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Molecular typing and *in vitro* resistance of *Cryptococcus neoformans* clinical isolates obtained in Germany between 2011 and 2017

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ABSTRACT

Cryptococcosis is a fungal infection of the central nervous system predominantly caused by *Cryptococcus neoformans* in immunocompromised patients. In several countries worldwide, up to 50% of isolates show *in vitro* resistance to clinically used antifungals including fluconazole. No prospective data on susceptibility to antifungal drugs are available for Germany. In this study, we characterised all *C. neoformans* isolates collected from individual patients' samples at the German reference laboratory for cryptococcosis 2011 and 2017 (n = 133) by multi-locus sequence typing and phenotypic drug susceptibility testing. We identified serotype A/genotype VNI isolates belonging to clonal complexes previously described from Europe, Africa, Asia and South America as the most prevalent agents of cryptococcosis in Germany. Overall, we observed minimal inhibitory concentrations (MICs) above the epidemiological cut-offs (ECVs) in 1.6% of isolates regarding fluconazole and 2.3% of isolates regarding 5-flucytosine. Here, two *C. neoformans* var. *grubii* isolates displayed decreased drug susceptibility to fluconazole, one of them additionally to 5-flucytosine. We also found 5-flucytosine MICs above the ECV for two *C. neoformans* var. *neoformans* isolates. We identified a novel mutation in the *ERG11* gene which might be associated with the elevated fluconazole MIC in one of the isolates. The clinical importance of the detected *in vitro* resistance is documented by patient histories showing relapsed infection or primary fatal disease. Of note, sertraline demonstrated antifungal activity comparable to previous reports. Systematic collection of susceptibility data in combination with molecular typing of *C. neoformans* is important to comprehensively assess the spread of isolates and to understand their drug resistance patterns.

1. Introduction

Cryptococcosis is caused primarily by two fungal species, i.e. *Cryptococcus neoformans* (serotypes A and D) and *Cryptococcus gattii* (serotypes B and C) (Perfect and Bicanic, 2015). These species have been sub-divided into several genotypes, i.e. VNI-VNIV and VNB for *C.*

Abbreviations: CC, clonal complex; *C.neoformans*, *Cryptococcus neoformans*; CSF, cerebrospinal fluid; ECV, epidemiological cut-off value; MLST, multi-locus sequence typing; ST, sequence type

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neoformans and VGI-VGIV for *C. gattii*. A novel classification system for the agents of cryptococcosis has been proposed (Hagen et al., 2015). However, there is ongoing discussion whether this is data is preliminary (Kwon-Chung et al., 2017; Hagen et al., 2017); therefore, in this manuscript we report mainly genotypes.

It has been estimated that more than 220,000 cases of cryptococcosis occur annually worldwide, resulting in up to 180,000 deaths predominantly among individuals infected with HIV in sub-Saharan Africa and Southeast Asia. Mortality from cryptococcosis infection is estimated to be 30% in patients in high-income countries despite antifungal combination treatment consisting of an induction therapy with amphotericin B and 5-flucytosine followed by fluconazole. In contrast, mortality approaches 70% in patients from low-income countries where many antifungals are not available and fluconazole is thus frequently prescribed as monotherapy (May et al., 2016; Rajasingham et al., 2017).

Reduced susceptibility to fluconazole has been connected to decreased survival of cryptococcosis patients and has also been associated with progressive infections and relapse (Aller et al., 2000; Ponzio et al., 2019). A meta-analysis of 25 studies performing *in vitro* resistance testing documented fluconazole resistance in 18.7% of clinical *C. neoformans* isolates (Bongomin et al., 2018). Resistance rates between 0 and 50% have been described in different countries, with highest rates found in South Africa, Taiwan and Spain.

In *Cryptococcus* isolates with decreased *in vitro* susceptibility to fluconazole, mutations in the cytochrome P450 lanosterol 14-alpha-demethylase encoded by the *ERG11* gene have been reported (Rodero et al., 2003; Sionov et al., 2012). This enzyme is the main target of azole drugs and is crucial for the biosynthesis of ergosterol, an important component of the fungal cell wall. In addition, other mechanisms conferring resistance to fluconazole have been described (Chang et al., 2018; Sionov et al., 2009). *In vitro* resistance to azoles may arise during antifungal therapy or might be associated with agricultural azole use (Bastos et al., 2018; Bicanic et al., 2006; Stone et al., 2019).

In Germany, *in vitro* susceptibility data on clinical *C. neoformans* isolates have not been reported previously. According to hospital discharge data, 50–60 cryptococcosis cases are diagnosed each year (Smith et al., 2015a). In this study, we performed drug susceptibility testing using the CLSI microdilution reference method on *C. neoformans* isolates collected from individual patients in Germany between 2011 and 2017 in order to report on the prevalence of drug resistant isolates. In addition, we performed molecular typing by multi-locus sequence typing (MLST) to understand the current epidemiology of cryptococcosis in Germany at that level (Meyer et al., 2009; Sanchini et al., 2014).

2. Material and methods

2.1. Inclusion criteria

Isolates of *Cryptococcus neoformans* from patients with cryptococcosis diagnosed in Germany between 2011 and 2017 were included (n = 133), which were received at the German reference laboratory for cryptococcosis and rare systemic mycoses at the Robert Koch Institute. Each isolate represented an individual cryptococcosis case; sequential or repeat isolates were excluded from the analysis. *C. neoformans* isolates were identified phenotypically by a brown colour effect on Niger Seed (*Guizotia abyssinica)* Extract Agar (in house, Robert Koch Institute, Berlin, Germany), urease activity (BBL urea agar base, BD Biosciences, Franklin Lakes, NJ) and the absence of blue colour production on L-canavanine glycine bromothymol blue agar (in house). The respective genotypes were determined amplifying the *STR1* gene (Feng et al., 2013) of *C. neoformans* and *C. gattii* after DNA extraction (see below). *C. gattii* (n = 2) and hybrid strains (n = 6) as detected by *STR1* genotyping were excluded from the analysis (Smith et al., 2015a). Isolates were stored at −70 °C (Microbank™ conservation system, Pro-Lab Diganostics™, Toronto, Canada). Patient histories were obtained from physicians for two isolates demonstrating *in vitro* resistance to fluconazole or double resistance to fluconazole and 5-flucytosine in order to describe the clinical course of the disease. The use of patient information was approved by the ethics committee of the Goethe University Frankfurt/Main, Department of Medicine (track number 198/18).

2.2. DNA extraction

DNA extraction was performed from isolates grown on Difco™ Sabouraud Dextrose Agar (BD Biosciences) for 2–3 days. The Master Pure™ Yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI) was used with an additional bead beating step with 0.25 mm silicon-carbide sharps in a Fast Prep FP120 machine as described previously (Sanchini et al., 2014). Extracted DNA was solved in TE buffer and stored at −20 °C until PCR amplification.

2.3. Multi-locus sequence typing (MLST) and genetic analysis

MLST of seven *C. neoformans* loci was performed according to previously described protocols (Meyer et al., 2009; Sanchini et al., 2014) except for the *sod*1 locus which was amplified using primers with the 5′-3′ sequences AAGCCTCTCATCCATATCTT (forward) and CGCAGCTGT TCGTCTGGATA (reverse). PCR products were purified using Exo Sap-IT™ (Affymetrix, High Wycombe, UK) according to the manufacturer's instructions. Forward and reverse strands of the PCR products were sequenced using an Applied Biosystems 3500 Genetic Analyser (Life Technologies, Carlsbad, CA). Sequences were analysed using Geneious Version 10.0.5 (Biomatters, Auckland, NZ). Allele and sequence types were assigned by comparison with the MLST database of the Molecular Mycology Research Laboratory at the University of Sydney (http://mlst.mycologylab.org). Novel alleles were submitted to this database.

For phylogenetic analysis, all seven MLST loci of each isolate were concatenated in Geneious. A 1000x bootstrap maximum likelihood analysis was performed with the software MEGA, version 7.0.26. Sequences of *C. neoformans* VNI strain H99 (serotype A), VNII strain CBS 10084 (serotype A), VNIV strain JEC21 (serotype D) and *C. gattii* VGII strain R265 (serotype B) were used to identify major genotypes or as outgroup and were available from previous reports (Sanchini et al., 2014; Smith et al., 2015a). Sequences of a VNB strain representing ST102 was taken from the fungal MLST database. In addition, allele types and sequence types of each isolate were imported into Phyloviz 2.0 in order to construct a minimum spanning tree.

Sequencing of the ERG11 gene was performed as described previously for isolates with decreased drug susceptibility to fluconazole and sequences were assembled on the basis of the ERG11 sequence of *C. neoformans var. grubii* H99 (accession number: AY265353)(Gago et al., 2017).

2.4. In vitro drug susceptibility testing

Drug susceptibility testing of all *Cryptococcus* isolates was performed according to the CLSI M27-A3 microdilution method. The following antifungal drugs were evaluated (concentrations in parenthesis): 5-flucytosine (0.125–64 µg/ml), amphotericin B (0.0313–16 µg/ml), fluconazole (0.125–64 µg/ml), posaconazole (0.0313–16 µg/ml), sertraline (0.125–64 µg/ml) and voriconazole (0.03–16 µg/ml).The inoculum and the absence of contaminations were controlled by plating on Columbia 5% sheep blood agar (BD Biosciences). Reference strains *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) documented the correct performance of the microdilution assays in each experiment (data not shown).

In order to calculate minimal inhibitory concentrations (MICs), optical densities of cultures in microdilution plates were read at 620 nm using a Tecan sunrise microplate reader (Tecan, Maennedorf, Switzerland) after 72 h of incubation. The drug concentration at 50%

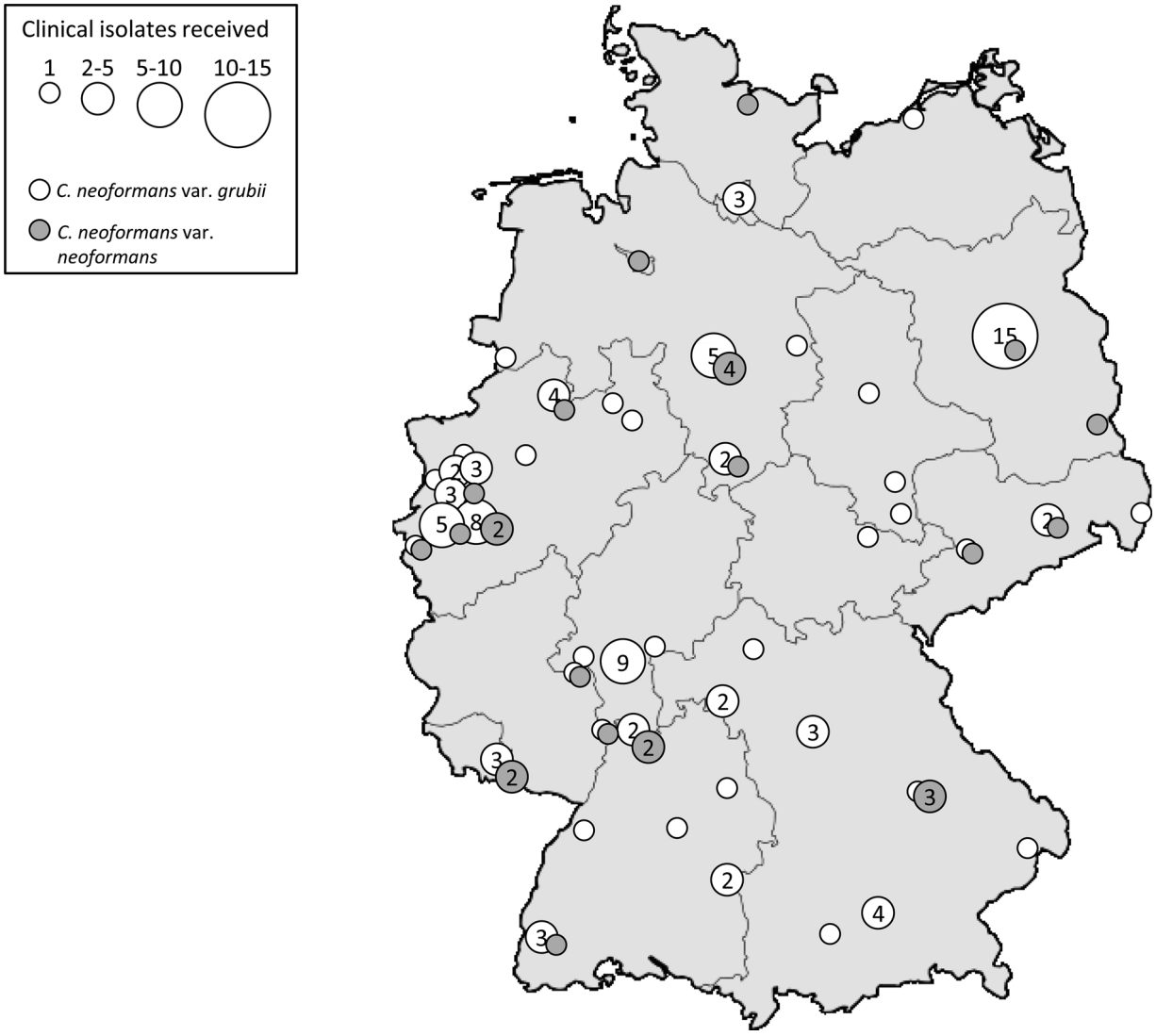

Fig. 1. Map of Germany and number of single patient isolates of *C. neoformans* from patients diagnosed with cryptococcosis in Germany between 2011 and 2017 obtained for each contributing laboratory. White circles: Location of contributing laboratories sending *C. neoformans* var. *grubii* (serotype A; n = 105) isolates. Grey circles: Location of contributing laboratories sending *C. neoformans* var. *neoformans* (serotype D; n = 28) isolates. Sizes of the circles represent the number of isolates provided from one laboratory. If more than one isolate was received, the numbers are indicated within the circles.

reduction in growth compared to growth control was evaluated for each antifungal except for amphotericin B, where 100% growth inhibition was measured. For sertraline, both 50% and 100% growth inhibition were determined.

MICs were compared to epidemiological cut-off values (Espinel-Ingroff et al., 2012a, b): amphotericin B: 0.5 µg/ml; 5-flucytosine: 8 µg/ml; fluconazole: 8 µg/ml; posaconazole and voriconazole: 0.25 µg/ml. For sertraline, a cut-off concentration of 4 µg/ml was used as previously described (Smith et al., 2015b).

3. Results

Isolates from patients with cryptococcosis (n = 133) were obtained from 48 German contributors, mostly representing microbiology laboratories (Fig. 1, Table S1). *C. neoformans var. grubii* (n = 105; 79%) and *C. neoformans var. neoformans* (n = 28; 21%) were cultivated from patients diagnosed all over the country. On average, we received 19 isolates each year (2011: n = 11, 2012: n = 12, 2013: n = 21, 2014: n = 13; 2015: n = 28, 2016: n = 13, 2017: n = 35).

3.1. Molecular typing

Molecular typing was performed for all 133 *C. neoformans* isolates. The maximum likelihood (ML) tree separated the previously defined genotypes VNI, VNII, VNIV and VNB (Fig. 2). Phylogenetic analysis of *C. neoformans var. grubii* (serotype A) suggested that the vast majority of isolates belonged to the molecular type VNI (n = 102; 97.1%), while two isolates clustered with VNII (ST40, 1.9%) and one isolate (ST232, 1%) did not cluster with any reference isolate included in the analysis (Fig. 2). Multi-locus sequence typing (MLST) analysis identified 18 sequence types for VNI, the most frequent sequence types being ST23 (n = 27), ST63 (n = 17), ST2 (n = 11), ST58 (n = 8) (Fig. 3), all belonging to the previously described clonal complex (CC) 23 (Cogliati et al., 2019). This complex is separated by three alleles from another cluster, consisting of ST69 and ST71, representatives of CC69. The identified isolates of sequence types ST5, ST4, ST6 and ST77 belonging to the CC5 (Cogliati et al., 2019) are separated from the above mentioned complexes by ST592 and the sequence types ST93 and ST32 that have been described as the VNI minor group (Ferreira-Paim et al., 2017). Isolates of *C. neoformans* var. *neoformans* (serotype D) could be assigned to a total of 18 sequence types, the most frequent being ST116

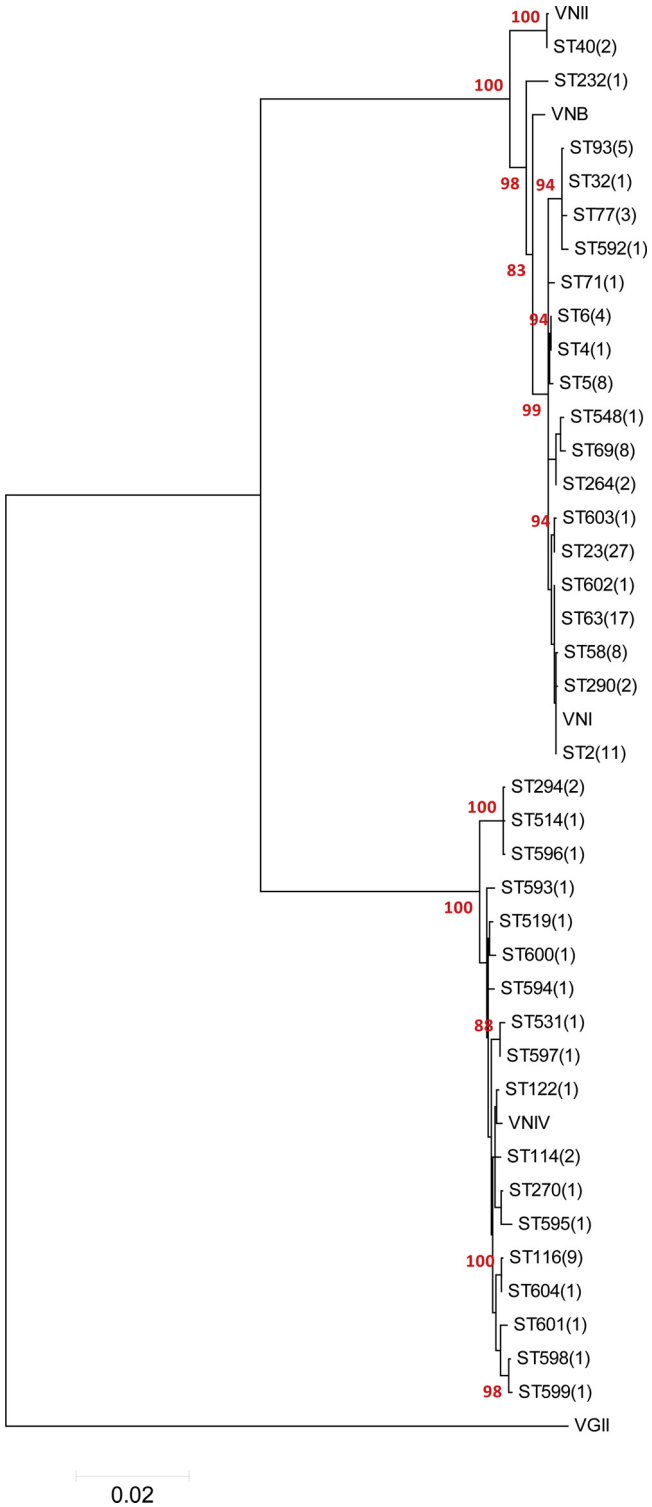

Fig. 2. Maximum-likelihood tree of 38 unique sequence types (STs) from *C. neoformans* single patient isolates. Detected sequence types (STs) are shown and the number of isolates for each type is depicted in brackets. Reference sequences of VNI, VNII, VNB, VNIV, and *C. gattii* VGII as outgroups are included. Red numbers next to branch lengths indicate branch support values. The scale bar indicates the number of substitutions per base. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(n = 9), ST114 and ST294 (2 isolates each). All other 15 sequence types of serotype D were detected only once. New sequence types not previously documented in the MLST database are summarized in the supplement (Table S2).

3.2. In vitro drug susceptibility testing and ERG11 genotyping

Drug susceptibility testing was performed with a total of 129 isolates, 102 *C. neoformans* var. *grubii* (serotype A; VNI: n = 99, VNII: n = 2, unknown: n = 1) and 27 *C. neoformans* var. *neoformans* (serotype D; VNIV). Four isolates could not be re-cultivated from frozen stocks and were therefore not tested. Detailed minimal inhibitory concentration (MIC) distributions of all tested clinical isolates are shown in Table 1. MICs above the epidemiological cut-off values (ECVs) (Espinel-Ingroff et al., 2012a, b) were detected in a total of four isolates, in 1.6% of isolates for fluconazole (n = 2) and 2.3% for 5-flucytosine (n = 3), including one isolate with decreased susceptibility to both drugs.

With the exception of one isolate, the MIC for amphotericin B was 0.5 µg/ml for serotype A. Approximately 30% of serotype D isolates (8/27) were slightly more susceptible to amphotericin B, displaying a MIC value of 0.25 µg/ml.

Serotype A isolates were more susceptible to 5-flucytosine than serotype D isolates (Table 1). One serotype A (ST58, received in 2017) and two serotype D isolates (ST514 and ST596, received in 2012 and 2013, respectively), were resistant to 5-flucytosine according to the epidemiological cut-off value (ECV) used, as their growth could not be inhibited with the tested 5-flucytosine concentrations up to 64 µg/ml.

For the ST58 isolate received in 2017, we additionally observed a fluconazole MIC above the ECV described (16 µg/ml). Moreover, one other serotype A isolate (ST5, received 2011) displayed a fluconazole MIC of 16 µg/ml. Overall, serotype A isolates were less susceptible to fluconazole than serotype D isolates (Table 1). The same was true for posaconazole and the MICs of two serotype A isolates were above the ECV for this drug (MIC = 0.5 µg/ml, Table 1). These were the same isolates displaying also fluconazole resistance. No differences between the serotypes could be observed for voriconazole (Table 1).

The observed MIC values for sertraline were similar for serotype A and serotype D isolates (Table 1).

In molecular characterisation of the *ERG11* gene of the fluconazole resistant ST5 isolate, a point mutation A to G was observed. This mutation led to an amino acid exchange of isoleucine at position 99 in the *C. neoformans var. grubii* reference sequence to valine in the *ERG11* sequence of the investigated isolate (I99 V). Other mutations previously described in *C. neoformans* isolates displaying fluconazole resistance, i.e. Y145 F, G470R and G484S, were not found (Rodero et al., 2003; Sionov et al., 2012). No mutation could be found in the *ERG11* gene of the other fluconazole resistant isolate (ST58).

3.3. Clinical manifestations of infections by fluconazole resistant isolates

A 43 year old male patient presented with a generalized seizure (day 1). HIV infection had been identified 8 years previously, and cerebral cryptococcosis had been diagnosed at that time. Additionally, a first relapse had occurred three months before admission (day 1), and cryptococcosis had been treated with liposomal amphotericin B and 5-flucytosine for 2 weeks, followed by fluconazole (5 mg/kg). No details were available on the laboratory findings of these two episodes. No travel history to Africa, Southeast Asia, Australia or the Americas was reported. On admission for the second relapse the patient was alert. He was on treatment with the antiretroviral drug Triumeq (dolutegravir 50 mg, abacavir 600 mg, lamivudine 300 mg) and fluconazole 400 mg daily. CD4 cell count was 238/µl and the HIV viral load was suppressed, i.e. below 20 copies/ml. An MRI scan on day 2 showed multiple parenchymal and meningeal inflammatory changes. A spinal tap documented an elevated opening pressure (29 cm H_2O). *C. neoformans var. grubii* (ST58) was cultivated from the cerebrospinal fluid (CSF). Susceptibility testing documented *in vitro* resistance to 5-flucytosine (> 64 µg/ml) and fluconazole (32 µg/ml). Antifungal therapy was started on day 1 with liposomal amphotericin B (5 mg/kg) and 5-

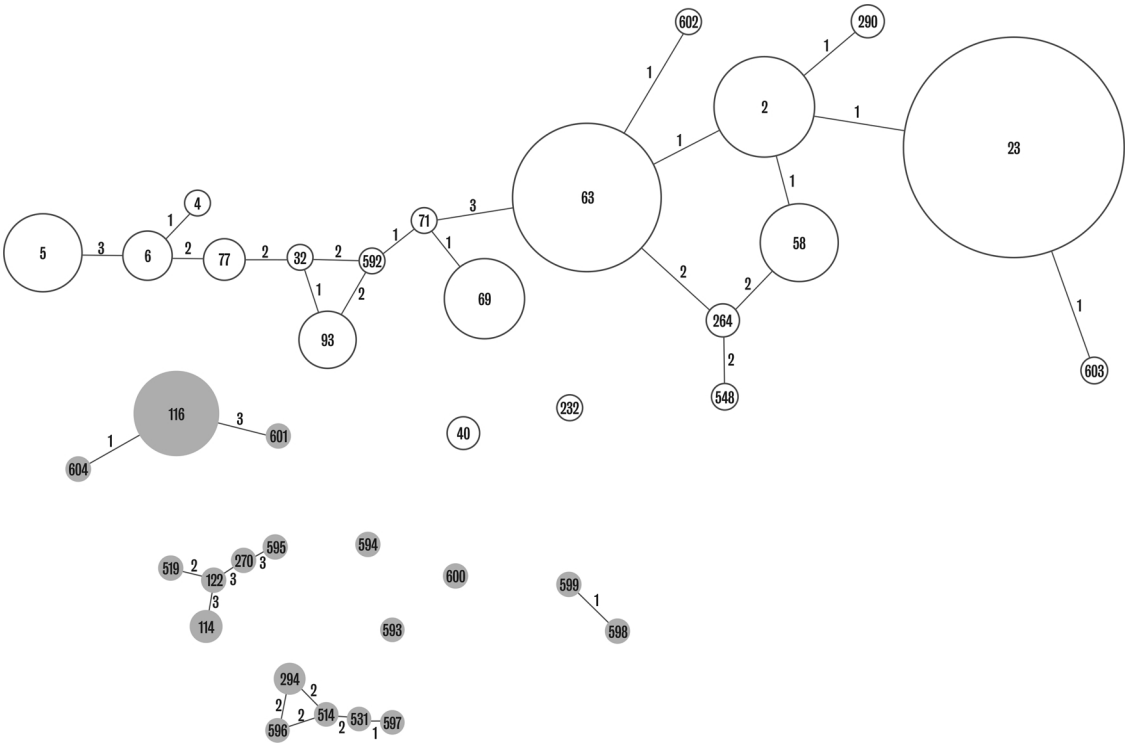

Fig. 3. Minimum spanning tree showing sequence types of *C. neoformans* from patients with cryptococcosis isolated in Germany between 2011 and 2017. *C. neoformans* var. *grubii* (20 STs; 105 isolates) are shown in white circles, *C. neoformans* var. *neoformans* (18 STs, 28 isolates) in grey circles. Each circle represents a unique sequence type (ST) with size proportional to the number of isolates within each ST. Numbers within circles represent sequence types, while numbers next to connecting lines indicate the number of allelic differences between two sequence types based on the 7 gene MLST scheme (a maximum of 3 allelic differences is shown).

C. neoformans var. *grubii* isolates have been described to belong to the following clonal complexes (CCs) previously (Cogliati et al., 2019): CC23 (ST2, ST23, ST58, ST63, ST264, ST290, ST548); CC5 (ST4, ST5, ST6, ST77), CC69 (ST69, ST71). VNI minor group: ST32, ST93 (Ferreira-Paim et al., 2017).

Table 1
Drug susceptibility described in minimum inhibitory concentrations (MICs) of *C. neoformans* var. *grubii* (serotype A) and var. *neoformans* (serotype D) isolates. AMB: amphotericin B, 5FC: 5-flucytosine, FCZ: fluconazole, PCZ: posaconazole, VCZ: voriconazole, SER50: 50% growth inhibition by sertraline, SER100: 100% growth inhibition by sertraline. MIC 50: Concentration that inhibits 50% of isolates, MIC90: Concentration that inhibits 90% of isolates.

			No of isolates for which MIC was (μg/ml)												
serotype	Antifungal	No. of isolates tested	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	> 64	MIC50	MIC90
A	AMB	102			1		101								0.5	0.5
D	AMB	27				8	19								0.5	0.5
A	5FC	102				4	33	56	8					1	1	1
D	5FC	27						3	10	7	5			2	4	8
A	FCZ	102					12	55	32	1		2			1	2
D	FCZ	27			2	5	9	8	3						0.5	2
A	PCZ	102	20	44	29	7	2								0.06	0.125
D	PCZ	27	18	6	3										0.03	0.06
A	VCZ	102	97	4	1										0.03	0.03
D	VCZ	27	27												0.03	0.03
A	SER 50	102							1	11	90				8	8
D	SER 50	27								22	5				4	8
A	SER 100	102									67	35			8	16
D	SER 100	27									26	1			8	8

flucytosine (125 mg/kg). Due to elevated opening pressure, repeated lumbar punctures were performed. Starting from day 7, CSF cultures remained negative. Antifungal therapy was switched to voriconazole on day 28 (5 mg/kg). The voriconazole trough level was 2 mg/kg on day 33 (5 days after initiation of voriconazole therapy). Voriconazole was switched to itraconazole due to side effects. No further relapse was documented during the next 2 years of follow up. No mutations were found in the *ERG11* gene of the patient's isolate.

A 69 year old female patient suffered from autoimmune hepatitis with cirrhosis, arterial hypertension, type II diabetes, coronary heart disease and combined aortic and mitral valve disease. Aortic valve surgery was performed for progressive heart failure (day 1). She was readmitted on day 20 from rehabilitation with reduced consciousness, hemiparesis and pleural effusions. *C. neoformans var. grubii* (ST5) was cultivated from the pleural effusion and blood cultures, but the pathogen was not detected in the CSF. Antimicrobial therapy included liposomal amphotericin B and fluconazole. However, she died on day 33 after heart surgery due to septic shock, renal and progressive hepatic failure. Antifungal susceptibility testing and characterisation of the *ERG11* gene were performed retrospectively and revealed *in vitro*

resistance to fluconazole (MIC 16 µg/ml) and the above described point mutation (I99 V), respectively.

4. Discussion

We report a retrospective analysis on the molecular epidemiology and *in vitro* susceptibility of *C. neoformans* isolated from cryptococcosis patients in Germany between 2011 and 2017. Elevated MICs above the ECV were rarely identified but selected patient histories suggest a potential for a serious impact on clinical outcomes including relapsed infections or lethal outcome for resistant strains.

Molecular typing was performed to get insights into the distribution of strains in Germany. Serotype A (*C. neoformans* var. *grubii*) isolates were confirmed as the most prevalent agents of cryptococcosis in Germany. The majority of these isolates belonged to the previously characterised CC23 (Cogliati et al., 2019). Previously, CC23 isolates have been reported from human cryptococcosis patients and from veterinary infections in several European countries. They were also cultivated from the environment, suggesting a wide distribution of these isolates in natural habitats that may serve as sources for infections (Cogliati et al., 2019; Ferreira-Paim et al., 2017). In contrast, isolates belonging to CC5 or CC69 have rarely been cultivated from environmental sources in Europe, suggesting that infections caused by these strains may be imported (Cogliati et al., 2019; Sanchini et al., 2014). Strains of CC69 are part of the genetically diverse *C. neoformans* population found in Sub-Saharan Africa (May et al., 2016; Thanh et al., 2018). In addition, ST93 and ST32 have been described in Africa, Asia and Brazil from environmental and clinical sources, but rarely from Europe and North-America, suggesting a wide distribution particularly in tropical countries (Ferreira-Paim et al., 2017). Also isolates belonging to CC5 are rarely cultivated from the European environment and have predominately been described in several Asian countries including Laos, China and Thailand (Thanh et al., 2018). Interestingly, *in vitro* resistance to fluconazole has been documented for up to 30% of clinical isolates in Asian countries (Chen et al., 2015; Fan et al., 2016). Of note, one fatal infection in our report in a HIV negative female was caused by a fluconazole-resistant isolate belonging to CC5. It is currently unknown if fungi belonging to this clonal cluster present increased virulence or are more likely to become resistant to azoles. However, the high proportion of CC5 isolates causing cryptococcosis in non-HIV infected individuals (Thanh et al., 2018) might support the theory that different sequence types may be associated with distinct clinical outcomes. Additionally, studies performed in Sub-Saharan Africa suggested significantly reduced survival when infections were caused by ST93 as compared to ST63, a frequent sequence type found in Europe (Wiesner et al., 2012). For these reasons, this raises the question if the sequence type may be predictive for the clinical outcome of cryptococcosis. This would allow for a targeted treatment with a potential for improved survival.

Compared to clinical serotype A isolates, serotype D isolates in our study were genetically more heterogeneous, illustrated by the high number of STs in relation to the number of isolates, pointing to more frequent recombination as suggested previously (Cogliati et al., 2016). Virulence differences among serotype D isolates have not been determined. However, cohort studies support differences in clinical presentation between serotype A and D isolates including increased likelihood of localized skin lesions and pulmonary infections in cryptococcosis caused by serotype D (Neuville et al., 2003; Sanchini et al., 2014). Serotype D / molecular type VNIV isolates were suggested to be mainly found in the European environment (Cogliati et al., 2019).

Determination of MICs documented differences in MIC distributions between serotype D and serotype A isolates in our study. We found higher 5-flucytosine MICs for serotype D in contrast to a previous study also using the CLSI reference method (Pfaller et al., 2011). This may reflect differences in intrinsic susceptibility of the diverse serotype D strains or difference in the therapeutic use of 5-flucytosine in different countries. However, only two serotype D and one serotype A (CC23) isolate in our study showed *in vitro* resistance to this drug, which may have played a role in the relapsed infection in one patient. Guidelines suggest that alternative agents such as fluconazole may be considered as combination drug for amphotericin B in case of 5-flucytosine resistance in the context of clinical relapse, together with a prolongation of induction therapy (Perfect et al., 2010). The observed higher MICs for serotype A regarding fluconazole and posaconazole are in accordance with a previous study (Espinel-Ingroff et al., 2012a). Compared to our results, the rate of fluconazole resistant isolates was substantially higher in other European studies, i.e. 29–46.6% in Spain. Others have used higher cut-offs than 8 µg/ml for fluconazole; however, our cut-off was also in line with a study determining ECVs by CLSI microdilution (Pfaller et al., 2011). In low-income countries, higher resistance rates may be caused by fluconazole monotherapy (Bicanic et al., 2006; Loyse et al., 2013; Stone et al., 2019). In Europe where combination therapy is readily available, exposure to fluconazole may be related to treatment of oropharyngeal candidiasis, a frequent AIDS-related fungal infection often occurring before cryptococcosis. In addition, environmental exposure of fungi to azoles has been described leading to *in vitro* resistance in treatment naïve patients with *Aspergillus fumigatus* infection (Berger et al., 2017; ECDC, 2013). It is currently unclear if differences in agricultural use of azoles amongst European countries result in different azole *in vitro* susceptibility rates of *C. neoformans*.

Resistance to azoles has been described as a result of mutations in the *ERG11* gene (Rodero et al., 2003; Sionov et al., 2012), encoding the target of azole antifungals. While we could not identify previously described mutations conferring resistance to fluconazole, we detected an amino acid exchange in one of two fluconazole resistant isolates. Further tests are necessary to clarify if this mutation is the reason for increased fluconazole MIC. Fluconazole resistance in the absence of *ERG11* mutations may be caused by a mechanism called heteroresistance (Sionov et al., 2009). Chromosomal aneuploidy, especially multiple copies of chromosome 1 (Chr1), were shown to be connected to this mechanism. Both the *ERG11* gene and the *AFR1* gene, an efflux pump able to reduce the concentration of intracellular fluconazole are encoded on Chr1 (Stone et al., 2019).This effect caused by subpopulations with higher MICs has also been described for other important fungal pathogens including *Candida albicans* and has been associated with persistent infections (Rosenberg et al., 2018). As it has been shown for *Candida* species, fluconazole might not only play an important role in the selection of resistant phenotypes, but might also promote originally clonal populations to mate and pass on the resistant phenotype to their progeny (Popp et al., 2019). For several reasons, heteroresistance is difficult to detect with microdilution methodology. First, microdilution may not be suitable to observe heterogeneous populations in a sample. Second, isolates received at the reference laboratory might have been passaged several times before *in vitro* susceptibility testing, making detection of this reversible phenotype difficult. It might therefore be valuable to implement a strategy testing primary samples (i.e. CSF) for resistant subpopulations in Germany (Stone et al., 2019). Guidelines for management of cryptococcosis advise fluconazole susceptibility testing in patients failing initial therapy, in relapsed infections, if previous exposure to fluconazole has occurred and if this drug is used for induction therapy. Here, both primary and secondary resistance might be an issue (Perfect et al., 2010). Management of infections by fluconazole-resistant strains may include prolonged induction therapy with amphotericin B and 5-flucytosine and the use of alternative agents including itra-, posa-or voriconazole as maintenance therapy.

The antifungal activity of the antidepressant sertraline was identified in an effort to screen for potential antifungals among compounds in clinical use. Good penetration to the central nervous system, fungicidal activity, favourable safety profile, *in vitro* activity against cryptococci and synergistic interaction with fluconazole argued for a potential use

of this drug in the treatment of cryptococcosis (Zhai et al., 2012). Initial clinical trials suggested faster CSF clearance of the pathogen and decreased relapse rates (Rhein et al., 2016). We confirm the *in vitro* activity for isolates from Germany, and the MIC values observed in our study were in line with a previous report (Smith et al., 2015b). However, it is currently undefined if this drug has a role in the treatment of cryptococcosis. Studies performed in HIV-infected patients suggest potential interaction with antiretrovirals leading to adverse outcomes (Rhein et al., 2018).

In conclusion, we showed that most cases of cryptococcosis in Germany are caused by serotype A/ VNI isolates, belonging to a limited number of CCs. Compared to serotype A, clinical *C. neoformans* var. *neoformans* (serotype D) isolates presented greater molecular diversity. Drug resistance in *C. neoformans* was only rarely detected using passaged isolates. We could identify a new mutation in the *ERG11* gene which might explain fluconazole resistance in one of two resistant isolates. Clinical consequences of resistance were highlighted by patient histories of progressive fatal disease and relapsed infection. The combination of susceptibility testing and molecular typing may further improve the knowledge on the spread of resistant isolates and may help to optimize patient management.

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Declaration of Competing Interest

E.A. Idelevich reports personal fees from Pfizer outside the submitted work.

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Appendix A. Supplementary data

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