In vitro antifungal susceptibilities and molecular typing of sequentially isolated clinical Cryptococcus neoformans strains from Croatia

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A collection of 48 clinical Cryptococcus neoformans isolates from Croatia was investigated retrospectively using in vitro antifungal susceptibility testing and molecular biological techniques to determine mating type and serotype by PCR and amplified fragment length polymorphism (AFLP) genotyping. These isolates were obtained from 15 patients: ten were human immunodeficiency virus (HIV)-negative (66.7 %) and five were HIV-positive (33.3 %). From five patients, only one isolate was available, whilst from the other ten patients, two to 11 isolates were isolated sequentially. Antifungal susceptibility was tested by a broth microdilution method. Serotype A (genotype AFLP1) and serotype D (genotype AFLP2) were both found in six patients (40 % each), and serotype AD (genotype AFLP3) in three (20.0 %) patients. Mating type a (n=12; 80.0 %) predominated and a/a hybrids were identified in 20.0 % of patients diagnosed with cryptococcosis. Two AFLP genotypes of C. neoformans were isolated during a single episode from one patient. The in vitro antifungal MIC90 and susceptibility ranges for C. neoformans isolates were 0.5 μg ml⁻¹ (range 0.031–0.5 μg ml⁻¹) for amphotericin B, 4 μg ml⁻¹ (range 1–4 μg ml⁻¹) for flucytosine and fluconazole, 0.25 μg ml⁻¹ (range 0.031–0.5 μg ml⁻¹) for itraconazole and 0.062 μg ml⁻¹ (range 0.031–0.25 μg ml⁻¹) for voriconazole.

INTRODUCTION

Cryptococcosis is a life-threatening systemic fungal infection caused by the opportunistic pathogenic yeast Cryptococcus neoformans or its primary pathogenic sibling Cryptococcus gattii. Worldwide, C. neoformans is the major cause of cryptococcosis in individuals with an underlying immunological disorder, such as patients that are infected with human immunodeficiency virus (HIV), who have undergone solid organ transplantation (Bovers et al., 2008; Warkentien & Crum-Cianflone, 2010) or who have haematological malignancies (Bassetti et al., 2009; Vigouroux et al., 2000). It has been estimated recently that the global burden of HIV-associated cryptococcosis approximates to 1 million cases annually (Park et al., 2009). In Croatia, since 1985, when HIV was diagnosed for the first time, up until December 2009, nearly 800 HIV-infected persons were registered (http://www.hzjz.hr; data accessed 24 December 2010). Croatia has a centralized system of healthcare for HIV/acquired immunodeficiency syndrome patients who are all treated at the University Hospital of Infectious Diseases (UHID) in Zagreb. Since 1998, HIV-positive patients at this hospital have been treated with highly active antiretroviral therapy (HAART). Extrapulmonary cryptococcosis is an important opportunistic infection and was found in 29 (8.7 %) of the 332 HIV-positive patients treated at the UHID in the period 1986–2010.

The most frequent and severe clinical presentation of cryptococcosis is a disseminated meningoencephalitis,
which is almost uniformly fatal if not diagnosed and treated appropriately. In addition to the traditional antifungal compounds amphotericin B, lipid amphotericin B formulations, flucytosine, fluconazole, itraconazole and their combinations, treatment options for cryptococcosis have been increased by the expanded-spectrum azoles voriconazole and posaconazole (Perfect et al., 2010).

The application of molecular biological methods, such as PCR fingerprinting, PLB1 and URA5 RFLP fingerprinting, amplified fragment length polymorphism (AFLP) fingerprinting and multi-locus sequence typing, has led to a better insight into the taxonomy and identification of the causative agents of cryptococcosis (Bovers et al., 2008; Meyer et al., 2009). Within C. neoformans, three varieties can be distinguished: var. grubii (serotype A, AFLP genotype 1), var. neoformans (serotype D, AFLP genotype 2) and a hybrid between these two varieties (serotype AD, AFLP genotype 3). A third variety, C. neoformans var. gattii, has been raised to the species level and named C. gattii (Kwon-Chung et al., 2002). C. gattii (serotypes B and C, AFLP genotypes 4–7 and 10) predominantly causes infections in apparently immunocompetent individuals (Bovers et al., 2008; Hagen et al., 2010).

Epidemiological surveys provide a better understanding of the population dynamics of C. neoformans and C. gattii, as visualized by the ongoing C. gattii outbreak (Kidd et al., 2004). The epidemiology of C. neoformans has been studied intensively in western and Mediterranean Europe (Desnos-Ollivier et al., 2010; Guinea et al., 2010; van Elden et al., 2000; Viviani et al., 2006). However, there has been a lack of epidemiological studies on the occurrence of cryptococcosis in eastern Europe. Therefore, we initiated a retrospective study to investigate the epidemiology of cryptococcosis among Croatian citizens, as well as the molecular characterization and in vitro antifungal susceptibility profiles of the Cryptococcus isolates obtained.

**METHODS**

**Isolates and patients.** In total, 48 C. neoformans isolates, obtained from cerebrospinal fluid (CSF; n=46), blood (n=1), and sputum samples (n=1) of 15 patients during the period February 2005 to February 2010, were studied retrospectively (Table 1). All cryptococcal infections were diagnosed at the Reference Centre for Diagnostics of Systemic Mycoses (RCDSM) at the Croatian National Institute of Public Health in Zagreb, Croatia. There were five HIV-infected patients and ten non-HIV-related immunodeficient patients. The latter were patients with haematological malignacies (n=9) and one patient with idiopathic CD4+ lymphocytopenia. The median age of the patients was 54 years (range 24–71 years). The five HIV-infected patients had a median CD4+ T-cell count of 27 mm−3 (range 15–51 mm−3). Fourteen patients had meningitis and one had cryptococcaemia without meningitis. The majority of the patients were treated at UHID (n=11), whilst two were treated at the Dubrava Clinical Hospital in Zagreb and two at the Varaždin General Hospital. The patients from UHID were treated initially with a combination of amphotericin B and flucytosine (n=5), sequential amphotericin B and fluconazole (n=4) or fluconazole only (n=2). The four HIV-negative patients hospitalized in two other hospitals did not receive antifungal treatment because cryptococcosis was diagnosed post-mortem.

Samples were taken in accordance with clinical indications. All available isolates from each patient were analysed. One C. neoformans isolate was obtained from five patients, whilst from ten patients, between two and 11 isolates were recovered (Table 1). From nine patients, 46 Cryptococcus isolates were obtained during a single episode of cryptococcosis. The longest time span between the isolation of the first and last C. neoformans isolate was 58 days, with a mean of 14 days. From one patient, two isolates were obtained during two episodes of cryptococcosis, which were separated by a 130 day period (Table 1).

Isolates kept at the Culture Collection of the RCDSM were stored at −80°C using the Microbank system (Pro-Lab Diagnostics). All isolates were deposited in the public culture collection of the CBS-KNAW Fungal Biodiversity Centre under accession numbers CBS 11956–11997 and CBS 11999–12004.

**Media and identification.** The initial diagnosis of cryptococcosis was made by direct examination of CSF samples with India ink preparation and by detection of capsular antigen in CSF and/or serum samples with a latex agglutination test (Pastorex Crypto Plus; Bio-Rad). Cryptococcal isolation was carried out using Sabouraud’s glucose agar and brain–heart infusion agar (BD Diagnostics). The ID32C method (bioMérieux) and morphology on cornmeal agar (BD Diagnostics) were used for the identification of C. neoformans isolates.

**Antifungal susceptibility testing procedures.** In vitro antifungal susceptibility profiles of Cryptococcus isolates were determined by a broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). The antifungal agents were obtained directly from the manufacturers and comprised amphotericin B and flucytosine (Sigma Aldrich), fluconazole and voriconazole (Pfizer Pharmaceuticals) and itraconazole (Jansen Pharmaceutica). Briefly, powdered forms of each antifungal compound were dissolved in appropriate solvents to prepare stock solutions following the recommendations of CLSI (2008). Final antifungal concentrations ranged from 0.062 to 64 μg ml−1 for fluconazole, from 0.031 to 16 μg ml−1 for amphotericin B and flucytosine, and from 0.016 to 8 μg ml−1 for itraconazole and voriconazole. The test inoculum was adjusted to 1×106 c.f.u. ml−1.

Microdilution plates were incubated at 35°C for 72 h and the MIC end points were subsequently determined visually as complete growth inhibition for amphotericin B and as a prominent decrease in turbidity (≥50%) for the other antifungal agents compared with that for the growth control. Candida parapsilosis ATCC 22019/CBS 604 and Candida krusei ATCC 6528/CBS 573 were included as quality-control strains.

According to recent studies, C. neoformans isolates can be considered resistant when the MIC values are ≥2 μg ml−1 for amphotericin B, ≥32 μg ml−1 for flucytosine, ≥16 μg ml−1 for fluconazole, and ≥1 μg ml−1 for itraconazole and voriconazole (Almeida et al., 2007; Guinea et al., 2010; Hagen et al., 2010; Perfect et al., 2010; Pfaffer et al., 2005; Souza et al., 2005).

**Mating type, serotype and genotype determination.** Four different PCRs, using primers specific for each of the four mating type and serotype combinations (aA, aA, aD and aD), were performed to determine the mating type and serotype of the C. neoformans isolates by partial amplification of the STE20 locus (Barreto de Oliveira et al., 2004). Amplification reactions were carried out in a mixture containing 12.9 μl ddH2O, 2.0 μl 10× PCR buffer (15 mM MgCl2, pH 8.3), 2.0 μl dNTPs (1 mM; Bioline), 1.0 μl each
primer (10 pmol l⁻¹), 0.1 μl Taq DNA polymerase (5 U μl⁻¹; Bioline) and 1.0 μl genomic DNA (100 ng μl⁻¹). Reference strains 125.91 (=CBS 10512; αA, AFLP1), H99 (=CBS 8710; αA, AFLP1), JEC20 (=CBS 10511; αD, AFLP2) and JEC21 (=CBS 10513; αD, AFLP2) were included as controls.

AFLP fingerprinting was performed to genotype the isolates as described previously (Barreto de Oliveira et al., 2004; Boekhout et al., 2001). The reference strains H99 (=CBS 8710; AFLP1), Bt63 (=CBS 11550; AFLP1A), WM626 (=CBS 10085; AFLP1B), JEC21 (=CBS 10513; AFLP2), CBS 132 (AFLP3), WM179 (=CBS 10078; AFLP4),

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<th>HIV status</th>
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<th>Hospitalized day</th>
<th>Serotype</th>
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Table 1. Clinical data, source, days since the first positive culture, serotype, AFLP genotype and MIC values (μg ml⁻¹) of the five tested antifungal compounds for each isolate.
WM161 (=CBS 10081; AFLP5), WM178 (=CBS 10082; AFLP6), WM779 (=CBS 10101; AFLP7), CBS 10488 (AFLP8), CBS 10496 (AFLP9) and CBS11687 (AFLP10) were included to assign AFLP genotypes to each of the clinical isolates (Hagen et al., 2010; Meyer et al., 2009).

Statistical analysis of results. The MIC₅₀, MIC₉₀ and geometric mean MIC values of each antifungal agent were calculated. Only the first isolate for every patient was used in the analysis of the relationship between in vitro susceptibility to the five tested antifungal compounds and the patients’ immune status and the different mating types, serotypes and genotypes of C. neoformans isolates. To compare two groups, the Mann–Whitney test was applied, whilst the Kruskal–Wallis test was used to compare more than two groups. Statistical analyses were performed using SPSS version 12 (SPSS), and statistical significance was determined when a value of P<0.05 was observed.

RESULTS

Mating type, serotype and AFLP genotype analyses

PCR-based mating type and serotype determination revealed that cryptococcosis in the majority of patients was caused by mating type a (n=12; 80.0 %), whilst the remaining three (20.0 %) patients were infected by cryptococcal isolates with the mating types a and α (Table 1). Combined with the serotype of these isolates, it was found that six (40.0 %) patients were infected with αA, another six (40.0 %) with αD, two (13.3 %) with αA-αD and one (6.7 %) patient with αD-αA. The frequency of cryptococcosis caused by genotype AFLP1 and AFLP2 isolates was 40.0 % each (six patients each), whilst genotype AFLP3 isolates were observed to have infected three patients (20.0 %).

AFLP fingerprinting revealed more diversity among isolates that were isolated from the same patient (Fig. 1). No discrepancies between the previously determined mating type and serotype results and the AFLP genotyping were observed.

None of the patients was found to be infected with C. neoformans isolates that had different mating types or serotypes (Fig. 1; Table 1). However, the AFLP fingerprint patterns showed that there were genotypic differences among isolates from the same patient. The majority of differences between ‘same-patient isolates’ were found to be minor changes in the AFLP fingerprint patterns, i.e. the absence or presence of minor fragments (Fig. 1). It was observed that patient 8 was infected with two different C. neoformans genotypes. The first two C. neoformans var. grubii isolates cultured from patient 8 were found to be genotypically similar, namely AFLP1B, whilst the third isolate from day 11 was found to belong to genotype AFLP1 (Fig. 1). Two out of 11 C. neoformans var. neoformans isolates from patient 7, isolated on days 1 and 2 of hospitalization, were found to be genotypically slightly different from the other isolates that were isolated on the following 56 days, as indicated by the absence of some major fragments (Fig. 1). However, the C. neoformans var. neoformans isolate cultured on the first 2 days persisted, as a similar genotypic isolate was found in the CSF sample taken on day 39. Similar observations were made for the C. neoformans isolates cultured from patients 1 and 6. Six isolates were available from patient 1: the isolate from the first day of hospitalization was found to be similar to those isolated 4 and 11 days after the first sample was taken. Genotypically slightly different isolates were cultured during the 3 days following the first sample. For patient 6, the isolate on the first day of hospitalization had additional fragments in its AFLP fingerprint pattern compared with isolates cultured during successive days of isolation. Interestingly, the absence of two minor fragments in the AFLP fingerprint profile of the C. neoformans genotype AFLP3 isolates CRO008 and CRO09 cultured from patient 3 caused an apparent problem for the AFLP fingerprint clustering software, as these isolates clustered in different clades (Fig. 1).

Antifungal susceptibility testing

Detailed results of the broth microdilution antifungal susceptibility testing for all 48 clinical isolates have been summarized in Tables 1 and 2. All five antifungal compounds demonstrated high in vitro activity against all C. neoformans isolates. The MIC₅₀ for amphotericin B was 0.5 µg ml⁻¹, for flucytosine and fluconazole was 4 µg ml⁻¹, for itraconazole was 0.25 µg ml⁻¹ and for voriconazole was 0.062 µg ml⁻¹. The MIC range for amphotericin B was 0.031–0.5 µg ml⁻¹ and for flucytosine and fluconazole was 1–4 µg ml⁻¹, whilst the MIC range of itraconazole was found to be broader (0.031–0.5 µg ml⁻¹) than that of voriconazole (0.031–0.25 µg ml⁻¹).

No significant differences in susceptibility to any of the five tested antifungal compounds were observed when the patients’ HIV status, or the mating type, serotype or AFLP genotype of the C. neoformans isolates were analysed (Table 3).

DISCUSSION

The present retrospective study describes the epidemiology and in vitro antifungal susceptibility profiles of sequentially isolated C. neoformans strains from 15 Croatian patients during a 5 year period (2005–2010). This study was initiated due to the absence of any epidemiological data regarding Cryptococcus infections in Eastern Europe.

In vitro antifungal susceptibility profiles

The tested antifungal compounds amphotericin B, flucytosine, fluconazole, itraconazole and voriconazole demonstrated high in vitro activity against C. neoformans isolates (Table 1). The highest geometric mean MIC was found for flucytosine (2.79 µg ml⁻¹) and the lowest for voriconazole (0.06 µg ml⁻¹) (Table 2). Despite the increase in in vitro
Fig. 1. AFLP fingerprint patterns of 48 Croatian *C. neoformans* isolates. AFLP genotype, mating type and serotype, patient number, source of isolation and HIV status of the patient, as well as the day of isolation during hospitalization, are indicated after the AFLP fingerprint pattern. Examples of differences in AFLP fingerprint patterns among sequential isolates of *C. neoformans* are indicated by a box. Three *C. neoformans* isolates that were isolated from patient 8 are indicated by asterisks to highlight the different AFLP genotypes that these isolates exhibited.
MIC values by 1–3 log₂ dilutions, the maximum MIC values for these sequential isolates obtained during the course of a single or multiple episodes of cryptococcosis remained susceptible in vitro to all tested antifungal agents (Table 1).

Similar to the clinical isolates of *C. neoformans* from Croatia, large percentages (~99%) of tested clinical isolates from Africa, Europe, North America, South America and the Pacific Region (Pfaller et al., 2005), Cuba (Illnait-Zaragozi et al., 2008), France (Dannaoui et al., 2006), Spain (Guinea et al., 2010; López-Jodra et al., 2000), Brazil (Almeida et al., 2007; Souza et al., 2005) and the USA (Brandt et al., 2001) were shown to be susceptible in vitro to amphotericin B (MIC ≤ 1 μg ml⁻¹). However, detection of *in vitro* resistance to amphotericin B (MIC ≥ 2 μg ml⁻¹) in 5.3% of the studied clinical *C. neoformans* isolates from a Spanish collection appeared to be different from the current study, as well as from the other aforementioned studies (Perkins et al., 2005).

In concordance with the results of the current study, previous studies have also found *in vitro* susceptibility of all investigated clinical *C. neoformans* isolates to itraconazole (MIC ≤ 0.5 μg ml⁻¹) (Datta et al., 2003; López-Jodra et al., 2000; Souza et al., 2005) and voriconazole (MIC ≤ 0.5 μg ml⁻¹) (Guinea et al., 2010; Illnait-Zaragozi et al., 2008; Perkins et al., 2005; Souza et al., 2005). Exceptions were the findings of *in vitro* resistance (MIC ≥ 1 μg ml⁻¹) to itraconazole for <10% of the studied *C. neoformans* isolates (Almeida et al., 2007; Brandt et al., 2001; Illnait-Zaragozi et al., 2008) and even up to 15.8% for a group of Spanish *C. neoformans* isolates (Perkins et al., 2005).

The results of the *in vitro* testing for susceptibility to fluconazole and flucytosine for the Croatian clinical *C. neoformans* isolates are in concordance with other recent studies. For fluconazole, we observed MICs of <16 μg ml⁻¹, which is similar to data given by Almeida et al. (2007), Dannaoui et al. (2006) and Souza et al. (2005), whilst the MIC of <32 μg ml⁻¹ for flucytosine was similar to those found by Dannaoui et al. (2006) and López-Jodra et al. (2000). In contrast to this, *in vitro* resistance to fluconazole (MIC ≥ 16 μg ml⁻¹) was proven in 3.4% of *C. neoformans* isolates originating from Spain (Guinea et al., 2010), 15.9% from India (Datta et al., 2003), 19% from the USA (Brandt et al., 2001), 25% from North America (Pfaller et al., 2005) and 46.6% from Spain (Perkins et al., 2005). The percentage of clinical *C. neoformans* isolates resistant to flucytosine (MIC ≥ 32 μg ml⁻¹) was lower and also dependent on geographical area. Whilst all Croatian *C. neoformans* isolates were found to be susceptible to flucytosine, 1% of African, European, Pacific and North and South American isolates (Pfaller et al., 2005), 3.8% of isolates from the USA (Brandt et al., 2001) and 4.7% of isolates from Spain (Perkins et al., 2005) were observed to be resistant.

The difference in *in vitro* antifungal susceptibility between the different genotypes and mating types within *C. neoformans* has not been studied extensively. To the best of our knowledge, the current study is the second known, after that of Guinea et al. (2010). The current study (summarized in Table 3) and that of Guinea et al. (2010) observed that the genotypic background of a cryptococcal isolate (genotype AFLP 1–3) or the mating type z had no statistically significant influence on the *in vitro* antifungal activity against fluconazole and voriconazole. In contrast to our results (Table 3), the Spanish epidemiological study of Guinea et al. (2010) observed a statistically significant correlation of *in vitro* susceptibility to amphotericin B with the AFLP genotypes of the *C. neoformans* isolates.

Several studies have evaluated the relationships between antifungal susceptibility and serotypes of *C. neoformans* isolates. There was no significant difference observed between the antifungal susceptibility values to the five antifungal compounds when compared with the serotype A, D and AD background of the isolates (Table 3). The French (Dannaoui et al., 2006) and Italian (Tortorano et al., 1997) studies showed that serotype A isolates were significantly less susceptible to fluconazole than serotype D. However, this discrepancy between serotypes A and D was not observed in the Spanish study (Guinea et al., 2010). The French (Dannaoui et al., 2006) and Spanish (Guinea et al., 2010) studies observed that *in vitro* antifungal susceptibility to amphotericin B was lower in serotype A than in serotype D isolates. The French *C. neoformans* serotype A isolates were also less susceptible to flucytosine than serotype D isolates (Dannaoui et al., 2006). In contrast to this, the susceptibility to fluconazole and voriconazole for

### Table 2. MIC ranges, geometric mean MICs, MIC₅₀, MIC₉₀ and percentages of *C. neoformans* isolates per MIC group

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different serotypes of *C. neoformans* was similar to those observed in the Spanish study (Guinea et al., 2010).

### Sequential *C. neoformans* isolates from the same patient

A handful of studies have been published that describe the occurrence of mixed *C. neoformans* infections or shifts in genotypic and antifungal susceptibility differences among sequentially isolated *C. neoformans* strains (Blasi et al., 2001; Casadevall et al., 1993; Desnos-Ollivier et al., 2010; Illnait-Zaragozí et al., 2010; Pfaller et al., 1998; Spitzer et al., 1993; Sullivan et al., 1996). It has been shown that genomic differences between sequential isolates of *C. neoformans* can influence virulence and persistence of a cryptococcal infection, as has been shown using a mouse pathogenicity model (Fries & Casadevall, 1998). Besides a change in the genotype of the initial *C. neoformans* isolate due to microevolution or karyotype instability, replacement of the initial isolate with another genotypic variant can influence the outcome of antifungal treatment or may cause a relapse (Blasi et al., 2001; Fries & Casadevall, 1998).

From the patients from whom we studied multiple *C. neoformans* isolates, only patient 8 was found to be infected with multiple genotypic *C. neoformans* variants, namely genotype AFLP1 and AFLP1B isolate (Fig. 1, indicated by asterisks). Several other patients (1, 6 and 7) were found to be infected with isolates that showed minor differences in the AFLP fingerprint patterns. Interestingly, it was observed that the 11 *C. neoformans* var. neoformans isolates from patient 7 became gradually less susceptible, when tested in vitro, to amphotericin B (0.062–0.5 µg ml⁻¹), fluycytosine (1–4 µg ml⁻¹), fluconazole (1–2 µg ml⁻¹) and itraconazole (0.031–0.125 µg ml⁻¹), whilst no change in voriconazole susceptibility was observed (Table 1). The gradual decrease in in vitro antifungal susceptibility of these isolates might be due to microevolution that took place in the *C. neoformans* isolates during the course of antifungal therapy undergone by this patient. Patient 7 was initially treated with intravenous (i.v.) colloidal amphotericin B (400 mg day⁻¹) and fluconazole (400 mg day⁻¹). After 3 weeks therapy, the dosage of fluconazole was doubled. Different formulations of amphotericin B were continuously given for a total of 2 months, after which a high dosage of fluconazole (1600 mg day⁻¹ i.v.) was administrated. HIV infection in patient 7 was confirmed after the diagnosis of cryptococcal meningitis. The patient had a CD4⁺ cell count of 25 mm⁻³ and a plasma RNA HIV-1 viral load of 956 000 copies ml⁻¹. HAART, with stavudine, lamivudine and nevirapine, was given after 3 weeks antifungal therapy. After 6 weeks HAART, the CD4⁺ cell count increased to 96 mm⁻³ and the plasma viral load decreased to 1490 HIV-1 RNA copies ml⁻¹. However, the patient did not recover and died 3 months after the diagnosis of cryptococcal meningitis was made. HIV-infected patient 7 was shown to be infected initially with a *C. neoformans* var. neoformans isolate that was replaced by...
another genotypic variant after day 2, whilst the initial genotypic variant was again isolated on day 39 (Fig. 1, Table 1).

The case of patient 7 is comparable to results observed by Spitzer et al. (1993), who showed that initial and recurrent C. neoformans isolates were clonally related. This confirms that an initial C. neoformans isolate can persist despite antifungal therapy, rather than that a novel infection is acquired. Thus, therapeutic failure might occur because an antifungal treatment strategy cannot eradicate the initial infection (Spitzer et al., 1993). In contrast, it was also observed that sequential C. neoformans isolates became increasingly susceptible in vitro to fluconazole (Casadevall et al., 1993). This provides evidence against antifungal resistance as the cause of recurrent C. neoformans infection, and it might be that an attenuated immune system can give rise to the persistence of cryptococcal infections (Casadevall et al., 1993).

For the C. neoformans cultures from patient 8, higher MIC values were found between the two initial and the last isolate for amphotericin B (0.062 and 0.125 µg ml⁻¹, respectively) (Table 1). This patient was treated initially with colloidal amphotericin B (400 mg day⁻¹ i.v.) plus fluconazole (800 mg day⁻¹ i.v.) and the dose of fluconazole was lowered (400 mg day⁻¹ i.v.). Five days later, the treatment was continued with amphotericin B deoxycholate (75 mg day⁻¹ i.v. and the same dosage of fluconazole (400 mg day⁻¹ i.v.). Different formulations of amphotericin B were given for a total of 3 weeks. Fluconazole (400 mg day⁻¹ orally), as maintenance therapy, was administered for 12 months. At follow-up, the patient had recovered fully.

Mixed infections have been described in detail recently by Desnos-Ollivier et al. (2010), who observed that nearly 20% of investigated patients were infected with multiple C. neoformans genotypic variants. They also found that serotype A and D isolates can form serotype AD hybrids in vivo using a mice model. Thus, it is likely that C. neoformans is able to form new genotypic variants in vivo that eventually might be less susceptible to antifungal compounds. In the current study, patient 8 was found to be infected initially with an isolate belonging to genotype AFLP1B, whilst 10 days later a genotypically different isolate with genotype AFLP1 was cultured. Despite the fact that only one C. neoformans colony was used for further characterization, the possibility exists that the initial C. neoformans AFLP1B isolate underwent genotypic changes to an AFLP1 genotype due to the selection pressure caused by the antifungal treatment. This is a plausible explanation, as it has been hypothesized that C. neoformans genotype AFLP1A and AFLP1B strains might be hybrid genotypes (Barreto de Oliveira et al., 2004; Boekhout et al., 2001). Alternatively, the patient could be infected with multiple C. neoformans genotypic variants but, due to the fact that only one colony was investigated per sample, an initial mixed infection might be overlooked. This explanation has also been given for a similar observation for sequential isolates of C. neoformans from Cuban patients, where four of 19 patients were found to be probably infected by more than one C. neoformans genotypic variant but only a single colony was investigated (Illnait-Zaragozì et al., 2010).

In conclusion, no in vitro resistance to amphotericin B, flucytosine, fluconazole, itraconazole or voriconazole was observed among the 48 clinical C. neoformans isolates from Croatia collected between 2005 and 2010. A statistically significant correlation was not demonstrated between in vitro antifungal susceptibility patterns and the HIV status of the patients, or the mating type, serotype and AFLP genotype of the studied C. neoformans isolates. Multiple C. neoformans genotypic variants might exist during the initial infection and therefore it is recommended that multiple single colonies are investigated during the course of infection when a patient is not responding to the antifungal treatment.

REFERENCES


