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Molecular Characterization and Antifungal Susceptibility Testing of Sequentially Obtained Clinical *Cryptococcus deneoformans* and *Cryptococcus neoformans* Isolates from Ljubljana, Slovenia

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Abstract
Aim To retrospectively investigate the epidemiology of cryptococcosis in Ljubljana, Slovenia.
Methodology Forty-six sequentially obtained isolates from 19 patients were subjected to amplified fragment length polymorphism (AFLP) genotyping, microsatellite typing, mating- and serotype PCRs and antifungal susceptibility testing.
Results Majority of the isolates were *Cryptococcus deneoformans* ($n = 29/46$; 63%) followed by *Cryptococcus neoformans* ($n = 16/46$; 34.8%) and their interspecies hybrid ($n = 1/46$; 2.2%). Mating-type α was predominant, two mating-type **a** *C. deneoformans* isolates and one mating-type **a**/α isolate were observed. Several mixed infections were found by microsatellite typing; one patient had a persisting *C. deneoformans* infection for > 2.5 years. For *C. deneoformans,* the in vitro antifungal MIC_{90} and susceptibility ranges were for amphotericin B 0.25 μg/ml (0.031–0.25 μg/ml), 5-fluorocytosine 0.25 μg/ml (0.063–4 μg/ml), fluconazole 8 μg/ml (0.5–16 μg/ml), voriconazole 0.063 μg/ml (0.008–0.125 μg/ml), posaconazole 0.063 μg/ml (0.008–0.063 μg/ml) and itraconazole 0.063 μg/ml (0.031–0.125 μg/ml). For *C. neoformans,* these values were for amphotericin B 0.25 μg/ml (0.063–0.5 μg/ml), 5-fluorocytosine 1 μg/ml (0.063–1 μg/ml), fluconazole 16 μg/ml (0.5–64 μg/ml), voriconazole 0.125 μg/ml (0.008–0.25 μg/ml), posaconazole 0.063 μg/ml (0.008–0.063 μg/ml) and itraconazole 0.063 μg/ml (0.031–0.125 μg/ml).
Conclusions Majority of the cases were caused by *C. deneoformans*; mating-type α was predominant. Several mixed infections were identified by AFLP genotyping and microsatellite typing. Despite antifungal therapy, a cryptococcal isolate could persist for years. Voriconazole, itraconazole and posaconazole were the most potent antifungal drugs.

Keywords Cryptococcosis · *Cryptococcus deneoformans* · *Cryptococcus neoformans* · AFLP genotyping · Microsatellite typing · Epidemiology

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Introduction

Cryptococcosis is caused by members of the basidiomycetous yeast genus *Cryptococcus*, mainly by species that belongs to the *Cryptococcus gattii*/*Cryptococcus neoformans* species complexes [1, 2]. Cryptococcal infections became one of the major fungal culprits of disease with the onset of the HIV-pandemic, although the introduction of antiretroviral therapy has turned the tide, the number of infections remains high in less developed countries [2]. The global burden of HIV-associated cryptococcal meningitis was estimated to be nearly 225,000 new infections each year mainly in sub-Saharan Africa, and a large number of these infected patients do not survive [3].

Cryptococcus neoformans sensu lato is globally the major cause of cryptococcal infection among immunosuppressed patients, while the sibling *C. gattii* sensu lato includes genotypes that are primarily observed among HIV-infected, while other genotypes have mostly been isolated from immunocompetent individuals [4, 5]. With the introduction of molecular techniques, such as PCR fingerprinting, restriction fragment length polymorphism (RFLP) analysis of *PLB1* and *URA5* genes, amplified fragment length polymorphism (AFLP) fingerprinting, multi-locus sequence typing (MLST) and whole genome sequencing, it became apparent that *C. gattii* and *C. neoformans* were both species complexes [5, 6]. After more than 15 years of debate, the taxonomy of the *C. gattii* and *C. neoformans* species complex was revised [5, 7, 8]. The varieties of *C. neoformans* were raised to the species level, with *C. neoformans* (formerly *C. neoformans* var. *grubii*; serotype A; genotype AFLP1/VNI, AFLP1A/VNB/VNII, AFLP1B/VNII) and *C. deneoformans* (formerly *C. neoformans* var. *neoformans*; serotype D; genotype AFLP2/VNIV) [5]. The five genotypes within *C. gattii* sensu lato were all raised to the species level as *C. gattii* sensu stricto (serotype B; genotype AFLP4/VGI), *C. bacillisporus* (serotype C; genotype AFLP5/VGIII), *C. deuterogattii* (serotype B; genotype AFLP6/VGII), *C. tetragattii* (serotype C; genotype AFLP7/VGIV) and *C. decagattii* (serotype B; genotype AFLP10/VGIV) [5]. Several interspecies hybrids have been described; *C. neoformans* × *C. deneoformans* (serotype AD; genotype AFLP3/VNIII) is the most encountered hybrid, while hybrids between *C. gattii* sensu lato and *C. neoformans* sensu lato have been found to be a rarity [4, 5, 9–12].

Epidemiological surveys performed in Europe have observed that *C. neoformans* sensu stricto (serotype A) is the major cause of cryptococcal disease, but that *C. deneoformans* (serotype D) and *C. neoformans* × *C. deneoformans* hybrids are often found in patients and the environment of Mediterranean Europe [4, 13, 14]. Despite the large number of epidemiological studies, little is known about differences in host predilection and clinical differences between *C. neoformans*, *C. deneoformans* and their interspecies hybrids. Although the latter two seems to be more often involved in cutaneous and soft tissue infections, *C. deneoformans* is more frequently a cause of infection in the elderly [15].

As epidemiological data from Eastern Europe and the Balkan region are sparse, the aim of this study was to investigate the epidemiology of cryptococcosis in Slovenia. For this purpose, cryptococcal isolates were molecularly characterized by using mating- and serotyping by qPCR's; the genetic relatedness was assessed by AFLP fingerprinting and microsatellite typing; and antifungal susceptibility testing was performed and included amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole and voriconazole.

Materials and Methods

Patients, Isolates and Media

Forty-six cryptococcal isolates were collected during the period of October 1998–April 2016 and involved 19 Slovenian patients (Fig. 1). From ten patients, one culture was obtained; from five patients, two isolates were cultured; four, six, seven and nine isolates were obtained each from one patient (Fig. 1). Patients were between 14 and 85 years old, with a median age of 44, 63.2% ($n = 12$) were male and 36.8% ($n = 7$) were female. Patients had different underlying diseases, with HIV infection being the most common ($n = 8$, 42.1%), followed by kidney transplant ($n = 3$, 15.8%), non-ST segment elevation myocardial infarction (NSTEMI; $n = 2$, 10.5%), lymphoma ($n = 1$, 5.3%), sepsis ($n = 1$, 5.3%), pneumonia ($n = 1$, 5.3%), ST segment elevation myocardial infarction (STEMI; $n = 1$, 5.3%) and cystic fibrosis ($n = 1$,

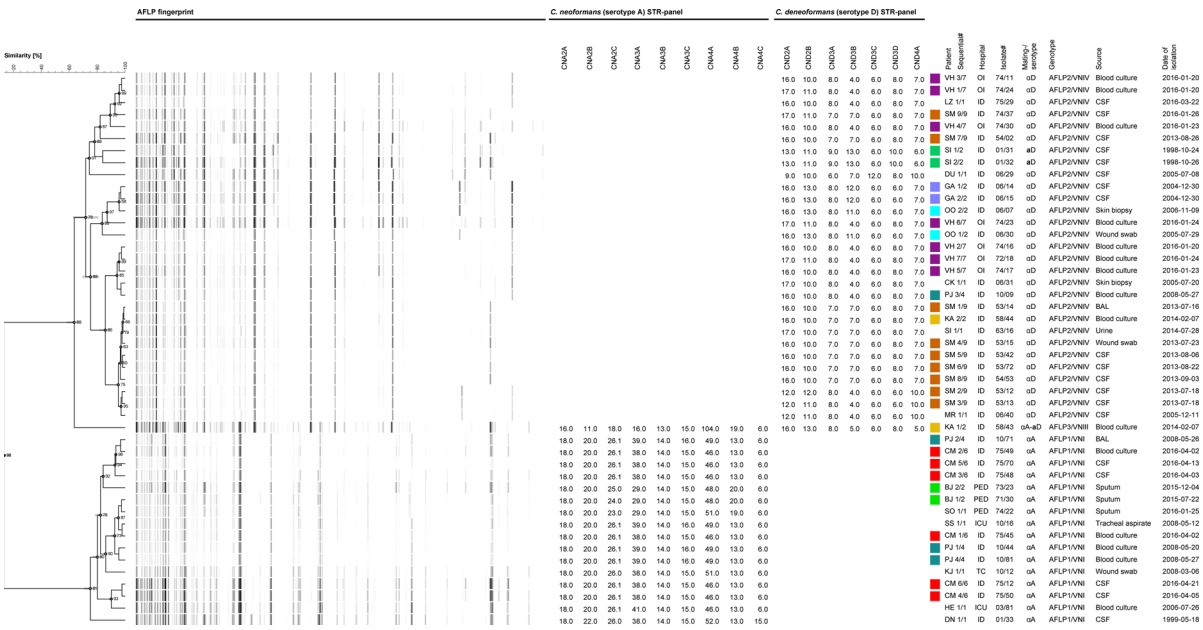

Fig. 1 AFLP genotyping, microsatellite typing and background information of studied cryptococcal isolates. The dendrogram was calculated by using the AFLP fingerprint data as input with the Pearson correlation similarity coefficient in combination with UPGMA cluster analysis; cophenetic correlation was applied to show branch qualities. AFLP fingerprint and microsatellite data are presented next to each other, followed by clinical background information

5.3%). One female patient had a combination of breast cancer, diabetes mellitus type 2, polymyalgia rheumatica and NSTEMI, which accounts for 5.3% ($n = 1$) of our patient population. All, except two, patients were regarded as having one episode of cryptococcal infection (range 1–135 days). One episode of disease was defined by having at least one positive culture; > 1 episode was defined as having two positive cultures with an interval ≥ 1 year. One patient had two episodes that were separated by 2.5 years; another patient had two episodes that were diagnosed 1.3 years after each other (Fig. 1).

Cryptococcal isolates were cultured from CSF ($n = 19$; 41.3%), blood culture ($n = 15$; 32.6%), sputum ($n = 3$; 6.5%), wound swab ($n = 3$; 6.5%), bronchial alveolar lavage ($n = 2$; 4.3%), skin biopsy ($n = 2$; 4.3%), tracheal aspirate ($n = 1$, 2.2%) and urine ($n = 1$, 2.2%).

Primary clinical specimens and positive blood cultures were plated onto Sabouraud dextrose agar supplemented with chloramphenicol and gentamycin (BioMérieux, Marcy l'Etoile, France) and chromogenic medium CHROMagar Candida (Mast Diagnostica, Reinfeld, Germany). Plates were incubated at 37 °C. Isolates were identified biochemically using ID 32C strips according to manufacturer's instructions (BioMérieux). Colonies suggestive for *Cryptococcus* species were subjected to ethanol–formiate extraction for identification with the MALDI-TOF Biotyper system, version 3.1, according to manufacturer's instructions (Bruker Daltonik, Bremen, Germany). Isolates were cultured onto Sabouraud dextrose agar supplemented with chloramphenicol (Oxoid, Basingstoke, UK) and incubated for 48 h at 30 °C. Stock cultures were kept at −80 °C for which the Microbank system was used (Pro-Lab Diagnostics, Richmond Hill, ON, Canada).

The isolates were deposited in the culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, with the accession numbers CBS15077-CBS15103, CBS15110-CBS15127 and CBS15149.

Extraction of Genomic DNA

A loopfull of cryptococcal cells were harvested from 48-h old cultures and suspended into Green Beads tubes that contain 300 µl bacterial lysis buffer followed by bead beating with a MagNA Lyser (all Roche Diagnostics, Almere, The Netherlands). After

2-min centrifugation at 14,000 rpm, the cell lysates were heat-inactivated by 100 °C for 10 min, and after cooling to room temperature 200 µl of the cell lysate was used as input for automatic genomic DNA extraction on a MagNA Pure 96 platform (Roche Diagnostics) for which the Pathogen Detection 200 protocol was used with a final elution volume of 100 µl. Genomic DNA samples were stored at −20 °C upon further use.

Molecular Characterization by AFLP, qPCR's and Microsatellite Typing

Genotypic characterization was performed by AFLP fingerprinting, as described previously, by using the restriction enzymes EcoR1 and MseI during the enzymatic reaction and the selective primers EcoR1-AC (fluorescently labelled with fluorescein at the 5′-side) and MseI-G for the AFLP-PCR [16, 17]. The reference strains for *C. neoformans* (CBS10512 and CBS10515, genotype AFLP1/VNI; Bt1, genotype AFLP1A/VNB/VNII; WM626, genotype AFLP1B/VNII), *C. deneoformans* (CBS10511 and CBS10513, genotype AFLP2/VNIV), *C. neoformans* × *C. deneoformans* hybrid (CBS132, genotype AFLP3/VNIII), *C. gattii* sensu stricto (WM179, genotype AFLP4/VGI), *C. bacillisporus* (WM161, genotype AFLP5/VGIII), *C. deuterogattii* (WM178, genotype AFLP6/VGII), *C. tetragattii* (CBS10101, genotype AFLP7/VGIV), *C. decagattii* (IHEM14941, genotype AFLP10/VGIV), *C. gattii* sensu stricto × *C. deneoformans* (CBS10488, genotype AFLP8) and *C. gattii* sensu stricto × *C. neoformans* (CBS10496, genotype AFLP9) were included as controls to assign the appropriate species and genotype. Fragment analysis was performed on an ABI3500xL Genetic Analyser according to the manufacturer's instructions (Applied Biosystems, Palo Alto, CA, U.S.A.); for this purpose, 1 µl 10 × diluted AFLP-PCR product was added to a mixture of 8.9 µl ddH_2O and 0.1 µl LIZ600 internal size marker (Applied Biosystems) and boiled for 1 min at 100 °C followed by cooling to 4 °C. Raw data were analysed with BioNumerics v7.5 (Applied Maths, St. Martem-Latum, Belgium), and fingerprints were compared by using UPGMA clustering after a matrix has been created with the similarity coefficient curve-based Pearson correlation.

The mating- and serotype of the isolates were determined by two previously described multiplex qPCRs that target a part of the *RUM1* gene, one qPCR specific for the mating-type **a** allele and the other for the mating-type α allele, while two hydrolysis probes specifically target *C. neoformans* (serotype A) and *C. deneoformans* (serotype D) [18]. The reference strains for *C. neoformans* (serotype A; CBS10512, mating-type **a**; CBS10515, mating-type α) and *C. deneoformans* (serotype D; CBS10511, mating-type **a**; CBS10513, mating-type α) were included as controls.

Microsatellite typing was performed to investigate the genetic relatedness of isolates that have a similar AFLP genotype; isolates that belong to *C. neoformans* (including hybrids) were subjected to a *C. neoformans*-specific microsatellite panel that includes nine loci [19]. Isolates that were observed to be *C. deneoformans* (including hybrids) were subjected to a *C. deneoformans*-specific microsatellite panel that consists of seven loci [20]. Multiplex PCRs were performed as described before [19, 20]. Fragment analysis was performed in a similar approach as described above for AFLP fingerprinting; briefly, 1 µl of 100 × diluted multiplex PCR product was added to a mixture of 8.9 µl ddH_2O and 0.1 µl CC-ROX500 internal size marker (Promega, Leiden, The Netherlands) and boiled for 1 min at 100 °C followed by cooling to 4 °C. Subsequently, the microsatellite fragments were determined on an ABI3500xL Genetic Analyser (Applied Biosystems); according to the manufacturer's instructions, raw data were imported into GeneMapper software (Applied Biosystems) and microsatellite repeat numbers were assigned. The microsatellite profiles, containing either nine or seven numbers for the *C. neoformans* and *C. deneoformans* panels, respectively, were imported into BioNumerics v7.5 (Applied Maths).

Antifungal Susceptibility Testing

Susceptibility testing of all 46 isolates was performed with the Micronaut-AM Antifungal Agents MIC broth microdilution (Merlin Diagnostika, Bornheim-Hersel, Germany) according to manufacturer's instructions. The test plates contain serial twofold dilutions of amphotericin B (AMB; 0.03–16 µg/ml), 5-fluorocytosine (5FC; 0.06–32 µg/ml), fluconazole (FLZ; 0.002–128 µg/ml), voriconazole (VCZ; 0.008–8.0 µg/ml), posaconazole (PCZ; 0.008–8 µg/ml), itraconazole (ITZ; 0.03–4 µg/ml), micafungin (0.002–8 µg/ml), anidulafungin (0.002–8 µg/ml) and

caspofungin (0.002–8 μg/ml). The latter three compounds are not effective against *Cryptococcus* species and are not further interpreted. In brief, isolates were grown on Sabouraud dextrose agar (BioMérieux) at 37 °C for 48 h. Isolate suspensions were prepared in 0.9% NaCl and adjusted to 0.5 McFarland turbidity standard. A 20-fold dilution was prepared in 0.9% NaCl, and a fivefold dilution was prepared in 11 mL of RPMI broth (Merlin Diagnostika) supplemented with 50 μL AST indicator. The test plates were then inoculated with 100 μL/well of suspension in RPMI broth and incubated at 30 °C for 48–72 h. Test results were determined by a photometric reading using Micronaut Software (Merlin Diagnostika). The reference strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality controls.

Results

Molecular Characterization

Two major groups were identified by AFLP fingerprinting; the largest one was formed by *C. deneoformans* (AFLP2/VNIV) that includes 29 isolates (63%); 16 isolates (34.8%) clustered with the *C. neoformans* (AFLP1/VNI) reference strain, and one isolate (2.2%) was found to be a hybrid between *C. neoformans* × *C. deneoformans* (AFLP3/VNIII) (Fig. 1).

Mating- and serotyping by qPCR showed that all *C. neoformans* isolates were mating-type α and serotype A; all *C. deneoformans* isolates were serotype D, and 27 of these were found to be mating-type α and two were mating-type **a**. The interspecies hybrid was found to be mating-type α serotype A and mating-type **a** serotype D (Fig. 1).

By microsatellite typing, 13 genotypes could be identified within the group of 29 *C. deneoformans* and the single serotype AD hybrid, while 9 microsatellite genotypes were observed among the 16 *C. neoformans* and the single serotype AD isolate (Fig. 1).

When taking the patient data into account, some interesting observations were made. Of the five patients from which 2 isolates were available, patient KA was found to be infected with *C. deneoformans* and the hybrid *C. neoformans* × *C. deneoformans*. The *C. deneoformans* genetic background of the latter differs from that of the former when considering the microsatellite profiles (Fig. 1). From patient PJ, four isolates were available; three of them were *C. neoformans* from blood cultures and BAL with an identical microsatellite profile, while the fourth was a *C. deneoformans* isolate from a blood culture. All isolates from this patient were collected within an 8-day period indicating a mixed cryptococcal infection (Fig. 1). From patient CM, six *C. neoformans* isolates were collected from CSF and blood cultures during a 3-week period; all isolates had a similar microsatellite profile, but were found to have minor differences in the AFLP fingerprints (Fig. 1). Seven isolates were available from patient VH and were collected during a 5-day period. During this period, two *C. deneoformans* microsatellite profiles were found, but these differences were found to be minor as only two of the seven loci had a one repeat unit difference (Fig. 1). Patient SM experienced multiple episodes of a *C. deneoformans* infection; the eight isolates from the first episode could be split into two major microsatellite profiles with six isolates that fell in one group and two isolates that belong to the second group which had only a one repeat unit difference for one out of seven microsatellite loci (Fig. 1). The ninth isolate from patient SM was obtained more than 2 years after the first episode; this isolate was found to be closely related to the isolates involved in the first episode as two of the seven microsatellite loci had a one repeat unit difference (Fig. 1).

Antifungal Susceptibility Testing

The MIC ranges, MIC_{50}, MIC_{90} and geometric mean MICs of six antifungal drugs are presented in Table 1. Susceptibility data are presented for *C. neoformans* AFLP1/VNI ($n = 16$), *C. deneoformans* AFLP2/VNIV ($n = 29$) and the serotype AD hybrid AFLP3/VNIII ($n = 1$). The overall MIC ranges for each of six antifungal drugs were 0.031–0.5 μg/ml for amphotericin B, 0.063–4 μg/ml for 5-fluorocytosine, 0.5–64 μg/ml for fluconazole, 0.008–0.25 μg/ml for voriconazole, 0.008–0.063 μg/ml for posaconazole and 0.008–0.25 μg/ml for itraconazole. Fluconazole and 5-fluorocytosine had the highest geometric mean MICs of 4.06 and 0.35 μg/ml, respectively, while voriconazole and posaconazole had the lowest geometric MICs with 0.03 and 0.02 μg/ml, respectively. Amphotericin B and itraconazole fall in between with 0.17 and 0.04 μg/ml, respectively. The highest MIC

Table 1 Antifungal susceptibility for each of the *Cryptococcus* species and genotypes

	MIC (μg/ml)					
	AMB	5FC	FLZ	VCZ	PCZ	ITZ
C. deneoformans AFLP2/VNIV ($n = 29$)						
Range	0.031–0.25	0.063–4	0.5–16	0.008–0.125	0.008–0.063	0.031–0.125
MIC_{50}	0.125	0.25	4	0.031	0.016	0.031
MIC_{90}	0.25	0.25	8	0.063	0.063	0.063
Geometric mean MIC	0.14	0.29	3.1	0.002	0.02	0.04
C. neoformans AFLP1/VNI ($n = 16$)						
Range	0.063–0.5	0.063–1	0.5–64	0.008–0.25	0.008–0.063	0.031–0.25
MIC_{50}	0.25	0.5	8	0.063	0.031	0.031
MIC_{90}	0.25	1	16	0.125	0.063	0.063
Geometric mean MIC	0.22	0.52	7.3	0.05	0.03	0.05
Cryptococcus hybrid AFLP3/VNIII ($n = 1$)	0.125	0.25	1	0.008	0.008	0.031

MIC minimum inhibitory concentration, *AMB* amphotericin B; *5FC* 5-fluorocytosine, *FLZ* fluconazole, *VCZ* voriconazole, *PCZ* posaconazole; *ITZ* itraconazole

values were observed for one *C. neoformans* AFLP1/VNI isolate from cerebrospinal fluid of an HIV-infected patient with an MIC of 64 μg/ml for fluconazole and three *C. deneoformans* AFLP2/VNIV isolates from cerebrospinal fluid of a single HIV-infected patient with an MIC of 4.0 μg/ml for 5-fluorocytosine (Tables 1, 2).

Discussion

The epidemiology of cryptococcal infections in Slovenia during the period 1998–2016 was retrospectively investigated by AFLP fingerprinting, microsatellite typing, mating- and serotyping by qPCRs and antifungal susceptibility testing that included amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, posaconazole and voriconazole.

The majority of the cryptococcal isolates were observed to be *C. deneoformans* (serotype D; genotype AFLP2/VNIV) that was represented by 63% ($n = 29/46$) isolates, followed by *C. neoformans* (serotype A; genotype AFLP1/VNI) with 34.8% ($n = 16/46$) and one isolate that was a hybrid between both species (serotype AD; genotype AFLP3/VNIII). *Cryptococcus deneoformans* remained the major culprit when the number of isolates per patient was 'clone corrected', as 11 patients were infected by this cryptococcal species. Eight patients had a *C. neoformans* infection, and one patient was infected with a serotype AD hybrid isolate (Fig. 1). It is known from epidemiological studies that the number of *C. deneoformans* and *C. neoformans* × *C. deneoformans* isolates is relatively higher around the Mediterranean basin compared to other geographical regions [13, 14]. But so far, there are only few studies, notably from the neighbouring countries Croatia and Italy, that reported equal numbers of clinical *C. neoformans* and *C. deneoformans* isolates ($n_{Croatia} = 6$ for both; $n_{Italy} = 45$ for both) [14, 21]. Although the number of cryptococcal infections is low in Slovenia, with an incidence of 20 episodes during the period October 1998–April 2016 resulting in 1.14 infections per year in a population of ~ 2 million inhabitants, it is remarkable that the number of *C. neoformans* is much lower compared to what has been observed in other epidemiological studies from the Mediterranean basin.

The mating- and serotypes were determined by qPCR, which showed no discrepancies between the determined serotypes versus the observed genotypes. Remarkably, nearly all isolates were mating-type α ($n = 43/46$; 93.5%), two *C. deneoformans* isolates from one patient were mating-type **a**, and one isolate was found to be a hybrid αA-**a**D (Fig. 1). The

Table 2 Antifungal susceptibility profiles for each *Cryptococcus* isolates from three patients with multiple isolates over time

Patient	Species and genotype	Date of isolation	Sequential isolate[a]	MIC (μg/ml)					
				AMB	5FC	FLZ	VCZ	POZ	ITZ
VH	*C. deneoformans* AFLP2/VNIV	2016-01-20	1/7	0.125	0.125	4	0.031	0.008	0.031
		2016-01-20	2/7	0.125	0.25	1	0.008	0.008	0.031
		2016-01-20	3/7	0.125	0.25	4	0.031	0.016	0.031
		2016-01-23	4/7	0.125	0.125	4	0.031	0.016	0.031
		2016-01-23	5/7	0.125	0.125	4	0.031	0.016	0.031
		2016-01-24	6/7	0.125	0.125	2	0.016	0.008	0.031
		2016-01-24	7/7	0.125	0.25	8	0.063	0.031	0.031
SM	*C. deneoformans* AFLP2/VNIV	2013-07-16	1/9	0.125	0.25	2	0.008	0.008	0.031
		2013-07-18	2/9	0.25	4	8	0.063	0.063	0.125
		2013-07-18	3/9	0.25	4	8	0.063	0.063	0.125
		2013-07-23	4/9	0.125	1	8	0.031	0.016	0.031
		2013-08-06	5/9	0.125	0.25	4	0.008	0.016	0.031
		2013-08-22	6/9	0.125	0.5	2	0.008	0.008	0.031
		2013-08-26	7/9	0.125	1	8	0.063	0.016	0.031
		2013-09-03	8/9	0.125	0.25	2	0.008	0.016	0.031
		2016-01-26	9/9	0.25	4	8	0.063	0.063	0.125
CM	*C. neoformans* AFLP1/VNI	2016-04-02	1/6	0.25	1	8	0.063	0.063	0.063
		2016-04-02	2/6	0.25	1	4	0.063	0.031	0.031
		2016-04-03	3/6	0.25	0.5	8	0.031	0.031	0.031
		2016-04-05	4/6	0.25	0.5	16	0.125	0.063	0.25
		2016-04-13	5/6	0.25	0.5	4	0.031	0.031	0.031
		2016-04-21	6/6	0.5	0.06	64	0.25	0.063	0.063

MIC minimum inhibitory concentration, *AMB* amphotericin B, *5FC* 5-fluorocytosine, *FLZ* fluconazole, *VCZ* voriconazole, *PCZ* posaconazole, *ITZ* itraconazole

[a] See Fig. 1 for more background information

phenomenon that mating-type α harbouring isolates are overrepresented among clinical samples seems to points towards a higher virulence potential for these isolates, as was several decades ago postulated by Kwon-Chung and co-workers [22]. However, genetic studies revealed that mating-type **a** and α congenic isolates had equivalent virulence potential, but that the parental mating-type α isolate was more virulent compared to the mating-type **a** parental, suggestive for the presence of other genetic markers outside the mating-type locus that influence cryptococcal virulence [23]. Mating-type α isolates were found to have an enhanced predilection to penetrate the central nervous system, compared to its mating-type **a** counterpart, which might explain the higher incidence of the former in cryptococcal infections [24]. A recent European environmental survey observed mating-type distributions similar to those from the current study, with mating-type α versus mating-type **a** isolates of 327 versus 6 for *C. neoformans* and 78 versus 29 for *C. deneoformans* isolates [13].

In the current study, we observed that several patients had a persisting infection or a infection with multiple *Cryptococcus* isolates or species. Infections with multiple *Cryptococcus* species have been described before, commonly *C. neoformans* with *C. deneoformans* and/or its interspecies hybrids [25, 26], but also a mixed *C. neoformans*/*C. deuterogattii* infection was described [27]. Infections with multiple isolates that have different genotypes were observed, mostly for *C. neoformans* [26, 28–32]. As in the current study, microsatellite typing has been a

valuable molecular technique to differentiate the involved *C. neoformans* isolates [28, 30, 31]; also, sequenced-based methods have been applied to study mixed and recurrent infections [25, 33]. It has been postulated that interspecies hybrid can be generated in vivo [25], but the single patient in the current cohort had isolates with different *C. deneoformans* genetic backgrounds (Fig. 1). One Slovenian patient experienced two episodes of cryptococcal infection; here, microsatellite typing showed that the initial isolate underwent microevolution as only few microsatellite markers differ one repeat unit (Fig. 1; Table 2). Microevolution of isolates involved in persistent infections may result in attenuated phenotypic properties, such as a decreased antifungal susceptibility [26, 28–32, 34]. However, the isolate from patient CM obtained from the second episode did not differ in its antifungal susceptibility compared to isolates that were obtained in the early phase of the first episode (Table 2).

According to antifungal susceptibility testing, the most potent antifungal drugs are voriconazole, itraconazole and posaconazole with MICs $\leq$ 0.125 μg/ml (Table 1). These drugs exhibit the lowest MIC_{50} in all three genotypes, generally being 1–2 dilution steps lower than reported in other studies [18, 20, 21]. By applying the genotype-specific epidemiologic cut-off values (ECV) proposed by Espinel-Ingroff and coworkers [35], these cryptococcal isolates probably do not possess acquired resistance mechanisms for these three triazoles. Amphotericin B also exhibits good in vitro activity with MICs $\leq$ 0.25 μg/ml, and similar to the Dutch study *C. neoformans* (AFLP1/VNI) has one dilution step higher MIC_{50} than *C. deneoformans* (AFLP2/VNIV) with values of 0.25 μg/ml versus 0.125 μg/ml, respectively [20]. Compared to other tested triazoles, fluconazole exhibits higher MICs with 89.1% (n = 41/46) *C. neoformans* sensu lato isolates having MICs $\geq$ 8.0 μg/ml. MIC_{50} values for *C. neoformans* (AFLP1/VNI) and *C. deneoformans* (AFLP2/VNIV) are 8.0 μg/ml and 4.0 μg/ml, respectively. MIC_{50} values for fluconazole are generally 1–2 dilution step higher than observed in other studies [18, 20, 21], but we also observed that *C. neoformans* (AFLP1/VNI) has higher MIC_{50}'s than *C. deneoformans* (AFLP2/VNIV), a similar trend as reported in other studies [5, 18, 20, 21, 35]. When ECVs are applied, 10.9% of all cryptococcal isolates express reduced susceptibility to fluconazole (MIC $\geq$ 16 μg/ml) [35, 36]. The majority (80.0%; n = 4/5) of these potentially resistant isolates belong to *C. neoformans* (AFLP1/VNI) and came from three different patients: one of these isolates with MIC 16 μg/ml came from a tracheal aspirate from a patient with NSTEMI and was not considered as cryptococcosis, and the other three isolates came from HIV-positive patients. One of these two patients had cryptococcemia caused by both species: *C. neoformans* (AFLP1/VNI) and *C. deneoformans* (AFLP2/VNIV), with MIC 16 μg/ml; the patient died during treatment with amphotericin B plus fluconazole. The other HIV-positive patient (CM) had cryptococcal meningoencephalitis caused by *C. neoformans* (AFLP1/VNI), and in the period of three weeks MICs of fluconazole went from 4.0 to 64 μg/ml, which may indicate acquired resistance mechanisms (Table 2). This is the only case in which we observed a transition from wildtype to non-wildtype during the course of the infection. There were two other patients that had several isolates: VH had seven *C. deneoformans* isolates in five days with MICs for fluconazole varying from 1.0 to 8.0 μg/ml, still in the wildtype range, and patient SM with the largest number of isolates (all *C. deneoformans*) that were isolated during July 2013 to January 2016 with MICs for fluconazole varying from 2.0 μg/ml to 8.0 μg/ml with no non-wildtypes observed.

In summary, in Slovenia 19 cases of cryptococcosis were reported during the study period of October 1998–April 2016. The majority of the cases were caused by *C. deneoformans*, followed by *C. neoformans* and the interspecies hybrids between the two species. Nearly, all isolates were found to be mating-type α, with the exception of three isolates that had the mating-type **a** allele. By AFLP genotyping and microsatellite typing, it was observed that several patients had a mixed cryptococcal infection, but also that a cryptococcal isolate can persist for years. Antifungal susceptibility testing showed that voriconazole, itraconazole and posaconazole were the most potent drugs with MICs $\leq$ 0.125 μg/ml. Overall, *C. neoformans* was found to have higher MIC_{50}'s than *C. deneoformans*.

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Compliance with Ethical Standards

Conflict of interest JF.M received grants from Astellas, Basilea, F2G and Merck. He has been a consultant to Astellas, Basilea and Merck and received speaker's fees from Merck, Gilead and United Medical.

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