




# Reduced Susceptibility to Azoles in *Cryptococcus gattii* Correlates with the Substitution R258L in a Substrate Recognition Site of the Lanosterol 14- $\alpha$ -Demethylase

Silvia Katherine Carvajal,<sup>a</sup> Javier Melendres,<sup>b\*</sup> Patricia Escandón,<sup>a</sup>  Carolina Firacative<sup>b</sup>

<sup>a</sup>Group of Microbiology, Instituto Nacional de Salud, Bogotá, Colombia

<sup>b</sup>Studies in Translational Microbiology and Emerging Diseases (MICROS) Research Group, School of Medicine and Health Sciences, Universidad del Rosario, Bogotá, Colombia

**ABSTRACT** *Cryptococcus neoformans* and *Cryptococcus gattii* cause cryptococcosis, a life-threatening fungal infection affecting mostly immunocompromised patients. In fact, cryptococcal meningitis accounts for about 19% of AIDS-related deaths in the world. Because of long-term azole therapies to treat this mycosis, resistance to fluconazole leading to treatment failure and poor prognosis has long been reported for both fungal species. Among the mechanisms implicated in resistance to azoles, mutations in the *ERG11* gene, encoding the azole target enzyme lanosterol 14- $\alpha$ -demethylase, have been described. This study aimed to establish the amino acid composition of ERG11 of Colombian clinical isolates of *C. neoformans* and *C. gattii* and to correlate any possible substitution with the *in vitro* susceptibility profile of the isolates to fluconazole, voriconazole, and itraconazole. Antifungal susceptibility testing results showed that *C. gattii* isolates are less susceptible to azoles than *C. neoformans* isolates, which could correlate with differences in the amino acid composition and structure of ERG11 of each species. In addition, in a *C. gattii* isolate with high MICs for fluconazole (64  $\mu$ g/mL) and voriconazole (1  $\mu$ g/mL), a G973T mutation resulting in the substitution R258L, located in substrate recognition site 3 of ERG11, was identified. This finding suggests the association of the newly reported substitution with the azole resistance phenotype in *C. gattii*. Further investigations are needed to determine the exact role that R258L plays in the decreased susceptibility to fluconazole and voriconazole, as well as to determine the participation of additional mechanisms of resistance to azole drugs.

**IMPORTANCE** The fungal species *Cryptococcus neoformans* and *C. gattii* are human pathogens for which drug resistance or other treatment and management challenges exist. Here, we report differential susceptibility to azoles among both species, with some isolates displaying resistant phenotypes. Azoles are among the most commonly used drugs to treat cryptococcal infections. Our findings underscore the necessity of testing antifungal susceptibility in the clinical setting in order to assist patient management and beneficial outcomes. In addition, we report an amino acid change in the sequence of the target protein of azoles, which suggests that this change might be implicated in resistance to these drugs. Identifying and understanding possible mechanisms that affect drug affinity will eventually aid the design of new drugs that overcome the global growing concern of antifungal resistance.

**KEYWORDS** Colombia, cryptococcosis, *Cryptococcus neoformans*, *Cryptococcus gattii*, *ERG11*, fluconazole resistance, antimicrobial resistance, azole resistance, voriconazole

**C** *ryptococcus neoformans* and *Cryptococcus gattii* are encapsulated basidiomycetous yeasts that can cause cryptococcosis, affecting the lungs and, more frequently, the central nervous system (CNS) (1, 2). *C. neoformans*, which causes the majority of cases

**Editor** Alexandre Alanio, Institut Pasteur

**Copyright** © 2023 Carvajal et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Carolina Firacative, cfiracative@gmail.com.

\*Present address: Javier Melendres, Clinical Laboratory, Hospital Infantil Universitario de San José, Bogotá, Colombia.

The authors declare no conflict of interest.

**Received** 31 March 2023

**Accepted** 1 June 2023

**Published** 21 June 2023

worldwide, primarily infects immunocompromised patients, mostly people living with HIV (PLHIV), but also cancer patients receiving chemotherapy as well as recipients of solid-organ and hematopoietic stem cell transplants (3–5). *C. gattii*, conversely, which accounts for less than 20% of cases globally, was long considered to infect immunocompetent hosts or those with unidentified risk factors (6). However, in the last decade, subtle alterations in people's immunity, such as anti-granulocyte-macrophage colony-stimulating factor autoantibodies, have been detected in most patients affected by this species (7, 8).

Annually, the global incidence of cryptococcal meningitis is estimated to be 152,000 cases, with ~54% of cases in sub-Saharan Africa alone, resulting in 112,100 deaths. This accounts for approximately 19% of AIDS-related mortality (9). In Colombia, the incidence of cryptococcosis has been estimated to be 0.23 case per million people in the general population, while this value increases to 1.1 cases per thousand PLHIV, with mortality rates of almost 50% (10). Although *C. neoformans* causes the majority of cases of cryptococcosis (~96%) in the country, *C. gattii* has long been recognized as an endemic pathogen. Notably, infection by this species usually needs longer periods and higher doses of antifungal treatment, requires additional clinical follow-up, and leaves more neurological sequelae (6, 11).

Treatment for cryptococcal infection remains a challenge due to the few therapeutic options currently available. Only three classes of antifungals, polyenes, flucytosine, and azoles, are used to treat this mycosis (12). For the first phase of treatment (induction), the combination of amphotericin B deoxycholate with 5-fluorocytosine presents favorable results (13). However, the toxicity of amphotericin B deoxycholate limits its use, and although there is a less toxic formulation of this polyene, the high price restricts its availability, as it occurs with 5-fluorocytosine (14, 15). Fluconazole, which can be used alone or in combination with 5-fluorocytosine in the induction phase, is as well the antifungal of choice for the consolidation and maintenance phases, which together can last up to 1 year (13). In addition, fluconazole is often used as monotherapy in the induction phase in countries with limited resources, including Colombia, and it is recommended as primary prophylaxis in HIV-positive adults and adolescents with CD4 counts less than 100 cells/ $\mu$ L (13–15). Despite its wide use, fluconazole is much less effective in eliminating fungi from the cerebrospinal fluid, due to its fungistatic nature, and has poorer clinical outcomes than does the amphotericin B-based induction regimen (16, 17). Furthermore, associated with its prolonged use, isolates with heteroresistance and decreased susceptibility to fluconazole have long been reported, leading to therapeutic failure and suboptimal outcomes (18–21).

Fluconazole and other triazoles interact with the lanosterol 14- $\alpha$ -demethylase (ERG11), a cytochrome P450 enzyme encoded by the *ERG11* gene and responsible for catalyzing the conversion of lanosterol to ergosterol, which leads to impaired cell membrane integrity and functionality (22). Even though the mechanisms implicated in resistance to azoles remain poorly understood, increased or decreased expression and mutations in the *ERG11* gene, as well as aneuploidy and overexpression of the membrane efflux pump proteins, have been described for cryptococcal isolates with reduced susceptibility to these antifungal drugs (23–25). Regarding amino acid substitution in ERG11, particularly, G484S has been reported for *C. neoformans* with MIC values for fluconazole of  $\geq 16 \mu\text{g/mL}$  (26, 27). Other substitutions in ERG11 that may confer decreased susceptibility to azoles have been identified in *C. neoformans*, such as Y145F, conferring resistance to voriconazole (28), G344S (corresponding to G417S), found in multiazole-resistant isolates (29), G470R, identified in two clinical isolates with the fluconazole resistance phenotype (30), and I99V, detected in isolates with high MICs for fluconazole (16 to 24  $\mu\text{g/mL}$ ) (31, 32). To our knowledge, in *C. gattii*, only the substitution N249D, which was suggested to correlate with azole resistance, has been reported (33).

Considering that in Colombia, cryptococcal isolates with high MIC values for fluconazole and other triazoles have been reported (34) and that the antifungal susceptibility varies between and within *C. neoformans* and *C. gattii* (35, 36), this study aimed to

**TABLE 1** Distribution of the MIC of clinical *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from Colombia

| Antifungal                | Species              | n  | GM <sup>b</sup> MIC ( $\mu\text{g/mL}$ ) | No. of isolates with indicated MIC ( $\mu\text{g/mL}$ ) <sup>a</sup> |      |           |      |     |   |          |   |    |           |          |    |   |
|---------------------------|----------------------|----|--|--|------|-----------|------|-----|---|----------|---|----|-----------|----------|----|---|
|                           |                      |    |  | 0.03   | 0.06 | 0.125     | 0.25 | 0.5 | 1 | 2        | 4 | 8  | 16        | 32       | 64 |   |
| Fluconazole <sup>c</sup>  | <i>C. neoformans</i> | 31 | 5.231                                    |  |      |           |      |     |   | 1        | 4 | 10 | <u>14</u> | 2        |    |   |
|                           | <i>C. gattii</i>     | 19 | 9.958                                    |  |      |           |      |     |   |          | 1 | 3  | 6         | <u>8</u> | 0  | 1 |
| Voriconazole <sup>c</sup> | <i>C. neoformans</i> | 31 | 0.2138                                   | 2  | 0    | <u>11</u> | 10   | 6   |   | <u>2</u> |   |    |           |          |    |   |
|                           | <i>C. gattii</i>     | 19 | 0.5378                                   |  |      | 1         | 4    | 6   |   | <u>8</u> |   |    |           |          |    |   |
| Itraconazole              | <i>C. neoformans</i> | 31 | 0.1999                                   |  |      | <u>20</u> | 3    | 6   |   | <u>2</u> |   |    |           |          |    |   |
|                           | <i>C. gattii</i>     | 19 | 0.2500                                   |  |      | <u>7</u>  | 6    | 5   |   | 1        |   |    |           |          |    |   |

<sup>a</sup>Modes are underlined. Non-wild-type isolates, as established elsewhere (38), are highlighted.

<sup>b</sup>GM, geometric mean.

<sup>c</sup>Statistically significant differences between species ( $P < 0.05$ ) were found.

help elucidate some of the molecular mechanisms that might be implicated in azole resistance. To achieve this, the *ERG11* genes of clinical isolates of both etiological agents of cryptococcosis were sequenced, to correlate *ERG11* amino acid composition and any possible amino acid substitutions with the *in vitro* antifungal susceptibility of the isolates to fluconazole, voriconazole, and itraconazole. Identifying and understanding how substitutions in antifungal targets affect drug affinity will eventually aid design of new drugs that overcome the growing concern of antifungal resistance.

## RESULTS

### *C. gattii* isolates are less susceptible to azoles than *C. neoformans* isolates.

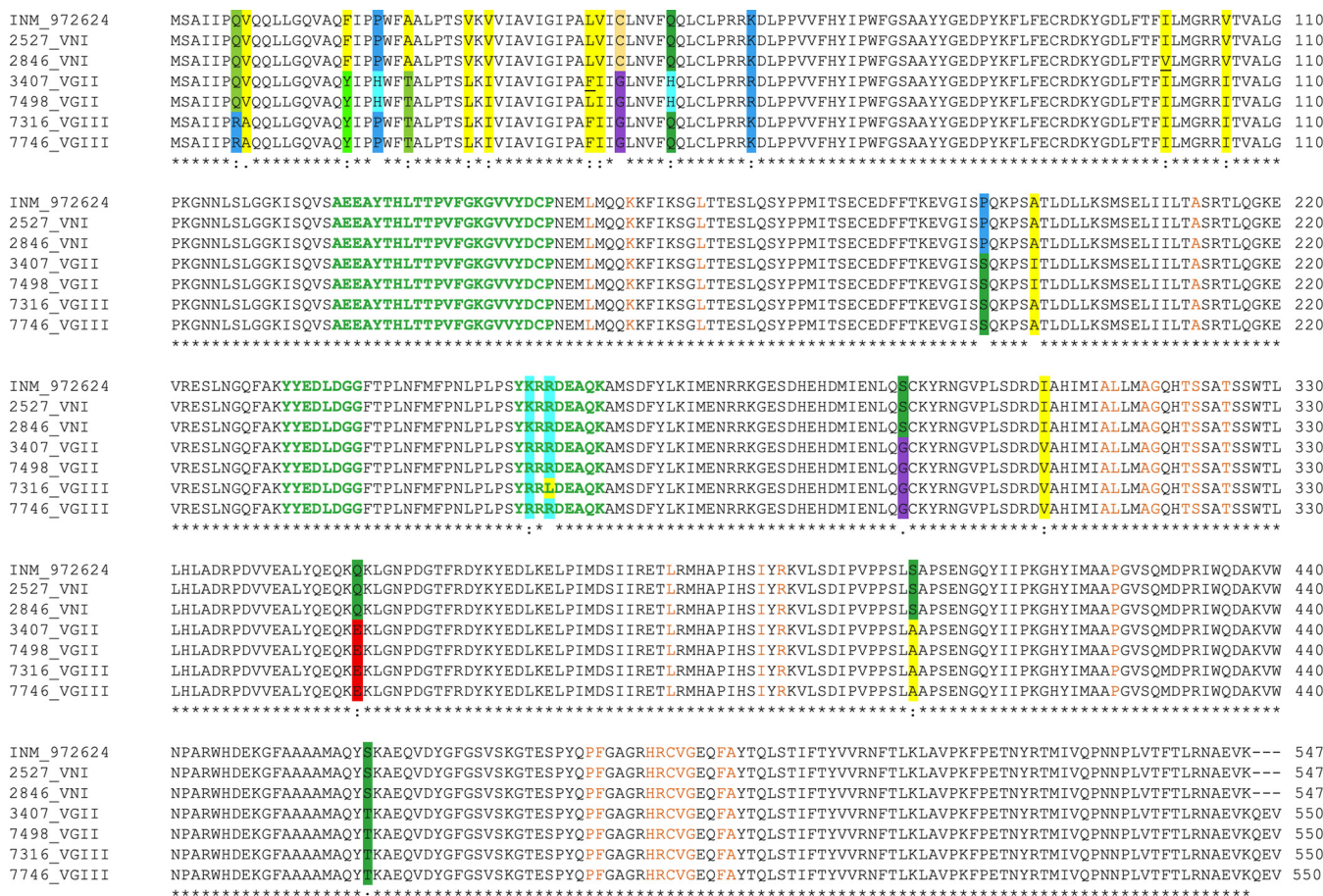
Antifungal susceptibility testing with fluconazole, voriconazole, and itraconazole allowed us to determine that the majority of studied isolates were distributed among the wild-type population of the species, per antifungal drug (Table 1). However, of the 31 *C. neoformans* isolates, 1 was identified as voriconazole non-wild type, 1 as itraconazole non-wild type, and 1 as simultaneously non-wild type to both azoles, since they presented a MIC that is higher than the epidemiological cutoff value that encompasses more than 99% of the wild-type population (ECV >99%) for these azoles (Table 1). Of the 19 *C. gattii* isolates, 8 (42.1%) were identified as voriconazole-non-wild-type isolates. Of these 8 isolates, 1 (5.3%) was, in addition, a fluconazole-non-wild-type isolate (Table 1).

When comparing the geometric mean MICs among species, per antifungal tested, it was found that statistically, *C. gattii* isolates were less susceptible to fluconazole (9.958  $\mu\text{g/mL}$  versus 5.231  $\mu\text{g/mL}$ ;  $P = 0.0025$ ) and voriconazole (0.5378  $\mu\text{g/mL}$  versus 0.2138  $\mu\text{g/mL}$ ;  $P = 0.0001$ ) than *C. neoformans* isolates. Moreover, *C. gattii* had a higher geometric mean MIC for itraconazole than *C. neoformans*, even though this difference was not statistically significant (0.2500  $\mu\text{g/mL}$  versus 0.1999  $\mu\text{g/mL}$ ;  $P = 0.1502$ ).

The correlation between MICs for fluconazole and voriconazole was strong in *C. neoformans* ( $\rho = 0.5040$ ;  $P = 0.0038$ ). In addition, the correlation between MICs for voriconazole and itraconazole was strong in *C. neoformans* ( $\rho = 0.7293$ ) and fair in *C. gattii* ( $\rho = 0.4931$ ) ( $P < 0.0001$  and  $P = 0.0319$ , respectively). However, no other associations between antifungals were found in any species.

**ERG11 amino acid composition and structure differ between *C. neoformans* and *C. gattii*.** Among the 31 *C. neoformans* isolates studied, two different *ERG11* protein sequences of 547 amino acids were identified. In 28 (90.3%) isolates, the amino acid composition was the same as that for the reference strains of *C. neoformans* var. *grubii*, H99 (GenBank accession no. [AEQ63271](#)) and INM 972624 (GenBank accession no. [AAP12370](#)), reported as fluconazole susceptible (26, 33). In the remaining 3 isolates (9.7%), a single nucleotide polymorphism (SNP), A393G, resulting in an amino acid substitution of isoleucine 99 for valine (I99V), was detected. These two amino acids have strongly similar biochemical properties; therefore, the substitution was classified as conservative (37).

Among the 19 *C. gattii* isolates studied, four different *ERG11* protein sequences of 550 amino acids were identified. Compared with the *C. neoformans* reference strains, H99 and INM 972624, all *C. gattii* isolates presented in common 14 amino acid substitutions and three amino acids extra in the C-terminal region (QEV) (Fig. 1). Most of these substitutions



**FIG 1** Complete alignment of the six different sequences of the lanosterol 14- $\alpha$ -demethylase found in 31 *Cryptococcus neoformans* and 19 *Cryptococcus gattii* isolates from Colombia. Two protein sequences were identified in this study in *C. neoformans* and are represented by the VNI isolates H0058-I-2527 and H0058-I-2846. Four protein sequences were identified in *C. gattii* and are represented by the VGII isolates H0058-I-3407 and H0058-I-7498 and the VGIII isolates H0058-I-7316 and H0058-I-7746. Amino acid substitutions, compared to the reference strain *C. neoformans* var. *grubii* INM 972624 (GenBank accession no. AAP12370), are highlighted in different colors. Amino acids of the three substrate recognition sites are written in green and heme binding sites in orange (39). Residues that are identical among all isolates are indicated with asterisks. Colons and periods indicate conservation between groups of strongly (conservative) and weakly (semiconservative) similar properties, respectively.

were classified as conservative (F18Y, A24T, V30L, V32I, V43I, V105I, K256R, I307V, Q349E, S404A, and S460T), one as semiconservative (S293G), and two as nonconservative (C45G, P191S) (37). Of the four proteins identified in *C. gattii*, two were from VGII and two from VGIII isolates. In the 15 VGII isolates, four additional amino acid substitutions were identified (Q50H and K58R [conservative] and P21H and A196I [nonconservative]), and, except for one isolate, a fifth extra conservative substitution, L42F, was detected. In the four VGIII isolates, apart from the common 14 amino acid substitutions and QEV in the C-terminal region already mentioned, three extra amino acid substitutions were identified (Q7R and L42F [conservative] and V8A [semiconservative]). From the four VGIII isolates, an additional nonconservative substitution, R258L, was identified in one isolate (Fig. 1).

The levels of identity between the reference strains and the two proteins identified in *C. neoformans* were 100% and 99.82%, whereas the levels of identity between the reference strains and the four proteins identified in *C. gattii* were 96.53% and 96.71% for the VGII isolates and 96.71% and 96.89% for the VGIII isolates (Fig. 1). Structural modeling of the ERG11 proteins identified in the studied *C. neoformans* and *C. gattii* isolates revealed various differences in their structures (see Fig. S1 in the supplemental material).

***C. gattii* with high MICs for fluconazole and voriconazole harbors the substitution R258L in ERG11.** Antifungal susceptibility testing allowed the identification of a *C. gattii* VGIII isolate, H0058-I-7316, with a MIC for fluconazole of 64  $\mu$ g/mL and a MIC for voriconazole of 1  $\mu$ g/mL. This indicates that the isolate does not distribute among the

|              |                                 |   |     |
|--------------|---------------------------------|---|-----|
| AAK73659     | <i>Aspergillus fumigatus</i>    | GFTPINFMLPWAPLPH <b>NK</b> KRDAAHARMRSIYVDIINQRRLDG           | 256 |
| AAK73660     | <i>Aspergillus fumigatus</i>    | GFAPINFMLPWAPLPH <b>NK</b> KRDA <b>AAQ</b> RKLTETYMEIIKARRQAG | 270 |
| AAA34546     | <i>Saccharomyces cerevisiae</i> | GFTPINFVFPNLPLEH <b>YR</b> KRDHA <b>Q</b> KAISGTYMSLIKERKNN   | 277 |
| AAB02329     | <i>Candida glabrata</i>         | GFTPINFVFPNLPLEH <b>YR</b> KRDHA <b>Q</b> QAISGTYMSLIKERREKN  | 278 |
| AAA53284     | <i>Candida tropicalis</i>       | GFTPINFVFPNLPLEH <b>Y</b> WRRD <b>AAQ</b> RKISAHYMKEIKRRRESG  | 270 |
| AAF00598     | <i>Candida albicans</i>         | GFTPINFVFPNLPLEH <b>Y</b> WRRD <b>AAQ</b> RKISATYMKEIKSRRDRG  | 270 |
| QBC74785     | <i>Candida auris</i>            | GFTPINFVFPNLPLEH <b>Y</b> WRRD <b>AAQ</b> RKISATYMSLINERRKTG  | 270 |
| 1742         | <i>Trichosporon asahii</i>      | GFTPVNFLFPNLPPLS <b>Y</b> KRRD <b>KAQ</b> KEMSDFYMSIIEKRRSGE  | 302 |
| 38441        | <i>Tremella mesenterica</i>     | GFTPLNFMFPNLPPLS <b>Y</b> KRRD <b>KAQ</b> KSMSDFYLNIMQKRREGA  | 267 |
| AAP12370     | <i>Cryptococcus neoformans</i>  | GFTPLNFMFPNLPPLS <b>Y</b> KRRD <b>EAQ</b> KAMSDFYLKIMENRRKGE  | 280 |
| AEQ63273     | <i>Cryptococcus gattii</i>      | GFTPLNFMFPNLPPLS <b>Y</b> KRRD <b>EAQ</b> KAMSDFYLKIMENRRKGE  | 280 |
| H0058-I-7316 | <i>Cryptococcus gattii</i>      | GFTPLNFMFPNLPPLS <b>Y</b> RRL <b>DEA</b> QKAMSDFYLKIMENRRKGE  | 280 |
|              |                                 | **:*:*:*:*:* ** : * * : : * . . : : **                        |     |

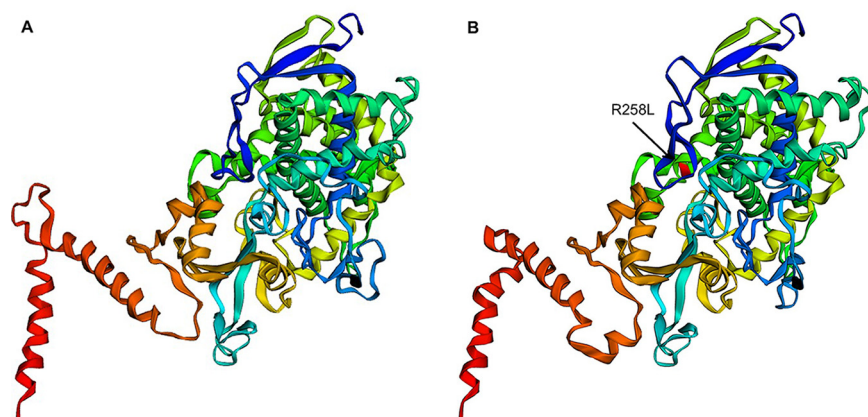
**FIG 2** Alignment of 42 amino acid residues of the cytochrome P450s from reference strains of *Aspergillus fumigatus*, *Saccharomyces cerevisiae*, *Candida glabrata*, *Candida tropicalis*, *Candida albicans*, *Candida auris*, and the Tremellomycetes *Trichosporon asahii*, *Tremella mesenterica*, *Cryptococcus neoformans* H99, and *Cryptococcus gattii* CDCR265, together with the fluconazole-resistant isolate *C. gattii* H0058-I-7316. Amino acids of substrate recognition site 3 (SRS3) are shown in green (39). The substituted residue of *C. gattii* H0058-I-7316 at position 258 is shown in red. Residues that are identical among all the species are indicated with asterisks. Colons and a period indicate conservation between groups of strongly (conservative) and weakly (semiconservative) similar properties, respectively.

wild-type population of the molecular type VGIII for either of the azoles. Notoriously, the MIC for fluconazole was 2 dilutions higher than the ECV >99% for this drug for this molecular type, which is 16 µg/mL (38). Sequencing of the *ERG11* gene of this isolate (GenBank accession no. [OP868692](https://www.ncbi.nlm.nih.gov/nuccore/OP868692)) detected an SNP, G973T, resulting in an amino acid substitution of arginine 258 for leucine (R258L), which is located in substrate recognition site 3 (SRS3), which was recently identified in cryptococcal species and other Tremellomycetes (39). In the cytochrome P450s of other yeasts and filamentous fungi, SRS3, conformed by nine residues, is rather conserved between groups, and this substitution has not been reported (Fig. 2). Apart from R258L, no other additional substitutions previously reported in *C. neoformans*, *C. gattii*, *Candida albicans*, *Candida auris*, and *Aspergillus fumigatus*, and which have been related with decreased susceptibility or resistance to azole drugs, were identified in the studied *C. neoformans* and *C. gattii* isolates (Fig. S2) (40–49).

Structural modeling of the ERG11 proteins from the reference strains of *C. neoformans*, INM 972624, and *C. gattii*, H0058-I-7316, pointed out notable differences in their structures, considering the different amino acid compositions between species (96.71% identity), and revealed the substitution at position 258 (Fig. 3).

**DISCUSSION**

*C. neoformans* and *C. gattii* are pathogens for which drug resistance or other treatment and management challenges exist; hence, these are currently ranked in the list of



**FIG 3** Structural modeling of conformational differences of the lanosterol 14-α-demethylase from *Cryptococcus neoformans* var. *grubii* INM 972624 (A) and *Cryptococcus gattii* H0058-I-7316 (B), indicating the nonconservative R258L amino acid substitution.

fungal priority pathogens to focus attention on their perceived public health importance (50). A large survey carried out with almost 5,000 cryptococcal isolates from around the world revealed an overall prevalence of fluconazole resistance of 12%, with a prevalence lower in incident isolates (10.6%) than in relapse isolates (24.1%) (51). In Colombia specifically, between 14% and 57% of *C. neoformans* and *C. gattii* isolates have been reported to have MICs for fluconazole above the ECVs (ranging from  $\geq 16$  to  $\geq 64$   $\mu\text{g/mL}$ ), including isolates from patients with treatment failure and relapse (34, 52, 53). In particular, resistance to fluconazole in these yeasts is most commonly arising given that patients undergo long-term azole consolidation and maintenance therapies (26, 28, 54). Moreover, in low-resource settings, where there are fewer therapeutic alternatives for the treatment of cryptococcosis, fluconazole is also used as monotherapy in the induction phase (2, 23, 55). The use of fluconazole as primary antifungal prophylaxis in the prevention of cryptococcal meningitis further drives the uncontrolled use of this azole, which could also potentially lead to the development of antifungal resistance (13). Recently, the exposure to azole pesticides used in agriculture was as well reported to be responsible for the appearance of resistance to medical azoles in *C. neoformans*, as has been well documented for other environmental pathogenic fungi, such as *A. fumigatus* (56).

Since there are no clinical breakpoints for *C. neoformans* and *C. gattii*, the establishment of MIC values and ECVs not only aids in determination of differential antifungal profiles among species or molecular types but also serves as an early indication of the emergence of isolates with acquired mechanisms of resistance to a particular antifungal drug (38). In this study, *C. gattii* isolates were less susceptible to fluconazole and voriconazole, with a trend to be as well less susceptible to itraconazole, than *C. neoformans* isolates. These findings coincide with several studies that revealed differential susceptibilities to azole drugs depending on species and molecular types (34–36) and studies showing the generally low susceptibility of *C. gattii* to azole-based antifungals, especially fluconazole (57–60). Interestingly, this might be associated with the differences in the protein compositions found between these sibling species, as *C. gattii* proteins differed from those of *C. neoformans* at 17 or more amino acids, including three extra residues (QEV) in the C-terminal region. Although the major mechanisms implicated in azole resistance are diverse, differential ERG11 compositions between *C. neoformans* and *C. gattii* have been described not only for VGII and VGIII isolates, as in the case of our study, but also for other VGII and VGI isolates (33). Our findings highlight, therefore, the importance of testing antifungal susceptibility of cryptococcal species when treating cryptococcosis, especially in recurrent isolates from patients with treatment failure, and encourage additional studies to establish the association between high MICs of azoles and treatment doses, duration of therapy, and clinical prognosis of patients.

The identification of non-wild-type isolates to azoles, through antifungal susceptibility testing, allowed us as well to further characterize some of the molecular basis of resistance to these drugs, which have been extensively studied in *C. albicans* (40–42, 61–64), *C. auris* (48, 49), and *A. fumigatus* (43–47, 64, 65), but not in *C. neoformans* (23–32), and to a lesser extent in *C. gattii* (33, 60). Particularly, sequencing of the *ERG11* genes of the studied isolates led to the identification of a newly reported mutation (G973T), resulting in the amino acid substitution R258L in a *C. gattii* isolate with high MICs for fluconazole and voriconazole, two structurally similar azoles that consist of short side chains. Although it is uncertain how the mutation R258L directly contributes to the decreased susceptibility of the studied isolate to both triazoles, previous studies on *C. neoformans*, *C. albicans*, and *Saccharomyces cerevisiae* have shown that the substitutions G484S, G464S, and Y140F/H in each species, respectively, are responsible for conformational changes in the 14- $\alpha$ -demethylase, which might decrease its affinity to azole drugs (63, 66–68). Arginine (R), a positively charged, polar amino acid, frequently plays an important role in structure, as it is quite frequent in protein active or binding sites. Consequently, a nonconservative change for leucine (L), an aliphatic, hydrophobic amino acid, could be disfavored, since leucine can play a role in

substrate recognition rather than being directly involved in protein function (37). Moreover, structural modeling of the protein harboring the mutation R258L shows various conformational differences from proteins of azole-susceptible isolates. Until now, none of the residues of substrate recognition site 3 (SRS3), where the mutation R258L is located, had been reported to be associated with resistance to azoles in other yeasts or filamentous fungi, yet there are reports of residue changes playing a role in azole resistance in other SRSs (39, 64). The substitutions Y132F, Y132H, and F126L in *C. albicans* (40, 41, 48) as well as F126T and Y132F in *C. auris* (48), which are all located in SRS1, have been described for isolates that are resistant to azoles and have even been considered potential predictive markers of azole resistance in these ascomycetous yeasts (62). Moreover, the substitution Y135F in *C. neoformans* and its equivalent Y136F in *Histoplasma capsulatum*, both in SRS1, have also been described as conferring resistance to the short-tailed triazoles, fluconazole and voriconazole (28, 69).

Regarding other substitutions reported to be associated with azole resistance, three of the *C. neoformans* isolates studied were found to harbor the conservative missense mutation, I99V, previously identified in *C. neoformans* isolates with MICs for fluconazole ranging between 16 and 24  $\mu\text{g}/\text{mL}$  (31, 32). However, in the Colombian isolates with this mutation, the MICs of fluconazole were rather low (1, 4, and 8  $\mu\text{g}/\text{mL}$ , respectively), which suggests that amino acid polymorphism is not always sufficient to predict azole susceptibility (33, 62). A substitution of amino acids with strongly similar properties, such as I99V, has been suggested to be unlikely to lead to disruption in function of the 14- $\alpha$ -demethylase but instead could result in changes in the levels of *ERG11* expression (32). Another conservative substitution, S460T, has been detected in both fluconazole-resistant and fluconazole-susceptible *C. neoformans* isolates, showing once again that mutations are not always linked to decreased fluconazole susceptibility (28, 70). In *C. auris*, amino acid substitutions that are not associated with antifungal resistance have been suggested to likely represent genetically evolved clade differences (71).

Similarly, the identification of voriconazole- and itraconazole-non-wild-type isolates that do not harbor any substitution that correlates with decreased susceptibility to azoles in cryptococcal species and other yeasts and filamentous fungi suggests that not only variations in *ERG11* coding sequences are responsible for the high azole MICs observed in these fungal pathogens and that there must be different mechanisms that contribute to azole resistance (33, 60, 70). Nevertheless, reduced susceptibility to voriconazole and itraconazole must be surveyed, since these azoles, although they are less effective, could be used as primary or salvage therapies when fluconazole is not available, when resistance appears, in cases of patient intolerance or drug toxicity, or among patients with refractory cryptococcosis (72).

To conclude, this study shows differential susceptibilities to azoles among *C. neoformans* and *C. gattii* isolates, with some presenting high MICs for the assessed drugs, which underscores the necessity of *in vitro* susceptibility testing of clinical isolates against different groups of azoles in order to assist patient management. This study also revealed a *C. gattii* isolate with high MICs for both fluconazole and voriconazole harboring a nonconservative amino acid substitution, R258L, located in SRS3 of the lanosterol 14- $\alpha$ -demethylase, which suggests the association of this substitution with the phenotype of decreased susceptibility to these triazoles, although the possibility of participation of other parallel molecular mechanisms of resistance to azoles cannot be ruled out and needs to be examined. Further experiments, such as reverse genetics and whole-genome sequencing, could help explore other mechanisms associated with the reduced susceptibility or resistance phenotype of the studied isolates.

## MATERIALS AND METHODS

**Isolates.** Thirty-one and 19 clinical isolates of *C. neoformans* and *C. gattii*, respectively, recovered between 2005 and 2019 from cerebrospinal fluid (94%), blood (4%), and skin lesions (2%) from 50 patients were studied. Among the patients, only 17 (34%) had data on antifungal treatment. Of these, 12 (24%) received monotherapy with amphotericin B and 1 (2%) with fluconazole, while 4 (8%) received combined therapy with these two antifungals. Among the isolates, 21 were recovered from Antioquia, 10 from Norte de Santander, 6 from Valle del Cauca, 5 from Bogota, 2 from Meta, and 1 each from

Atlántico, Boyacá, Cauca, Cesar, Cundinamarca, and Santander as part of the National Surveillance Program for *Cryptococcus* and Cryptococcosis led by the Instituto Nacional de Salud (INS), in Bogotá, Colombia. All isolates had data on species and molecular type, determined by glycine assimilation on L-canavanine–glycine–bromothymol blue medium and *URA5* restriction fragment length polymorphism (RFLP), respectively, as previously reported (73, 74). All *C. neoformans* isolates were VNI, while among the *C. gattii* isolates, 15 were VGII and 4 were VGIII. Isolates, maintained in 10% glycerol at  $-80^{\circ}\text{C}$ , were cultured on Sabouraud dextrose agar and incubated for 48 h at  $35^{\circ}\text{C}$  prior to antifungal susceptibility testing and DNA extraction.

**Antifungal susceptibility to azoles.** The MICs of fluconazole, voriconazole, and itraconazole was determined for all studied isolates, using broth microdilution and following the M27M44S guideline of the Clinical and Laboratory Standards Institute (CLSI) (75). Plates were incubated at  $35^{\circ}\text{C}$  and read after 72 h. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were used as quality control strains. The ranges of drug concentrations tested by 2-fold serial dilutions were 0.25 to 128  $\mu\text{g}/\text{mL}$  for fluconazole and 0.03125 to 16  $\mu\text{g}/\text{mL}$  for voriconazole and itraconazole. Mode and geometric mean MICs were calculated per drug and species. MIC values were compared with ECV  $>99\%$ , when available, to determine if the isolates distributed among the wild-type population of each species or molecular type, per drug, as established elsewhere (38).

Etest for fluconazole was performed for one isolate of *C. gattii*, H0058-I-7316, which presented a high MIC for this antifungal, in order to corroborate decreased susceptibility to fluconazole and to visualize its growth in solid medium (Fig. S3). Etest was also done for the quality control strains, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019. Briefly, 90-mm-diameter plates containing solidified RPMI 1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) with 2% glucose, at a depth of 4.0 mm, were inoculated with a cell suspension adjusted spectrophotometrically to the turbidity of a 0.5 McFarland, by using a cotton swab. After the inoculum was absorbed completely into the agar, an Etest strip (bioMérieux SA, Marcy-l'Étoile, France) was placed on each plate. The plates were incubated at  $35^{\circ}\text{C}$  and read at 24 and 48 h. The MIC was established as the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the Etest strip. Any growth, such as microcolonies, throughout a discernible inhibition ellipse was ignored (76).

**Determination of the lanosterol 14- $\alpha$ -demethylase amino acid composition.** Genomic DNA of all isolates was extracted as described previously (77). Amplification of the *ERG11* gene was done as reported before (26), with some modifications. For *C. neoformans* isolates, the primers CnERG11A (5'-TCGTCGAACCATCTTCG-3') and CnERG11B (5'-CGTCTATGACTTCATGACC-3') were used (26). However, for *C. gattii* isolates, the forward primer CnERG11A was used together with a new reverse primer, CgERG11R (5'-CGTCTATTAATTTCTGACT-3'), which was designed in this study. All primers were synthesized by Macrogen Inc., Seoul, South Korea. PCRs were carried out in 50- $\mu\text{L}$  reaction volumes containing  $1 \times$  Taq buffer, 3 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  deoxynucleoside triphosphates (dNTPs), 2.5 U of Taq polymerase (Invitrogen, Life Technologies, Carlsbad, CA, USA), a 0.5  $\mu\text{M}$  concentration of each primer, and 10 ng of genomic DNA. Thermocycling conditions consisted of 1 cycle of initial denaturation for 5 min at  $94^{\circ}\text{C}$ , followed by 30 cycles of 30 s at  $94^{\circ}\text{C}$ , 45 s at  $58^{\circ}\text{C}$ , and 2 min at  $72^{\circ}\text{C}$ , and 1 final cycle of 10 min at  $72^{\circ}\text{C}$ . After amplification, PCR products of  $\sim 2,200$  bp were commercially purified and sequenced, both forward and reverse strands, by Macrogen Inc., Seoul, South Korea.

Sequences were edited and contigs were assembled using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). DNA alignments, annotation, and translation to amino acids were done using the program MEGA 11 (78). The reference strains of *C. neoformans* var. *grubii*, H99 (GenBank accession no. [AEQ63271](#)) and INM 972624 (GenBank accession no. [AAP12370](#)), reported as fluconazole susceptible (26, 33), were used to annotate the obtained sequences and to identify single nucleotide polymorphisms (SNPs) and amino acid substitutions. An additional alignment with the cytochrome P450 sequences from *Aspergillus fumigatus* (GenBank accession no. [AAK73659](#) and [AAK73660](#)), *Saccharomyces cerevisiae* (GenBank accession no. [AAA34546](#)), *Candida glabrata* (GenBank accession no. [AAB02329](#)), *Candida tropicalis* (GenBank accession no. [AAA53284](#)), *Candida albicans* (GenBank accession no. [AAF00598](#)), *Candida auris* (GenBank accession no. [QBC74785](#)), *Trichosporon asahii* 1742, *Tremella mesenterica* 38441 (33), and *C. gattii* molecular type VGII, strain CDCR265 (GenBank accession no. [AEQ63272](#)), was also carried out to identify amino acid substitutions related with azole resistance in other yeasts and filamentous fungi.

Multiple-sequence alignments were carried out using Clustal Omega, and the percent identity between proteins was calculated by Clustal 2.1 (79).

**Structural modeling of the lanosterol 14- $\alpha$ -demethylase.** With the two types of proteins identified in *C. neoformans* and the four in *C. gattii*, structural modeling was carried out using one-to-one threading to model the sequences against an in-house structure in the Phyre2 web portal for protein modeling, prediction, and analysis (80). For this, chain A of the experimental structure (PDB code [4LXJ](#)) from *Saccharomyces cerevisiae* lanosterol 14- $\alpha$ -demethylase with lanosterol bound (81) was used. The files generated in Phyre2 were visualized using EzMol, a web server for the rapid visualization of protein structure (82).

**Statistical analysis.** MIC differences between species were compared, per drug, by using the Mann-Whitney test. Association between MICs of fluconazole and voriconazole, fluconazole and itraconazole, and voriconazole and itraconazole were assessed, per species, using the Pearson correlation coefficient ( $\rho$ ). Correlation was judged very strong at values from 1 to 0.8, strong from 0.8 to 0.5, fair from 0.5 to 0.2, and poor from 0.2 to 0. Alpha risk was set to 5% ( $\alpha = 0.05$ ). Statistical analysis was performed with GraphPad (La Jolla, CA, USA) Prism v 9.4.1.

**Data availability.** Nucleotide sequences of all studied isolates were deposited in GenBank under the following accession numbers: [OP823165](#) to [OP823195](#) for *C. neoformans* and [OP868674](#) to [OP868692](#) for *C. gattii*.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, DOCX file, 5.3 MB.

## ACKNOWLEDGMENTS

We thank the National Reference Laboratory of the National Institutes of Health for allowing the use of the strains. We also thank the public health laboratories in Colombia that take part in the National Surveillance Program of *Cryptococcus* and Cryptococcosis as well as the clinicians and epidemiologists of the participating hospitals.

Carolina Firacative was supported by small grant no. IV-FPC014 from the Dirección de Investigación e Innovación, Universidad del Rosario.

We report no conflicts of interest.

## REFERENCES

- Maziarsz EK, Perfect JR. 2016. Cryptococcosis. *Infect Dis Clin North Am* 30: 179–206. <https://doi.org/10.1016/j.idc.2015.10.006>.
- Kwon-Chung KJ, Fraser JA, Doering TL, Wang Z, Janbon G, Idnum A, Bahn YS. 2014. Cryptococcus neoformans and Cryptococcus gattii, the etiologic agents of cryptococcosis. *Cold Spring Harb Perspect Med* 4:a019760. <https://doi.org/10.1101/cshperspect.a019760>.
- Mitchell TG, Perfect JR. 1995. Cryptococcosis in the era of AIDS—100 years after the discovery of Cryptococcus neoformans. *Clin Microbiol Rev* 8: 515–548. <https://doi.org/10.1128/CMR.8.4.515>.
- Pappas PG. 2013. Cryptococcal infections in non-HIV-infected patients. *Trans Am Clin Climatol Assoc* 124:61–79.
- Firacative C, Carvajal SK, Escandon P, Lizarazo J. 2020. Cryptococcosis in hematopoietic stem cell transplant recipients: a rare presentation warranting recognition. *Can J Infect Dis Med Microbiol* 2020:3713241. <https://doi.org/10.1155/2020/3713241>.
- Chen SC, Meyer W, Sorrell TC. 2014. Cryptococcus gattii infections. *Clin Microbiol Rev* 27:980–1024. <https://doi.org/10.1128/CMR.00126-13>.
- Rosen LB, Freeman AF, Yang LM, Jutivorakool K, Olivier KN, Angkasekwinai N, Suputtamongkol Y, Bennett JE, Pyrgos V, Williamson PR, Ding L, Holland SM, Browne SK. 2013. Anti-GM-CSF autoantibodies in patients with cryptococcal meningitis. *J Immunol* 190:3959–3966. <https://doi.org/10.4049/jimmunol.1202526>.
- Saijo T, Chen J, Chen SC, Rosen LB, Yi J, Sorrell TC, Bennett JE, Holland SM, Browne SK, Kwon-Chung KJ. 2014. Anti-granulocyte-macrophage colony-stimulating factor autoantibodies are a risk factor for central nervous system infection by Cryptococcus gattii in otherwise immunocompetent patients. *mBio* 5:e00912-14. <https://doi.org/10.1128/mBio.00912-14>.
- Rajasingham R, Govender NP, Jordan A, Loyse A, Shroufi A, Denning DW, Meya DB, Chiller TM, Boulware DR. 2022. The global burden of HIV-associated cryptococcal infection in adults in 2020: a modelling analysis. *Lancet Infect Dis* 22:1748–1755. [https://doi.org/10.1016/S1473-3099\(22\)00499-6](https://doi.org/10.1016/S1473-3099(22)00499-6).
- Escandón P, Lizarazo J, Agudelo CI, Castañeda E. 2018. Cryptococcosis in Colombia: compilation and analysis of data from laboratory-based surveillance. *J Fungi (Basel)* 4:32. <https://doi.org/10.3390/jof4010032>.
- Lizarazo J, Escandon P, Agudelo CI, Firacative C, Meyer W, Castaneda E. 2014. Retrospective study of the epidemiology and clinical manifestations of Cryptococcus gattii infections in Colombia from 1997–2011. *PLoS Negl Trop Dis* 8:e3272. <https://doi.org/10.1371/journal.pntd.0003272>.
- Mourad A, Perfect JR. 2018. The war on cryptococcosis: a review of the antifungal arsenal. *Mem Inst Oswaldo Cruz* 113:e170391. <https://doi.org/10.1590/0074-02760170391>.
- WHO. 2022. Guidelines for diagnosing, preventing and managing cryptococcal disease among adults, adolescents and children living with HIV. WHO, Geneva, Switzerland.
- Loyse A, Dromer F, Day J, Lortholary O, Harrison TS. 2013. Flucytosine and cryptococcosis: time to urgently address the worldwide accessibility of a 50-year-old antifungal. *J Antimicrob Chemother* 68:2435–2444. <https://doi.org/10.1093/jac/dkt221>.
- Perfect JR, Bicanic T. 2015. Cryptococcosis diagnosis and treatment: what do we know now. *Fungal Genet Biol* 78:49–54. <https://doi.org/10.1016/j.fgb.2014.10.003>.
- van der Horst CM, Saag MS, Cloud GA, Hamill RJ, Graybill JR, Sobel JD, Johnson PC, Tuazon CU, Kerkering T, Moskovitz BL, Powderly WG, Dismukes WE. 1997. Treatment of cryptococcal meningitis associated with the acquired immunodeficiency syndrome. *N Engl J Med* 337:15–21. <https://doi.org/10.1056/NEJM199707033370103>.
- Schiave LA, Nascimento E, Vilar FC, Haes TM, Takayanagui OM, Gaitani CM, Martinez R. 2018. Fluconazole levels in serum and cerebrospinal fluid according to daily dosage in patients with cryptococcosis and other fungal infections. *Braz J Infect Dis* 22:11–15. <https://doi.org/10.1016/j.bjid.2017.11.003>.
- Sionov E, Chang YC, Garraffo HM, Kwon-Chung KJ. 2009. Heteroresistance to fluconazole in Cryptococcus neoformans is intrinsic and associated with virulence. *Antimicrob Agents Chemother* 53:2804–2815. <https://doi.org/10.1128/AAC.00295-09>.
- Mpoza E, Rhein J, Abassi M. 2018. Emerging fluconazole resistance: implications for the management of cryptococcal meningitis. *Med Mycol Case Rep* 19:30–32. <https://doi.org/10.1016/j.mmcr.2017.11.004>.
- Moreira I, Cortez ACA, de Souza ÉS, Pinheiro SB, de Souza Oliveira JG, Sadahiro A, Cruz KS, Matsuura ABJ, Melhem M, Frickmann H, de Souza JVB. 2022. Investigation of fluconazole heteroresistance in clinical and environmental isolates of Cryptococcus neoformans complex and Cryptococcus gattii complex in the state of Amazonas, Brazil. *Med Mycol* 60:myac005. <https://doi.org/10.1093/mmy/myac005>.
- Firacative C. 2023. Antifungal resistance: a growing concern. *Acta Biol Colomb*, in Press.
- Scorzoni L, de Paula ESAC, Marcos CM, Assato PA, de Melo WC, de Oliveira HC, Costa-Orlandi CB, Mendes-Giannini MJ, Fusco-Almeida AM. 2017. Antifungal therapy: new advances in the understanding and treatment of mycosis. *Front Microbiol* 8:36. <https://doi.org/10.3389/fmicb.2017.00036>.
- Iyer KR, Revie NM, Fu C, Robbins N, Cowen LE. 2021. Treatment strategies for cryptococcal infection: challenges, advances and future outlook. *Nat Rev Microbiol* 19:454–466. <https://doi.org/10.1038/s41579-021-00511-0>.
- Cheong JW, McCormack J. 2013. Fluconazole resistance in cryptococcal disease: emerging or intrinsic? *Med Mycol* 51:261–269. <https://doi.org/10.3109/13693786.2012.715763>.
- Bermas A, Geddes-McAlister J. 2020. Combatting the evolution of antifungal resistance in Cryptococcus neoformans. *Mol Microbiol* 114:721–734. <https://doi.org/10.1111/mmi.14565>.
- Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P, Cuenca-Estrella M, Davel G, Rodriguez-Tudela JL. 2003. 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* 47:3653–3656. <https://doi.org/10.1128/AAC.47.11.3653-3656.2003>.
- Bosco-Borgeat ME, Mazza M, Taverna CG, Cordoba S, Murisengo OA, Vivot W, Davel G. 2016. Amino acid substitution in Cryptococcus neoformans lanosterol 14-alpha-demethylase involved in fluconazole resistance in clinical isolates. *Rev Argent Microbiol* 48:137–142. <https://doi.org/10.1016/j.ram.2016.03.003>.
- Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung KJ. 2012. Identification of a Cryptococcus neoformans cytochrome P450 lanosterol 14alpha-demethylase (Erg11) residue critical for differential susceptibility between fluconazole/voriconazole and itraconazole/posaconazole. *Antimicrob Agents Chemother* 56:1162–1169. <https://doi.org/10.1128/AAC.05502-11>.
- Kano R, Okubo M, Hasegawa A, Kamata H. 2017. Multi-azole-resistant strains of Cryptococcus neoformans var. grubii isolated from a FLZ-

- resistant strain by culturing in medium containing voriconazole. *Med Mycol* 55:877–882. <https://doi.org/10.1093/mmy/myw101>.
30. Gago S, Serrano C, Alastruey-Izquierdo A, Cuesta I, Martin-Mazuelos E, Aller AI, Gomez-Lopez A, Mellado E. 2017. Molecular identification, antifungal resistance and virulence of *Cryptococcus neoformans* and *Cryptococcus deneoformans* isolated in Seville, Spain. *Mycoses* 60:40–50. <https://doi.org/10.1111/myc.12543>.
  31. Selb R, Fuchs V, Graf B, Hamprecht A, Hogardt M, Sedlacek L, Schwarz R, Idevlevich EA, Becker SL, Held J, Kupper-Tetzel CP, McCormick-Smith I, Heckmann D, Gerkrath J, Han CO, Wilmes D, Rickerts V. 2019. Molecular typing and in vitro resistance of *Cryptococcus neoformans* clinical isolates obtained in Germany between 2011 and 2017. *Int J Med Microbiol* 309:151336. <https://doi.org/10.1016/j.ijmm.2019.151336>.
  32. Albehaijani SHI, Macreadie I, Morrissey CO, Boyce KJ. 2022. Molecular mechanisms underlying the emergence of polygenetic antifungal drug resistance in *msh2* mismatch repair mutants of *Cryptococcus*. *JAC Antimicrob Resist* 4:dla033. <https://doi.org/10.1093/jacamr/dlac033>.
  33. Gast CE, Basso LR, Jr, Bruzual I, Wong B. 2013. Azole resistance in *Cryptococcus gattii* from the Pacific Northwest: investigation of the role of ERG11. *Antimicrob Agents Chemother* 57:5478–5485. <https://doi.org/10.1128/AAC.02287-12>.
  34. Firacative C, Escandon P. 2021. Antifungal susceptibility of clinical *Cryptococcus gattii* isolates from Colombia varies among molecular types. *Med Mycol* 59:1122–1125. <https://doi.org/10.1093/mmy/myab041>.
  35. Chong HS, Dagg R, Malik R, Chen S, Carter D. 2010. In vitro susceptibility of the yeast pathogen *Cryptococcus* to fluconazole and other azoles varies with molecular genotype. *J Clin Microbiol* 48:4115–4120. <https://doi.org/10.1128/JCM.01271-10>.
  36. Trilles L, Meyer W, Wanke B, Guarro J, Lazera M. 2012. Correlation of antifungal susceptibility and molecular type within the *Cryptococcus neoformans/C. gattii* species complex. *Med Mycol* 50:328–332. <https://doi.org/10.3109/13693786.2011.602126>.
  37. Betts MJ, Russell RB. 2003. Amino acid properties and consequences of substitutions, p 289–316. In Barnes MR, Gray IC (ed), *Bioinformatics for geneticists*. John Wiley & Sons, Ltd, Chichester, England. <https://doi.org/10.1002/0470867302>.
  38. Espinel-Ingroff A, Aller AI, Canton E, Castanon-Olivares LR, Chowdhary A, Cordoba S, Cuenca-Estrella M, Fothergill A, Fuller J, Govender N, Hagen F, Illnait-Zaragoza MT, Johnson E, Kidd S, Lass-Flörl C, Lockhart SR, Martins MA, Meis JF, Melhem MS, Ostrosky-Zeichner L, Pelaez T, Pfaller MA, Schell WA, St-Germain G, Trilles L, Turnidge J. 2012. *Cryptococcus neoformans-Cryptococcus gattii* species complex: an international study of wild-type susceptibility endpoint distributions and epidemiological cutoff values for fluconazole, itraconazole, posaconazole, and voriconazole. *Antimicrob Agents Chemother* 56:5898–5906. <https://doi.org/10.1128/AAC.01115-12>.
  39. Akapo OO, Macnar JM, Krys JD, Syed PR, Syed K, Gront D. 2021. In silico structural modeling and analysis of interactions of Tremellomycetes cytochrome P450 monooxygenases CYP51s with substrates and azoles. *Int J Mol Sci* 22:7811. <https://doi.org/10.3390/ijms22157811>.
  40. Feng W, Yang J, Xi Z, Qiao Z, Lv Y, Wang Y, Ma Y, Wang Y, Cen W. 2017. Mutations and/or overexpressions of ERG4 and ERG11 genes in clinical azole-resistant isolates of *Candida albicans*. *Microb Drug Resist* 23:563–570. <https://doi.org/10.1089/mdr.2016.0095>.
  41. Mane A, Vidhate P, Kusro C, Waman V, Saxena V, Kulkarni-Kale U, Risbud A. 2016. Molecular mechanisms associated with fluconazole resistance in clinical *Candida albicans* isolates from India. *Mycoses* 59:93–100. <https://doi.org/10.1111/myc.12439>.
  42. Warrillow AG, Mullins JG, Hull CM, Parker JE, Lamb DC, Kelly DE, Kelly SL. 2012. S279 point mutations in *Candida albicans* sterol 14- $\alpha$  demethylase (CYP51) reduce in vitro inhibition by fluconazole. *Antimicrob Agents Chemother* 56:2099–2107. <https://doi.org/10.1128/AAC.05389-11>.
  43. Bader O, Weig M, Reichard U, Lugert R, Kuhns M, Christner M, Held J, Peter S, Schumacher U, Buchheidt D, Tintelnot K, Gross U, MykoLabNet DP, MykoLabNet-D Partners. 2013. *cyp51A*-based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. *Antimicrob Agents Chemother* 57:3513–3517. <https://doi.org/10.1128/AAC.00167-13>.
  44. Ballard E, Melchers WJG, Zoll J, Brown AJ, Verweij PE, Warris A. 2018. In-host microevolution of *Aspergillus fumigatus*: a phenotypic and genotypic analysis. *Fungal Genet Biol* 113:1–13. <https://doi.org/10.1016/j.fgb.2018.02.003>.
  45. Chen P, Liu M, Zeng Q, Zhang Z, Liu W, Sang H, Lu L. 2019. Uncovering new mutations conferring azole resistance in the *Aspergillus fumigatus cyp51A* gene. *Front Microbiol* 10:3127. <https://doi.org/10.3389/fmicb.2019.03127>.
  46. Leonardelli F, Theill L, Nardin ME, Macedo D, Dudiuk C, Mendez E, Gamarra S, Garcia-Effron G. 2017. First itraconazole resistant *Aspergillus fumigatus* clinical isolate harbouring a G54E substitution in *Cyp51A* in South America. *Rev Iberoam Micol* 34:46–48. <https://doi.org/10.1016/j.riam.2016.05.005>.
  47. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. 2004. Substitutions at methionine 220 in the 14 $\alpha$ -sterol demethylase (*Cyp51A*) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. *Antimicrob Agents Chemother* 48:2747–2750. <https://doi.org/10.1128/AAC.48.7.2747-2750.2004>.
  48. Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP, Colombo AL, Calvo B, Cuomo CA, Desjardins CA, Berkow EL, Castanheira M, Magobo RE, Jabeen K, Asghar RJ, Meis JF, Jackson B, Chiller T, Litvintseva AP. 2017. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. *Clin Infect Dis* 64:134–140. <https://doi.org/10.1093/cid/ciw691>.
  49. Li J, Coste AT, Liechti M, Bachmann D, Sanglard D, Lamoth F. 2023. Novel ERG11 and TAC1b mutations associated with azole resistance in *Candida auris*. *Antimicrob Agents Chemother* 65:e02663-20. <https://doi.org/10.1128/AAC.02663-20>.
  50. WHO. 2022. WHO fungal priority pathogens list to guide research, development and public health action. World Health Organization, Geneva, Switzerland.
  51. Bongomin F, Oladele RO, Gago S, Moore CB, Richardson MD. 2018. A systematic review of fluconazole resistance in clinical isolates of *Cryptococcus* species. *Mycoses* 61:290–297. <https://doi.org/10.1111/myc.12747>.
  52. Agudelo CA, Munoz C, Ramirez A, Tobon AM, de Bedout Bact C, Cano LE, Restrepo A. 2015. Response to therapy in patients with cryptococcosis and AIDS: association with in vitro susceptibility to fluconazole. *Rev Iberoam Micol* 32:214–220. <https://doi.org/10.1016/j.riam.2014.07.006>.
  53. Torres I, Gallo JE, Gomez OM, Rua-Giraldo A, McEwen JG, Garcia AM. 2022. Gene expression profiles of ERG11, MDR1 and AFR1 in *Cryptococcus neoformans* var. *grubbi* from HIV patients. *Biomedica* 42:697–706. <https://doi.org/10.7705/biomedica.6519>.
  54. Paugam A, Dupouy-Camet J, Blanche P, Gangneux JP, Tourte-Schaefer C, Sicard D. 1994. Increased fluconazole resistance of *Cryptococcus neoformans* isolated from a patient with AIDS and recurrent meningitis. *Clin Infect Dis* 19:975–976. <https://doi.org/10.1093/clinids/19.5.975-a>.
  55. Brandt ME, Pfaller MA, Hajjeh RA, Hamill RJ, Pappas PG, Reingold AL, Rimland D, Warnock DW, Cryptococcal Disease Active Surveillance Group. 2001. Trends in antifungal drug susceptibility of *Cryptococcus neoformans* isolates in the United States: 1992 to 1994 and 1996 to 1998. *Antimicrob Agents Chemother* 45:3065–3069. <https://doi.org/10.1128/AAC.45.11.3065-3069.2001>.
  56. Drakulovski P, Krasteva D, Bellet V, Randazzo S, Roger F, Pottier C, Bertout S. 2023. Exposure of *Cryptococcus neoformans* to seven commonly used agricultural azole fungicides induces resistance to fluconazole as well as cross-resistance to voriconazole, posaconazole, itraconazole and isavuconazole. *Pathogens* 12:662. <https://doi.org/10.3390/pathogens12050662>.
  57. Gomez-Lopez A, Zaragoza O, Dos Anjos Martins M, Melhem MC, Rodriguez-Tudela JL, Cuenca-Estrella M. 2008. In vitro susceptibility of *Cryptococcus gattii* clinical isolates. *Clin Microbiol Infect* 14:727–730. <https://doi.org/10.1111/j.1469-0691.2008.02021.x>.
  58. Grizante BP, Tonani L, Cocio TA, Martinez R, Nascimento E, von Zeska Kress MR. 2020. Molecular typing, in vitro susceptibility and virulence of *Cryptococcus neoformans/Cryptococcus gattii* species complex clinical isolates from south-eastern Brazil. *Mycoses* 63:1341–1351. <https://doi.org/10.1111/myc.13174>.
  59. Trilles L, Fernández-Torres B, Lazera MdS, Wanke B, Guarro J. 2004. In vitro antifungal susceptibility of *Cryptococcus gattii*. *J Clin Microbiol* 42:4815–4817. <https://doi.org/10.1128/JCM.42.10.4815-4817.2004>.
  60. Bertout S, Roger F, Drakulovski P, Martin AS, Gouveia T, Kassi F, Menan H, Krasteva D, Delaporte E, Bellet V. 2020. African ST173 *Cryptococcus deuterogattii* strains are commonly less susceptible to fluconazole: an unclear mechanism of resistance. *J Glob Antimicrob Resist* 21:262–269. <https://doi.org/10.1016/j.jgar.2019.10.017>.
  61. Xiang M-J, Liu J-Y, Ni P-H, Wang S, Shi C, Wei B, Ni Y-X, Ge H-L. 2013. Erg11 mutations associated with azole resistance in clinical isolates of *Candida albicans*. *FEMS Yeast Res* 13:386–393. <https://doi.org/10.1111/1567-1364.12042>.
  62. Morio F, Loge C, Besse B, Hennequin C, Le Pape P. 2010. Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. *Diagn Microbiol Infect Dis* 66:373–384. <https://doi.org/10.1016/j.diagmicrobio.2009.11.006>.

63. Sanglard D, Ischer F, Koymans L, Bille J. 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14 $\alpha$ -demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob Agents Chemother* 42:241–253. <https://doi.org/10.1128/AAC.42.2.241>.
64. Zhang J, Li L, Lv Q, Yan L, Wang Y, Jiang Y. 2019. The fungal CYP51s: their functions, structures, related drug resistance, and inhibitors. *Front Microbiol* 10:691. <https://doi.org/10.3389/fmicb.2019.00691>.
65. Hargrove TY, Wawrzak Z, Lamb DC, Guengerich FP, Lepesheva GI. 2015. Structure-functional characterization of cytochrome P450 sterol 14 $\alpha$ -demethylase (CYP51B) from *Aspergillus fumigatus* and molecular basis for the development of antifungal drugs. *J Biol Chem* 290:23916–23934. <https://doi.org/10.1074/jbc.M115.677310>.
66. Kelly SL, Lamb DC, Loeffler J, Einsele H, Kelly DE. 1999. The G464S amino acid substitution in *Candida albicans* sterol 14 $\alpha$ -demethylase causes fluconazole resistance in the clinic through reduced affinity. *Biochem Biophys Res Commun* 262:174–179. <https://doi.org/10.1006/bbrc.1999.1136>.
67. Sheng C, Miao Z, Ji H, Yao J, Wang W, Che X, Dong G, Lu J, Guo W, Zhang W. 2009. Three-dimensional model of lanosterol 14  $\alpha$ -demethylase from *Cryptococcus neoformans*: active-site characterization and insights into azole binding. *Antimicrob Agents Chemother* 53:3487–3495. <https://doi.org/10.1128/AAC.01630-08>.
68. Sagatova AA, Keniya MV, Wilson RK, Sabherwal M, Tyndall JDA, Monk BC. 2016. Triazole resistance mediated by mutations of a conserved active site tyrosine in fungal lanosterol 14 $\alpha$ -demethylase. *Sci Rep* 6:26213. <https://doi.org/10.1038/srep26213>.
69. Wheat LJ, Connolly P, Smedema M, Durkin M, Brizendine E, Mann P, Patel R, McNicholas PM, Goldman M. 2006. Activity of newer triazoles against *Histoplasma capsulatum* from patients with AIDS who failed fluconazole. *J Antimicrob Chemother* 57:1235–1239. <https://doi.org/10.1093/jac/dkl133>.
70. Atim PB, Meya DB, Gerlach ES, Muhanguzi D, Male A, Kanamwanji B, Nielsen K. 2022. Lack of association between fluconazole susceptibility and ERG11 nucleotide polymorphisms in *Cryptococcus neoformans* clinical isolates from Uganda. *J Fungi (Basel)* 8:508. <https://doi.org/10.3390/jof8050508>.
71. Healey KR, Kordalewska M, Jimenez OC, Singh A, Berrio I, Chowdhary A, Perlin DS. 2018. Limited ERG11 mutations identified in isolates of *Candida auris* directly contribute to reduced azole susceptibility. *Antimicrob Agents Chemother* 62:e01427-18. <https://doi.org/10.1128/AAC.01427-18>.
72. Perfect JR, Dismukes WE, Dromer F, Goldman DL, Graybill JR, Hamill RJ, Harrison TS, Larsen RA, Lortholary O, Nguyen MH, Pappas PG, Powderly WG, Singh N, Sobel JD, Sorrell TC. 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. *Clin Infect Dis* 50:291–322. <https://doi.org/10.1086/649858>.
73. Meyer W, Castañeda A, Jackson S, Huynh M, Castañeda E, Group ICS, Ibero-American Cryptococcal Study Group. 2003. Molecular typing of Ibero-American *Cryptococcus neoformans* isolates. *Emerg Infect Dis* 9:189–195. <https://doi.org/10.3201/eid0902.020246>.
74. Castañeda E, Lizarazo J, Firacative C. 2018. *Criptococcosis*, p 157–173. In González A, Gómez BL, Tobón A, Restrepo A (ed), *Fundamentos de las micosis humanas*, 1st ed. CIB—Universidad de Antioquia, Medellín, Colombia.
75. CLSI. 2022. Performance standards for antifungal susceptibility testing of yeasts, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
76. Espinel-Ingroff A, Pfaller M, Erwin ME, Jones RN. 1996. Interlaboratory evaluation of Etest method for testing antifungal susceptibilities of pathogenic yeasts to five antifungal agents by using Casitone agar and solidified RPMI 1640 medium with 2% glucose. *J Clin Microbiol* 34:848–852. <https://doi.org/10.1128/jcm.34.4.848-852.1996>.
77. Ferrer C, Colom F, Frases S, Mulet E, Abad JL, Alio JL. 2001. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J Clin Microbiol* 39:2873–2879. <https://doi.org/10.1128/JCM.39.8.2873-2879.2001>.
78. Tamura K, Stecher G, Kumar S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Mol Biol Evol* 38:3022–3027. <https://doi.org/10.1093/molbev/msab120>.
79. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7:539. <https://doi.org/10.1038/msb.2011.75>.
80. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 10:845–858. <https://doi.org/10.1038/nprot.2015.053>.
81. Monk BC, Tomasiak TM, Keniya MV, Huschmann FU, Tyndall JD, O'Connell JD, Cannon RD, McDonald JG, Rodriguez A, Finer-Moore JS, Stroud RM. 2014. Architecture of a single membrane spanning cytochrome P450 suggests constraints that orient the catalytic domain relative to a bilayer. *Proc Natl Acad Sci U S A* 111:3865–3870. <https://doi.org/10.1073/pnas.1324245111>.
82. Reynolds CR, Islam SA, Sternberg MJE. 2018. EzMol: a web server wizard for the rapid visualization and image production of protein and nucleic acid structures. *J Mol Biol* 430:2244–2248. <https://doi.org/10.1016/j.jmb.2018.01.013>.