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 α ($n=12$; 80.0%) predominated and α/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 $\mu\text{g ml}^{-1}$ (range 0.031–0.5 $\mu\text{g ml}^{-1}$) for amphotericin B, 4 $\mu\text{g ml}^{-1}$ (range 1–4 $\mu\text{g ml}^{-1}$) for flucytosine and fluconazole, 0.25 $\mu\text{g ml}^{-1}$ (range 0.031–0.5 $\mu\text{g ml}^{-1}$) for itraconazole and 0.062 $\mu\text{g ml}^{-1}$ (range 0.031–0.25 $\mu\text{g ml}^{-1}$) for voriconazole.

Received 13 February 2011

Accepted 26 May 2011

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).

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Abbreviations: AFLP, amplified fragment length polymorphisms; CSF, cerebrospinal fluid; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; i.v., intravenous; RCDSM, Reference Centre for Diagnostics of Systemic Mycoses; UHID, University Hospital of Infectious Diseases.

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, from 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 malignancies ($n=9$) 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 fluconazole ($n=5$), 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 $\mu\text{g ml}^{-1}$ for fluconazole, from 0.031 to 16 $\mu\text{g ml}^{-1}$ for amphotericin B and flucytosine, and from 0.016 to 8 $\mu\text{g ml}^{-1}$ for itraconazole and voriconazole. The test inoculum was adjusted to 1×10^3 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 ($\geq 50\%$) for the other antifungal agents compared with that for the growth control. *Candida parapsilosis* ATCC 22019/CBS 604 and *Candida krusei* ATCC 6258/CBS 573 were included as quality-control strains.

According to recent studies, *C. neoformans* isolates can be considered resistant when the MIC values are $\geq 2 \mu\text{g ml}^{-1}$ for amphotericin B, $\geq 32 \mu\text{g ml}^{-1}$ for flucytosine, $\geq 16 \mu\text{g ml}^{-1}$ for fluconazole, and $\geq 1 \mu\text{g ml}^{-1}$ for itraconazole and voriconazole (Almeida *et al.*, 2007; Guinea *et al.*, 2010; Hagen *et al.*, 2010; Perfect *et al.*, 2010; Pfaller *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 (αA , αA , αD and αD), 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 $10 \times$ PCR buffer (15 mM MgCl₂, pH 8.3), 2.0 μl dNTPs (1 mM; Bioline), 1.0 μl each

Table 1. Clinical data, source, days since the first positive culture, serotype, AFLP genotype and MIC values ($\mu\text{g ml}^{-1}$) of the five tested antifungal compounds for each isolate

Isolate	Patient	Sex	HIV status	Source	Hospitalized day	Serotype	AFLP	AMB	5FC	FLU	ITR	VOR
1	1	F	HIV ⁺	CSF	0	α A	1	0.062	1	2	0.250	0.062
2				CSF	1	α A	1	0.062	1	2	0.250	0.062
3				CSF	2	α A	1	0.062	1	2	0.250	0.062
4				CSF	3	α A	1	0.062	1	2	0.250	0.062
5				CSF	4	α A	1	0.062	1	4	0.250	0.062
6				CSF	11	α A	1	0.062	2	4	0.500	0.062
7	2	M	HIV ⁻	CSF	0	α A	1	0.500	1	4	0.500	0.031
8	3	M	HIV ⁻	CSF	0	α D- aA	3	0.062	2	4	0.125	0.062
9				CSF	130	α D- aA	3	0.500	4	4	0.125	0.125
10	4	M	HIV ⁻	CSF	0	α D	2	0.125	4	2	0.125	0.031
11				CSF	1	α D	2	0.125	4	2	0.125	0.031
12				CSF	2	α D	2	0.125	4	2	0.125	0.031
13				CSF	3	α D	2	0.125	4	2	0.125	0.031
14	5	M	HIV ⁻	CSF	0	α A- aD	3	0.062	4	1	0.125	0.062
15	6	M	HIV ⁻	CSF	0	α D	2	0.125	4	1	0.125	0.062
16				CSF	1	α D	2	0.125	4	1	0.125	0.062
17				CSF	2	α D	2	0.125	4	1	0.125	0.062
18				CSF	6	α D	2	0.125	4	1	0.125	0.062
19	7	M	HIV ⁺	CSF	0	α D	2	0.062	1	1	0.031	0.062
20				CSF	1	α D	2	0.062	1	1	0.031	0.062
21				CSF	2	α D	2	0.062	1	1	0.031	0.062
22				CSF	14	α D	2	0.062	2	2	0.125	0.062
23				CSF	21	α D	2	0.125	2	2	0.125	0.062
24				CSF	28	α D	2	0.125	4	2	0.125	0.062
25				CSF	34	α D	2	0.250	4	2	0.125	0.062
26				CSF	39	α D	2	0.250	4	2	0.125	0.062
27				CSF	45	α D	2	0.250	4	2	0.125	0.062
28				CSF	53	α D	2	0.500	4	2	0.125	0.062
29				CSF	57	α D	2	0.500	4	2	0.125	0.062
30	8	M	HIV ⁺	CSF	0	α A	1B	0.062	2	1	0.125	0.062
31				CSF	1	α A	1B	0.062	2	1	0.125	0.062
32				CSF	11	α A	1	0.125	2	1	0.125	0.062
33	9	F	HIV ⁻	Blood	0	α A	1B	0.031	4	2	0.125	0.031
34	10	M	HIV ⁺	CSF	0	α D	2	0.062	4	1	0.062	0.062
35				Sputum	13	α D	2	0.125	4	1	0.062	0.062
36	11	F	HIV ⁻	CSF	0	α A	1	0.062	4	1	0.062	0.062
37				CSF	1	α A	1	0.062	4	1	0.062	0.062
38				CSF	2	α A	1	0.062	4	1	0.062	0.062
39				CSF	4	α A	1	0.062	4	1	0.062	0.062
40				CSF	5	α A	1	0.062	4	1	0.062	0.062
41	12	M	HIV ⁻	CSF	0	α A	1	0.500	4	4	0.500	0.031
42	13	F	HIV ⁻	CSF	0	α D	2	0.062	4	1	0.062	0.062
43				CSF	1	α D	2	0.062	4	1	0.062	0.062
44				CSF	13	α D	2	0.125	4	1	0.062	0.062
45	14	M	HIV ⁻	CSF	0	α D	2	0.500	4	2	0.062	0.031
46	15	M	HIV ⁺	CSF	0	α A- aD	3	0.062	4	2	0.062	0.125
47				CSF	12	α A- aD	3	0.062	4	4	0.125	0.125
48				CSF	18	α A- aD	3	0.062	4	4	0.125	0.250

primer ($10 \text{ pmol } \mu\text{l}^{-1}$), $0.1 \text{ } \mu\text{l}$ *Taq* DNA polymerase ($5 \text{ U } \mu\text{l}^{-1}$; Bioline) and $1.0 \text{ } \mu\text{l}$ genomic DNA ($100 \text{ ng } \mu\text{l}^{-1}$). Reference strains 125.91 (=CBS 10512; **aA**, AFLP1), H99 (=CBS 8710; α A, AFLP1), JEC20 (=CBS 10511; **aD**, AFLP2) and JEC21 (=CBS 10513; α D, 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 10083; AFLP1B), JEC21 (=CBS 10513; AFLP2), CBS 132 (AFLP3), WM179 (=CBS 10078; AFLP4),

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 α ($n=12$; 80.0%), whilst the remaining three (20.0%) patients were found to be infected by cryptococcal isolates with the mating types α and **a** (Table 1). Combined with the serotype of these isolates, it was found that six (40.0%) patients were infected with α A, another six (40.0%) with α D, two (13.3%) with α A- α D and one (6.7%) patient with α D-**a**A. 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 CRO08 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 MIC₉₀ for amphotericin B was 0.5 $\mu\text{g ml}^{-1}$, for flucytosine and fluconazole was 4 $\mu\text{g ml}^{-1}$, for itraconazole was 0.25 $\mu\text{g ml}^{-1}$ and for voriconazole was 0.062 $\mu\text{g ml}^{-1}$. The MIC range for amphotericin B was 0.031–0.5 $\mu\text{g ml}^{-1}$ and for flucytosine and fluconazole was 1–4 $\mu\text{g ml}^{-1}$, whilst the MIC range of itraconazole was found to be broader (0.031–0.5 $\mu\text{g ml}^{-1}$) than that of voriconazole (0.031–0.25 $\mu\text{g ml}^{-1}$).

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 $\mu\text{g ml}^{-1}$) and the lowest for voriconazole (0.06 $\mu\text{g ml}^{-1}$) (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.

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

Antifungal	MIC values ($\mu\text{g ml}^{-1}$)				Percentage per MIC group ($\mu\text{g ml}^{-1}$)							
	Range	MIC ₅₀	Geometric mean MIC	MIC ₉₀	0.031	0.062	0.125	0.25	0.5	1	2	4
Amphotericin B	0.031–0.5	0.062	0.100	0.5	2.1	52.1	27.1	6.2	12.5	–	–	–
Flucytosine	1–4	4	2.788	4	–	–	–	–	–	18.7	14.6	66.7
Fluconazole	1–4	2	1.658	4	–	–	–	–	–	43.7	39.6	16.7
Itraconazole	0.031–0.5	0.125	0.113	0.25	6.2	25.0	52.1	10.4	6.2	–	–	–
Voriconazole	0.031–0.25	0.062	0.059	0.062	16.7	75.0	6.2	2.1	–	–	–	–

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-Zaragozí *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 $\leq 1 \mu\text{g ml}^{-1}$). However, detection of *in vitro* resistance to amphotericin B (MIC $\geq 2 \mu\text{g ml}^{-1}$) 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 $\leq 0.5 \mu\text{g ml}^{-1}$) (Datta *et al.*, 2003; López-Jodra *et al.*, 2000; Souza *et al.*, 2005) and voriconazole (MIC $\leq 0.5 \mu\text{g ml}^{-1}$) (Guinea *et al.*, 2010; Illnait-Zaragozí *et al.*, 2008; Perkins *et al.*, 2005; Souza *et al.*, 2005). Exceptions were the findings of *in vitro* resistance (MIC $\geq 1 \mu\text{g ml}^{-1}$) to itraconazole for <10 % of the studied *C. neoformans* isolates (Almeida *et al.*, 2007; Brandt *et al.*, 2001; Illnait-Zaragozí *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 $<16 \mu\text{g ml}^{-1}$, which is similar to data given by Almeida *et al.* (2007), Dannaoui *et al.* (2006) and Souza *et al.* (2005), whilst the MIC of $<32 \mu\text{g ml}^{-1}$ 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 $\geq 16 \mu\text{g ml}^{-1}$) 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 $\geq 32 \mu\text{g ml}^{-1}$) 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 α 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 concordance with the results of the current study (Table 3), the susceptibility to fluconazole and voriconazole for

Table 3. Relationships between *in vitro* susceptibility to five antifungal compounds and patients' immune status and the different mating types, serotypes and genotypes of *C. neoformans* isolates

Category	Patients' immune status			Isolate mating type			Isolate serotype/AFLP genotype			
	HIV positive	HIV negative	P value	α	α/a	P value	A/AFLP1	D/AFLP2	AD/AFLP3	P value
No. patients	5	10	–	12	3	–	6	6	3	–
Amphotericin B	0.062	0.100	0.242	0.091	0.062	0.624	0.111	0.076	0.062	0.866
Flucytosine	2.000	3.250	0.159	2.670	3.170	0.795	2.240	3.170	3.170	0.516
Fluconazole	1.320	1.870	0.346	1.590	2.000	0.533	2.000	1.260	2.000	0.311
Itraconazole	0.082	0.134	0.294	0.118	0.099	0.879	0.198	0.070	0.098	0.079
Voriconazole	0.071	0.044	0.051	0.047	0.078	0.067	0.044	0.049	0.078	0.163

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 $\mu\text{g ml}^{-1}$), flucytosine (1–4 $\mu\text{g ml}^{-1}$), fluconazole (1–2 $\mu\text{g ml}^{-1}$) and itraconazole (0.031–0.125 $\mu\text{g ml}^{-1}$), 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 administered. 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.). After 5 days, colloidal amphotericin B was replaced by liposomal amphotericin B (300 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-Zaragozí *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.

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