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Research Paper

Correlation of antifungal susceptibility and sequence types within *Cryptococcus neoformans* VNI from HIV patients, and *ERG11* gene polymorphism

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ABSTRACT

Introduction: Here we tested the correlation between minimum inhibitory concentrations (MICs) of major antifungal agents and sequence types (STs) within *Cryptococcus neoformans* VNI isolates, and explored the *ERG11* gene of included strains.
Materials and methods: We analysed 23 *C. neoformans* strains categorised into two groups according to the distribution of the ST profile in Kinshasa clinics (Democratic Republic of Congo): major ST [ST93 (n = 15)], and less common STs [ST659 (n = 2), ST5 (n = 2), ST4 (n = 1), ST 53 (n = 1), ST31 (n = 1), and ST69 (n = 1)]. The MICs of the major antifungal agents [amphotericin B (AMB), 5-fluorocytosine (5FC) and fluconazole (FCZ)] were determined following EUCAST guidelines. *ERG11* gene sequences were extracted from whole genome sequence of the isolates and compared with the wild-type gene sequence of the *C. neoformans* VNI.
Results: Although major ST isolates appeared to have lower median MICs for AMB and 5FU than less common ST isolates (0.50 vs. 0.75 mg/L for AMB, 2 vs. 4 mg/L for 5FU, respectively), FCZ susceptibility was similar in both groups (4 mg/L) (p-value >0.05). The susceptibility profile of *C. neoformans* strains separately considered did not significantly affect the patients' clinical outcomes (p-value >0.05). Furthermore, two structural modalities of the *ERG11* gene were observed: (1) that of the reference gene, and (2) that containing two exonic silent point substitutions, and one intronic point substitution located in a sequence potentially involved in pre-mRNA splicing (c.337-22C > T); with no association with the MICs of the isolates (p-value >0.05).
Conclusions: The lack of association/correlation found in this study calls for further investigations to better understand the mechanisms of *C. neoformans* resistance to antifungal agents.

Introduction

Cryptococcus neoformans sensu stricto is the main yeast responsible for cryptococcal meningitis (CM) in people living with HIV (PLHIV) [1,2]. In the multilocus sequence typing (MLST) scheme for characterisation of *Cryptococcus neoformans/C. gattii* isolates proposed by the international society of human and animal mycology (ISHAM), it is possible to determine the molecular type and sequence type (ST) of each species from the seven loci sequences of interest in its genome [3].

CM causes more than 112,000 deaths each year among PLHIV worldwide, and about 63% (71,000) of them occur in sub-Saharan Africa [4]. Efforts to end deaths from CM in HIV patients include active screening for cryptococcosis in antiretroviral (ARV)-naïve PLHIV or advanced HIV disease patients, followed by pre-emptive antifungal treatment for those who test positive [5]. For CM active cases, the world health organisation (WHO) guidelines recommend the combined use of a single high-dose of liposomal amphotericin B (AMB) on the first day of treatment, followed by 14 days of 5-flucytosine (5FC) and fluconazole (FCZ) in the induction phase, FCZ in consolidation phase and later in the maintenance phase [6]. In developing countries such as the Democratic Republic of Congo

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(DRC), the scarcity and high cost of the first two antifungals (AMB and 5FC), as well as the difficulty of managing the side effects of some of them (mainly AMB), limit therapy to FCZ monotherapy [7]. This situation is conducive to resistance development.

To date, the global prevalence of FCZ-resistant *Cryptococcus* strains has been estimated at 12.1%; and this phenomenon has been reported in several countries around the world [8–12]. Among the main molecular mechanisms involved in the resistance of fungal pathogens to triazoles, especially FCZ, are 1) alterations or overexpression of azoles' target enzyme: lanosterol 14-α-demethylase whose biosynthesis is mediated by the *ERG11* gene; 2) disturbances of efflux membrane proteins (e.g. MDR = multidrug resistance) whose expression is regulated by *AFR1* gene among many others; and 3) the duplication of chromosome 1 which houses the *ERG11* and *AFR1* genes [9,12–14]. Interestingly, the first mechanism often generates strains with low levels of resistance, whereas the second and third mechanisms are more likely to result in isolates with high levels of resistance [15].

About 9,265 PLHIV suffer from CM each year in the DRC. From 1953 to 2021, the main treatment administered for CM remained FCZ monotherapy (80.6%). Subsequently, more than one in two patients died in the same period [16]. The long delay in CM diagnosis due to the lack of laboratory tools, the advanced stage of the patients' HIV disease on arrival at the clinics, the genetic diversity of *Cryptococcus* spp. and the low sensitivity of some species to common antifungal agents as previously described in DRC could partially explain this fatal outcome [17,18].

Although a correlation between antifungal susceptibility and molecular type within the *C. neoformans/C. gattii* species complexes has previously been described [19], comparative data at the ST level of *C. neoformans* sensu lato (s.l) and/or *C. gattii* s.l are insufficiently described.

In the present study, we aimed to verify the correlation between minimum inhibitory concentrations (MICs) of major antifungal drugs and STs within *C. neoformans* VNI isolates, and to explore the nucleotide sequences (and corresponding amino acid sequences) of the *ERG11* gene in all included strains.

Materials and methods

Cryptococcus neoformans *isolates*

We included 23 *C. neoformans* VNI strains cross-sectionally isolated from the cerebrospinal fluid (CSF) of PLHIV hospitalized in three Kinshasa public hospitals from February 2019 to February 2020. In addition to the samples collected as part of the study, clinical data (demographics, HIV history, clinical presentation on admission, antifungal treatment administered as well as doses, duration of treatment, notified complications and therapeutic outcomes) and biological data on CSF analysis were also collected on a survey form from these meningitis patients for analysis. The overall data from this survey were included in our previous article. The MLST characterisation of the isolates carried out previously and allowed identification of seven different STs, including one main ST (ST93, n = 15) and six less common STs [ST659 (n = 2), ST5 (n = 2), ST4 (n = 1), ST 53 (n = 1), ST31 (n = 1), and ST69 (n = 1)] [17].

Antifungal susceptibility testing

Antifungal drug MICs were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) E.Def 7.3.1 procedure [20]. Inoculum suspensions of 0.5 McFarland standard were prepared and diluted 1:10 with sterile distilled water (Sensititre tm demineralized water, Thermo Fisher Scientific, USA). After inoculation, the following final concentration range was targeted: 0.008–8 mg/L for AMB, and 0.06–64 mg/L for 5FC and FCZ. The reading of the MIC50 value (drug concentration resulting in 50% inhibition of microorganisms) for 5FC and FCZ, and MIC90 for AMB, was done according to the described recommendations using a visual and automated reading at 405 nm with a Multiscan FC spectrophotometer (Thermo Scientific, MA, USA). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as the quality control strains for the tests. The interpretation criteria for AMB were those defined in the antifungals EUCAST breakpoint tables' version 10.0: susceptible, ≤1 mg/L; resistant, >1 mg/L. Not being defined in the EUCAST breakpoint tables, the FCZ and 5FC interpretation criteria were based on the epidemiological cutoff values for *in vitro* susceptibility testing provided by the Clinical and Laboratory Standards Institute (CLSI) as follows: for both FCZ and 5FC, sensitive, ≤8 mg/L; resistant, >8 mg/L [21].

ERG11 *gene sequence analysis*

From DNA extracts, cryptococcal genomes were sequenced using Illumina HiSeq (Illumina, San Diego, California, USA) as previously described [22], and the raw contig sequences were paired, cleared of duplicate reads, trimmed, and assembled by mapping to the *C. neoformans* H99 reference genome. The *ERG11* gene of the isolates were then extracted by mapping of the reconstructed genomes to the *C. neoformans* wild-type *ERG11* gene (Gen-Bank accession no. AY265353). Nucleotide translation into protein and multiple sequence alignment has subsequently been performed and analysed. All bioinformatics analyses were carried out using the Geneious Prime 64_2021_1 software (https://www.geneious.com).

Statistics

R-cmdr version 2.6-1 (R Foundation for Statistical Computing, Vienna, Austria) was used for statistical analysis. The Wilcoxon test was used for median MICs comparisons among the *C. neoformans* ST strains. Moreover, Pearson's chi-square test or Fisher's exact test was applied to estimate the association between the antifungal susceptibility of strains and the patients' clinical outcomes/and the *ERG11* gene polymorphism. Two categories of ST (main ST *versus* less common STs) and clinical outcome [good (recovery and discharge from hospital) *versus* poor therapeutic outcome (death, status quo, and discharge against medical advice or transfer due to complications)] were considered. P-value < 0.05 was considered to define significance.

Ethics statement

The present work involved *Cryptococcus neoformans* strains collected after ethical approval from the School of Public Health, Faculty of Medicine, University of Kinshasa (approval number: ESP/CE/071/2019). All patients included in the study gave written consent after the information session on the risks associated with participation. The anonymity of the included patients was guaranteed and the data obtained were stored and processed only by the research team.

Results

MICs and Cryptococcus neoformans *VNI ST correlation*

While all strains tested were susceptible to AMB, 4.3% (1/23) were resistant to 5FC and 8.7% (2/23) of the strains had high MICs to FCZ. The antifungal MICs of 23 included *C. neoformans* strains (MIC90 for AMB, and MIC50 for FCZ and 5FC), *ERG11* gene allele type of isolates, and the patients' clinical outcome are shown in Table 1. Although the major ST isolates appeared to have lower median MICs for AMB and 5FC than the less common ST isolates (0.50 versus 0.75 mg/L for AMB and 2 versus 4 mg/L for 5FC, respectively), FCZ MICs were similar in both groups (4 mg/L). This was verified by a non-significant Wilcoxon

Table 1
Minimum inhibitory concentrations (MICs) of three major antifungal agents tested against 23 *Cryptococcus neoformans* strains by EUCAST method, *ERG11* allele type of isolates, and the patients' clinical outcome.

				Minimal inhibitory concentrations (MICs) mg/L				
Strain no.	Strain ID	Molecular type	Sequence type	AMB $S \leq 1, R > 1$	FCZ $S \leq 8, R > 8$	5FC $S \leq 8, R > 8$	*ERG11* allele type	Clinical outcome
1	BZ-3NGA	VNI	ST93	0.5	8	8	Non-wt	Pejorative
2	BZ-6NGA	VNI	ST93	1	4	2	Non-wt	Favourable
3	BZ-9NGA	VNI	ST93	0.5	8	2	Non-wt	Favourable
4	BZ-13NGA	VNI	ST31	0.5	4	1	Wt	Pejorative
5	BZ-24NGA	VNI	ST53	1	8	1	Wt	Pejorative
6	BZ-44NGA	VNI	ST53	0.5	0.25	4	Wt	Pejorative
7	BZ-46NGA	VNI	ST93	1	8	4	Non-wt	Favourable
8	BZ-73NGA	VNI	ST93	0.5	8	2	Non-wt	Favourable
9	BZ-97NGA	VNI	ST93	0.5	2	16	Non-wt	Pejorative
10	BZ-103NGA	VNI	ST93	1	4	2	Non-wt	Favourable
11	BZ-110NGA	VNI	ST93	1	2	1	Non-wt	Favourable
12	BZ-124NGA	VNI	ST93	0.25	2	8	Non-wt	Favourable
13	BZ-4RB	VNI	ST659	1	2	8	Wt	Pejorative
14	BZ-12RB	VNI	ST5	1	4	4	Wt	Pejorative
15	BZ-22RB	VNI	ST93	0.5	8	0.5	Non-wt	Pejorative
16	BZ-27RB	VNI	ST659	1	4	4	Wt	Favourable
17	BZ-28RB	VNI	ST93	1	2	2	Non-wt	Favourable
18	BZ-55RB	VNI	ST93	0.25	2	4	Non-wt	Pejorative
19	BZ-68RB	VNI	ST4	0.5	1	0.25	Wt	Pejorative
20	BZ-78RB	VNI	ST93	1	32	1	Non-wt	Pejorative
21	BZ-88RB	VNI	ST93	1	4	4	Non-wt	Favourable
22	BZ-91RB	VNI	ST93	0.25	4	0.125	Non-wt	Pejorative
23	BZ-14LU	VNI	ST69	0.5	16	4	Wt	Pejorative

Pejorative outcome: Death, status quo, discharge against medical advice, or transfer due to complications. Favourable outcome**:** Recovery and discharge from hospital. VNI: Variety *neoformans* I. Wt: wild-type sequence of *ERG11* gene. Non-wt: non wild type sequence of *ERG11* gene.

test (*P*-value >0.05). Despite the high AMB susceptibility of the strains, all the infected patients with FCZ-resistant and separately 5FC-resistant strains had pejorative therapeutic outcomes [2 and one patient out of 13 pejorative therapeutic outcomes patients, respectively]. Observations found without statistical significance (*P*-value >0.05).

ERG11 *gene sequences analysis*

Analysis of the nucleotide sequences of the *ERG11* gene revealed two structural modalities (allele type) of the gene: the first is similar to the reference gene sequence (wild-type sequence), and the second contains two silent point substitutions in the Coding DNA Sequence (CDS) [**1)** c.831A > G, corresponding to the codon change AGA (*R* = arginine) > AGG (*R* = arginine), and **2)** c.1374A > G, equivalent to the codon change CAA (*Q* = glutamine) > CAG (*Q* = glutamine)], and a point substitution in the first intron sequence (c.337-22C > T), located at 22 nucleotides upstream from the 2nd exon (Gene allele sequence submitted to GenBank, ID: 2695595). The latter represents one of two potential sequences containing the branching site for pre-mRNA splicing (Fig. 1). No significant association was found between these two allele types and the MICs of the antifungal drugs against

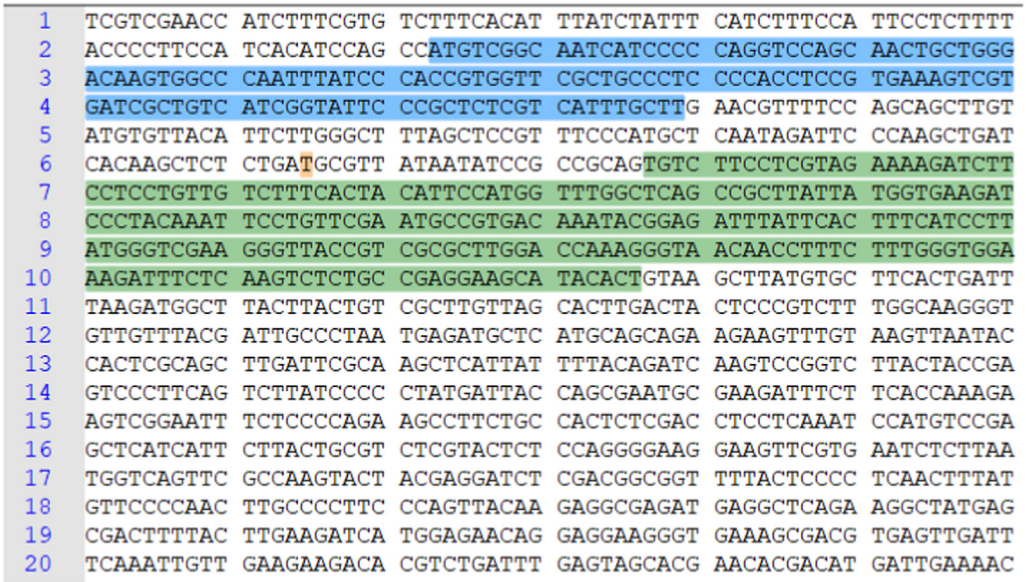

Fig. 1. . Nucleotide sequence part of *ERG11* gene from BZ-78RB strain (FCZ MIC=32 mg/L), showing the intronic point substitution (orange), exon 1 (blue), and exon 2 (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the isolates (*P*-value >0.05). In addition, no substantial difference was observed in the clinical outcome of patients infected with either *ERG11* gene allele type, although all patients were treated with the usual triple antifungal therapy (AMB, 5FC and FCZ) except one (BZ-27RB) because of the lack of diagnostic confirmation at the time of treatment in the clinic.

Discussion

Among the parameters determining the antifungal therapeutic success, drug resistance, host comorbidities, drug intolerance, poor drug compliance and pharmacokinetics are the best identified [23]. In the present study, the correlation of AMB, 5FC and FCZ MICs and ST within *C. neoformans* VNI isolates was verified, as well as the association between the antifungal MICs and the patients' clinical outcome. In addition, *ERG11* gene sequences were explored for defects that could explain the FCZ high MICs of the strains.

While all strains had low MICs to AMB, one isolate (strain no. 9) exhibited resistance to 5FC and two isolates were resistant to FCZ (strains nos. 20 and 23). Although 5FU resistance appears to be rarer than FCZ resistance in *Cryptococcus*, some studies have also focused on cases of 5FU resistance. As early as 1976, in the USA and Europe, 1.8% of 279 clinical isolates of *C. neoformans* tested in an epidemiological survey were resistant to 5FC [24]. In Spain, in an unavailability context of oral and intravenous 5FC forms, primary resistance to this compound has been estimated to 6.9% (MIC ≥32 mg/L) apart from 39.5% of strains that showed dose-dependent susceptibility (MIC 8 −16 mg/L) [25]. However, the beneficial effect of combining 5FC with AMB against *Cryptococcus* infections (mainly meningeal presentations), even those caused by 5FC-resistant strains, is well documented [26]. Referring to the antifungal mechanism of 5FC as previously detailed [27], most of the resistances described in *Cryptococcus* spp. are due to defects or mutations in the gene and/or protein associated with uridine-5-monophosphate pyrophosphorylase or uracil phosphoribosyltransferase. Uncommonly, mutational defects in genes encoding cytosine-specific permease or cytosine deaminase have been speculated in relapsing strains [23]. This background opens up prospects for research into the genetic markers of resistance to this drug, an area that is currently little explored.

FCZ remains the most widely used antifungal agent against cryptococcosis in developing countries, including the DRC [7,16]. In the present study, 8.7% (2/23) of *C. neoformans* strains exhibited high MICs values for FCZ (MICs: 16 mg/L and 32 mg/L). Numerous studies have surprisingly described high proportions of FCZ-resistant *C. neoformans* strains [8,9,28]. A proportion of 18.7% (936 / 4995) of strains with a MIC above the epidemiological threshold value was described in a large systematic review [8]. Particularly in Spain, 29% of *C. neoformans* strains were reported to be resistant to FCZ, with a strain carrying a mutation in the *ERG11* gene (G470R) that would be partially responsible for this resistance [9]. Prophylactic indication of FCZ against opportunistic infections in PLHIV as adopted in various countries has been described to increase the selection and emergence of *Cryptococcus* strains resistant to FCZ. Thus, in careful studies, the previous episodes notion of FCZ treatment has been reported before the resistance development [12]. In the DRC, about one fifth (21.7%) of patients are diagnosed with HIV infection and CM at the same time, not counting here patients with no indication or subvention (money) for FCZ prophylaxis. This suggests that many of the patients in our series had no significant exposure to FCZ prior to the current cryptococcosis. This may explain the relatively low proportion of FCZ-resistant strains found in this study, compared to those in other studies, and to what might be expected.

The difference in antifungal MICs values among ST *C. neoformans* categorised was evaluated as not significant. Also, the association between antifungal susceptibility and the patients' clinical outcomes was not proven. Therapeutic protocol combining three antifungal drugs as applied in the Kinshasa clinics supported by Doctors without Borders, case of the sample collection sites in this study, would be the annihilation basis of the ineffectiveness of certain drugs considered separately. While previous studies have indicated a clear correlation between antifungal susceptibilities and species or genotypes of the cryptococcosis causative agents, our study did not validate this trend at the ST level of *C. neoformans* strains [19]. Despite the small number of samples analysed in the present study, these results should be considered and call for further large-scale studies.

Of the three main mechanisms described in the azoles resistance phenomenon for yeast strains, *ERG11* gene mutations have widely been documented in several fungal species, including *Aspergillus fumigatus, Candida albicans* and *C. neoformans* [29,30]. This mechanism is constantly associated with a moderate level of FCZ resistance in *C. neoformans* strains [31], as our findings have shown. In this register, Laura Rodero *et al*. indexed a G484S protein point mutation in the FCZ-resistant *Cryptococcus* strain from an AIDS patient in the 5th episode of recurrent CM previously treated with FCZ at different doses [12]. The G470R protein point mutation has been described in clinical strains of *Cryptococcus deneoformans* (VNIV) from PLHIV, showing *in vitro* resistance to FCZ developed during the FCZ exposure [9]. Amino-acid substitutions induce phenotypic expressions when they localise in or near the interaction sites of the *ERG11* enzyme. For example, the catalytic domain and the conserved hemo-binding domain have been suspected to being impacted by these substitutions [9,12,32]. Furthermore, despite identifying mutations in the coding DNA sequences (CDS) of *ERG11* gene, with repercussions on the protein sequence, Priscilla Belbir Atim *et al.* found no association between these mutations and high MIC to FCZ [33].

In this study, a variant harbouring an intronic substitution in the *ERG11* gene was found (c.337-22C > T), at 22 nucleotides upstream from the 2nd exon. Although it was also identified in the strain with the highest MIC for FCZ (BZ-78RB, 32 mg/L), this polymorphism was not associated with resistance among all strains. This site coincides with one of the two potential CURAY sequences in intron 1. Indeed, splicing in Eukaryotes requires the presence of functional splicing sites, including the 5′ and 3′ splice sites at the 5′ and 3′ ends of an intron, and CURAY sequence (*R* = purine nucleotide, *Y* = pyrimidine nucleotide) on which the A (adenine) branching site is located [34]. To our best knowledge, this specific mechanism has not yet been implicated in triazole resistance in fungi. As described in human diseases, genetic events occurring at these sites can disrupt the splicing process and compromise the synthesis of a structural and active protein [35]. Menkes disease and occipital horn syndrome are two of many examples [34]. In yeast, deletion mutants in the 3′ intron region of one of the actin genes have provided insight into the role of intronic sequences in defining new intro-exon boundaries and hence in the recruitment of branching sites [36]. In addition, it has been demonstrated that alterations of an intronic sequence of the yeast cycloheximide resistance gene (CYH2m) lead to changes in the normal position of the branching site in the intron RNA lariats produced during pre-mRNA splicing and prevents splicing in vivo. Thus, the mutated CYH2m pre-mRNA is not specifically excised and spliced in the correct way [37].

Conclusions

The MICs of *Cryptococcus neoformans* VNI strains described in this study did not show significant variation according to the sequence types involved in CM. Due to the combined use of antifungal agents in CM management, the susceptibility profile of strains to individual drug did not affect the patients' outcomes. Furthermore, we have identified a variant in the *ERG11* gene harbouring an intronic substitution that could be incriminated in the FCZ resistance phenomenon of the strains, although it is not statistically associated with high MICs among all strains.

Study limitations

Out of the small sample size, the lack of analysis of potential protein variants arising from *ERG11* gene with intronic substitution (by software prediction or western-blot) are the main study limitations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Bive Zono Bive: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing – original draft. **Rosalie Sacheli:** Formal analysis, Writing – review & editing. **Celestin Nzanzu Mudogo:** Writing – review & editing. **Pius Kabututu Zakayi:** Writing – review & editing. **Sébastien Bontems:** Writing – review & editing. **Georges Mvumbi Lelo:** Supervision, Validation, Writing – review & editing. **Marie-Pierre Hayette:** Supervision, Validation, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.mycmed.2023.101428.

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