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Lack of Association between Fluconazole Susceptibility and *ERG11* Nucleotide Polymorphisms in *Cryptococcus neoformans* Clinical Isolates from Uganda

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Abstract: Fluconazole is the drug of choice for cryptococcal meningitis (CM) monoprophylaxis in resource-limited settings such as Uganda. Emerging fluconazole resistance linked to mutations in the *Cryptococcus neoformans ERG11* gene (CYP51) has been observed in clinical isolates. Currently, the single nucleotide polymorphisms [SNPs] in the *Cryptococcus* spp. *ERG11* gene that could be responsible for fluconazole resistance are poorly characterized within Ugandan *C. neoformans* clinical isolates. If available, this information would be useful in the management of cryptococcosis among HIV patients. This cross-sectional study investigates the SNPs present in the coding region of the *C. neoformans ERG11* gene to determine the relationship between the SNPs identified and fluconazole susceptibility of the clinical isolates. 310 *C. neoformans* isolates recovered from the Cerebrospinal Fluid (CSF) of patients with HIV and cryptococcal meningitis were examined. The fluconazole half-maximal inhibitory concentrations (IC_{50} range: 0.25–32 μg/mL) was determined using the microbroth dilution method. A total of 56.1% of the isolates had low IC_{50} values of <8 μg/mL while 43.9% had high IC_{50} values $\geq$ 8 μg/mL. We amplified and sequenced 600 bp of the *ERG11* coding sequence from 40 of the clinical isolates. Novel synonymous and 2 missense mutations, S460T and A457V, were identified in the *ERG11* gene. The identified SNPs were not associated with differences in fluconazole IC_{50} values in vitro (p = 0.179).

Keywords: *ERG11*; *Cryptococcus neoformans*; single nucleotide polymorphisms; fluconazole; IC_{50}; prophylaxis; cryptococcal meningitis

1. Introduction

Fluconazole is a major prophylaxis used both before and during antiretroviral treatment against cryptococcosis [1]. The recommended antifungal treatment for acute cryptococcal meningitis (CM) is a combination of amphotericin B with flucytosine for the induction treatment phase, followed by fluconazole for the consolidation and maintenance treatment phases [2]. In resource-limited settings, such as in sub-Saharan Africa, fluconazole is a commonly available drug that is used for induction monotherapy [3]; however, is not recommended due to poor survival rates, slow fungal clearance and the emergence of fluconazole resistance [3,4].

Despite the adoption of rigorous approaches towards managing cryptococcosis among HIV patients, HIV-related *Cryptococcus neoformans* mortality is estimated at 2412 persons

per year in Uganda [5] due to probable antifungal resistance and late diagnosis [6,7]. An increase in the basal fluconazole MIC has been observed in Ugandan clinical isolates that is associated with the widespread use of fluconazole in HIV patients [8]. Similarly, in South Africa, a two-fold increase in fluconazole minimum inhibitory concentrations (MICs) was observed in clinical *Cryptococcus* spp. isolates over a decade [9]. Thus, the emergence of fluconazole-resistant *Cryptococcus* spp. isolates poses a challenge for the effective management of cryptococcal infections [8,9]. Currently, there are no interpretive breakpoints for in vitro antifungal susceptibility testing of *C. neoformans* and thus it is difficult to define phenotypes associated with fluconazole susceptibility and resistance [10]. Instead, clinical isolates are typically described as having low or high IC_{50} values [11].

Fluconazole inhibits ergosterol synthesis by interfering with the $14\text{-}\alpha$ lanosterol demethylase enzyme *ERG11* [12]. Importantly, the mechanisms of fluconazole resistance in *Cryptococcus* spp. have been linked to increased or decreased expression of the *ERG11* gene [13], aneuploidy [14,15], overexpression of the membrane efflux pump proteins [16] and mutations in the *ERG11* gene. These *ERG11* gene alterations affect the ability of the drug to bind to the Erg11p protein [17,18]. An increase in the fluconazole MIC was also observed in *C. neoformans* clinical isolates with mutations at the fluconazole binding site on the Erg11p protein [19]. The specific mutations in Erg11p that were identified among fluconazole-resistant *C. neoformans* isolates include G484S, G470R, Y145 F, and I99V [18–20]. Factors that have exacerbated the emergence of fluconazole resistance include increased clinical use of fluconazole [8] and widespread use of triazole fungicides [21].

In this study, SNPs in the *ERG11* gene of clinical *C. neoformans* isolates from HIV-infected individuals in Uganda were investigated to identify possible genetic changes that are associated with the observed increasing fluconazole MICs in Uganda. These datasets are important to characterize SNPs in the *ERG11* gene in the context of the fluconazole susceptibility of *C. neoformans*.

2. Materials and Methods

The *C. neoformans* clinical isolates used in this study were collected as part of the ASTRO (Adjunctive Sertraline for the Treatment of HIV-Associated Cryptococcal Meningitis) clinical trials [22–24] and were obtained from individuals with HIV and cryptococcal meningitis co-infections. The isolates were obtained from cerebrospinal fluid (CSF) and stored in a 20% glycerol solution at $-80\,^\circ\text{C}$ in the Department of Medical Microbiology at Makerere University. The clinical isolates were cultured from the glycerol stocks onto Sabouraud Dextrose Agar (SDA) plates (Difco, Sparks, MD, USA). The extracted DNA samples were stored at $-20\,^\circ\text{C}$ until use. The clinical *C. neoformans* cultures were either shipped as glycerol stocks or the culture was placed on filter paper and shipped at room temperature to the University of Minnesota, where they were subsequently stored as $-80\,^\circ\text{C}$ glycerol stocks.

2.1. Fluconazole Minimum Inhibitory Concentration (MIC) Broth Microdilution Assays

The Fluconazole IC_{50} values of 310 *C. neoformans* isolates were determined using the EUCAST microbroth dilution assay, as described in [25]. Briefly, the *C. neoformans* clinical isolates were plated onto yeast-peptone-dextrose (YPD) plates containing 0.10 mg/mL chloramphenicol and incubated at $30\,^\circ\text{C}$ for 48 h. Overnight cultures were then prepared in YPD broth containing 10 μg/mL chloramphenicol and incubated at $30\,^\circ\text{C}$ with shaking. The resulting cultures were centrifuged and washed 3 times with sterile water, resuspended, and a 1:100 dilution was prepared for cell quantification using a hemocytometer. The final inoculum of each isolate for the microbroth dilution MIC assay was prepared to EUCAST specifications in sterile water. A 50 mg/mL stock solution of fluconazole (Sigma-Aldrich, St. Louis, MO, USA) was prepared in DMSO. Fluconazole test concentrations ranged from 0.25–128 μg/mL as described by the EUCAST microbroth dilution assay [25].

All of the broth microdilution assays were carried out using a 2% glucose RPMI-1640 medium (Sigma R-8755, St. Louis, MO, USA) with a final inoculum concentration of

0.5×10^5–2.5×10^5 [25]. Immediately after inoculation, the optical density was measured at 600 nm (OD600) using a Biotek Synergy H1 Hybrid reader (Winooski, VT, USA). Plates were then incubated 72 h at 37 °C, and a second OD600 measurement was taken. The IC$_{50}$ for each strain was determined based on analysis of the well turbidity measurements, using the OD600, as described in [8]. A KN99α reference strain [26], with a known fluconazole IC$_{50}$ of 2 μg/mL was included as an inter-assay calibration reference in every MIC plate to verify accuracy across all of tthe MIC plates. IC$_{50}$ was defined as the first fluconazole drug concentration at which ≥50% of the growth was inhibited [11].

2.2. DNA Extraction, Amplification, and Sequencing

Single colony isolates were plated on SDA for 40 of the *C. neoformans* clinical isolates. DNA was extracted from 3 independent single colonies for each of the 40 isolates. The colonies were suspended in 150 μL of 1X TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and pH 8.0) in a 1.5 mL microcentrifuge tube and vortexed for 2 min. Thereafter, the suspension was heated in a microwave for 2 min, cooled to room temperature and centrifuged at 13,000 rpm for 2 min. The supernatant was then transferred to a fresh 0.5 mL tube from which 2 μL was used as amplicon in a 25 μL PCR reaction.

A 600 bp fragment within the *ERG11* gene-coding region, centered on the known G484 SNP site, was amplified using a single pair of primers ERGF-5′-AGTTGCCCATCATGG-ACTCTA-3′ and ERGR-5′-GAAGACTTACACGGTAATTGG-3′ in a final PCR volume of 25 μL. The amplification reactions were performed using an Eppendorf Mastercycler Thermal Cycler (Eppendorf AG, Hamburg, Germany). The PCR reaction contained 1X PCR buffer, 1U Taq DNA polymerase (New England BioLabs, Ipswich, MA, USA), 0.5 μM of each primer, 0.5 μM dNTPs (New England BioLabs, Ipswich, MA), and 2 μL DNA. The amplification program was as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles, each consisting of denaturation at 94 °C for 20 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. The program ended with a final extension step at 72 °C for 10 min. The amplicons were resolved on a 1.6 % agarose gel at 90 V for 1 h in 1X TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8.2). The gel was soaked in 0.5 μg/mL ethidium bromide for 20 min to stain and imaged using a Syngene G: BOX gel documentation system (Fredrick, MD, USA).

Exonuclease 1-Shrimp Alkaline Phosphatase (ExoSAP-IT) (Applied Biosystems, Vilnius, Lithuania) was used to clean-up the PCR products for sequencing. ExoSAP-IT (Applied Biosystems, Vilnius, Lithuania) was diluted (1:3) in PCR grade water. The cleanup reaction was comprised of 2 μL of diluted ExoSAP-IT and 3.5 μL of PCR product. The cleanup reaction was performed using a SimpliAmpTM Thermal Cycler (Applied Biosystems). The program for cleanup was 37 °C for 45 min then 80 °C for 15 min. The cleanup products were stored at −20 °C until sequencing.

The sequencing reaction mixture contained 1 μL BigDyeTM terminator (Applied Biosystems), 1.5 μL 5X buffer (Applied Biosystems), 1 μL 10μM of either the ERGF or ERGR primer, 1 μL ExoSAP-IT treated PCR product, and 5.5 μL PCR grade water (10 μL in total). The cycle sequencing reaction was performed using a SimpliAmpTM Thermal Cycler (Applied Biosystems) using the following program: 25 cycles each consisting of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min. The gene products were then sequenced using the Sanger method [27] on an ABI 3730 automated DNA sequencer (Applied Biosystems). The generated sequences were analyzed using Sequencing Analysis v.5.3 software (Applied Biosystems). The *C. neoformans* partial coding region *ERG11* gene nucleotide sequences were each entered into the BLASTn [28] sequence analysis program on NCBI and SNPs were identified. A graphical representation of the nucleotide changes in the coding region of the *ERG11* gene was generated using Weblogo version 2.8.2 [29].The exon sequences were then analyzed using AUGUSTUS for protein prediction [30]. The subsequent protein fragments were aligned to the clinical *C. neoformans* reference strain INM 972624, NCBI accession ID AAP12370.1, using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) [31] software package

and amino acid changes were identified in the multiple sequence alignment. All of the sequences from this study were deposited in the NCBI GenBank with accession number IDs MZ673051-MZ673090.

2.3. Analysis

To compare the association of the SNPs and the Fluconazole IC_{50} values and human mortality, a linear regression analysis was performed, using STATA SE 15 (StataCorp LLC, College Station, TX, USA)software.

3. Results

3.1. In Vitro Fluconazole Susceptibility

This study analyzed 310 isolates with fluconazole IC_{50} values ranging from low values of <8 µg/mL (n = 174) to high IC_{50} values of 8 µg/mL(n = 93), 16 µg/mL(n = 39) and 64 µg/mL(n = 4) (Table 1). The geometric mean for the isolates was 5.1 µg/mL. Recommended fluconazole breakpoints for *C. neoformans* are not defined yet and as such, we considered $IC_{50} < 8$ µg/mL low, and $IC_{50} \geq 8$ µg/mL high [11].

Table 1. *C. neoformans* clinical Isolates Fluconazole IC_{50}.

Fluconazole IC_{50} (µg/mL)	Low IC_{50}					High IC_{50}			
	0.25	0.5	1	2	4	8	16	32	64
N [proportion]	0 (0)	7 (2.2)	16 (5.16)	44 (14.2)	107 (34.5)	93 (30)	39 (12.6)	0 (0)	4 (1.29)

A 600 bp DNA fragment was amplified and sequenced from the region of the *ERG11* gene coding region that has previously been shown to be a "hot spot" for SNPs Sequencing was performed on a subset of 40 clinical isolates, derived from 37 patients Table 2 provides a summary of the characteristics of the 37 patients from whom the isolates were taken. 24.3% (9/37) of the patients died from CM and 8.1% (3/37) of the patients experienced a CM relapse.

Table 2. Characteristics of the 37 study patients whose isolates were selected for *ERG11* sequencing.

Parameter	Results n = 37
Age in years: median (range)	35.9 (20–65)
Gender	
Male	21 (56.7%)
Female	16 (43.2%)
CM Relapse	
History of CM	3 (8.1%)
No History of CM	34 (91.9%)
Mortality	
Alive	28 (75.7%)
Dead	9 (24.3%)

3.2. SNP Analysis of the ERG11 Gene

SNPs found within the *ERG11* coding region are presented in Table 3 and their relative abundance within the population is shown in Figure 1. The largest number of SNPs were found in *C. neoformans* clinical isolate 11420 (MZ673090) (Table 3).

Table 3. Single nucleotide polymorphisms (SNPs) in the partial *ERG11* gene-coding region from 40 *C. neoformans* clinical isolates show no association with fluconazole IC_{50} values.

Isolate	SNP Type	SNP	IC_{50} (µg/mL)	GenBank Codes
110159	A1861G	Synonymous	8	MZ673063
110166	A1861G	Synonymous	8	MZ673065
110174	A1861G	Synonymous	16	MZ673067
110180	C1741T	Synonymous	8	MZ673055
110183	A1861G	Synonymous	2	MZ673066
110242	A1861G	Synonymous	4	MZ673077
110246	A1861G	Synonymous	8	MZ673064
110252	A1861G	Synonymous	0.5	MZ673070
110271	A1861G	Synonymous	2	MZ673058
110288	C1741T, A1861G	Synonymous	2	MZ673080
110290	C1741T, A1861G	Synonymous	4	MZ673084
110301	A1861G	Synonymous	8	MZ673071
110352	A1861G	Synonymous	4	MZ673060
110353	A1861G	Synonymous	4	MZ673083
110355	A1861G	Synonymous	8	MZ673074
110389	A1861G	Synonymous	8	MZ673059
110390	A1861G	Synonymous	2	MZ673052
110395	A1861G	Synonymous	2	MZ673078
110399	C1684T, T1699C, T1753C, A1801G, C1882T, ‡ G1866C	**Missense mutation S460T** [1]	4	MZ673089
110404	A1861G	Synonymous	4	MZ673054
110413	A1861G	Synonymous	8	MZ673081
110414 D1	C1684T, T1699C, T1753C, C1882T, T1885C, ‡ G1866C	**Missense mutation S460T** [1]	2	MZ673086
110414 D3	C1684T, T1699C, T1753C, A1801G, C1882T, T1885C,	Synonymous	2	MZ673087
110416	A1861G	Synonymous	8	MZ673056
110418	A1861G	Synonymous	4	MZ673079
110420	G1651C, C1657T, T1675C, T1699C, C1705T, A1720G, C1741T, T1753C, T1765C, T1768Y, A1801G, T1849C, ‡ C1857T, C1873G, T1903C, A1933T, T1939C, T1960C, C1963T, A1984C	**Missense mutation A457V** [1]	4	MZ673090
110422	A1861G	Synonymous	4	MZ673068
110428	-	Identical to reference wild-type *C. neoforman* sequence AY265353.1	4	MZ673075
110429	A1861G	Synonymous	4	MZ673085
110433	A1861G	Synonymous	8	MZ673076
110433 *	A1861G	Synonymous	4	MZ673057
110435	A1861G	Synonymous	4	MZ673073

Table 3. *Cont.*

Isolate	SNP Type	SNP	IC$_{50}$ (µg/mL)	GenBank Codes
110439	A1861G	Synonymous	4	MZ673082
110441	A1861G	Synonymous	4	MZ673062
110444 D1	A1861G	Synonymous	4	MZ673061
110444 D7	A1861G	Synonymous	8	MZ673051
110449	-	Identical to reference wild-type *C.neoforman* sequence AY265353.1	1	MZ673088
110450	A1861G	Synonymous	8	MZ673053
110451	A1861G	Synonymous	4	MZ673072
110461	A1861G	Synonymous	8	MZ673069

‡ Indicates non-synonymous mutations that change the amino acid sequence of Erg11p. [1] Isolates with mutations that resulted in amino acid changes had low fluconazole IC$_{50}$ < 8 µg/mL. *Additional isolate from the same patient with identical SNP but different IC$_{50}$.
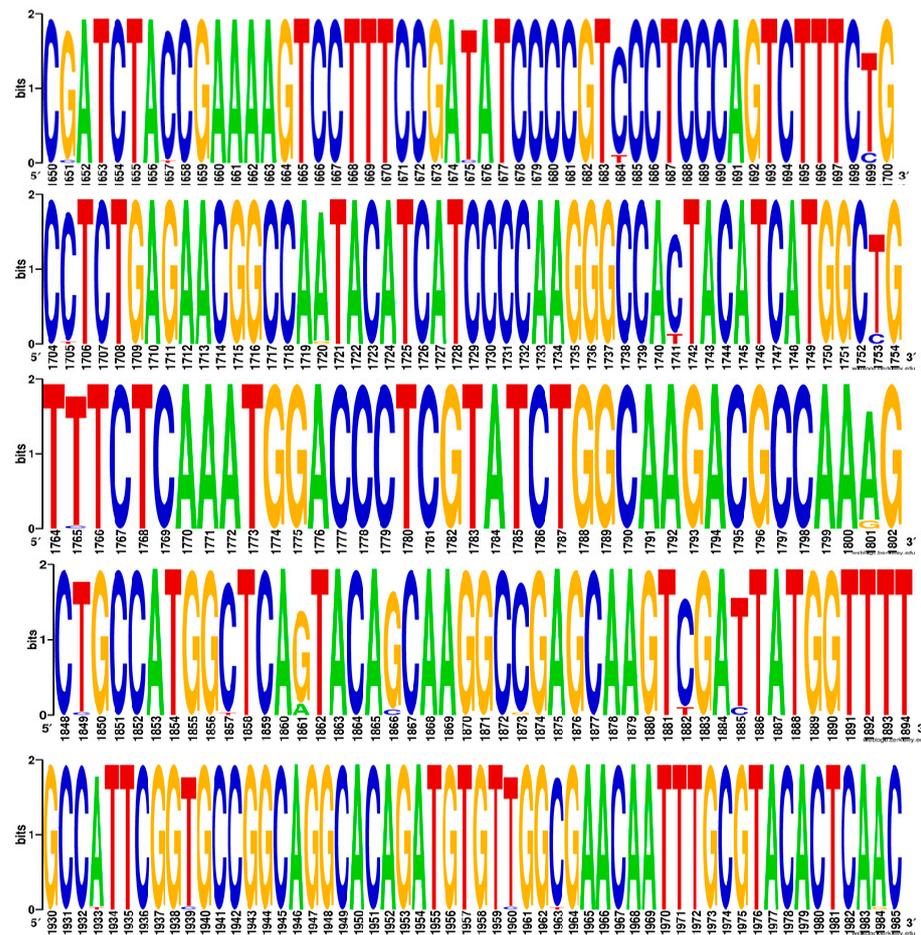
Figure 1. Sequence logo showing the SNPs within the *C. neoformans ERG11* gene.

The synonymous polymorphism A1861G was present in all the clinical isolate sequences except isolate numbers 110414 (MZ673086), 110414 D3 (MZ673087), 110399 (MZ673-089), and 110420 (MZ673090). Two non-synonymous SNPs (indicated with a ‡ in Table 3) resulted in the amino acid changes A457V and S460T. Novel synonymous SNPs found in the *C. neoformans* clinical isolates that had not been previously reported were also identified. Surprisingly, low fluconazole IC$_{50}$s < 8 µg/mL were observed in isolates with amino acid

changes; and isolates with high fluconazole IC_{50}s did not contain *ERG11* SNPs ($p = 0.179$, -1.37 *t*-test).

4. Discussion

The goal of the present study was to determine the fluconazole susceptibility of *C. neoformans* clinical isolates from a Ugandan patient cohort and investigate the presence of SNPs in a highly conserved coding region of the *ERG11* gene. The *C. neoformans* isolates in this study were classified as having either low IC_{50} values (56.1%) or high IC_{50} values (43.9%) based on the classification recommended by Gerlach et al. [11]. The high incidence of fluconazole IC_{50} values ≥ 8 μg/mL in this group of *C. neoformans* clinical isolates is consistent with surveillance data in sub-Saharan Africa and elsewhere over the past two decades, with an increase in MICs across many geographical regions [9,32–34]. This increased resistance poses a public health challenge, especially in Africa where fluconazole is widely prescribed and frequently used as a monotherapy for both consolidation and maintenance CM therapy [9]. In addition, the increasing trend of high fluconazole IC_{50} values observed among *C. neoformans* clinical isolates in Uganda [8,11] underscores the need to review the current recommended fluconazole dosages for optimal therapeutic outcomes [33]. Studies have recommended 800 mg/day for consolidation therapy of patients infected with isolates that have high MICs as a mechanism to improve clinical outcomes [33]. While there are no standardized breakpoints for *C. neoformans*, IC_{50}s ≥ 8 μg/mL have been associated with poor clinical outcomes [10,35].

The subset of 40 sequenced isolates, which represents 13% of the entire population, was representative of the fluconazole susceptibility and patient outcomes observed in the larger 310 ASTRO clinical trial isolate set [22–24]. Additionally, we showed that this subset readily contained isolates with polymorphisms in *ERG11* but no fluconazole sensitivity. Based on our observation of multiple isolates with polymorphisms that were not linked with high fluconazole IC_{50}s, the subset we analyzed is sufficiently large to show the necessary diversity. In the subset of patients for which we preformed *ERG11* sequencing of their clinical isolate, there was a 24.3% (9/37) CM mortality rate. Moreover, 77. 8% (7/9) of the clinical isolates from the patients who died had low fluconazole IC_{50} of <8 μg/mL, while only 22.2% (2/9) had a high fluconazole IC_{50} of $\geq$8 μg/mL. Although 8.1% (3/37) of the patients were relapse cases, only 1 of these patients had an IC_{50} of $\geq$8 μg/mL.

The disparity between the overall clinical outcomes in patients with low in vitro fluconazole IC_{50} has been consistently observed across studies and has been attributed to possible antifungal drug tolerance [36]. Tolerance in fungi is defined as slow growth of a subpopulation of cells at drug concentrations above the IC_{50}, with this growth often observed after longer incubation periods beyond those used for the standard MIC assays [36]. However, the characterization of fluconazole tolerance in *Cryptococcus* spp. remains poorly defined. Fluconazole tolerance may be underestimated when MICs are performed in 2% glucose due to the ability of *Cryptococcus* spp. to exhibit in vivo fluconazole tolerance in the low glucose host environment [37,38]. In addition, host factors such as patient drug adherence, pharmacokinetic data [4] and host-specific immune reconstitution inflammatory syndrome [39] can collectively cause treatment failure.

We observed 2 amino acid changes (A457V, S460T), along with other synonymous nucleotide changes, in the *ERG11* gene coding region. These differences in sequence could be due to natural variations in the *ERG11* genetic code. However, sequence analysis of the Ergl1p protein across different fungi has previously shown that the enzyme ligand-binding pocket site amino acid domains are highly conserved (Figure 2) [40]. This conservation is most likely required for the integrity and protein function of the 14α-demethylase activity of the protein. [41]. Polymorphisms in the *ERG11* gene, with or without resulting amino acid changes have previously been described as possible mechanism for fluconazole resistance among clinical isolates of *C. neoformans* [42]. The presence of these polymorphisms suggests there is genetic diversity within *C. neoformans* and may highlight allelic variations in the *ERG11* gene. *ERG11* gene SNPs have been observed in other yeasts of medical importance,

such as the *Candida* spp. [43]. It is unknown whether synonymous polymorphisms, such as those we observed in our population, are directly causing fluconazole resistance. Another possibility is that the accumulation of these nucleotide polymorphisms and mutations, coupled with other factors such as recombination and selective environmental pressure, may indirectly affect Erg11p function [41].

MSAIIPQVQQLLGQVAQFIPPWFAALPTSVKVVIAVIGIPALVICLNVFQQLCLPRRKDLPPV

VFHYIPWFGSAAYYGEDPYKFLFECRDKYGDLFTFILMGRRVTVALGPKGNNLSLGGKISQ

VSAEEAYTHLTTPVFGKGVVYDCPNEMLMQQKKFIKSGLTTESLQSYPPMITSEC

EDFFTKEVGISPQKPSATLDLLKSMSELIILTASRTLQGKEVRESLNGQFAKYYEDLDGGF

TPLNFMFPNLPLPSYKRRDEAQKAMSDFYLKIMENRRKGESDHEHDMIENLQSCKYRN

GVPLSDRDIAHIMIALLMAGQHTSSATSSWTLLHLADRPDVVEALYQEQKQKLGNPDG

TFRDYKYEDLKELPIMDSIIRETLRMHAPIHSIYRKVLSDIPVPPSLSAPSENGQYIIPKGHYI

MAAPGVSQMDPRIWQDAKVWNPARWHDEKGFAAAAMAQYSKAEQVDYGFGSVSKGT

ESPYQPFGAGRHRCVGEQFAYTQLSTIFTYVVRNFTLKLAVPKFPETNYRTMIVQPNNP

LVTFTLRNAEVK

Figure 2. Amino acid sequence of the *C. neorfomans ERG11* gene coding sequence indicating the region sequenced in this study. Thered arrows show the gene region which was sequenced that covers the "hotspot" region at position 484 (indicated red) previously associated with azole resistance [44]. Heme binding sites are highlighted in purple [20]. Substrate recognition sites are highlighted in green [45].

For example, the missense mutation S460T that we observed was also previously observed in fluconazole-resistant *C. neoformans* isolates [18]. Although specific *C. neoformans ERG11* gene amino acid mutations are known to cause high fluconazole MICs [17,20], our study showed that the S460T and A457V mutations were not linked to increased fluconazole IC$_{50}$ in our clinical isolates. Based on this observation, we conclude that the *Cryptococcus ERG11* gene is polymorphic and the SNPs we identified in our study are not the main cause of the high fluconazole IC$_{50}$ observed in our and previous studies.

This study had several limitations. First, our *ERG11* SNP investigation was limited to 600 bp of the gene coding region. Analysis of the entire ERG11 gene region, including the promoter, could yield additional data and identify SNPs outside of the region we analyzed that associate with drug resistance. Second, the patient clinical data (CD4 count) collected in the parent ASTRO clinical trial had missing data. Specifically, only 19/37 (51.3%) of the patient CD4 counts were collected at the time of CSF culture collection. In addition, while the clinical trial did not exclude patients that had or were receiving HIV therapy, the current status of that therapy was not provided with the isolates. Ultimately, Cryptococcus-related HIV fatalities remain high in sub-Saharan Africa. Emerging fluconazole resistance is a major public health challenge and effective antifungal therapy is critical [9]. Yet this study suggests that this increased resistance may not be linked to SNPs within the *C.neoformans* "hot spot" region of the Erg11p protein [44]. Other mechanisms of resistance, such as aneuploidy

(heteroresistance) and over-expression of *ERG11* or ABC fluconazole transporter genes, may be critically important in *C. neoformans* fluconazole resistance and need to be examined in clinical isolate cohorts. Until then, management of CM needs to incorporate development of new non-azole drugs as well as combination therapy approaches that utilize drugs with different modes of action [11].

5. Conclusions

This study revealed nonsynonymous polymorphisms and a novel synonymous polymorphism in the *ERG11* gene-coding region from clinical isolates of *C. neoformans*. Our results suggest that these SNPs are not associated with the high fluconazole IC_{50} observed in some of the isolates. Larger studies involving more clinical isolates and genome-wide association studies on these isolates is needed to investigate the genetic variations within the high and low fluconazole IC_{50} isolates. Future studies of the virulence of isolates with high and low fluconazole IC_{50} should be performed in animal models of cryptococcosis to determine the association between fluconazole IC_{50} and isolate virulence potential. In addition, studies on the functional impact of SNPs on *ERG11* gene expression, along with alternative molecular mechanisms for the increasing fluconazole resistance, such as intrinsic heteroresistance and over-expression of the ABC fluconazole transporter genes, should be performed.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the Makerere University School of Biomedical Sciences (SBS-702 approved on 19 Dec. 2019).

Informed Consent Statement: Patient consent was waived because patients in the primary study consented to have their culture isolate samples stored and used for further genetic research studies on meningitis.

Data Availability Statement: *ERG11* gene sequences for the clinical isolates are deposited in GenBank under accession numbers MZ673051-MZ673090.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Parkes-Ratanshi, R.; Wakeham, K.; Levin, J.; Namusoke, D.; Whitworth, J.; Coutinho, A.; Mugisha, N.K.; Grosskurth, H.; Kamali, A.; Lalloo, D.G. Primary prophylaxis of cryptococcal disease with fluconazole in HIV-positive Ugandan adults: A double-blind, randomised, placebo-controlled trial. *Lancet Infect. Dis.* **2011**, *11*, 933–941. [CrossRef]
2. Perfect, J.R.; Dismukes, W.E.; Dromer, F.; Goldman, D.L.; Graybill, J.R.; Hamill, R.J.; Harrison, T.S.; Larsen, R.A.; Lortholary, O.; Nguyen, M.-H.; et al. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of america. *Clin. Infect. Dis.* **2010**, *50*, 291–322. [CrossRef] [PubMed]
3. Loyse, A.; Burry, J.; Cohn, J.; Ford, N.; Chiller, T.; Ribeiro, I.; Koulla-Shiro, S.; Mghamba, J.; Ramadhani, A.; Nyirenda, R.; et al. Leave no one behind: Response to new evidence and guidelines for the management of cryptococcal meningitis in low-income and middle-income countries. *Lancet Infect. Dis.* **2019**, *19*, 143–147. [CrossRef]

4. Hope, W.; Stone, N.R.H.; Johnson, A.; McEntee, L.; Farrington, N.; Santoro-Castelazo, A.; Liu, X.; Lucaci, A.; Hughes, M.; Oliver, J.D.; et al. Fluconazole monotherapy is a suboptimal option for initial treatment of cryptococcal meningitis because of emergence of resistance. *MBio* **2019**, *10*, e02575-19. Available online: https://www.meta.org/papers/fluconazole-monotherapy-is-a-suboptimal-option/31796539 (accessed on 8 February 2021). [CrossRef] [PubMed]

5. Parkes-Ratanshi, R.; Achan, B.; Kwizera, R.; Kambugu, A.; Meya, D.; Denning, D.W. Cryptococcal disease and the burden of other fungal diseases in Uganda; Where are the knowledge gaps and how can we fill them? *Mycoses* **2015**, *58*, 85–93. [CrossRef] [PubMed]

6. Abassi, M.; Rhein, J.; Meya, D.B.; Boulware, D.R. Cryptococcal disease in the era of "test and treat": Is there cause for concern? *Open Forum Infect. Dis.* **2018**, *5*, ofx274. [CrossRef] [PubMed]

7. Williamson, P.R. The relentless march of cryptococcal meningitis. *Lancet Infect. Dis.* **2017**, *8*, 790–791. [CrossRef]

8. Smith, K.D.; Achan, B.; Hullsiek, K.H.; McDonald, T.R.; Okagaki, L.H.; Alhadab, A.A.; Akampurira, A.; Rhein, J.R.; Meya, D.B.; Boulware, D.R.; et al. Increased antifungal drug resistance in clinical isolates of *Cryptococcus neoformans* in Uganda. *Antimicrob. Agents Chemother.* **2015**, *59*, 7197–7204. [CrossRef]

9. Naicker, S.D.; Mpembe, R.S.; Maphanga, T.G.; Zulu, T.G.; Desanto, D.; Wadula, J.; Mvelase, N.; Maluleka, C.; Reddy, K.; Dawood, H.; et al. Decreasing fluconazole susceptibility of clinical south african *Cryptococcus neoformans* isolates over a decade. *PLoS Negl. Trop. Dis.* **2020**, *14*, e0008137. [CrossRef]

10. Cheong, J.W.S.; Mccormack, J. Fluconazole resistance in cryptococcal disease: Emerging or intrinsic? *Med. Mycol.* **2013**, *51*, 261–269. [CrossRef]

11. Gerlach, E.S.; Altamirano, S.; Yoder, J.M.; Luggya, T.S.; Akampurira, A.; Meya, D.B.; Boulware, D.R.; Rhein, J.; Nielsen, K. ATI-2307 exhibits equivalent antifungal activity in *Cryptococcus neoformans* clinical isolates with High and low Fluconazole IC50. *Front. Cell. Infect. Microbiol.* **2021**, *11*. [CrossRef] [PubMed]

12. Goa, K.L.; Barradell, L.B. Fluconazole: An update of its pharmacodynamic and pharmacokinetic properties and therapeutic use in major superficial and systemic mycoses in immunocompromised patients. *Drugs* **1995**, *50*, 658–690. [CrossRef] [PubMed]

13. Sykes, J.E.; Hodge, G.; Singapuri, A.; Yang, M.L.; Gelli, A.; Thompson, G.R. In vivo development of fluconazole resistance in serial *Cryptococcus gattii* isolates from a cat. *Med. Mycol.* **2017**, *55*, 396–401. [CrossRef] [PubMed]

14. Sionov, E.; Lee, H.; Chang, Y.C.; Kwon-Chung, K.J. *Cryptococcus neoformans* overcomes stress of azole drugs by formation of disomy in specific multiple chromosomes. *PLoS Pathog.* **2010**, *6*, 1–13. [CrossRef] [PubMed]

15. Sionov, E.; Chang, Y.C.; Garraffo, H.M.; Kwon-Chung, K.J. Heteroresistance to fluconazole in *Cryptococcus neoformans* is intrinsic and associated with virulence. *Antimicrob. Agents Chemother.* **2009**, *53*, 2804–2815. [CrossRef] [PubMed]

16. Basso, L.R.; Gast, C.E.; Bruzual, I.; Wong, B. Identification and properties of plasma membrane azole efflux pumps from the pathogenic fungi *Cryptococcus gattii* and *Cryptococcus neoformans*. *J. Antimicrob. Chemother.* **2015**, *70*, 1396–1407. [CrossRef]

17. Kano, R.; Okubo, M.; Hasegawa, A.; Kamata, H. Multi-azole-resistant strains of *Cryptococcus neoformans* var. *grubii* isolated from a FLZ-resistant strain by culturing in medium containing voriconazole. *Med. Mycol.* **2017**, *55*, 877–882. [CrossRef]

18. Sionov, E.; Chang, Y.C.; Garraffo, H.M.; Dolan, M.A.; Ghannoum, M.A.; Kwon-Chung, K.J. Identification of a *Cryptococcus neoformans* Cytochrome P450 Lanosterol 14 -Demethylase (Erg11) residue critical for differential susceptibility between Fluconazole/Voriconazole and Itraconazole/Posaconazole. *Antimicrob. Agents Chemother.* **2011**, *56*, 1162–1169. [CrossRef]

19. Selb, R.; Fuchs, V.; Graf, B.; Hamprecht, A.; Hogardt, M.; Sedlacek, L.; Schwarz, R.; Idelevich, E.A.; Becker, S.L.; Held, J.; et al. Molecular typing and in vitro resistance of *Cryptococcus neoformans* clinical isolates obtained in Germany between 2011 and 2017. *Int. J. Med. Microbiol.* **2019**, *309*, 151336. [CrossRef]

20. Rodero, L.; Mellado, E.; Rodriguez, A.C.; Salve, A.; Guelfand, L.; Cahn, P.; Cuenca-Estrella, M.; Davel, G.; Rodriguez-Tudela, J.L. G484S amino acid substitution in lanosterol 14-alpha demethylase (ERG11) is related to fluconazole resistance in a recurrent *Cryptococcus neoformans* clinical isolate. *Antimicrob. Agents Chemother.* **2003**, *47*, 3653–3656. [CrossRef]

21. Carneiro, H.C.S.; Bastos, R.W.; Ribeiro, N.Q.; Gouveia-Eufrasio, L.; Costa, M.C.; Magalhães, T.F.F.; Oliveira, L.V.N.; Paixão, T.A.; Joffe, L.S.; Rodrigues, M.L.; et al. Hypervirulence and cross-resistance to a clinical antifungal are induced by an environmental fungicide in *Cryptococcus gattii*. *Sci. Total Environ.* **2020**, *740*, 140135. [CrossRef] [PubMed]

22. Ellis, J.; Bangdiwala, A.S.; Cresswell, F.V.; Rhein, J.; Nuwagira, E.; Ssebambulidde, K.; Tugume, L.; Rajasingham, R.; Bridge, S.C.; Muzoora, C.; et al. The changing epidemiology of HIV-Associated Adult Meningitis, Uganda 2015–2017. *Open Forum Infect. Dis.* **2019**, *6*, ofz419. [CrossRef] [PubMed]

23. Rhein, J.; Morawski, B.M.; Hullsiek, K.H.; Nabeta, H.W.; Kiggundu, R.; Tugume, L.; Musubire, A.; Akampurira, A.; Smith, K.D.; Alhadab, A.; et al. Efficacy of adjunctive sertraline for the treatment of HIV-associated cryptococcal meningitis: An open-label dose-ranging study. *Lancet. Infect. Dis.* **2016**, *16*, 809–818. [CrossRef]

24. Rhein, J.; Hullsiek, K.H.; Evans, E.E.; Tugume, L.; Nuwagira, E.; Ssebambulidde, K.; Kiggundu, R.; Mpoza, E.; Musubire, A.K.; Bangdiwala, A.S.; et al. Detrimental outcomes of unmasking Cryptococcal Meningitis with recent ART initiation. *Open Forum Infect. Dis.* **2018**, *5*, ofy122. [CrossRef] [PubMed]

25. Arendrup, M.C.; Meletiadis, J.; Mouton, J.W.; Lagrou, K.; Hamal, P.; Guinea, J. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. EUCAST definitive document E. *Def* **2017**, *7*, E246–E247.

26. Nielsen, K.; Cox, G.M.; Wang, P.; Toffaletti, D.L.; Perfect, J.R.; Heitman, J. Sexual cycle of Cryptococcus neoformans var. grubii and Virulence of congenic a and α isolates. *Infect. Immun.* **2003**, *71*, 4831–4841. [CrossRef]

27. Sanger, F.; Nicklen, S.; Coulson, A.R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 5463–5467. [CrossRef] [PubMed]

28. Coordinators, N.R. Database resources of the national center for biotechnology information. *Nucleic Acids Res.* **2016**, *44*, D7–D19. [CrossRef]

29. Crooks, G.E.; Hon, G.; Chandonia, J.-M.; Brenner, S.E. WebLogo: A sequence logo generator. *Genome Res.* **2004**, *14*, 1188–1190. [CrossRef]

30. Stanke, M.; Morgenstern, B. Augustus: A web server for gene prediction in eukaryotes that allows user-defined constraints. *Nucleic Acids Res.* **2005**, *33*, W465–W467. [CrossRef]

31. Edgar, R.C. Muscle: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [CrossRef] [PubMed]

32. Bongomin, F.; Oladele, R.O.; Gago, S.; Moore, C.B.; Richardson, M.D. A systematic review of fluconazole resistance in clinical isolates of Cryptococcus species. *Mycoses* **2018**, *61*, 290–297. [CrossRef] [PubMed]

33. Chesdachai, S.; Rajasingham, R.; Nicol, M.R.; Meya, D.B.; Bongomin, F.; Abassi, M.; Skipper, C.; Kwizera, R.; Rhein, J.; Boulware, D.R. Minimum inhibitory concentration distribution of Fluconazole against Cryptococcus species and the Fluconazole exposure prediction model. *Open Forum Infect. Dis.* **2019**, *6*, ofz369. [CrossRef] [PubMed]

34. Pfaller, M.A.; Diekema, D.J.; Gibbs, D.L.; Newell, V.A.; Bijie, H.; Dzierzanowska, D.; Klimko, N.N.; Letscher-Bru, V.; Lisalova, M.; Muehlethaler, K.; et al. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: 10.5-year analysis of susceptibilities of noncandidal yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J. Clin. Microbiol.* **2009**, *47*, 117–123. [CrossRef] [PubMed]

35. Aller, A.I.; Martin-Mazuelos, E.; Lozano, F.; Gomez-Mateos, J.; Steele-Moore, L.; Holloway, W.J.; Gutiérrez, M.J.; Recio, F.J.; Espinel-Ingroff, A. Correlation of fluconazole MICs with clinical outcome in cryptococcal infection. *Antimicrob. Agents Chemother.* **2000**, *44*, 1544–1548. [CrossRef] [PubMed]

36. Berman, J.; Krysan, D.J. Drug resistance and tolerance in fungi. *Nat. Rev. Microbiol.* **2020**, *18*, 319–331. [CrossRef] [PubMed]

37. Bhattacharya, S.; Oliveira, N.K.; Savitt, A.G.; Silva, V.K.A.; Krausert, R.B.; Ghebrehiwet, B.; Fries, B.C. Low glucose mediated Fluconazole tolerance in *Cryptococcus neoformans*. *J. Fungi* **2021**, *7*, 489. [CrossRef] [PubMed]

38. Carlson, T.; Lupinacci, E.; Moseley, K.; Chandrasekaran, S. Effects of environmental factors on sensitivity of *Cryptococcus neoformans* to fluconazole and amphotericin B. *FEMS Microbiol. Lett.* **2021**, *368*, fnab040. [CrossRef] [PubMed]

39. Castelnuovo, B.; Manabe, Y.C.; Kiragga, A.; Kamya, M.; Easterbrook, P.; Kambugu, A. Cause-Specific mortality and the contribution of immune reconstitution inflammatory Syndrome in the first 3 years after Antiretroviral therapy initiation in an urban african cohort. *Clin. Infect. Dis.* **2009**, *49*, 965–972. [CrossRef] [PubMed]

40. Ceita, G.D.O.; Ceita, G.D.O.; Vilas-Boas, L.A.; Castilho, M.S.; Carazzolle, M.F.; Pirovani, C.P.; Selbach-Schnadelbach, A.; Gramacho, K.P.; Ramos, P.I.P.; Barbosa, L.V.; et al. Analysis of the ergosterol biosynthesis pathway cloning, Molecular characterization and phylogeny of lanosterol 14 α-demethylase (ERG11) gene of moniliophthora perniciosa. *Genet. Mol. Biol.* **2014**, *37*, 683–693. [CrossRef]

41. Dos Santos Silva, D.B.; Carbonera Rodrigues, L.M.; De Almeida, A.A.; de Oliveira, K.M.P.; Grisolia, A.B. Novel point mutations in the ERG11 gene in clinical isolates of azole resistant Candida species. *Mem. Inst. Oswaldo Cruz* **2016**, *111*, 192–199. [CrossRef] [PubMed]

42. Xu, J.; Onyewu, C.; Yoell, H.J.; Ali, R.Y.; Vilgalys, R.J.; Mitchell, T.G. Dynamic and heterogeneous mutations to fluconazole resistance in *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* **2001**, *45*, 420–427. [CrossRef] [PubMed]

43. Paul, S.; Dadwal, R.; Singh, S.; Shaw, D.; Chakrabarti, A.; Rudramurthy, S.M.; Ghosh, A.K. Rapid detection of ERG11 polymorphism associated azole resistance in Candida tropicalis. *PLoS ONE* **2021**, *16*, e0245160. [CrossRef] [PubMed]

44. Bosco-Borgeat, M.E.; Mazza, M.; Taverna, C.G.; Córdoba, S.; Murisengo, O.A.; Vivot, W.; Davel, G. Amino acid substitution in *Cryptococcus neoformans* lanosterol 14-α-demethylase involved in fluconazole resistance in clinical isolates. *Rev. Argent. Microbiol.* **2016**, *48*, 137–142. [CrossRef] [PubMed]

45. Akapo, O.O.; Macnar, J.M.; Kryś, J.D.; Syed, P.R.; Syed, K.; Gront, D. In silico structural modeling and analysis of interactions of tremellomycetes cytochrome p450 monooxygenases cyp51s with substrates and azoles. *Int. J. Mol. Sci.* **2021**, *22*, 7811. [CrossRef] [PubMed]