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Bimodal distribution of azole susceptibility in *Sporothrix brasiliensis* isolates in Brazil

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ABSTRACT *Sporothrix brasiliensis* is an emerging zoonotic fungal pathogen that can be difficult to treat. Antifungal susceptibility testing was performed on the mold phase of a convenience sample of 61 *Sporothrix* spp. isolates from human and cat sporotrichosis cases in Brazil using the Clinical and Laboratory Standards Institute standard M38. A bimodal distribution of azole susceptibility was observed with 50% (28/56) of *S. brasiliensis* isolates showing elevated itraconazole minimum inhibitory concentrations ≥16 µg/mL. Phylogenetic analysis found the *in vitro* resistant isolates were not clonal and were distributed across three different *S. brasiliensis* clades. Single nucleotide polymorphism (SNP) analysis was performed to identify potential mechanisms of *in vitro* resistance. Two of the 28 resistant isolates (MIC ≥16 mg/L) had a polymorphism in the cytochrome P450 gene, *cyp51*, corresponding to the well-known G448S substitution inducing azole resistance in *Aspergillus fumigatus*. SNPs corresponding to other known mechanisms of azole resistance were not identified in the remaining 26 *in vitro* resistant isolates.

KEYWORDS sporotrichosis, antifungal agents, antifungal resistance

A large sporotrichosis epidemic, driven by *Sporothrix brasiliensis*, is ongoing in Brazil with rising cat-to-cat and cat-to-human transmission in 24 of 25 Brazilian states (1). More recently, cat cases with sporotrichosis caused by *S. brasiliensis* have been reported in Argentina (2, 3), Paraguay, Chile (4), and Uruguay (5). *S. brasiliensis* is characterized by high virulence, increased zoonotic transmissibility, and high antifungal resistance and can have a wide spectrum of clinical presentations (6–8). Treatment of infected cats is essential for sporotrichosis transmission control (9). Diverse antifungal therapeutic strategies are available to treat infections caused by *Sporothrix* spp.; itraconazole is the first-choice antifungal drug with terbinafine and potassium iodide as alternatives and amphotericin B used in cases of severe infections (10). The high number of feral cats and treatment interruption due to the high cost of itraconazole are the main challenges to effective cat treatment in Brazil (11). In this context, reports of antifungal-resistant isolates recovered from cat cases have been reported (8, 12). Despite the number of cases, the molecular mechanisms of antifungal resistance among *Sporothrix* spp. are still poorly understood.

Several studies have reported azole minimum inhibitory concentration (MIC) values for *S. brasiliensis* (7, 8, 13, 14), along with a multicenter international study with proposed *S. brasiliensis* epidemiologic cutoff values (ECVs) to azoles (14). The generation of additional *S. brasiliensis* MIC values would facilitate the establishment of *S. brasiliensis* ECVs by the Clinical and Laboratory Standards Institute (CLSI). The possibility of identifying isolates with reduced susceptibility to antifungals, in the context of the zoonotic sporotrichosis epidemic that is ongoing in Brazil, is essential for treatment

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management and will facilitate the tracking of emergence and spread of azole resistance. Here, we determined the MICs for both azoles and echinocandins against *Sporothrix* spp. isolates recovered from cat and humans living in Brazil and the United States (15). All isolates were tested in the mold phase using the CLSI broth microdilution method. Phylogenetic analysis reported in a previous study (15) was correlated with susceptibility to itraconazole and was used to investigate single nucleotide polymorphisms (SNPs) in the target gene for the azoles, *cyp51*.

RESULTS

Antifungal susceptibility testing (AFST) was performed by broth microdilution as outlined in the CLSI reference standard M38 for filamentous fungi (16), for 56 *S. brasiliensis*, six *S. schenckii*, and one *S. globosa* isolates. As shown in Table 1, 50% (28/56) of the *S. brasiliensis* isolates had an MIC ranging from 0.03mg/L to 4 mg/L, and 50% had MICs of ≥16 mg/L to itraconazole (Fig. 1A). The same elevated MIC distribution was observed for the other azoles as follows: 32% (18/56) of isolates showed posaconazole MICs of ≥16 mg/L; 64% (36/56) of isolates showed isavuconazole MICs of >8 mg/L; and 39% (22/56) of isolates showed voriconazole MICs of ≥16 mg/L. Of the 28 *S. brasiliensis* with MIC ≥ 16 mg/L to itraconazole, 43% (12/28) also showed the highest MIC to posaconazole (>16 mg/L), 96% (27/28) to isavuconazole (≥16 mg/L), and 46% (13/28) to voriconazole (≥16 mg/L). Similarly, *S. schenckii* isolates showed a bimodal MIC distribution for itraconazole (Fig. S1), and elevated MIC values for isavuconazole and voriconazole (Table 1). For the echinocandins, the distribution of minimum effective concentration (MEC) values was low, ranging from ≤0.008mg/L to 0.03 mg/L for all isolates (Fig. S2).

To better understand the relatedness of *S. brasiliensis* isolates with high and low MIC values to itraconazole, a maximum likelihood (ML) tree based on whole-genome sequencing (WGS) data (15) was visualized with susceptibility results for itraconazole using Microreact (http://microreact.org) (Fig. 1). *In vitro* itraconazole-resistant *S. brasiliensis* isolates were found in both animal and human cases. Fifty-two percent (23/44) human and 42% (5/12) of cat isolates had itraconazole MIC values of ≥16 mg/L. The ML tree showed that the tested isolates were distributed in four genetically distinct *S. brasiliensis* clades (A–D) supported by bootstrap values of 100% (Fig. 1A). There was no correlation between the *S. brasiliensis* MIC distribution to itraconazole and the phylogenetic clustering patterns in either human or animal cases. Isolates with high MIC were often highly genetically related to isolates with low MIC and were found within all three major clades on *S. brasiliensis* ML phylogeny. However, clade C had the highest number of isolates with high MIC (Fig. 1B).

SNP analysis was performed to identify potential mechanisms of resistance to itraconazole. SNP analysis of the *S. brasiliensis cyp51* gene sequences revealed two isolates (B22074 and B22140) with a glycine to serine amino acid substitution in position 462 (G462S) of *cyp51* and itraconazole MIC values of ≥16 mg/L (Fig. 1B; Table S1). The amino acid substitution in position 462 corresponds to the c*yp51*A G448S mutation in *Aspergillus fumigatus* that is known to be linked to azole resistance (17, 18). *Cyp51*

TABLE 1 Distribution of MIC values for azole activity against 56 clinical isolates of *Sporothrix* spp. according to species[a]

Antifungal drug	*Sporothrix* spp.	0.03 mg/L	0.06 mg/L	0.12 mg/L	0.25 mg/L	0.5 mg/L	1 mg/L	2 mg/L	4 mg/L	8 mg/L	≥16 mg/L
Itraconazole	*S. brasiliensis*	2	2	5	7	8	1	2	1		28
	S. schenckii					1				1	4
Posaconazole	*S. brasiliensis*	2	4	1	9	9	3	7	2	1	18
	S. schenckii					2	2	1			1
Isavuconazole	*S. brasiliensis*			1			6	6	7	36	
	S. schenckii									6	
Voriconazole	*S. brasiliensis*					2	3	3	8	17	22
	S. schenckii										6

[a]No isolates showed MIC of 0.004 mg/L, 0.008 mg/L, or 0.015 mg/L to the azoles represented in the table.

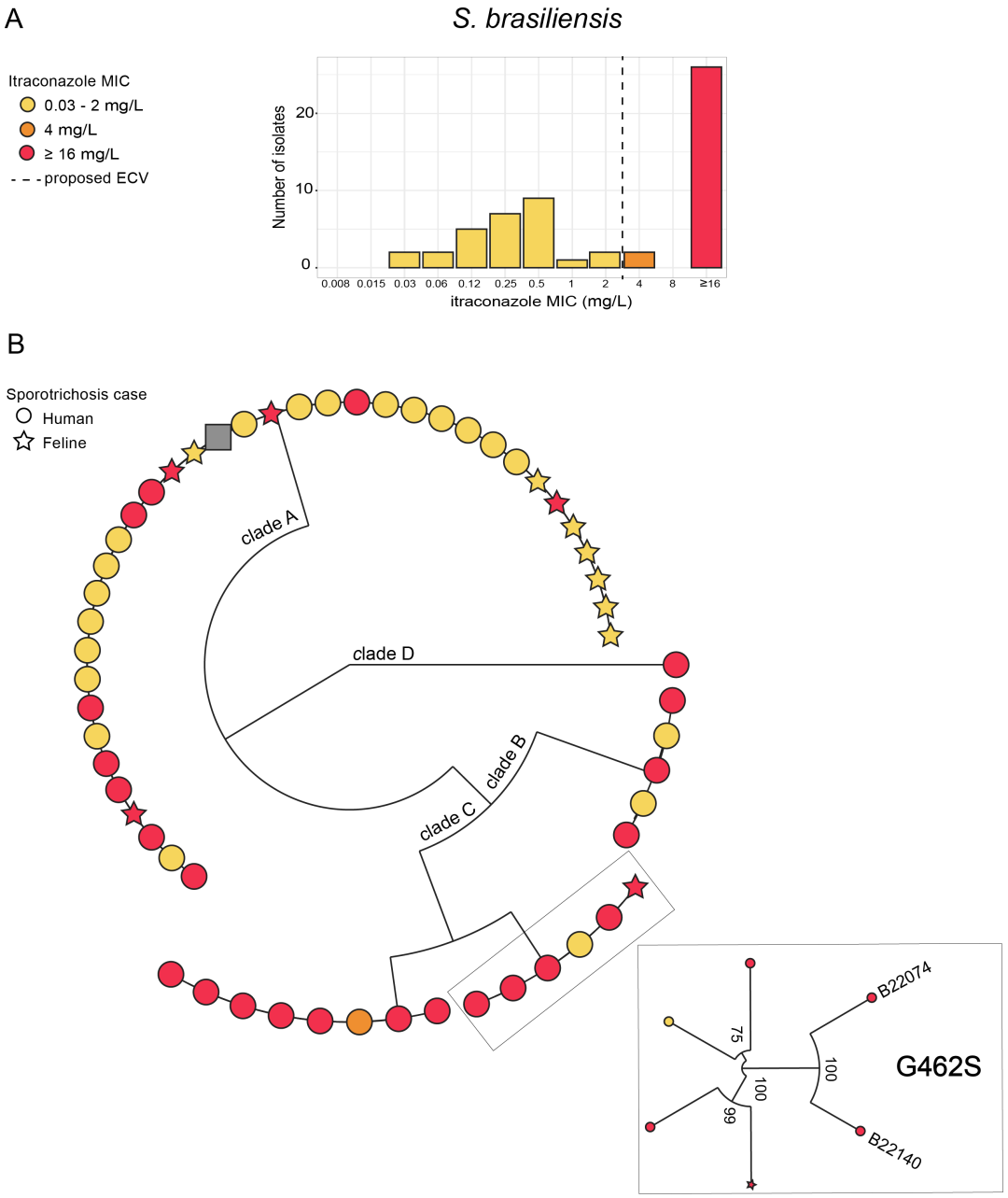

FIG 1 MIC distribution to itraconazole and genomic diversity among *S. brasiliensis* isolates. (A) MIC distribution of *S. brasiliensis* isolates to itraconazole. (B) Maximum likelihood (ML) phylogenetic tree of the *S. brasiliensis* whole-genome sequecing (WGS) (15). Colors correspond to the MIC values to itraconazole. The shape represents the *S. brasiliensis* host (human or cat), with the diamond representing the *S. brasiliensis* strain 5110 reference genome (NCBI: txid1398154). The ML tree and MIC values were visualized together with metadata containing additional epidemiologic data (NCBI BioProject ID: PRJNA957313) for each sample using Microreact (http://microreact.org).

sequencing of the six *S. shenckii* isolates revealed one resistant isolate (B10282) with the amino acid substitution N48K in *cyp51*. (Table S1).

DISCUSSION

Cat-associated sporotrichosis is an emerging fungal disease of increasing public health concern. In this study, we determined MIC values for eight different antifungals among *S. brasiliensis* and *S. schenckii* isolates. We report a bimodal distribution of *S. brasiliensis* MICs to itraconazole, which is the first-choice drug for the treatment of sporotrichosis,

and high MICs to most other azoles. Isolates resistant to itraconazole (MIC ≥16 µg/mL) *in vitro* originated from both human and animal cases and were distributed across three genetically distinct *S. brasiliensis* clades often clustering closely with susceptible isolates. This suggests multiple independent selection events for itraconazole resistance *in vitro*. Similar results were observed for *S. schenckii*. In addition, this was the first study to report *S. brasiliensis* isolates *in vitro* resistant to azoles containing a missense polymorphism in the cytochrome P450 gene (*cyp51*).

Currently, there are neither clinical breakpoints nor ECVs for *Sporothrix* spp. against any antifungal agent. An international multicenter study reporting antifungal susceptibility results of 306 *S. brasiliensis* isolates generated using CLSI M38-A2 broth microdilution found an itraconazole MIC distribution of ≤0.03 mg/L to ≥32 mg/L with a modal MIC of 1 mg/L (48% of isolates). As a result, the proposed ECV for itraconazole was 2 mg/L for both *S. brasiliensis* and *S. schenckii* (14, 19). According to this interpretation, 52% (29/56) of *S. brasiliensis* isolates included in our study would be considered "non-wild-type." Other studies using *S. brasiliensis* isolates from Brazil have reported considerable variability in MIC range for itraconazole and posaconazole (0.12 mg/L to >8 mg/L) (7, 8, 13, 14, 19–21). Considering these previous studies, we found a considerable number of *S. brasiliensis* isolates showing high MIC values to itraconazole, which could indicate a potential increasing resistance to this drug. However, additional longitudinal studies tracking the antifungal susceptibility of clinical isolates are essential to better understand this point.

Genomic analysis of isolates in our study did not identify an association between MIC values to itraconazole and phylogenetic structure; isolates with MICs to itraconazole above 16 mg/L clustered with isolates with MICs as low as 0.03 µg/mL. These results suggest ongoing selective pressure for reduced susceptibility to itraconazole and recent emergence of this phenotype. However, previous studies using a less discriminatory method of strain typing, multilocus sequence typing, found that isolates with high itraconazole MIC values could be genetically differentiated from susceptible isolates (22). Furthermore, no association between azole resistance and previous drug exposure has been reported by others (7, 8, 23). Although our phylogenetic and gene sequencing results suggest that *in vitro* resistance arose on multiple independent occasions, more studies associating clinical and genomic data are needed.

Several mechanisms have been associated with antifungal resistance including chromosome rearrangement, differential gene expression, and nucleotide substitutions in the target promoter genes. There has been little effort to characterize the genetic mechanisms of antifungal resistance in the genus *Sporothrix* (24–26). A recent study explored molecular mechanisms involved in antifungal drug resistance in four *S. brasiliensis* strains in Brazil and provided a working hypothesis for linking *S. brasiliensis* resistance profile to chromosomal variation (26). Multiple substitutions in the *cyp51* gene are associated with resistance to azoles in *A. fumigatus* (27, 28). In addition, an *in silico* study has recently suggested that intrinsic resistance to ketoconazole in *S. schenckii* complex could be explained by the fixed substitutions in the *cyp51* gene (25). We found two isolates of *S. brasiliensis* with high MIC values to itraconazole that had the c*yp51* amino acid substitution G462S. This substitution directly corresponds to the G448S mutation in *A. fumigatus* known to be linked to azole resistance (17, 18). Additional work is necessary to better understand the mechanisms of azole resistance in *S. brasiliensis*, but we demonstrate that more than one mechanism is involved.

There are several limitations to this study. Only the *cyp51* gene, rather than the entire genome, was explored for the possibility of polymorphisms linked to *in vitro* itraconazole resistance, in which other possible resistance mechanisms may have been missed. In the future, genetic mechanisms of resistance can be investigated by comparing genomes of isolates with different AFST profiles; however, larger collections of resistant and susceptible are needed for the genome-wide association studies. Another limitation is that the number of isolates from Brazil was relatively small and constituted a convenience sample with a disproportionate distribution of the three different *Sporothrix*

species, which may not accurately reflect the distribution of susceptibility pattern for the different species or within a species. Finally, clinical information for the sporotrichosis cases was not available, and the *in vitro* AFST results may not correspond to the clinical response to the antifungal treatment. Our study did not evaluate the MIC distribution to terbinafine, an allylamine widely used to treat sporotrichosis in combination with itraconazole, or when itraconazole or KI is not tolerated or cannot be used. Other studies evaluating the *S. brasiliensis* MIC distribution to terbinafine have shown low MIC values, ranging from ≤0.01 mg/L to 1 mg/L (14).

Altogether, our results suggest that more than one mechanism is involved in *in vitro* itraconazole resistance in *S. brasiliensis*. Further studies linking population structure with *S. brasiliensis* strains, antifungal susceptibility data, and clinical outcome of sporotrichosis would enhance understanding of the spread and possible emergence of antifungal resistance in the zoonotic sporotrichosis context.

MATERIALS AND METHODS

Isolates

Species identification and genomic epidemiology were previously reported (15). Among the 63 *Sporothrix* spp. isolates included in this study, 61 were from Brazil and 2 were from the United States. Isolates from Brazil were received by three different laboratories: Microbiology Section of Grupo Fleury (São Paulo, Brazil), Parasitology and Mycology Center of Adolfo Lutz Institute (São Paulo, Brazil), and the Central Public Health Laboratory of Mato Grosso do Sul (Campo Grande, Brazil). These isolates were from clinical samples received by the three reference laboratories for diagnosis purposes and were kept as part of the isolate collection bank for each laboratory. Clinical isolates were obtained from human (*N* = 48) and cat (*N* = 13) cases, and those with collection site information (*N* = 40) were from five different Brazilian states: São Paulo (*N* = 24), Rio de Janeiro (*N* = 2), Mato Grosso do Sul (9), Rio Grande do Norte (*N* = 3), Pernambuco (*N* = 1), and Bahia (*N* = 1). All *Sporothrix* spp. isolates collected from 2013 to 2022 by the three different laboratories in Brazil were included in this study. The two *Sporothrix* spp. isolates from the United States were obtained from human (*N* = 2) cases received by the U.S. Centers for Disease Control and Prevention (CDC) Mycotic Diseases Branch (MDB) laboratory (Atlanta, United States) for diagnosis purposes and were randomly chosen to be included in the study. Details of phylogenetic analysis, epidemiologic, and demographic information of cat and human cases associated with the isolates were previously described (15) and can be found at NCBI BioProject ID: PRJNA957313.

Antifungal susceptibility testing

Fifty-six *S. brasiliensis*, six *S. schenckii*, and one *S. globosa* isolates were used in this study. AFST was performed by broth microdilution as outlined in the CLSI reference standard M38 for filamentous fungi (16). Isolates *Aspergillus fumigatus* ATCC MYA-3626 and *A. fumigatus* MYA-3627 were included as quality controls. Fluconazole, voriconazole, itraconazole, isavuconazole, posaconazole, anidulafungin, caspofungin, and micafungin were tested. The MICs were determined visually after 48–72 hours of incubation at 35°C. For isavuconazole, itraconazole, posaconazole, and voriconazole, the MIC endpoint was the lowest concentration that produced complete inhibition of growth and for echinocandins, it was the lowest concentration producing a visual change in the appearance of the growth (MEC). Although there are no breakpoints for *Sporothrix* and itraconazole, an MIC value of ≥16 µg/mL was presumed to be resistant. Data visualizations were created with ggplot2 and ggpubr R packages.

Single-nucleotide polymorphism and phylogenetic analysis

SNPs were identified from WGS data using MycoSNP v 1.4 (https://github.com/CDCgov/mycosnp-nf/) as described by Bagal et al. (29) . The MycoSNP pipeline generated

the ML tree and VCFs from the filtered SNPs calling file. ML tree was visualized together with metadata containing additional epidemiologic data for each sample using Microreact (http://microreact.org). Using the VCFs and SnpEff (v 5.1) (30), we searched for SNPs in the *cyp51* gene orthologue (SPBR_08369 for *S. brasiliensis* and SPSK_09044 for *S. schenckii*) (31) in all analyzed genomes. All nonsynonymous polymorphisms in the respective *cyp51* genes present only in resistant isolates were retrieved. Protein cytochrome P450, family 51 (Sterol 14-demethylase) orthologues in *S. brasiliensis*, *S. schenckii*, and *A. fumigatus* were aligned using Geneious Prime v 2021.0.3 software to find the corresponding amino acid positions for each species.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1, Fig. S2, and Table S1 (AAC01620-23-s0001.docx). This file contains supplementary analysis done for *Sporothrix schenckii* isolates, as well as complete results of MIC of each isolate included in this study.

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