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SUSCEPTIBILITY

# Antifungal Susceptibility Profiles and Drug Resistance Mechanisms of Clinical *Lomentospora prolificans* Isolates

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**ABSTRACT** *Lomentospora prolificans* is an opportunistic fungal pathogen with low susceptibility to current antifungal drugs. Here, we tested the *in vitro* susceptibility of 8 drugs against 42 clinical *L. prolificans* isolates. All isolates showed high MICs to voriconazole ($MIC_{90}$>16 $\mu$g/ml), itraconazole ($MIC_{90}$>16 $\mu$g/ml), posaconazole ($MIC_{90}$>16 $\mu$g/ml), isavuconazole ($MIC_{90}$>16 $\mu$g/ml), amphotericin B ($MIC_{90}$>16 $\mu$g/ml), and terbinafine ($MIC_{90}$>64 $\mu$g/ml) and high minimum effective concentrations (MECs) to micafungin ($MEC_{90}$>8 $\mu$g/ml), with the exception of miltefosine showing an $MIC_{90}$ value of 4 $\mu$g/ml. We examined six different *in vitro* drug combinations and found that the combination of voriconazole and terbinafine achieved the most synergistic effort against *L. prolificans*. We then annotated the *L. prolificans* whole genome and located its *Cyp51* and *Fks1* genes. We completely sequenced the two genes to determine if any mutation would be related to azole and echinocandin resistance in *L. prolificans*. We found no amino acid changes in Cyp51 protein and no tandem repeats in the 5′ upstream region of the *Cyp51* gene. However, we identified three intrinsic amino acid residues (G138S, M220I, and T289A) in the Cyp51 protein that were linked to azole resistance. Likewise, two intrinsic amino acid residues (F639Y, W695F) that have reported to confer echinocandin resistance were found in Fks1 hot spot regions. In addition, three new amino acid alterations (D440A, S634R, and H1245R) were found outside Fks1 hot spot regions, and their contributions to echinocandin resistance need future investigation. Overall, our findings support the notion that *L. prolificans* is intrinsically resistant to azoles and echinocandins.

**KEYWORDS** *Lomentospora prolificans*, antifungal susceptibility, synergy, resistance mechanisms, antifungal agents, antifungal resistance, antifungal susceptibility testing

*Lomentospora prolificans*, formerly known as *Scedosporium prolificans*, is an opportunistic fungal pathogen that causes superficial to invasive fungal infections in immunocompromised and occasionally in immunocompetent people (1–3). *L. prolificans* has been reported to be resistant to a wide range of antifungal drugs, and its infection is often accompanied by high mortality rates (3, 4). Therefore, new effective antifungal strategies are urgently needed.

Antifungal drug combination therapy may be a useful approach. Several studies displayed that combinations of terbinafine with azoles or micafungin with amphotericin B had achieved a synergistic effect *in vitro* in some *L. prolificans* isolates (5–7) as well as demonstrated a therapeutic response in several clinical cases (8–10). Miltefosine, a kind of alkyl-phospholipid analogue, could be another option for treating *L. prolificans* infection. Although the drug was originally developed as an antileishmanial agent, it was found to possess antifungal activity (11–13). *In vitro* studies also demonstrated that

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combinations of miltefosine with antifungal drugs had a synergistic effect against some clinical mold isolates (13–15).

The *antifungal* drug-resistant mechanism in *L. prolificans* is poorly understood, and more studies are needed to understand its resistance mechanisms to azoles and echinocandins (16). Amino acid substitutions of the Cyp51 protein and tandem repeats in the promoter region of the *Cyp51* gene are the common azole resistance mechanisms in filamentous fungal pathogens (17). A partial protein sequence of Cyp51 in *L. prolificans* was previously reported, and several intrinsic amino acid residues associated with azole resistance in *Aspergillus fumigatus* were identified by a conserved residues amino acid alignment (18). Similarly, amino acid substitutions of Fks1 are a common echinocandin-resistant mechanism in other fungal pathogens (19). By hybrid expression of the partial *Fks1* sequence of *L. prolificans* in *Saccharomyces cerevisiae*, it was demonstrated that the innate or intrinsic amino acid residues in *L. prolificans* Fks1 equivalent to *S. cerevisiae* W695F and F639Y mutations in Fks1 hot spot regions may be linked to the intrinsic echinocandin resistance in *L. prolificans* (20, 21). However, both the complete open reading frames of *Cyp51* and *Fks1* genes are not revealed, and no data are shown about the gene polymorphisms of *Cyp51* and *Fks1* in clinical *L. prolificans* isolates.

In this study, we tested the antifungal susceptibility of 42 clinical isolates, including testing the efficacy of various *in vitro* drug combinations. In addition, we sequenced both complete open reading frames of *Cyp51* and *Fks1* genes and analyzed the azole and echinocandin resistance mechanisms in clinical *L. prolificans* isolates.

## RESULTS

**Clinical information of isolates and their antifungal susceptibility.** A total of 42 clinical isolates was collected from 13 patients (Table 1). Seven patients had multiple isolates recovered from different dates. Most isolates were isolated from pulmonary sources, followed by two isolates from blood, one isolate from a cornea, and one from a wound. As for antifungal susceptibility profiles, all isolates except one isolate (ZX2) were found to have the highest MICs for azoles and terbinafine (Table 1). For itraconazole, voriconazole, posaconazole, and isavuconazole, the values of median MIC, mode MIC, $MIC_{50}$, and $MIC_{90}$ were all over 16 $\mu$g/ml, and values of the geometric mean MIC were 30.97, 27.58, 30.45, and 28.51 $\mu$g/ml, respectively. Although micafungin and amphotericin B showed little activity against some clinical isolates (Table 1), their values of median MIC, mode MIC, minimum effective concentration at which 50% of the isolates tested are effective ($MEC_{50}$) or $MIC_{50}$, and $MEC_{90}$ or $MIC_{90}$ were all $>$8 and $>$16 $\mu$g/ml, respectively, and their geometric mean MIC values were 8.98 and 20.84 $\mu$g/ml, respectively. Terbinafine had the highest MIC values with median MIC, mode MIC, $MIC_{50}$, and $MIC_{90}$ all $>$64 $\mu$g/ml and geometric mean MIC of 125.90 $\mu$l/ml. Miltefosine was the only drug that showed the lowest MICs against all *L. prolificans* isolates, with median MIC, mode MIC, $MIC_{50}$, and $MIC_{90}$ values all of 4 $\mu$g/ml and geometric mean MIC of 3.12 $\mu$g/ml, suggesting that miltefosine may possess some good activity against *L. prolificans* compared to these antifungal drugs.

**Antifungal synergy testing.** Fifteen *L. prolificans* isolates were tested *in vitro* for drug susceptibility for different drug combinations (Tables 2 to 4). A synergistic effect of terbinafine-voriconazole was observed in seven clinical isolates (47%). Notably, the MIC of terbinafine was reduced over 32-fold (from $>$64 to 2 $\mu$g/ml) in isolate 176, and the MIC of voriconazole was reduced 4-fold (16 to 4 $\mu$g/ml) in isolate 35 (Table 2). However, the synergy effect of the terbinafine-amphotericin B combination was only observed in one isolate (isolate 23) (Table 2). When the voriconazole-amphotericin B combination was tested, only two clinical isolates demonstrated a synergistic effect (isolates 117 and 23); no synergistic effect was observed in the voriconazole-micafungin combination (Table 3). When the amphotericin B-miltefosine combination was tested, the amphotericin B MIC decreased at least 64-fold (from $>$16 to 0.25 $\mu$g/ml) in three isolates (117, 119, and ZX12), but a synergistic effect was only observed in two isolates

**TABLE 1** Clinical information of isolates and their drug susceptibility profiles

| | | | | | MIC ($\mu$g/ml) of: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P[a] | Disease | ID[b] | Date (yr/mo/day)[c] | Source | AMB | ITC | VOR | POS | ISA | TB | MIL | MICA MEC ($\mu$g/ml) |
| P1 | CF | 117[d,e] | 2014/8/17 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | >8 |
| | | 119[d] | 2014/8/21 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | >8 |
| | | 120 | 2014/8/21 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| | | 126[e] | 2014/9/11 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 2 |
| | | 128[d,e] | 2014/9/11 | S | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| | | 143[d,e] | 2014/12/23 | S | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| | | ZX1[e] | 2015/1/20 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 2 |
| | | 144[d,e] | 2015/3/17 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | >8 |
| | | 163 | 2015/7/13 | BAL | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| | | 166[d,e] | 2015/8/18 | S | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| | | 179[e] | 2015/12/17 | S | 16 | >16 | >16 | >16 | >16 | >64 | 4 | 4 |
| | | 181[d,e] | 2015/12/17 | S | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| | | 182[d] | 2015/12/17 | S | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| P2 | CF | 17 | 2012/6/8 | S | 4 | >16 | 16 | >16 | >16 | >64 | 4 | >8 |
| | | 19 | 2012/6/8 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| | | 27 | 2012/6/27 | S | >16 | >16 | 16 | >16 | >16 | >64 | 4 | >8 |
| | | 35[d] | 2012/7/25 | T | >16 | >16 | 16 | >16 | 16 | >64 | 2 | >8 |
| | | 72[d,e] | 2013/1/5 | E | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| P3 | CF | 23[d] | 2012/5/31 | BAL | >16 | >16 | >16 | >16 | >16 | >64 | 4 | >8 |
| | | 24[e] | 2012/6/13 | BAL | 8 | >16 | >16 | >16 | 16 | >64 | 2 | 2 |
| | | 22[e] | 2012/6/25 | BAL | 4 | >16 | 16 | >16 | 16 | >64 | 2 | >8 |
| | | 29[e] | 2012/6/25 | BAL | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 4 |
| | | 25 | 2012/7/2 | S | 4 | >16 | 16 | >16 | >16 | >64 | 4 | >8 |
| P4 | CF | ZX2[e] | 2014/10/1 | S | 16 | 8 | 4 | 4 | 4 | 64 | 2 | 2 |
| | | 145[e] | 2015/3/6 | BAL | 8 | >16 | 16 | >16 | 16 | >64 | 4 | 1 |
| | | ZX3 | 2018/1/5 | S | 2 | >16 | >16 | >16 | >16 | >64 | 1 | >8 |
| | | ZX4 | 2018/4/20 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | >8 |
| | | ZX5 | 2018/4/20 | S | 16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| | | ZX6[e] | 2018/4/20 | S | 4 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| P5 | MC | ZX8 | 2018/9/5 | Tb | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| | | ZX9[e] | 2018/9/5 | Tb | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 4 |
| | | ZX10 | 2018/9/5 | BAL | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| P6 | AML | ZX11[d,e] | 2018/5/22 | Blood | >16 | >16 | >16 | >16 | >16 | >64 | 4 | >8 |
| | | ZX12[d] | 2018/5/22 | Blood | >16 | >16 | >16 | >16 | >16 | >64 | 4 | >8 |
| P7 | CF | 216 | 2016/11/3 | T | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| | | 220 | 2017/2/9 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| P8 | CF | ZX16 | 2019/4/9 | S | 4 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| P9 | CU | ZX13[d,e] | 2014/2/11 | Cornea | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| P10 | AIDS | 84 | 2013/12/16 | S | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| P11 | CS | 176[d,e] | 2015/12/2 | E | >16 | >16 | >16 | >16 | >16 | >64 | 2 | >8 |
| P12 | N | ZX14 | 2015/5/7 | Wound | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 8 |
| P13 | SOD | ZX15[e] | 2018/7/8 | E | >16 | >16 | >16 | >16 | >16 | >64 | 4 | 4 |

[a]P, patient; AMB, amphotericin B; ITC, itraconazole; VOR, voriconazole; POS, posaconazole; ISA, isavuconazole; TB, terbinafine; MIL, miltefosine; MICA, micafungin; CF, cystic fibrosis; MC, multiple cancers; AML, acute myeloblastic leukemia; CU, corneal ulcer; CS, chronic sinusitis; N, not available; SOD, solid organ transplantation; S, sputum (expectorated); T, throat swabs; E, endotrachial aspirate; BAL fluid, bronchoalveolar lavage fluid; Tb, transbronchial biopsy.
[b]ID, isolate number.
[c]Sample collection date.
[d]Isolates (a total of 15 isolates) were tested *in vitro* susceptibility for different antifungal drug combinations shown in Table 2 to 4.
[e]Isolates (a total of 21 isolates) were sequenced for *Cyp51* and *FKs1* genes.

(isolate 23 and ZX11); no synergistic effect was observed in the voriconazole-miltefosine combination (Table 4).

**Sequence analysis of the *Cyp51* gene and promoter region in clinical isolates.** The complete open reading frame of the *L. prolificans Cyp51* gene was determined by bioinformatics analysis and showed that the *Cyp51* gene has 1,725 bp and 2 predicted exons (Fig. 1A). In order to identify amino acid substitutions in the Cyp51 protein that could be associated with azole resistance, the *Cyp51* gene was sequenced in 21 *L. prolificans* clinical isolates (Table 5). None of these isolates displayed any amino acid substitutions in the Cyp51 protein compared to the ones that are sequence-annotated from the *L. prolificans* whole-genome sequencing (GenPept accession no. PKS10573.1). However, the synonymous mutation T1530A was found in two isolates. The isolate ZX2 showed relatively the lowest MICs of azoles compared to other isolates, yet its Cyp51

**TABLE 2** Synergy test results of AMB, TB, and VOR against *L. prolificans* based on FICI values

| Isolate | MIC (μg/ml) of: | | | | MIC (μg/ml) of: | | | |
|---|---|---|---|---|---|---|---|---|
| | AMB[b] | TB | AMB/TB | FICI[a] | VOR | TB | VOR/TB | FICI[a] |
| 35 | >16 | >64 | >16/>64 | 2 | 16 | >64 | 4/32 | **0.5** |
| 117 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 8/8 | **0.317** |
| 119 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 8/32 | **0.5** |
| ZX11 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 16/16 | 0.625 |
| 23 | >16 | >64 | 4/8 | **0.281** | >16 | >64 | 8/32 | **0.5** |
| ZX12 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 16/8 | 0.563 |
| 143 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 16/32 | 0.75 |
| 128 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 8/8 | **0.313** |
| 182 | >16 | >64 | >16/>64 | 2 | >16 | >64 | >16/>64 | 2 |
| 181 | >16 | >64 | >16/>64 | 2 | >16 | >64 | >16/>64 | 2 |
| 166 | >16 | >64 | >16/>64 | 2 | >16 | >64 | >16/>64 | 2 |
| 144 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 16/32 | 0.75 |
| 72 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 16/0.5 | 0.504 |
| ZX13 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 8/32 | **0.5** |
| 176 | >16 | >64 | >16/>64 | 2 | >16 | >64 | 8/2 | **0.266** |

[a]FICI values in bold indicate synergy.
[b]AMB, amphotericin B; TB, terbinafine; VOR, voriconazole.

protein sequence did not differ from that of the others isolates with high MICs to azoles. By aligning the amino acid sequence of Cyp51 from *A. fumigatus*, *Scedosporium apiospermum*, and *L. prolificans*, we found the identity between Cyp51 of *S. apiospermum* and Cyp51 of *L. prolificans* was 86.6% (Fig. 1B). Additionally, regions of substrate recognition sites (SRSs) of P450 and the heme-binding region (HBR) among these species were conserved. Notably, the *L. prolificans* Cyp51 protein contained residues at three locations (S153, I235, and A302) that corresponded to three residues (G138, M220, and T289) in *A. fumigatus* Cyp51A (Fig. 1B) whose alterations (G138S, M220I, and T289A) have been reported to be associated with azole resistance (22–24). These innate or intrinsic amino acid residues presented in *L. prolificans* at the above locations corresponded to respective mutations found in azole-resistant *A. fumigatus* Cyp51A. In addition, we sequenced the promoter region (1,950 nucleotides upstream of the start codon) of *Cyp51* in 21 *L. prolificans* isolates. We did not identify any tandem repeats in the promoter region, although 4 single nucleotide polymorphisms (SNPs) (C-164G, C-174A, G-1525A, and A-1888G) were found (Table 5).

**TABLE 3** Synergy test results of AMB, VOR, and MICA against *L. prolificans* based on FICI values

| Isolate | MIC (μg/ml) of: | | | | MIC/MEC (μg/ml) of: | | | |
|---|---|---|---|---|---|---|---|---|
| | AMB[c] | VOR | AMB/VOR | FICI[a] | VOR | MICA | VOR/MICA[b] | FICI[a] |
| 35 | >16 | 16 | 16/4 | 0.75 | 16 | >16 | 16/8 | 1.25 |
| 117 | >16 | >16 | 8/4 | **0.375** | >16 | 16 | >16/>16 | 3 |
| 119 | >16 | >16 | 1/16 | 0.531 | >16 | >16 | >16/>16 | 2 |
| ZX11 | >16 | >16 | 4/16 | 0.625 | >16 | >16 | >16/>16 | 2 |
| 23 | >16 | >16 | 1/8 | **0.281** | >16 | 16 | 16/8 | 1 |
| ZX12 | >16 | >16 | 4/16 | 0.625 | >16 | >16 | >16/>16 | 2 |
| 143 | >16 | >16 | 8/16 | 0.75 | >16 | >16 | >16/>16 | 2 |
| 128 | >16 | >16 | 2/16 | 0.562 | >16 | 16 | >16/>16 | 3 |
| 182 | >16 | >16 | >16/>16 | 2 | >16 | >16 | >16/>16 | 2 |
| 181 | >16 | >16 | >16/>16 | 2 | >16 | >16 | >16/>16 | 2 |
| 166 | >16 | >16 | >16/>16 | 2 | >16 | >16 | >16/>16 | 2 |
| 144 | >16 | >16 | >16/>16 | 2 | >16 | 16 | 16/16 | 1.5 |
| 72 | >16 | >16 | >16/>16 | 2 | >16 | >16 | >16/>16 | 2 |
| ZX13 | >16 | >16 | >16/>16 | 2 | >16 | >16 | >16/>16 | 2 |
| 176 | >16 | >16 | >16/>16 | 2 | >16 | >16 | >16/>16 | 2 |

[a]FICI values in bold indicate synergy.
[b]MICs obtained in combination in checkerboard assays.
[c]AMB, amphotericin B; VOR, voriconazole; MICA, micafungin.

**TABLE 4** Synergy test results of AMB, MIL, and VOR against *L. prolificans* based on FICI values

| Isolate | MIC (μg/ml) of: | | | | MIC (μg/ml) of: | | | |
|---|---|---|---|---|---|---|---|---|
| | AMB[b] | MIL | AMB/MIL | FICI[a] | VOR | MIL | VOR/MIL | FICI[a] |
| 35 | >16 | 2 | >16/>1 | 2 | 16 | 2 | 16/>1 | 2 |
| 117 | >16 | 4 | 0.25/2 | 0.508 | >16 | 4 | 16/2 | 1 |
| 119 | >16 | 4 | 0.25/2 | 0.508 | >16 | 4 | 16/2 | 1 |
| ZX11 | >16 | 4 | 8/0.5 | **0.375** | >16 | 4 | 16/2 | 1 |
| 23 | >16 | 4 | 8/0.5 | **0.375** | >16 | 4 | 16/0.06 | 0.515 |
| ZX12 | >16 | 4 | 0.25/2 | 0.508 | >16 | 4 | 16/2 | 1 |
| 143 | >16 | 2 | 4/1 | 0.625 | >16 | 2 | >16/>1 | 2 |
| 128 | >16 | 2 | >16/>1 | 2 | >16 | 2 | 16/1 | 1 |
| 182 | >16 | 2 | >16/>1 | 2 | >16 | 2 | >16/>1 | 2 |
| 181 | >16 | 2 | >16/>1 | 2 | >16 | 2 | >16/>1 | 2 |
| 166 | >16 | 2 | >16/>1 | 2 | >16 | 2 | >16/>1 | 2 |
| 144 | >16 | 4 | 0.5/2 | 0.516 | >16 | 4 | 16/2 | 1 |
| 72 | >16 | 2 | >16/>1 | 2 | >16 | 2 | 16/0.5 | 0.75 |
| ZX13 | >16 | 2 | 16/1 | 1 | >16 | 2 | >16/>1 | 2 |
| 176 | >16 | 2 | >16/>1 | 2 | >16 | 2 | >16/>1 | 2 |

[a]FICI values in bold indicate synergy.
[b]AMB, amphotericin B; MIL, miltefosine; VOR, voriconazole.

**Sequence analysis of the FKS1 gene in clinical isolates.** The complete open reading frame of *L. prolificans Fks1* gene was determined by bioinformatics analysis and showed that the *Fks1* gene has 5,841 bp and 3 predicted exons (Fig. 2A). In order to identify amino acid substitutions in the Fks1 protein that could be associated with echinocandin resistance, the *Fks1* gene was sequenced in the 21 *L. prolificans* clinical isolates. We found 9 SNPs (T164G, A1372C, C1496T, C1955T, A3787C, C3788T, C4202T, C5124T, and C5769A) (Table 5). The SNP T164G in the intron 1 region was found in an isolate with a high micafungin MEC (>8 μg/ml). By aligning amino acid sequences of Fks1 from *S. cerevisiae*, *Candida albicans*, *A. fumigatus*, and *L. prolificans* (the identity between Fks1 of *L. prolificans* and Fks1 of *S. cerevisiae* was 61.9%), we found 3 resulting amino acid substitutions (D440A, S634R, and H1245P) of the Fks1 protein in the conserved regions (Fig. 2B; Table 5) (*S. cerevisiae* Fks1 amino acid numbering is used). Amino acid substitution D440A was found in one isolate (isolate ZX11) with a micafungin MEC of >8 μg/ml; substitution H1245P was found in a separate isolate (isolate 22) with a micafungin MEC of >8 μg/ml. Additionally, an amino acid substitution S634R was found in one isolate (isolate 24) with an MEC of 2 μg/ml and one isolate (isolate 166) with a micafungin MEC of >8 μg/ml. Notably, all 21 isolates contained two intrinsic amino acid residues in two hot spot regions of Fks1 proteins, F639Y in hot spot 1 and W695F in hot spot 3 (Fig. 2B). Substitutions to these residues have previously been demonstrated to cause echinocandin resistance in other fungi (20, 21).

## DISCUSSION

In this study, all *L. prolificans* clinical isolates were shown to be resistant to all antifungal drugs. Miltefosine may possess good activity against *L. prolificans*. Although the drug has been described to act against leishmania by inhibiting the biosynthesis of phospholipids, affecting alkyl-lipid metabolism, inducing an apoptosis-like cell death, and disrupting the parasite's calcium homeostasis (25–27), the exact mechanism of action of miltefosine against fungi is not well understood. Miltefosine likely has different antifungal mechanisms of action from azoles, echinocandins, and polyenes, and it could be a potential drug of choice to treat *L. prolificans* infections.

Consistent with a previous study (5), the combination of terbinafine and voriconazole has demonstrated the most synergistic effect against *L. prolificans* in this study. This drug combination regimen has been reported in successfully treating *L. prolificans*-infected cases (8, 10, 28). A minimal synergistic effect was observed in the drug combination of amphotericin B with voriconazole or miltefosine. The combination of terbinafine and amphotericin B was also tested, but we did not find any good activity

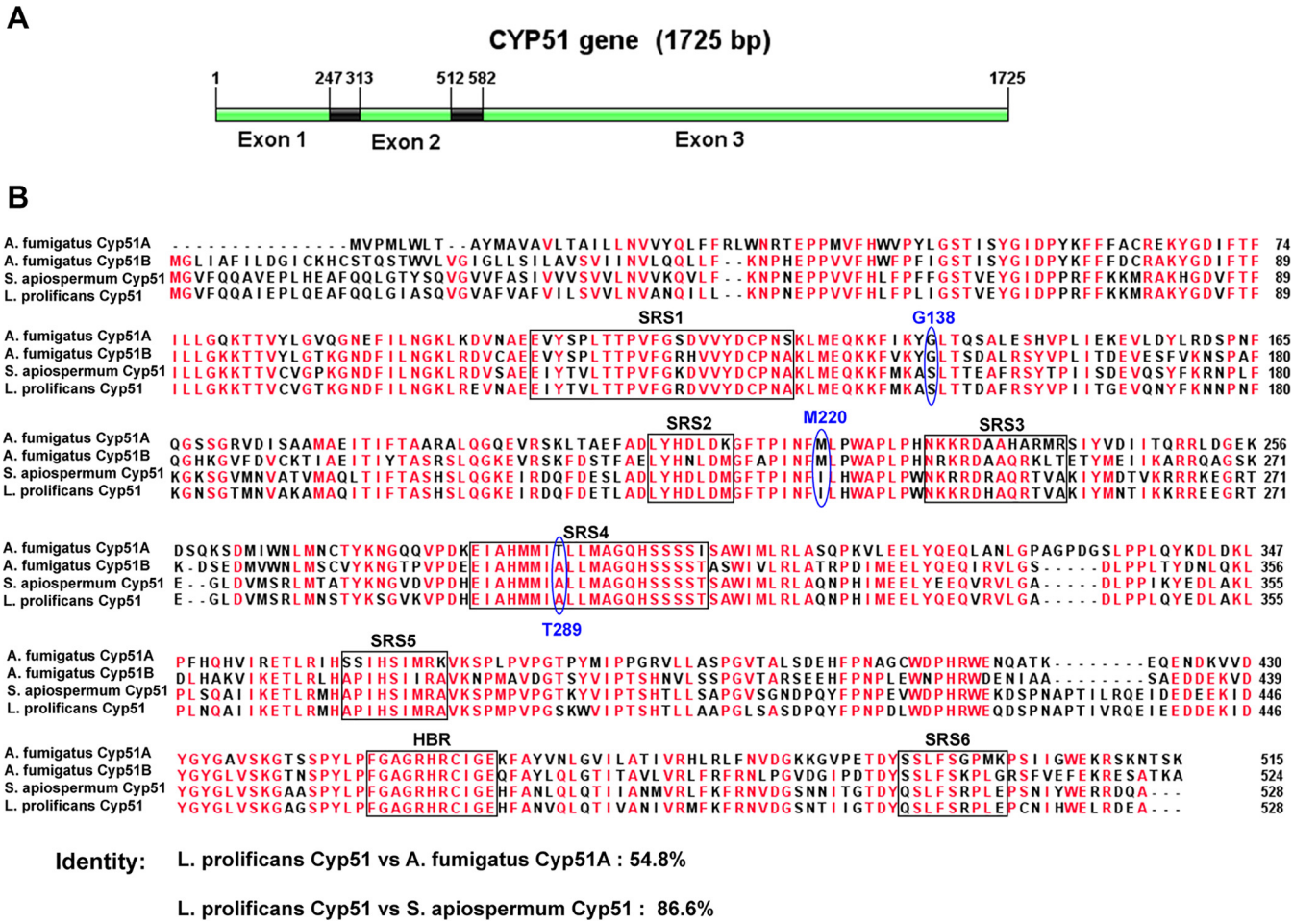


**FIG 1** (A) Schematic map of the *Cyp51* gene open reading frame of *L. prolificans*. (B) Amino acid sequence alignment of Cyp51 from *A. fumigatus*, *Scedosporium apiospermum*, and *L. prolificans*. The Cyp51 protein sequences shown here originated from NCBI GenBank database (accession nos. XP_752137.1, XP_749134.1, MH120909.1, and MN329109). Residues with the similarity of 75% and above are in red. The conserved substrate recognition sites (SRSs) of P450 and heme-binding region (HBR) are encircled with black boxes. Three amino acid residues whose alterations had been linked to azole resistance in *A. fumigatus* are circled with blue oval frames. *A. fumigatus* Cyp51 amino acid numbering is used in this picture.

against *L. prolificans* isolates. The azole-echinocandin combination against *L. prolificans* has not been well explored previously, although this combination displayed a synergistic effect against *Aspergillus* species (29, 30). Under this combination, we found voriconazole at a high concentration could cause abnormal hyphae growth of *L. prolificans* strains (data not shown), similar to what was described previously (31); thus, it is not appropriate to use MECs for evaluating the synergistic effect of the combination of voriconazole and micafungin, and we chose to read the MIC endpoint instead of MEC endpoint for this combination. However, we did not find any synergistic effect of this combination. Likewise, we did not find synergistic activity in the combination of miltefosine and voriconazole in this study, consistent with previous studies (14, 15).

**TABLE 5** Variability of *Cyp51* and *Fks1* genes in clinical *L. prolificans* isolates

| | Characteristics of DNA sequences | | | Characteristics of protein sequences | |
|---|---|---|---|---|---|
| Gene or region | Length (nt) | No. of SNPs | No. of allele types | Amino acid changes[a] | No. of protein types |
| *Cyp51* | 1,725 | 1 | 2 | | 1 |
| 5′ UTR[b] | 1,950 | 4 | 5 | | |
| *Fks1* | 5,841 | 9 | 8 | D440A, S634R, H1245P | 4 |

[a]5′ UTR, 5′ untranslated region of the *Cyp51* gene (promoter region).
[b]Amino acid changes compared with the protein sequence with the highest frequency of prevalence.

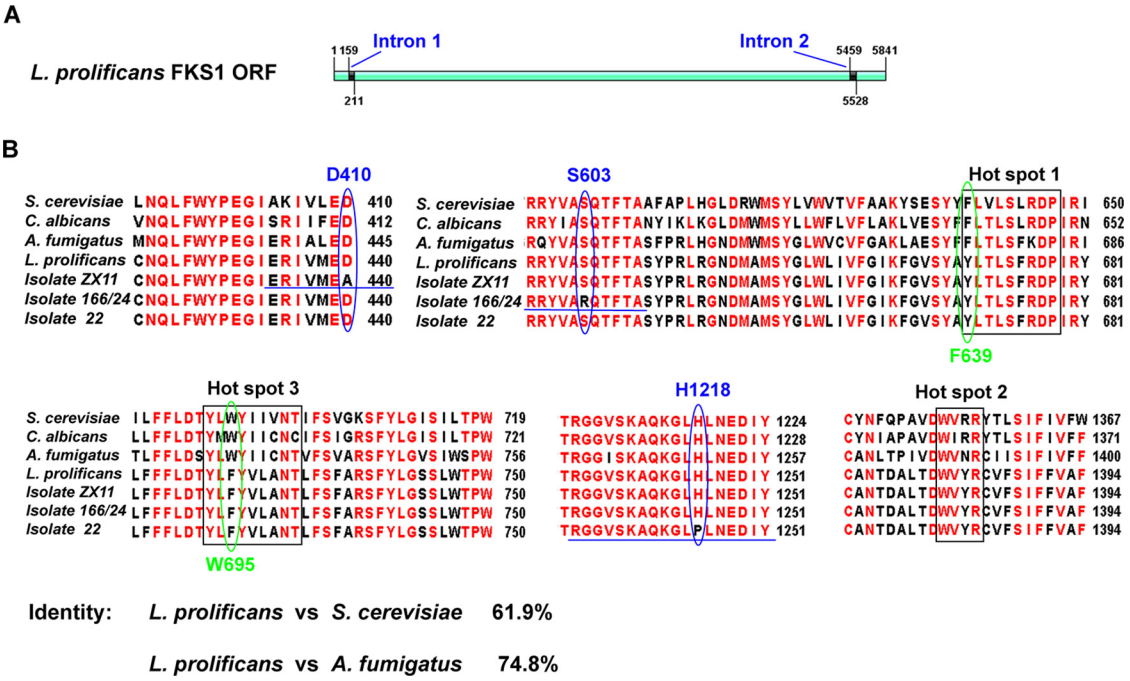


**FIG 2** (A) Schematic map of *Fks1* gene open reading frame of *L. prolificans*. (B) Amino acid sequence alignment of Fks1 from *S. cerevisiae*, *C. albicans*, *A. fumigatus*, and *L. prolificans*. The Fks1 protein sequences shown here originated from NCBI GenBank database and our sequenced isolates (accession nos. NC_001144.5, XP_721429.2, XP_751118.1, and MN329116). Residues with the similarity of 75% and above are in red. Three hot spot regions of Fks1 in which amino acid changes introduce echinocandin resistance are encircled with black boxes. Two amino acid residues whose alterations had been linked to echinocandin resistance in *L. prolificans* are circled with green oval frames. The amino acid residues circled with blue oval frames were equivalent substations (D440A, S634R, and H1245P) found in a few of our clinical isolates. *S. cerevisiae* Fks1 amino acid numbering is used in this picture.

Amino acid alterations in Cyp51 or Erg11 in fungi are a commonly occurring mechanism of azole resistance. Although we did not find any amino acid alterations of Cyp51 in our *L. prolificans* clinical isolates, we identified several azole resistance-related innate or intrinsic amino acid residues in Cyp51 of *L. prolificans*. Alteration to these residues has been reported to be associated with azole resistance in *A. fumigatus* (18). Amino acid substitutions G138S, M220I, and T289A of Cyp51A have been reported to be associated with azole resistance in *A. fumigatus*, with T289A/Y121F/$TR_{46}$ being the most common azole resistance-related genotype (22, 32). The T440A mutation was noted in our study. Although such a mutation associated with azole resistance in *Aspergillus* was suggested previously (33), there was no molecular data to support this notion. While the G138C was previously indicated (34), the association of G138S with azole resistance in *A. fumigatus* was recently reported (22). M220 substitutions conferred high itraconazole MIC and elevated MICs for the other azoles in *A. fumigatus* (24). Although these substitutions each alone in *Cyp51* are not enough to confer azole resistance in *Aspergillus* (23, 35) or their substitutions could represent a silent mutation (36, 37), the simultaneous presence of three azole resistance-related innate or intrinsic amino acid residues (G138S, M220I, and T289A) in Cyp51 of clinical *L. prolificans* isolates may support the notion that *L. prolificans* is intrinsically resistant to azoles. It happened that Cyp51 protein sequence of *L. prolificans* contained these intrinsic amino acid residues at the respective positions in Cyp51 of azole-resistant *A. fumigatus*, suggesting that reduced azole susceptibility of *L. prolificans* may result from the presence of these intrinsic amino acid residues of Cyp51 protein, leading to a reduced affinity to azoles. However, mutations of *Cyp51* are not the sole mechanism accounting for azole resistance in *Aspergillus*. Azole resistance without *Cyp51* mutations has been reported in clinical *Aspergillus* isolates (38–41). Therefore, the intrinsic azole resistance in *L. prolificans* could be associated with mechanisms other than *Cyp51*, and that probably needs further study.

Echinocandin resistance commonly involves amino acid mutations in *Fks1* hot spot regions during antifungal therapy (19). Innate or intrinsic residues in *L. prolificans* Fks1 equivalent to *S. cerevisiae* W695F and F639Y alterations in hot spot regions were confirmed to contribute to echinocandin resistance in *S. cerevisiae* (20, 21). All 21 isolates in this study possessed these amino acid residues (W695F, F638Y) in hot spot regions (Fig. 2B), possibly playing a role in causing high MECs ($MEC_{90} > 8$ $\mu$g/ml) to micafungin in these *L. prolificans* isolates. Other amino acid substitutions in Fks1, such as D440A and H1245R found in isolates with high micafungin MECs, may also play a role in echinocandin resistance, but this will need to be further studied. Nevertheless, echinocandin resistance in *L. prolificans* could be due to mechanisms other than the *Fks1* mutation, since echinocandin resistance in *A. fumigatus* has been demonstrated by non-*Fks1*-related mechanisms (42, 43).

One limitation of our study was the lack of genotyping data of these isolates collected from the same patient to determine if these were the same strain. When we chose these isolates from the same patient (Table 1), we considered them as different strains based on the following criteria: (i) collected at different time points (longitudinal collection), (ii) collected from a different source (bronchoalveolar lavage [BAL] fluid, sputum, tissues, etc.), and (iii) presented with different colony morphologies if collected at the same time. These criteria are commonly applied in clinical microbiology laboratories to label these isolates as different strains, and they often trigger antimicrobial susceptibility testing for each strain to determine if different susceptibility profiling presence would impact patient treatment. In fact, some of our strains collected from the same patient did present with different antifungal drug susceptibility profiles (Table 1). For example, isolate 22 and isolate 24 from patient 3 not only showed different antifungal susceptibility profiles but also demonstrated different amino acid substitutions in FKS1. Therefore, it would be reasonable to consider that these strains were different. However, genotyping would be a definitive way to confirm if they were the same strain or different clades, but this approach would not be feasibly accomplished in the manuscript since such a genotyping method for *L. prolificans* has not been developed and reported in the literature.

In summary, our study demonstrated that none of the antifungal drugs tested *in vitro* showed any activity against *L. prolificans*. However, some synergistic activity was observed in drug combinations, with the combination of voriconazole and terbinafine showing the most synergistic effect. Miltefosine, an antileishmania drug, may possess activity against this fungal pathogen. Studies on *Cyp51* and *Fsk1*, the two genes whose mutations are associated with resistance in azole and echinocandin, respectively, revealed that *L. prolificans* contained innate or intrinsic amino acid residues in which substitutions to these residues at the respective positions have been reported to cause azole and echinocandin resistance in other fungi, supporting the notion that *L. prolificans* is intrinsically resistant to azoles and echinocandins.

## MATERIALS AND METHODS

**Isolate identification and growth.** A total of 42 *L. prolificans* clinical isolates were isolated from 13 patients at the Johns Hopkins Hospital from May 2012 to April 2019. Multiple isolates from the same patient were collected using the following criteria: (i) collected at different time points, (ii) collected from different sources, and (iii) different colony morphologies if collected at the same time (Table 1). Strain typing was not performed in these isolates. All isolates were confirmed as *L. prolificans* based on their macroscopic and microscopic morphologies, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker biotype), and DNA sequencing targeting ITS and D1D2 regions. All isolates were grown on Sabouraud and potato flake agar plates (PFA) (BD, Sparks, MD) at 30°C to achieve adequate sporulation before antifungal susceptibility testing.

**Antifungal susceptibility testing.** The drug MICs or the minimum effective concentrations (MECs) of the 42 clinical isolates were determined using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute M38-A3 and M61 guidelines. The MIC was determined as the lowest concentration producing 100% growth inhibition and was used for all drugs tested except for micafungin. The MEC was determined as the lowest concentration that

causes a hyphal structural change from confluent to granular appearance and was used for micafungin. All isolates were grown in pure culture on PFA slants until sufficient conidia were present to prepare an inoculum. Conidial suspensions were incubated at 35°C, and results were read at 72 h for all drugs. Quality control was performed for each drug set every time using the following ATCC strains: *Paecilomyces variotii* MYA-3630, *Candida parapsilosis* 22019, and *Candida krusei* 6258. For MIC and MEC geometric mean calculations, concentrations >16 $\mu$g/ml were set to 32 $\mu$g/ml, and concentrations >8 $\mu$g/ml were set to 16 $\mu$g/ml.

**Antifungal synergism testing.** Fifteen *L. prolificans* isolates with the highest MICs for azoles and echinocandins were tested with *in vitro* antifungal synergy testing for different drug combinations. Testing for antifungal drug interactions was performed in accordance with the broth microdilution checkerboard method. The final concentration of antifungal drugs ranged from 0.015 to 16 $\mu$g/ml for voriconazole, amphotericin B, micafungin, and miltefosine and 0.06 to 64 $\mu$g/ml for terbinafine. MIC and MEC readings were carried out at 72 h of incubation. For all drug combination groups, the 100% inhibition endpoint was used for the combination of two drugs, including for the combination of voriconazole and micafungin. The MEC endpoint was not chosen for reading the voriconazole and micafungin combination because abnormal hyphae growth was observed in *L. prolificans* under a high concentration of voriconazole, as described previously (31) and as well as from our own experiences. To assess the *in vitro* interactions between antifungal drugs, the fractional inhibitory concentration index (FICI) was calculated. The high off-scale MIC was converted into the next highest concentration for calculation of the FICI, such as >16 $\mu$g/ml, was converted into 32 $\mu$g/ml. The synergy testing was repeated three times, and the higher FICI value of the two close results was reported. The interactions were defined as synergistic if the FICI was $\leq$0.5, indifferent if 0.5 $<$ FICI $\leq$ 4.0, and antagonistic if a value of FICI was >4.0 (44).

**Identification of *L. prolificans Cyp51* and *Fks1* orthologues through alignment.** Comparing partially published amino acid sequence of *Cyp51* (GenBank accession no. MH120874.1) and *Fks1* (GenBank accession no. EU337013.1) with *L. prolificans* strain JHH-5317 whole-genome sequence (GenBank accession no. NLAX01000008.1) plus gene annotation, we identified the *Cyp51* homologous protein (GenPept accession no. PKS10573.1) in *L. prolificans* annotated as "similar to Eburicol 14-alpha-demethylase" and *Fks1* homologous protein (GenPept accession no. PKS10859.1) annotated as "similar to 1, 3-beta-glucan synthase component Fks1" (18, 21, 45). Amino acid alignments of *Cyp51* and *Fks1* from published data and whole-genome sequencing analyzed data are shown in Fig. S1 in the supplemental material.

***Cyp51*, the promoter of *Cyp51*, *and Fks1* amplification and sequencing.** Genomic DNA of 21 selected *L. prolificans* isolates from 9 patients (Table 1) (12 isolates whose MICs for azoles and micafungin were all greater than the highest MIC values and 9 isolates whose MICs for azoles and micafungin were less than or equal to the highest MIC values) was extracted from the hyphal mass according to the manufacturer's instructions provided in Zymo Research Quick-DNA fungal/bacterial miniprep kit (Irvine, CA) and then was used as the template for amplification of the target sequences. The primers used for PCR amplification and sequencing are shown in Table S1. PrimeStar HS DNA polymerase (TaKaRa Bio, CA) was used for PCR amplification to get high-fidelity target sequences, and the PCR products were sequenced by Sanger sequencing (Thermo Fisher Scientific, Waltham, MA).

**Data availability.** The *L. prolificans Cyp51* sequence was deposited in GenBank with accession nos. MN329109 and MN329110. The 5′ untranscribed region (UTR) region sequence of the *Cyp51* gene was deposited in GenBank with accession nos. MN329111 through MN329115. The *Fks1* sequence was deposited in GenBank with accession nos. MN329116 through MN329123.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.6 MB.

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