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African ST173 *Cryptococcus deuterogattii* strains are commonly less susceptible to fluconazole: An unclear mechanism of resistance

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ABSTRACT

Objectives: Fluconazole (FCZ), either alone or in combination, is often administered for treatment of cryptococcal meningitis, especially in sub-Saharan Africa. Its extensive use has led to the emergence of FCZ-resistant strains. The mechanisms underlying FCZ resistance are poorly documented for yeasts belonging to the *Cryptococcus gattii* species complex. The literature suggests that resistance could be due to mutations in and/or overexpression of the *ERG11* gene (encoding the 14-α-demethylase) and efflux pumps such as *MDR* and *AFR* (two subclasses of ABC transporters). Here we highlight the presence of genotype VGII strains (*Cryptococcus deuterogattii*) from the Ivory Coast with a rare sequence type (ST173) associated with high FCZ minimum inhibitory concentrations (MICs) compared with strains originating from the Pacific Northwest (USA).
Methods: Mechanisms of FCZ resistance were investigated in 28 Ivorian clinical *C. deuterogattii* isolates recovered from three patients during their antifungal treatment and follow-up.
Results: The results demonstrated that: (i) these strains exhibited no mutations in the *ERG11* gene; (ii) some strains had increased *ERG11* and *MDR1* mRNA expression, whilst *AFR1* and *AFR2* were not overexpressed in strains with high FCZ MICs compared with the expression levels for strains with low FCZ MICs; and (iii) exposure to FCZ in strains with high MICs induced *AFR1* mRNA overexpression.
Conclusion: This study demonstrated that the FCZ resistance mechanism commonly described in *Cryptococcus neoformans* was not responsible for resistance to FCZ in rare subtype strains.

1. Introduction

Cryptococcus gattii and *Cryptococcus neoformans* are the main aetiological agents of cryptococcal meningitis (CM). These pathogens infect humans and other animals that inhale the desiccated environmental propagules and/or spores from the environment, resulting in pneumonia and/or severe meningitis. In 2014, annual fatalities from CM were estimated at 181,100 deaths globally, with 135,900 of these occurring in sub-Saharan Africa [1]. CM causes 15% of acquired immune deficiency syndrome (AIDS)-related mortality in sub-Saharan Africa, which has the most people bearing the burden of CM [2]. Until 2017, the therapeutic regimen recommended for the control of neurocryptococcosis during the consolidation and maintenance phases involved amphotericin B (AmB) and 5-fluorocytosine (5FC). Recently, the World Health Organization (WHO) published new recommendations in the event that AmB is unavailable, proposing the use of fluconazole (FCZ) at a high dose combined with oral 5FC. In sub-Saharan Africa, including the Ivory Coast, FCZ is the most commonly administered drug for cryptococcosis treatment; 80% of *Cryptococcus* infections are treated with FCZ monotherapy [3]. However, recurrence of cryptococcosis during FCZ maintenance therapy has been reported [4,5]. This drug arsenal leads to variations in prognosis and poor survival outcomes. Furthermore, different antifungal susceptibility patterns have been observed among cryptococcal species. Isolation of *Cryptococcus* strains with increased resistance to azole antifungals is often reported. In general, the *C. gattii* species complex shows higher minimum inhibitory concentrations (MICs)

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to azoles compared with isolates from the *C. neoformans* species complex [6–8].

The mechanisms of resistance to azole antifungals are (i) overexpression of the *ERG11* gene encoding lanosterol 14-α demethylase, (ii) mutations in *ERG11* that result in decreased susceptibility of the enzyme (Erg11p) to azole-induced inhibition, and (iii) overexpression of plasma membrane proteins that pump azoles out of the cell. The FCZ-resistant phenotype has been associated with two point mutations in the *ERG11* gene that are responsible for the amino acid substitutions G470R [9] and G484S [10] in *C. neoformans* strains. Other studies have demonstrated the role of efflux pumps by overexpressing the two ATP-binding cassette (ABC) transporters AFR1 and MDR1 [4,11,12]. Among the resistance mechanisms, innate heteroresistance to FCZ in vitro and in vivo was also reported for *C. neoformans* in which minor, highly resistant subpopulations have been produced due to adaptive formation of disomic chromosomes [13–15]. The five lineages within the *C. gattii* species complex have the ability to adapt to higher concentrations of FCZ in a stepwise manner, resulting in homogeneous resistant populations in vitro. The mechanisms of azole resistance in the *C. gattii* species complex were essentially studied in genotype VGII strains (*Cryptococcus deuterogattii*) from a North American outbreak or in the *C. gattii* reference strain R265. In these strains, neither overexpression of the azole target gene *ERG11* nor mutations in the *ERG11* coding sequences could explain the high MICs of azole observed [16].

In this study, we explored the mechanisms leading to the lower susceptibility of Ivorian *C. deuterogattii* isolates to FCZ. After determining the susceptibility profile of 28 Ivorian clinical *C. deuterogattii* strains to five azole antifungals [FCZ, itraconazole (ITR), posaconazole (PSC), voriconazole (VRZ) and isavuconazole (ISA)] as well as AmB and 5FC, the *ERG11* genes in all strains were sequenced to search for point mutations. Then, *ERG11*, *MDR1*, *AFR1* and *AFR2* mRNA levels were compared with those of the reference strain R265. The aim of this study was to determine whether the mechanisms commonly described in FCZ resistance in yeast are also involved in Ivorian strains of *C. deuterogattii*.

2. Materials and methods

2.1. Strains and growth media

A total of 28 clinical isolates of *C. deuterogattii* were analysed in this study. The isolates were from cerebrospinal fluid samples of three Ivorian human immunodeficiency virus (HIV)-positive patients during their follow-up. None of the patients received systemic antifungal treatment prior to their hospitalisation. All of the isolates were recovered during antifungal treatment.

Cryptococcus neoformans serotype A reference strain H99 (genotype VNI) and *C. deuterogattii* serotype B reference strain CBS10514 (R265; genotype VGIIa) were used as control strains. The two quality control strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used for in vitro antifungal susceptibility testing as recommended by the Clinical and Laboratory Standards Institute (CLSI) [17].

The media used in this study included yeast extract–peptone–dextrose (YEPD) liquid medium or Sabouraud chloramphenicol agar. RPMI medium was used for antifungal microdilution susceptibility testing.

2.2. Antifungal susceptibility testing

The in vitro susceptibility profile of *Cryptococcus* spp. against AmB, 5FC, FCZ, VRZ, PSC, ITR and ISA was determined using the reference broth microdilution method in accordance with the CLSI [17]. The final antifungal concentration ranges were as follows: 0.125–16 μg/mL for AmB; 0.25–64 μg/mL for FCZ and 5FC; and 0.03–16 μg/mL for VRZ, ITR, PSC and ISA. MICs for FCZ, 5FC and VRZ were defined as concentrations causing a 50% reduction in turbidity compared with growth of the control strain at 72 h. For AmB, the MIC was defined as the concentration resulting in 100% inhibition relative to the growth of the control.

No breakpoints are available for *C. neoformans* and the five lineages within the *C. gattii* species complex, in which case the epidemiological cut-off values (ECVs) were used to discriminate wild-type (WT) strains from non-WT strains with reduced susceptibility to some antifungals [18,19].

2.3. DNA extraction

Genomic DNA was extracted from each strain using a NucleoSpin® Blood Quick Extraction Kit (Macherey-Nagel GmbH and Co. KG, Duren, Germany) with modifications as previously described [20]. One aliquot was used for each of the experiments described in this study.

2.4. Molecular typing

2.4.1. Serotyping by multiplex PCR

To determine the molecular serotype of the isolates, four primers designed for cloning the *LAC1* gene and a pair of primers for the *CAP64* gene [21] were used in a slightly modified method as previously described [20,22].

2.4.2. Restriction fragment length polymorphism–PCR (PCR-RFLP) genotyping of the URA5 gene

PCR-RFLP analyses were also performed using the URA5 and SJ01 primers. The PCR conditions were as follows: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 61 °C for 1 min and extension at 72 °C for 2 min; and a final extension cycle at 72 °C for 10 min. Then, 10 μL of each PCR product was double-digested using *Sau*96I (15 U) and *Hha*I (15 U) for 5 h at 37 °C and the digested fragments were visualised on 1.5% agarose gels stained with ethidium bromide. Migration patterns were captured with an InGenius LR apparatus (Syngene, Cambridge, UK). Molecular profiles obtained via PCR fingerprinting were analysed based on the presence or absence of readily apparent and well-defined bands in the digitised gel images using GeneSnap and GeneTool software and were integrated in a database using GeneDirectory software (http://www.syngene.com/genedirectory-2/).

2.4.3. Multilocus sequence typing (MLST) and analysis

The International Society for Human and Animal Mycology (ISHAM) MLST consensus schemes described for the *C. neoformans* and *C. gattii* species complexes was used in this study. The six genes *CAP59*, *GPD*, *LAC1*, *PLB1*, *SOD1* and *URA5* as well as the IGS1 region were partially amplified [23]. PCR amplicons were purified and were sequenced using forward primers by GENEWIZ (London, UK). Sequences were manually edited and aligned using BioEdit software. Alleles types and sequences types (STs) were assigned by sequence comparison with the *C. neoformans* and *C. gattii* databases at http://mlst.mycologylab.org/.

2.4.4. ERG11 sequencing

The whole *ERG11* gene was amplified by PCR as followed: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s and extension at 72 °C for 1 min; and a final extension cycle at 72 °C for 10 min with specific primers for *C. neoformans* and *C. deuterogattii*, respectively (Table 1). Amplicons were purified and sequenced by GENEWIZ (Leipzig, Germany) using the four primers a, b, d and e for *C. neoformans* and primers a, c, d and f

Table 1
Primers used for *ERG11* PCR amplification and sequencing.

	Cryptococcus spp.	Primer sequence
PCR amplification	*C. neoformans*	5′-ATGTCGGCAATCATCCCCCA-3′ a 5′-CATACTAAAACTCGCACCATC-3′ b
	C. deuterogattii	5′-ATGTCGGCAATCATCCCCCA-3′ a 5′-TACACCTCCTGCTTGACCTC-3′ c
Sequencing	*C. neoformans*	5′-ATGTCGGCAATCATCCCCCA-3′ a 5′-CATACTAAAACTCGCACCATC-3′ b 5′-ATGAGCGACTTTTACTTGAA-3′ d 5′-GTGTTCGTGCTACTCAAATC-3′ e
	C. deuterogattii	5′-ATGTCGGCAATCATCCCCCA-3′ a 5′-TACACCTCCTGCTTGACCTC-3′ c 5′-ATGAGCGACTTTTACTTGAA-3′ d 5′-AGCAGATGAAGTGTGTTGGC-3′ f

Primers a and b and primers a and c are used for PCR amplification of the entire *ERG11* gene for *C. neoformans* and *C. deuterogattii*, respectively. Primers a, b, d and e and primers a, c, d and f are used to sequence the entire *ERG11* gene for *C. neoformans* and *C. deuterogattii*, respectively.

for *C. deuterogattii* to sequence the whole *ERG11* gene (Table 1). Sequences were manually edited and aligned using BioEdit software.

2.5. RNA isolation and reverse transcription quantitative PCR (qRT-PCR) analysis

For RNA extraction, cells were grown overnight in 10 mL of YEPD medium on a shaker at 30 °C. Following 24 h of growth, cells were harvested by centrifugation to obtain ~1×10^8 cells.

For RNA extraction following FCZ exposure, each strain was grown for 5 days in 10 mL of YEPD medium supplemented with a subinhibitory FCZ concentration (0.5 × MIC). After 5 days, cells were harvested by centrifugation to obtain ~1×10^8 cells.

Total RNA was extracted using an Ambion RiboPure™ Yeast Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations for RNA isolation. RNA samples were treated with DNAse to remove genomic DNA contamination. Following extraction, RNA was quantified using a Nanodrop 2000 spectrophotometer and the quality of the purified RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA was synthesised using a SuperScript® III First-Strand Synthesis System (Invitrogen) followed by reverse-transcription quantitative PCR (qRT-PCR) using a LightCycler® 480 Real-Time PCR Instrument (Roche). Forward and reverse primer sequences for *ERG11*, *MDR1*, *AFR1*, *AFR2* and actin are shown in Table 2 and were used at an annealing temperature of 57 °C. Data were normalised to levels of the actin gene and were expressed as the amount of mRNA relative to that of strain R265.

3. Results

All of the strains studied were *C. deuterogattii*. They all exhibited a VGII genotype (serotype B) and were ST173 (GenBank accession nos. MN431744 for CG Cap59 allele4, MN431749 for CG_GPD1_allele21, MN431753 for CG_IGS1_allele21, MN431758 for CG_LAC1_allele4, MN431763 for CG_PLB1_allele16, MN431766 for CG_SOD1_allele93 and MN431771 for CG_URA5_allele2). The strains were recovered from three patients during their follow-up.

Table 2
Primers used for reverse transcription quantitative PCR (qRT-PCR).

Gene	Primer sequence
ERG11	5′-CCATGTCCGAGCTCATCATTCTT-3′ 5′-ACTGGGAAGGGGCAAGTTGG-3′
MDR1	5′-GTCTTCACCTTCGTCCCGGAT-3′ 5′-CAGTACTCTCACTCCCGGCCTG-3′
AFR1	5′-TTCCCTGCTCCTTCAGGACAGA-3′ 5′-AGGCTTGGCCAGTTCGGTACT-3′
AFR2	5′-CGATATGGGATTTCACTGCCCT-3′ 5′-GCTCCTGATATTTGTCGCTCTGC-3′
Actin	5′-CCAAGCAGAACCGAGAGAAGATG-3′ 5′-GGACAGTGTGGGTGACACCGT-3′

Table 3
Minimum inhibitory concentrations (MICs) of antifungals in 28 *Cryptococcus deuterogattii* clinical isolates and two reference strains (H99 and R265).

Isolate code[a]	MIC (μg/mL)						
	FCZ	ITR	PSC	VRZ	ISA	AmB	5FC
Cg1	8	0.25	0.25	0.06	ND	1	2
Cg2	8	0.25	0.25	0.03	ND	1	4
Cg3	4	0.03	0.25	0.06	0.03	0.5	64
Cg4	4	0.03	0.06	0.03	0.03	0.5	1
Cg5	2	0.03	0.03	0.03	ND	0.25	1
Cg6	0.5	0.03	0.03	0.03	0.06	0.25	1
Cg7	4	0.25	0.125	0.03	0.03	0.25	1
Cg8	4	0.25	0.25	0.03	0.03	0.5	1
Cg9	8	0.03	0.25	0.03	0.03	0.5	0.5
Cg10	8	0.03	0.03	0.03	0.03	0.25	0.25
Cg11	64	0.125	1	0.5	0.03	0.5	4
Cg12	16	0.25	0.5	0.25	0.03	0.5	4
Cg13	16	0.125	1	0.25	0.03	0.5	4
Cg14	32	0.125	0.25	0.25	0.03	0.5	4
Cg15	16	0.03	0.5	0.06	0.06	0.5	0.25
Cg16	16	0.03	0.25	0.03	0.03	1	8
Cg17	32	0.03	0.5	0.03	0.03	0.5	4
Cg18	32	0.03	0.5	0.25	0.03	0.5	2
Cg19	16	0.125	0.5	0.25	0.03	1	8
Cg20	16	0.125	1	0.25	0.03	0.5	2
Cg21	32	0.25	0.5	0.25	0.03	1	4
Cg22	16	0.25	0.5	0.25	0.06	1	8
Cg23	32	0.03	0.25	0.03	0.03	1	8
Cg24	32	0.03	0.25	0.03	0.03	0.5	4
Cg25	16	0.03	1	0.03	0.03	1	2
Cg26	16	0.03	1	0.03	0.03	0.5	2
Cg27	16	0.03	1	0.125	0.03	0.5	4
Cg28	32	0.25	0.25	0.5	0.03	0.5	4
GM	12.19	0.07	0.30	0.08	0.03	0.55	2.56
Mode	16	0.03	0.25	0.03	0.03	0.5	4
H99	4	0.03	ND	0.031	0.03	0.1225	2
R265	8	0.063	ND	0.063	0.125	0.5	4

FCZ, fluconazole; ITR, itraconazole; PSC, posaconazole; VRZ, voriconazole; ISA, isavuconazole; AmB, amphotericin B, 5FC, 50fluorocytosine; ND, not determined. Epidemiological cut-off values (ECVs) for *C. deuterogattii* were 0.5/1 μg/mL for AmB, 0.5 μg/mL for ITR and PSC, 0.25 μg/mL for ISA and VRZ, 16 μg/mL for 5FC and 32 μg/mL for FCZ.

[a] Isolates Cg 1, 2, 11, 13, 14, 15, 16, 17, 18, 19, 21, 22, 24, 27 and 28 were recovered from Patient 1; isolates Cg 3, 4, 5, 6, 7, 8, 9 and 10 were from Patient 2; and isolates Cg 12, 20, 23, 25 and 26 were from Patient 3.

3.1. Antifungal susceptibility profiles

The MIC distributions for the azole compounds (FCZ, ITR, PSC, VRZ and ISA) and AmB and 5FC are shown in Tables 3 and 4 for the 28 clinical *C. deuterogattii* strains and the two reference strains [CBS10514 (R265) (*C. deuterogattii* B/VGII) and H99 (*C. neoformans* A/VNI)].

The FCZ MIC distribution among the 28 clinical strains was as follows: 10 (35.7%) had an MIC ≤8 μg/mL (Cg1 to Cg10); and 18 (64.3%) had an MIC ≥16 μg/mL (Cg11 to Cg28). The FCZ MICs were distributed as follows: 0.5 μg/mL (1; 3.6%); 2 μg/mL (1; 3.6%); 4 μg/mL (4; 14.3%); 8 μg/mL (4; 14.3%); 16 μg/mL (10; 35.7%); 32 μg/mL (7; 25.0%); and 64 μg/mL (1; 3.6%). According to the ECVs, only 1 isolate (3.6%) was non-WT to FCZ. The MIC distributions of other azole compounds (VRZ, PSC, ITR and ISA) were also analysed. For ITR, all of the strains had a MIC < 0.5 μg/mL; for VRZ, 26 (92.8%) had an MIC < 0.5 μg/mL and 2 (7.1%) (Cg11 and Cg28) had an MIC of 0.5 μg/mL; for PSC, 22 (78.6%) had an MIC ≤ 0.5 μg/mL and 6 (21.4%) had an MIC of 1 μg/mL; and for ISA, all of the strains exhibited MICs of 0.03–0.06 μg/mL.

Table 4
Minimum inhibitory concentration (MIC) distribution of fluconazole, itraconazole, posaconazole, voriconazole, isavuconazole, amphotericin B and 5-fluorcytosine for 28 *Cryptococcus deuterogattii* clinical strains obtained using the reference broth microdilution method.

Antifungal agent	No. of isolates at MIC (μg/mL) of:												
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	ND
Fluconazole					1		1	4	4	10	7	1	
Itraconazole	15		5	8									
Posaconazole	3	1	1	10	7	6							
Voriconazole	14	3	1	8	2								
Isavuconazole	22	3											3
Amphotericin B				4	16	8							
5-Fluorocytosine				2	1	5	5	10	4			1	

ND, not determined.

For AmB, all of the clinical strains tested had an MIC of 0.25–1 μg/mL. Regarding the MIC of 5FC, only one clinical strain had an MIC of 64 μg/mL; all of the other strains had 5FC MICs of 0.25–8 μg/mL.

3.2. No mutations in the ERG11 gene of Ivorian clinical Cryptococcus deuterogattii *strains are responsible for fluconazole resistance*

The *ERG11* gene from *C. deuterogattii* strain R265, *C. neoformans* strain H99 and 28 Ivorian clinical *C. deuterogattii* strains (GenBank accession no. MN447654) was sequenced. The *ERG11* gene sequences of the Ivorian strains were compared with those of the two reference strains using BioEdit software (Table 5). A single mutation (C101A) in the Ivorian *C. deuterogattii* strains was detected, regardless of whether they had high or low FCZ MICs. This point mutation was not present in the two reference strains R265 or H99. This mutation results in substitution of histidine at position 50 with asparagine (H50N).

3.3. ERG11 mRNA overexpression was observed in some strains of Cryptococcus deuterogattii *with high fluconazole MICs, but expression of AFR1 and AFR2 mRNA did not change*

Fig. 1 shows the expression levels of *ERG11*, *MDR1*, *AFR1* and *AFR2* mRNA for all of the clinical strains studied. Fold changes were obtained by comparing the ratio (gene of interest/housekeeping gene) for each Ivorian *C. deuterogattii* strain with that of the reference strain R265. mRNA expression levels for *ERG11* and the three efflux pump genes (*MDR1*, *AFR1* and *AFR2*) were higher in all of the Ivorian *C. deuterogattii* strains compared with strain R265. Fig. 1A shows that for strains Cg11 to Cg28 (FCZ MIC ≥ 16 μg/mL) changes for *ERG11* varied between 3.9- and 23.8-fold, whereas for strains Cg1 to Cg10 (MIC ≤ 8 μg/mL) they varied between 3.3- and 6-fold. Expression levels of the *ERG11* gene were higher in strains with high FCZ MICs (>16 μg/mL) than in strains with low FCZ MICs (≤8 μg/mL). Six strains (Cg11, Cg12, Cg13, Cg14, Cg18 and Cg24) showed an increase in *ERG11* mRNA expression with a fold change >10. Overall expression of *ERG11* mRNA varied within the strains with high MICs of FCZ (Cg10 to Cg28) but remained constant for the 10 strains with low FCZ MICs (Cg1 to Cg10).

Finally, for the three efflux pump genes (*MDR1*, *AFR1* and *AFR2*) there was no change in gene expression between the strains (Fig. 1B–D).

The fold change of each gene was also compared with the MIC of FCZ for each corresponding strain (Fig. 2.1). It was confirmed that the most important *ERG11* gene fold change was obtained in strains with FCZ MICs ≥16 μg/mL. In strains with high FCZ MICs, *ERG11* mRNA expression was increased, however this was not observed for the other studied genes.

3.4. Fluconazole exposure induced an increase in the mRNA expression of AFR1 *in strains with high fluconazole MICs (≥16 μg/mL)*

The effect of exposure to a subinhibitory FCZ concentration for each strain on the expression level of *ERG11*, *MDR1*, *AFR1* and *AFR2* mRNA in the 28 Ivorian clinical strains was also studied. Fig. 3 shows the fold changes in expression obtained by comparing mRNA expression of the studied gene in each strain with or without exposure to FCZ.

Exposure to FCZ induced an increase in *ERG11* mRNA expression in all strains (Fig. 3A). One strain (Cg17) showed a very large increase in *ERG11* mRNA expression following exposure to FCZ (28.9-fold).

For *MDR1* (Fig. 3B), the most important fold change was observed for strains with a FCZ MIC ≥ 16 μg/mL (e.g. Cg12, Cg15, Cg16, Cg17, Cg25 and Cg27).

For *AFR1*, an increase in mRNA expression was observed for strains Cg11 to Cg28 (FCZ MIC ≥ 16 μg/mL) and the fold change varied between 3.9 and 23.8. There was an increase in *AFR1* mRNA expression following exposure to FCZ in this group of strains compared with strains Cg1 to Cg10.

For *AFR2*, only 7 (25.0%) of the 28 strains showed an increase in mRNA expression, with a fold change between 1.27 and 4.1. We can conclude that mRNA expression of *ERG11* and *AFR1* was increased in 77.8% (14/18) of the strains with FCZ MICs ≥ 16 μg/mL following FCZ exposure. FCZ exposure had a different effect on gene expression for strains with MICs of FCZ ≥ 16 μg/mL compared with the other strains.

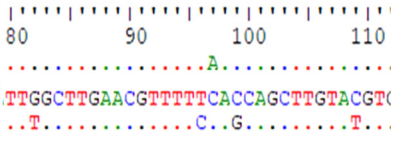
The fold change of each gene, obtained by comparing mRNA expression levels with or without FCZ exposure, was also compared with the FCZ MIC of the respective strain (Fig. 2.2). It was confirmed that exposure to FCZ induced an increase in *AFR1* expression in strains with a FCZ MIC ≥ 16 μg/mL.

4. Discussion

Cryptococcus deuterogattii clinical strains were first reported in the Ivory Coast in 2016 [8,20,22,24]. The strains were all identified as ST173 by MLST. ST173 was previously described only once [25] and it was hypothesised that it had originated in Africa.

Table 5
Nucleotide mutations in the *ERG11* gene and amino acid substitutions from Ivorian *Cryptococcus deuterogattii* isolates compared to ERG11 sequences from reference strains H99 and R265.

Isolate No	Nucleotide mutation	Amino acid substitution
Cg1 to Cg28	C101A	H50N
CBS10514 (R265)	x	x
C. neoformans H99	x	x



C, cytosine; A, adenine; H, histidine; N, asparagine.
Base numbers are with respect to the first ATG codon of the *ERG11* gene.

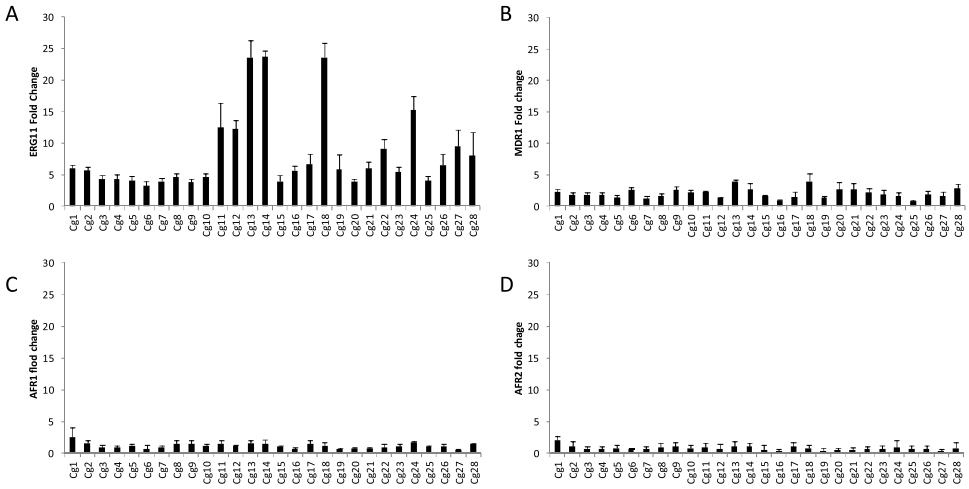


Fig. 1. mRNA expression level of (A) *ERG11*, (B) *MDR1*, (C) *AFR1* and (D) *AFR2* in 28 clinical *Cryptococcus deuterogattii* isolates. Expression levels of each gene were determined by reverse transcription quantitative PCR (qRT-PCR). The expression level of each gene was normalised to that of the actin gene and was compared with the expression level of the reference strain CBS10514 (R265).

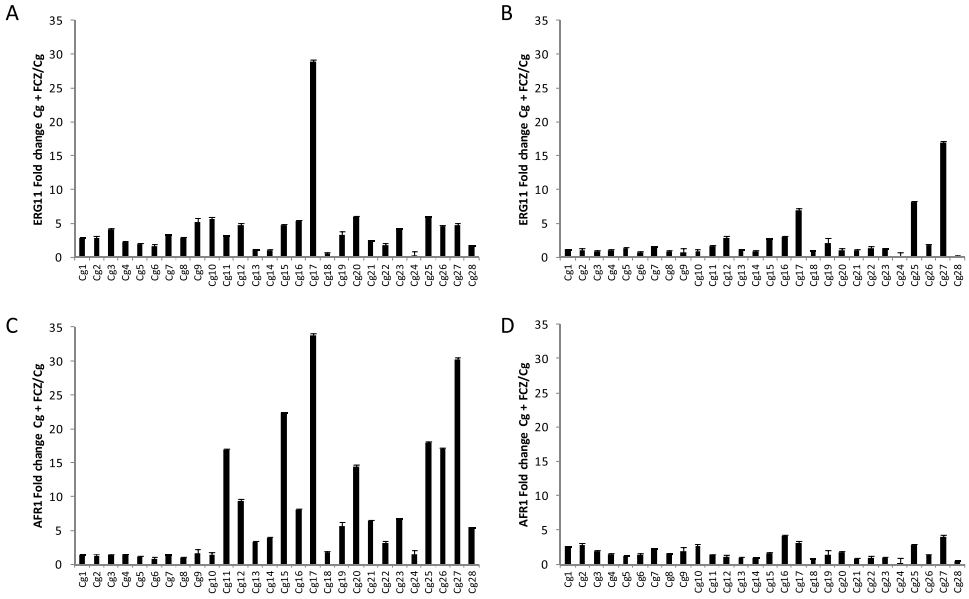


Fig. 2. (1) Minimum inhibitory concentrations (MICs) of fluconazole (FCZ) compared with mRNA fold change/CBS10514 for (A) *ERG11*, (B) *MDR1*, (C) *AFR1* and (D) *AFR2* for 28 clinical *Cryptococcus deuterogattii* isolates. (2) Fold change for (A) *ERG11*, (B) *MDR1*, (C) *AFR1* and (D) *AFR2* in *C. deuterogattii* isolates following exposure to FCZ for 5 days compared with strains without FCZ exposure according to the FCZ MIC for each isolate.

The antifungal susceptibility profile of the strains to azoles, AmB and 5FC was analysed. In the literature, the ECVs of FCZ for *C. deuterogattii* (B; AFLP6/VGII) strains varied. The most studied *C. deuterogattii* strains were from Vancouver Island (USA). By MLST, these strains, which are composed of three clonal expansions (a, b and c), were characterised as ST20, ST7, ST6 and ST49 [26,27]. The *C. deuterogattii* strains from the North American outbreak were separated according to their ECVs for FCZ. The ECV for the *C. deuterogattii* genotype AFLP6A/VGIIa was 8 μg/mL, whilst it was 32 μg/mL for the other strains (VGIIb, c and others) [18,28,29]. No ECVs were published for the Ivorian *C. deuterogattii* ST173 strains. There is growing evidence that antifungal drug susceptibility may vary by molecular type or geographical location [30,31]. Among the Ivorian *C. deuterogattii* population, we found that 28.6% (8/28) of the *Cryptococcus* strains had elevated FCZ MICs (≥32 μg/mL). The entire population had WT MICs for the other azole compounds, i.e. ITR, VRZ, PSC and ISA. Recently, ECVs were proposed for ISA, a new water-soluble triazole used for the treatment of invasive aspergillosis and other mycoses (0.25 μg/mL for *C. gattii* without variance according to genotype) [32–34]. All of the Ivorian strains were WT isolates. It is interesting that they were susceptible to ISA. Information regarding the activity of ISA is scarce with regard to emerging yeast pathogens that exhibit various susceptibilities to clinically available antifungals.

Many mutations in *ERG11* are related to FCZ resistance and their effect on ERG11p has been reported. The first was the amino acid substitution G484S in a clinical strain of *C. neoformans* [10]. The second involved the replacement of glycine with asparagine at amino acid 470 (G470R), which is located in the proximity of the catalytic domain of the protein and contributes to a decrease in azole affinity for ERG11p [9]. The third was a missense mutation in ERG11p (Y145F) that is responsible for the differential susceptibilities between two different kinds of triazoles: FCZ/VRZ and ITR/PSC [35]. By culturing in medium containing VRZ, multi-azole-resistant strains of *C. neoformans* were obtained and these strains encoded a protein with a G344S substitution [36]. In 2013, a study of two

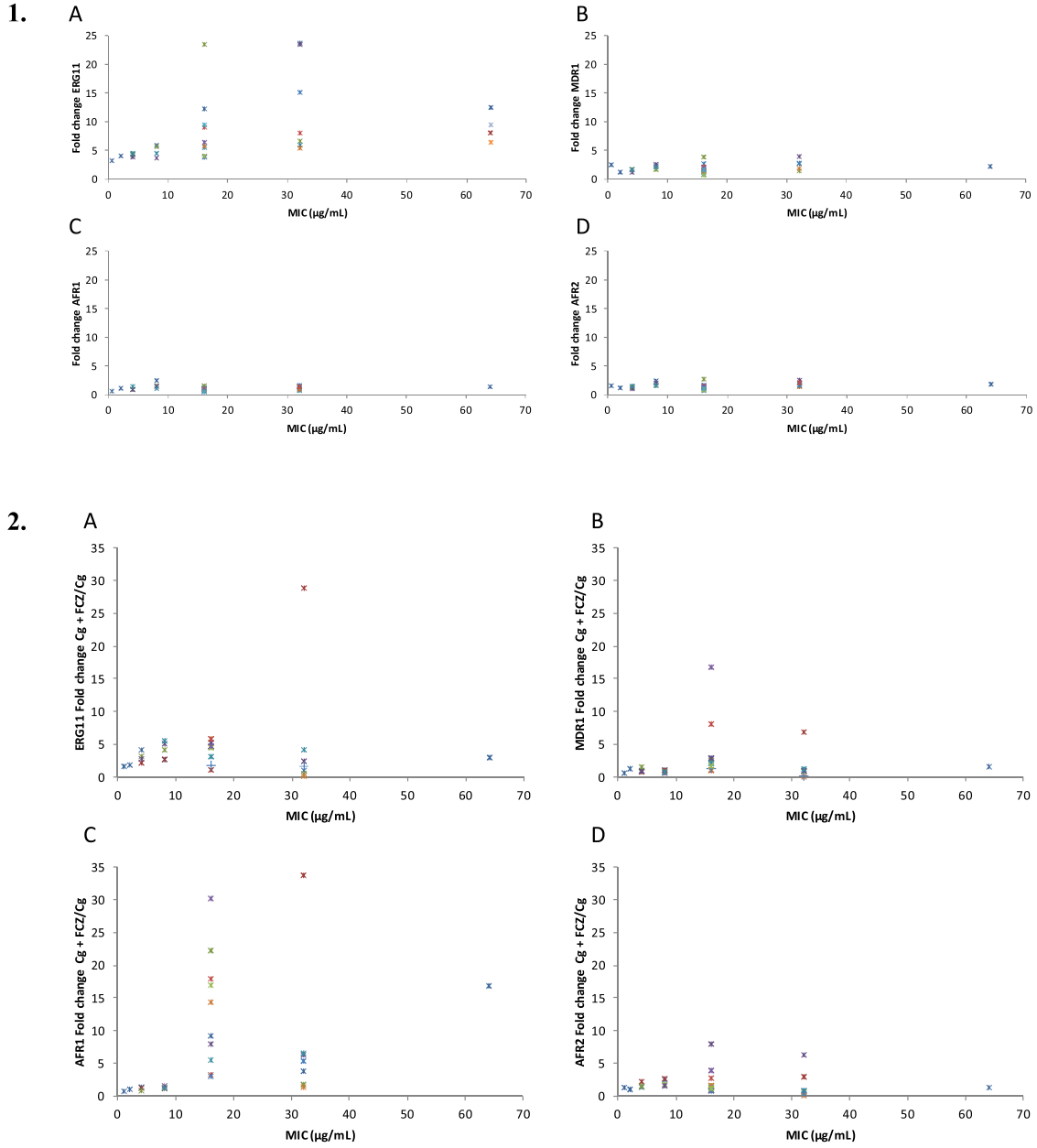


Fig. 3. mRNA expression level of (A) *ERG11*, (B) *MDR1*, (C) *AFR1* and (D) *AFR2* in isolates after exposure to fluconazole (FCZ) over 5 days. The expression level of each gene was determined by reverse transcription quantitative PCR (qRT-PCR) and was normalised to that of the actin gene. The fold change was obtained by comparing the expression level of each gene in strains exposed to FCZ with the expression level of each gene in the same strain not exposed to FCZ. Values represent the mean ± standard deviation of three biological replicates.

VGIIc strains with high FCZ MICs (32 µg/mL and 64 µg/mL) showed ERG11p sequences that contained a single amino acid substitution (N249D) not present in the deduced ERG11p sequence of any other *C. deuterogattii* strains studied. These variations in *ERG11* coding sequences were not responsible for the high azole MICs observed for the *C. deuterogattii* strains from the North American outbreak studied [16]. This finding is in agreement with those from the present study. A point mutation was found in the *ERG11* gene, the H50N substitution in ERG11p, that was not present in reference strains R265 or H99. It appears that the Ivorian *C. deuterogattii* VGII ST173 strains possessed a point mutation in the *ERG11* gene regardless of their FCZ MIC. Only two mutations in ERG11p have been shown to be responsible for FCZ resistance in *C. neoformans* and, to date, no mutation in *ERG11* has been shown to be involved in FCZ resistance in strains of the *C. gattii* species complex, even among the most virulent strains described, i.e. the *C. deuterogattii* strains from the North American outbreak.

The other major mechanism of FCZ resistance involves overexpression of multidrug efflux transporters (*MDR1*, *AFR1/PDR11* and *AFR2*) [37,38]. Overexpression of these drug efflux transporters has been linked to azole resistance [39]. More recently, it was suggested that *PDR11* alone may be responsible for triazole resistance in *C. deuterogattii* isolates from the North American outbreak [38], and deletion of *PDR11*, but not *MDR1*, has a significant effect on susceptibility to FCZ. Therefore, involvement of *MDR1* in azole resistance appears to be controversial, especially in *C. deuterogattii* strains [4,16,40]. To explain the high FCZ MICs in the Ivorian *C. deuterogattii* strains, the expression levels of *ERG11*, *AFR1*, *AFR2* and *MDR1* mRNA were studied. Some strains had a high level of *ERG11* and *MDR1* expression without a correlating high FCZ

MIC. This increase was not observed for all strains and it appears to be strain-specific. On the other hand, neither of the efflux pumps *AFR1* or *AFR2* were implicated, which is in contrast to the findings described in the literature. It was also observed that exposure to FCZ induced an increase in *AFR1* mRNA expression, especially in strains with high FCZ MICs. The mechanisms commonly described in *C. neoformans* were not found to be involved in the increased MICs FCZ in the Ivorian *C. deuterogattii* strains. We conclude that the mechanisms do not appear to be the same as the mechanisms described for *C. deuterogattii* from the North American outbreak. These results could be explained because the sequence type of the Ivorian strains (ST173) differs from that of the reference strain R265 (VGIIa; ST20) and strains from the North American outbreak.

It remains important to discover why theses specific strains isolated in Ivory Coast exhibit elevated FCZ MICs. To better characterise these strains, whole-genome or transcriptome-sequencing analysis could be used to find genes and proteins responsible for the increased FCZ MICs in the Ivorian *C. deuterogattii* strains, and the virulence of the strains in animal models should be studied.

Competing interests

None declared.

Ethical approval

This study was approved by the Ethical Sciences Committees of Life and Health of the Ivory Coast [021/MSLS/CNER-kp]. Written informed consent was provided by the patient or a family member prior to sample collection, and data were anonymised.

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