J Clin Microbiol* **42**: 1356–1359.

Berg J, Clancy CJ & Nguyen MH (1998) The hidden danger of primary fluconazole prophylaxis in patients with acquired immunodeficiency syndrome. *Clin Infect Dis* **26**: 186–187.

Boekhout T, van Belkum A, Leenders AC, Verbrugh HA, Mukamurangwa P, Swinne D & Scheffers WA (1997) Molecular typing of *Cryptococcus neoformans*, taxonomic and epidemiological aspects. *Int J Syst Bacteriol* **47**: 432–442.

Branchini MLM & Papaiordanou PMO (2000) Destaque para as infecções fúngicas em pacientes com Aids e no hospedeiro imunocomprometido no Brasil. *J Bras AIDS* **1**: 31–34.

Brandt ME, Hutwagner LC & the Cryptococcal Disease Active Surveillance Group, *et al.* (1996a) Molecular subtype distribution of *Cryptococcus neoformans* in four areas of the United States. *J Clin Microbiol* **34**: 912–917.

Brandt ME, Pfaller MA, Hajjeh RA, Graviss EA, Rees J, Spitzer ED, Pinner RW, Meyer LW & the Cryptococcal Disease Active Surveillance Group (1996b) Molecular subtypes and antifungal susceptibilities of serial *Cryptococcus neoformans* isolates in human immunodeficiency virus-associated cryptococcosis. *J Infect Dis* **174**: 812–820.

Brandt ME, Pfaller MA, Hajjeh RA, Hamill RJ, Pappas PG, Reingold AL, Rimland D, Warnock DW for the Cryptococcal

Disease Active Surveillance Group (2001) Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992–1994 and 1996–1998. *Antimicrob Agents Chemother* **45**: 3065–3069.

Calvo BM, Colombo AL, Fischman O, *et al.* (2001) Antifungal susceptibilities, varieties and electrophoretic karyotypes of clinical isolates of *Cryptococcus neoformans* from Brasil, Chile, and Venezuela. *J Clin Microbiol* **39**: 2348–2350.

Casadevall A & Perfect JR (1998) Epidemiology. *Cryptococcus neoformans*, pp. 351–380. The American Society for Microbiology, Washington, DC.

Casali AK, Goulart L, Rosa e Silva LK, Ribeiro AM, Amaral AA, Alves SH, Schrank A, Meyer W & Vainstein MH (2003) Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Res* **3**: 405–415.

Colombo AL, Barchiesi F, McCough DA & Rinaldi MG (1995) Comparison of E-test and National Committee for Clinical Laboratory Standards broth macrodiluition method for azole antifungal susceptibility testing. *J Clin Microbiol* **33**: 535–540.

Cuenca-Estrella M & Rodriguez-Tudela JL (2002) Should antifungal treatments be based upon results of antifungal susceptibility testing? *Rev Iberoam Micol* **19**: 133–138.

Currie BP, Ghannoum M, Bessen L & Casadevall A (1995) Decreased fluconazole susceptibility of a relapse *Cryptococcus neoformans* isolate after fluconazole treatment. *Infect Dis Clin Pract* **4**: 318–319.

Delgado AC, Taguchi H, Mikami Y, Myiajy M, Villares MC & Moretti ML (2005) Human cryptococcosis: relationship of environmental and clinical strains of *Cryptococcus neoformans* var. *neoformans* from urban and rural areas. *Mycopathologia* **159**: 7–11.

Franzot SP & Hamdan JS (1996) *In vitro* susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* to five antifungal drugs. *Antimicrob Agents Chemother* **40**: 822–824.

Franzot SP, Hamdan JS, Currie BP & Casadevall A (1997) Molecular epidemiology of *Cryptococcus neoformans* in Brazil and the United States: evidence for both local genetic differences and a global clonal population structure. *J Clin Microbiol* **35**: 2243–2251.

Franzot SP, Mukherjee J, Cherniak R, Chen LC, Hamdan JS & Casadevall A (1998) Microevolution of a standard of *Cryptococcus neoformans* resulting in differences in virulence and other phenotypes. *Infect Immun* **66**: 89–97.

Fries BC & Casadevall A (1998) Serial isolates of *Cryptococcus neoformans* from patients with AIDS differ in virulence for mice. *J Infect Dis* **178**: 1761–1766.

Fries BC, Chen F, Currie BP & Casadevall A (1996) Karyotype instability in *Cryptococcus neoformans* infection. *J Clin Microbiol* **34**: 1531–1534.

Friese G, Discher T, Fussle R, Schmalreck A & Lohmeyer J (2001) Development of azole resistance during fluconazole maintenance therapy for AIDS-associated cryptococcal disease. *AIDS* **15**: 2344–2345.

Horta JA, Staats CC, Casali AR, Ribeiro AM, Schrank IS, Schrank A & Vainstein MH (2002) Epidemiological aspects of clinical and environmental *Cryptococcoccus neoformans* isolates in the Brazilian state Rio Grande do Sul. *Med Mycol* **40**: 565–571.

Igreja RP, Lazera MS, Wanke B, Galhardo MC, Kidd SE & Meyer W (2004) Molecular epidemiology of *Cryptococcus neoformans* isolates from AIDS patients of the Brazilian city, Rio de Janeiro. *Med Mycol* **42**: 229–238.

Jain N, Wickes BL, Keller SM, Fu J, Casadevall A, Jain P, Ragan MA, Banerjee U & Fries BC (2005) Molecular epidemiology of clinical *Cryptococcus neoformans* strains from India. *J Clin Microbiol* **43**: 5733–5742.

Klepser ME & Pfaller MA (1998) Variation in electrophoretic karyotype and antifungal susceptibility of clinical isolates of *Cryptococcus neoformans* at a university-affiliated teaching hospital from 1987–1994. *J Clin Microbiol* **36**: 3653–3656.

Kwon-Chung KJ, Wickes BL, Stockman L, Roberts GD, Ellis E & Howard HH (1992) Virulence, serotype, and molecular characteristics of environmental strains of *Cryptococcus neoformans* var. *gattii*. *Infect Immun* **60**: 1869–1874.

Lasker B, Page LS, Lott TJ & Kobayashi GS (1992) Isolation, characterization, and sequencing of *Candida albicans* repetitive element 2. *Gene* **116**: 51–57.

Lazera MS, Cavalcanti MAS, Londero AT, Trilles L, Nishikawa MM & Wanke B (2000) Possible primary ecological niche of *Cryptococcus neoformans*. *Med Mycol* **38**: 379–383.

Maenza JR, Merz WG, Romagnoli MJ, Keruly JC, Moore RD & Gallant JE (1997) Infection due to fluconazole-resistant *Candida* in patients with AIDS: prevalence and microbiology. *Clin Infect Dis* **24**: 28–34.

Meyer W, Castaneda A, Jackson S, Huynh M & Castaneda E and the Ibero American Cryptococcal Study Group (2003) Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerg Infect Dis* **9**: 189–195.

Ministério da Saúde do Brasil (2002) Dados e pesquisa em DST e Aids. Coordenação do programa nacional de DTS/Aids. Brasília, DF.

Mitchell TG & Perfect JR (1995) Cryptococcosis in the era of AIDS – 100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev* **8**: 515–548.

Mondon P, Petter R, Amalfitano G, Luzzati R, Concia E, Polacheck I & Kwon-Chung KJ (1999) Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans*. *Antimicrob Agents Chemother* **43**: 1856–1861.

National Committee for Clinical Laboratory Standards (NCCLS) (1997) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved standard M27-A*. National Committee for Clinical Laboratory Standards, Wayne, PA.

National Committee for Clinical Laboratory Standards (NCCLS) (2002) *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved standard. NCCLS document M27-A2*. National Committee for Clinical Laboratory Standards, Wayne, PA.

Nguyen MH & Yu CY (1998) In vitro comparative efficacy of voriconazole and itraconazole against fluconazole-susceptible and resistance *Cryptococcus neoformans* isolates. *Antimicrob Agents Chemother* **42**: 471–472.

Pappalardo MCSM (2002) *Criptococose em Aids: estudo clínico e microbiológico em 35 pacientes acompanhados no Instituto de Infectologia Emílio Ribas, São Paulo, entre 1995 a 1997*, MSc Thesis, Coordenação dos Institutos de Pesquisa da Secretaria da Saúde de São Paulo, São Paulo, SP.

Pappalardo MCSM & Melhem MSC (2003) Cryptococcosis: a review of the Brazilian experience for the disease. *Rev Inst Med Trop S Paulo* **45**: 299–305.

Perfect JR & Cox GM (1999) Drug resistance in *Cryptococcus neoformans*. *Drug Resist Updat* **2**: 259–269.

Perkins A, Gomez-Lopez A, Mellado E, Rodriguez-Tudela JL & Cuenca-Estrella M (2005) Rates of antifungal resistance among Spanish clinical isolates of *Cryptococcus neoformans* var. *neoformans*. *J Antimicrob Chemother* **56**: 1144–1147.

Pfaller MA, Zhang J, Messer S, Tumberland M, Mbidde E, Jessup C & Ghannoum M (1998) Molecular epidemiology and antifungal susceptibility of *Cryptococcus neoformans* isolates from Ugandan AIDS patients. *Diagn Microb Infect Dis* **32**: 191–199.

Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Doern GV & Diekema DJ (2005) Global trends in the antifungal susceptibility of *Cryptococcus neoformans* (1990 to 2004). *J Clin Microbiol* **43**: 2163–2167.

Powderly WG, Finkelstein DM, Feinberg J, *et al.* (1995) A randomized trial comparing fluconazole with clotrimazole troches for the prevention of fungal infections in patients with advanced human immunodeficiency virus infection. *N Engl J Med* **332**: 700–705.

Rezende C (2002) *Aspectos fenotípicos e moleculares de amostras de Cryptococcus neoformans da região de Araraquara e Ribeirão Preto, relacionados aos perfis de suscetibilidade*, MSc Thesis, Universidade Estadual Paulista, Araraquara, SP, Brasil.

Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P, Cuenca-Estrella M, Davel G & Rodriguez-Tudela JL (2003) G484S amino acid substitution in lanosterol 14-alpha demethylase (ERG11) is related to fluconazole resistance in a recurrent *Cryptococcus neoformans* clinical isolate. *Antimicrob Agents Chemother* **47**: 3653–3656.

Rodriguez-Tudela JL, Berenguer J, Martinez-Suarez JV & Sanchez R (1996) Comparison of a spectrophotometric microdilution method with RPMI-2% glucose with the National Committee for Clinical Laboratory Standards reference macrodilution method M27-P for *in vitro* susceptibility testing of amphotericin B, flucytosine, and fluconazole against *Candida albicans*. *Antimicrob Agents Chemother* **40**: 1998–2003.

Rozenbaum R & Gonçalves AJ (1994) Clinical epidemiological study of 171 cases of cryptococcosis. *Clin Infect Dis* **18**: 369–380.

Sanati H, Messer SA, Pfaller MA, Witt M, Larsen R, Espinel-Ingroff A & Ghannoum M (1996) Multicenter evaluation of broth microdilution method for susceptibility testing of *Cryptococcus neoformans* against fluconazole. *J Clin Microbiol* **34**: 1280–1282.

Sar B, Monchy D, Vann M, *et al.* (2004) Increasing *in vitro* resistance to fluconazole in *Cryptococcus neoformans* Cambodian isolates: April 2000 to March 2002. *J Antimicrob Chemother* **54**: 563–565.

Soll DR (2000) The ins and outs of DNA fingerprinting the infectious fungi. *Clin Microbiol Rev* **13**: 332–370.

Sukroongreung S, Lim S, Tantimavanich S, Eampokalap B, Carter D, Nilakui C, Kulderatiyut S & Tanshyphaswadikui S (2001) Phenotypic switching and genetic diversity of *Cryptococcus neoformans*. *J Clin Microbiol* **39**: 2060–2064.