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Refractory sporotrichosis due to *Sporothrix brasiliensis* in humans appears to be unrelated to *in vivo* resistance

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Abstract

Sporotrichosis is a subacute to chronic infection caused by members of the *Sporothrix schenckii* complex. Itraconazole is the first choice antifungal drug for treating this infection, with terbinafine and potassium iodide as alternatives and amphotericin B used in cases of severe infections. Correlation of antifungal susceptibility data with the clinical outcome of the patients is scarce. The aim of this study was to correlate clinical and mycological data in patients with refractory sporotrichosis. In this work, antifungal susceptibilities, determined according to the reference M38-A2 CLSI protocol, of 25 *Sporothrix* strains, isolated from seven human cases of sporotrichosis with adversities in the treatment, are presented. Tested drugs included itraconazole, ketoconazole, posaconazole, voriconazole, terbinafine, and amphotericin B. Fungi were identified using the T3B PCR fingerprinting. This method identified all strains as *Sporothrix brasiliensis* and also demonstrated a high degree of similarity between the strains. In general, voriconazole was ineffective against all strains, and elevated minimal inhibitory concentrations (MICs) were observed for amphotericin B. High itraconazole and terbinafine MICs were not observed in *S. brasiliensis* isolates from patients of this study. Moreover, a significant increase in itraconazole and terbinafine MIC values from strains isolated from the same patient in different periods was not observed. The results suggest that the antifungal susceptibility to terbinafine and itraconazole determined by the reference method does not play an important role in therapeutic failure of sporotrichosis and that

acquisition of resistance during prolonged antifungal treatment is not likely to occur in *S. brasiliensis*.

Key words: antifungal susceptibility, sporotrichosis, *Sporothrix brasiliensis*, therapeutic failure.

Introduction

Sporotrichosis is a worldwide subcutaneous mycotic infection caused by dimorphic fungi belonging to the *Sporothrix schenckii* complex. The main agents of sporotrichosis are *Sporothrix schenckii* sensu stricto, *Sporothrix brasiliensis*, and *Sporothrix globosa*, and rare cases of infection are caused by *Sporothrix luriei*, *Sporothrix mexicana*, and *Sporothrix pallida*.[1]

After implantation of the fungus through the skin, usually after an injury, nodules, ulcers, and verrucous plaques with or without lymphatic dissemination occur in patients with normal immunity.[2] Pulmonary and disseminated sporotrichosis are not common, and usually are related to immunosuppression, especially human immunodeficiency virus (HIV) coinfection.[3,4] Treatment options include itraconazole, as a first choice antifungal drug,[5] with saturated solution of potassium iodide[6] and terbinafine[7] as an alternative for human patients. Amphotericin B is indicated in cases of pulmonary and disseminated infection.[8] The success rate for these drugs is typically high;[9–11] however a few patients have a slow response or do not respond to the treatment and develop a chronic infection in which the fungus can be isolated from the patients during prolonged periods.[12]

In recent years, there has been an increasing interest in the *in vitro* susceptibilities of yeasts and filamentous fungi to antifungal drugs,[13] including those for the dimorphic fungi belonging to the *S. schenckii* complex. Some of these studies have indicated that the geographic origin of the strains, the morphological form of the fungus (yeast or conidia), and the fungal species are associated with differences in minimal inhibitory concentrations (MIC).[14–16]

In this study, the *in vitro* antifungal susceptibility profile of *S. brasiliensis* strains isolated from patients with refractory sporotrichosis are reviewed, in order to determine if they were less susceptible than other described *Sporothrix* strains, and to screen for the development of *in vivo* resistance during prolonged antifungal treatment of patients.

Material and methods

Ethics statement

This study was approved by the Research Ethics Committee of the Instituto Nacional de Infectologia Evandro Chagas/Fundação Oswaldo Cruz (INI/Fiocruz), under the number CAAE-26637014.8.0000.5262. All patient strains and data were evaluated anonymously after getting a random number in the database.

Patients

A retrospective search for cases of sporotrichosis with slow responsiveness or therapeutic failure was conducted in the database of INI/Fiocruz from 2000 to 2013. Briefly, the protocol of sporotrichosis treatment in our institution is itraconazole, administered orally at a dosage of 100 mg/day or terbinafine, when itraconazole is contraindicated. Higher doses or the use of a combination of antifungals are performed when there is no improvement or worsening of the clinical picture after at least 2 months of treatment. Deoxycholate amphotericin B is indicated in severe disseminated cases. Posaconazole is not a standard drug in our institution, and it is difficult to use in a routine clinical setting due to its cost. In patients with severe unresponsive sporotrichosis, however, an effort is made to procure posaconazole, especially for those with HIV infection. Adjuvant therapies such as cryosurgery with liquid nitrogen in sequential sessions is also performed in vegetative lesions or curettage in crusted lesions when disease still persists after 2 to 3 months treatment, despite significant improvement in lesions elsewhere. The length of treatment is determined by the clinical cure rate, defined by the healing of skin lesions and absence of crusts. For extracutaneous sporotrichosis, cure is defined when the fungus is no longer detected in clinical samples from the infected sites.

Slow responsiveness or therapeutic failure was defined in this study when higher doses or the use of a combination of antifungals were used, adjuvant therapy was administered, or posaconazole was employed. Inclusion criteria included one of the above mentioned conditions and the availability of at least two viable *Sporothrix* strains from the same patient, isolated with a minimal interval of 2 months. Patients that underwent treatment exclusively with nonantifungal drugs, that is, those treated with saturated solution of potassium iodide, or those treated with local hyperthermia, were excluded from this study.

Strains

In sum, 25 strains, isolated from 2000 to 2013, were included in this study. They were all collected from human

patients living in the endemic area of zoonotic sporotrichosis in Rio de Janeiro, Brazil.[1,2] They were preserved by the lyophilization method and reactivated in Sabouraud Dextrose Agar (Difco Laboratories, Sparks, MD, USA) for molecular and susceptibility tests.

Molecular characterization by T3B PCR fingerprinting

Genomic DNA was extracted from the filamentous form of the strains as previously described.[17] For species identification, the T3B primer (5′-AGGTCGCGGGTTCGAATCC-3′) was used[18] and band profiles in a 1.2% agarose gel were compared to those generated by control strains CBS 120339 (*S. brasiliensis*), IOC 1226 (*S. schenckii*), IPEC 27135 (*S. globosa*), and MUM 11.02 (*S. mexicana*). Number and molecular weights of the bands were further recorded to assess the genetic similarity between the strains. The T3B fingerprinting profiles were analyzed applying unweighted pair-group arithmetic average (UPGMA) method using the SHAN subroutine through NTSYS-pc (numerical taxonomy system, 2.2 version) (numerical taxonomy system, Applied Biostatistics, NY).

Antifungal susceptibility test

Amphotericin B, terbinafine, ketoconazole, voriconazole, posaconazole, and itraconazole (Sigma Chemical Corporation, St. Louis, MO, USA) were tested. Stock and diluted antifungal solutions were prepared according to the CLSI M38-A2 document,[19] ranging from 0.03 to 8.0 mg/l. An inoculum of 1–5 $\times$ 10^4 conidia/ml was prepared for each strain after incubation in potato dextrose agar (Difco Laboratories, Sparks, MD, USA) during 7 days at 37°C. The strains ATCC 204304 (*Aspergillus flavus*) and ATCC 6258 (*Candida krusei*) were used as controls. The MICs were determined visually after 48–72 hours of incubation at 37°C. For amphotericin B, itraconazole, posaconazole, and voriconazole, the MIC endpoint was the lowest concentration that produced complete inhibition of growth. For ketoconazole, MIC was the lowest concentration producing a 50% reduction in growth, and for terbinafine it was the lowest concentration producing at least 80% of reduction in growth. When no differences in fungal growth relative to the control without drugs were observed in the highest antifungal concentration tested (8.0 mg/l), MICs were displayed as $\geq$16 mg/l. Antifungal susceptibility tests were performed at least twice and were validated by the determination of the same MICs in different experiments.

Literature search

In order to compare the results of this study with other *S. brasiliensis* susceptibilities studies, a search of the available literature was conducted in PubMed and Scopus. The search terms included "*Sporothrix brasiliensis,*" "*S. brasiliensis,*" "sporotrichosis," "antifungal susceptibility," "antifungal susceptibilities" with the following search strategy: [("*Sporothrix brasiliensis*" or "*S. brasiliensis*") or "sporotrichosis" AND ("antifungal susceptibility" or "antifungal susceptibilities")]. Studies published before 2007, the year of *S. brasiliensis* species description,[20] were discarded. In addition, papers without molecular support of *S. brasiliensis* identification were not taken in consideration in the analysis.

Analysis of results

Essential agreement between MICs from strains isolated from the same patient was defined if discrepancies of no more than two dilutions among the MIC endpoints were observed. Therefore, an increase of at least three dilutions of the MICs among strains indicated resistance acquisition. Descriptive statistics were performed with the Statistical Package for the Social Sciences (SPSS) for Windows®, version 17.0, to obtain the MIC range, MIC50 and MIC90 values, and geometric means. The MIC50 and MIC90 values correspond to the MIC of the drug capable to inhibit the growth of 50% and 90% of all fungal isolates, respectively. The Mann–Whitney test was applied, using the Prism for Windows 5.0 (GraphPad Software, Inc.) to compare MIC values of this work with those described in other publications. A value of $P < .05$ was considered significant.

Results

Patients

Seven patients were included in this study. Their main clinical and therapeutic characteristics are described on Table 1. The group included four female and three male patients, with ages ranging from 20 to 68 years; however, all except one patient were older than 60 years. Six of them reported contact with cats. Three patients presented with lymphocutaneous sporotrichosis, two presented with disseminated cutaneous sporotrichosis and two with disseminated sporotrichosis with extracutaneous involvement, one of which was infected with HIV. Initial treatment was itraconazole 100 mg/day for patients 1–3, terbinafine 250 mg/day for patients 4–6, and amphotericin B-deoxycholate for patient 7. Patients were followed for periods ranging from 19 to 96 months. Patients 1, 2, 3, and 5 were cured after undergoing alternative therapies such as

Table 1. Clinical and therapeutic characteristics of seven patients with sporotrichosis included in this study.

Patient	Transmission	Clinical form	HIV status	Treatment (weeks)		Follow-up (months)	Outcome
				Initial	Subsequent		
1	Cat bite	DC[g]	Negative	ITC[a] 100 mg/day (8)	ITC 400 mg/day (28) ITC 400 mg/day and FLC[b] 200 mg day (56) Curettage	23	Cure
2	Cat contact	LC[h]	Negative	ITC 100 mg/day (60)	Cryosurgery Curettage	19	Cure
3	Cat scratch	DC	Negative	ITC 100 mg/day (60)	Cryosurgery Curettage	23	Cure
4	Cat bite	LC	Negative	TRB[c] 250mg/day (42)	TRB 500 mg/day (21) Cryosurgery Curettage	36	Lost
5	Cat scratch and bite	LC	Negative	TRB 250 mg/day (16)	ITC 100 mg/day (24) ITC 200 mg/day (16) Cryosurgery	53	Cure
6	Injury at work	D/E[i]	Negative	TRB 250 mg/day (12)	TRB 500 mg/day (172) ITC 200 mg/day (14) ITC 300 mg/day (39) Curettage	96	Treating
7	Cat scratch	D/E	Positive	AmB-d[d] (5)	AmB-l[e], ITC 200 mg/day, and TRB 250 mg/day (8) PSC[f] 800 mg/day (64) Cryosurgery	20	Death

[a]ITC: itraconazole.
[b]FLC: fluconazole.
[c]TRB: terbinafine.
[d]AmB-d: amphotericin B deoxycolate.
[e]AmB-l: liposomal amphotericin B.
[f]PSC: posaconazole.
[g]DC: disseminated cutaneous.
[h]LC: lymphocutaneous.
[i]D/E: disseminated/extracutaneous.

curettage and cryotherapy, patient 6 is still receiving antifungal treatment with us, patient 4 abandoned treatment and was lost to follow-up, and the patient 7, the one with HIV coinfection, died.

Species identification and typing

All 25 strains presented band patterns on agarose gels similar to the *S. brasiliensis* type strain CBS 120339. Moreover, no significant differences in band number or size were verified in each patient, indicating a genetic similarity among the strains isolated from the same patient at different times (Fig. 1).

Minimal inhibitory concentrations and clinical correlation

Table 2 presents MIC data of the 25 *S. brasiliensis* strains included in this study. Two patients had five strains, two had four strains, one had three strains, and two had two strains collected in intervals ranging from 2 to 54 months. Terbinafine showed a good inhibitory activity against all tested isolates, even on the three patients that were initially treated with terbinafine at a 250 mg/day dosage (Table 1, patients 4, 5, and 6). MICs for terbinafine ranged from 0.03 to 0.12 mg/l. Itraconazole was also effective against the majority of the tested strains, with a MIC50 of 1.0 mg/l. Of the three patients initially treated with itraconazole 100 mg/day, one had an initial *S. brasiliensis* strain with a MIC of 2.0 mg/l; however, all but one *S. brasiliensis* strain isolated thereafter demonstrated a MIC of 1.0 mg/l, indicating an essential agreement between all five other isolates. Among the other azole drugs tested, voriconazole demonstrated poor inhibitory activity, with MICs ranging from 2.0 to >16 mg/l. It is worth mentioning that in only one patient (Table 1, patient 7) was a MIC of 2.0 mg/l observed for voriconazole. Ketoconazole and posaconazole, however, showed good activity, with MICs ranging from

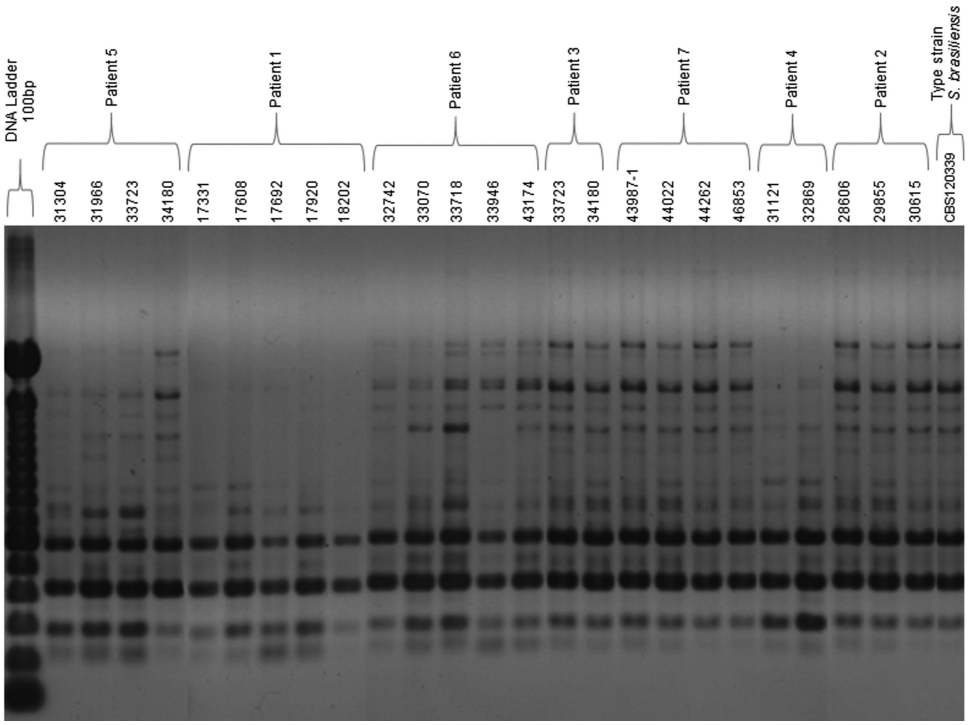

Figure 1. T3B PCR fingerprinting profiles of the twenty-five *Sporothrix* isolates from 7 patients. Molecular marker DNA ladder, 100 bp (Invitrogen) and Type strain *S. brasiliensis* (CBS120339).

0.5 to 2.0 mg/l and 0.5 to1.0 mg/l, respectively. All strains from patient 7, treated for 13 weeks with 800 mg/day of posaconazole after her diagnosis of sporotrichosis, showed a MIC of 1.0 mg/l for posaconazole. Amphotericin B yielded MICs ranging from 0.5 to 4.0 mg/l. The HIV-coinfected patient was initially treated with amphotericin B but with no improvement in her clinical condition. Two strains had MICs of 2.0 mg/l, while another two had MICs of 4.0 mg/l for this polyene agent.

Comparison of *S. brasiliensis* MICs

A search for papers describing *S. brasiliensis* antifungal susceptibility to the antifungal drugs tested in this study retrieved seven publications.[15,21–26] Taken together, data from 162 strains (range of strains per publication 1–48; mean = 23 strains per publication) were presented in these seven studies. The results of MIC ranges, MIC50, and MIC90 from this study and from these other publications, when available, are described on Table 3. Comparisons between the MICs of this study and MICs described in these publications showed no differences when strains were tested against posaconazole or voriconazole (P values of .4899, and .2774, respectively). MICs for itraconazole, ketoconazole, and amphotericin B from this study were higher than those previously published ($P < .001$), and MICs for terbinafine herein presented were lower than those published on the six selected references ($P < .001$).

Discussion

The refractoriness of the patients presented here raised the possibility of acquired antifungal resistance. Mechanisms that lead to therapeutic failure in sporotrichosis are not well understood. In previous studies only 6% of 562 patients that underwent itraconazole 100 mg/day needed higher doses of this drug to achieve sporotrichosis cure, and 1.2% needed to switch to other drugs, such as terbinafine or potassium iodide.[9] Failure of sporotrichosis treatment with terbinafine is also rare. Cure was achieved with a dose of 250 mg/day of terbinafine in almost 96% of patients in a cohort of 50 patients.[7] This study investigated three other patients with itraconazole failure and three patients with terbinafine failure. All these six patients were older than 60 years and presented negative HIV serology.

This study included patients with all clinical forms of the disease with the exception of the fixed localized form. All except one patient with severe immunosuppression (patient 7) were in good health; however, they did not respond as expected with the initial therapy.

All strains were identified as *S. brasiliensis*, the major agent of sporotrichosis in Rio de Janeiro, Brazil,[27] by the

Table 2. Mycological information and minimal inhibitory concentrations of 25 *Sporothrix brasiliensis* strains to six antifungal drugs.

			MIC (mg/l)					
Patient	Strain	Isolation date	Ketoconazole	Itraconazole	Posaconazole	Voriconazole	Terbinafine	Amphotericin B
1	17331	03/23/2000	1	2	1	4	0.06	1
	17608	06/01/2000	1	1	1	4	0.06	0.5
	17692	06/16/2000	2	1	1	>16	0.12	2
	17920	08/17/2000	2	2	1	>16	0.03	2
	18202	10/19/2000	1	1	1	4	0.03	1
2	28606	10/11/2005	0.5	1	1	8	0.06	4
	29855	05/12/2006	0.5	1	1	8	0.06	2
	30615	10/02/2007	0.5	1	1	4	0.12	1
3	30070	06/23/2006	1	1	0.5	>16	0.03	4
	30467	08/31/2006	1	1	1	8	0.06	4
4	31121	01/11/2007	0.5	1	1	8	0.03	2
	32869	10/18/2007	0.5	1	1	8	0.03	2
5	31304	02/07/2007	0.5	1	1	8	0.03	4
	31966	05/31/2007	1	1	1	8	0.06	2
	33723	03/20/2008	1	1	1	8	0.06	4
	34180	05/29/2008	1	1	1	8	0.06	4
6	32742	09/27/2007	2	2	1	>16	0.06	2
	33070	11/22/2007	2	2	1	>16	0.06	2
	33718	03/20/2008	2	2	1	>16	0.06	2
	33946	04/25/2008	2	2	1	>16	0.06	2
	43174	02/09/2012	2	2	1	>16	0.06	2
7	43987-1	06/14/2012	0.5	2	1	2	0.03	2
	44022	06/20/2012	0.5	2	1	2	0.06	4
	44262	06/24/2012	1	2	1	4	0.06	4
	46853	10/13/2013	1	2	1	2	0.03	2
Control	ATCC 204304	Unknown	1	0.5	0.25	2	0.12	1
Control	ATCC 6258	Unknown	0.25	0.5	0.5	0.25	0.12	2

T3B fingerprinting method.[18] This method was chosen due to its relative ease of performance and low cost, and because it permits the observation of some intraspecific variation within a single species. Methods for *S. brasiliensis* typing are few,[28] and with this technique differences in band patterns of sequential strains were not observed. In our previous study we observed some intraspecific variation (around 80%) within *S. brasiliensis*.[18] However, that study analyzed 29 *S. brasiliensis* strains from different patients. As the present study includes strains isolated from only seven patients, a lower variability should be expected.

Although a previous study showed that treatment time with itraconazole 100 mg/day was lower in sporotrichosis cases caused by *S. brasiliensis* (median 16 weeks), when compared to patients infected with *S. schenckii* (median 24 weeks),[29] our results suggest otherwise as three patients in the current study needed prolonged treatment times with itraconazole (60–92 weeks), in addition to other therapeutic strategies to resolve their infection.

Among the six drugs that were tested, terbinafine was the one with the lowest MICs, as described by other authors.[14–16,21] Two of the three patients had their terbinafine dosage increased to 500 mg/day, one was lost to follow-up, and another is still undergoing treatment, making it difficult to ascertain if terbinafine alone at higher doses is sufficient to cure sporotrichosis cases unresponsive to terbinafine at 250 mg/day. Patient 6 had disseminated disease (skin and bone), and it is not known to what extent terbinafine penetrated the bone. Furthermore, an increase of *S. brasiliensis* virulence throughout the years was observed in this case.[12] It is interesting to note that MIC values of all *S. brasiliensis* strains from all these three patients were very low (0.03–0.06 mg/l), not correlating with the unresponsiveness to treatment in these cases. Moreover, the terbinafine MICs in this study, even in therapeutic failures, are lower than

Table 3. Statistical parameters of *Sporothrix brasiliensis* antifungal susceptibilities to six different drugs obtained from seven different works.

Antifungal drug	Reference	Range[a]		MIC50[a]	MIC90[a]	Geometric mean[a]
		Minimum	Maximum			
Ketoconazole	This study	0.5	2.0	1.0	2.0	0.97
	Brilhante et al. 2016	0.03	1.0	0.25	1.0	0.27
	Stopiglia et al. 2014	0.03	1.0	0.12	0.5	0.16
	Stopiglia et al. 2012	0.12	0.5	NI[b]	NI	0.22
	Oliveira et al. 2011	0.5	0.5	NC[c]	NC	NC
	Marimon et al. 2008	0.06	0.5	0.12	0.25	0.15
Itraconazole	This study	1.0	2.0	1.0	2.0	1.36
	Brilhante et al. 2016	0.12	2.0	1.0	2.0	0.77
	Borba-Santos et al. 2015[d]	NI	NI	NI	NI	3.1 / 2.0
	Rodrigues et al. 2014	0.25	4.0	1.0	2.0	NI
	Stopiglia et al. 2014	0.06	2	0.5	0.5	0.36
	Stopiglia et al. 2012	0.06	2	NI	NI	0.33
	Oliveira et al. 2011	0.25	0.25	NC	NC	NC
	Marimon et al. 2008	0.5	2	0.5	1.0	0.70
Posaconazole	This study	0.5	1.0	1.0	1.0	0.97
	Borba-Santos et al. 2015	NI	NI	NI	NI	1.1 / 0.3
	Rodrigues et al. 2014	0.5	2.0	1.0	2.0	NI
	Marimon et al. 2008	0.25	1.0	0.5	1.0	0.62
Voriconazole	This study	2	>16	8.0	>16	7.36
	Brilhante et al. 2016	2	64	16	64	20.16
	Borba-Santos et al. 2015	NI	NI	NI	NI	5.9 / 6.4
	Rodrigues et al. 2014	2	>16	16	>16	NI
	Stopiglia et al. 2014	1	16	8	16	6.10
	Oliveira et al. 2011	8	8	NC	NC	NC
	Marimon et al. 2008	0.5	16	4	8	3.88
Terbinafine	This study	0.03	0.12	0.06	0.06	0.05
	Borba-Santos et al. 2015	NI	NI	NI	NI	0.1/0.1
	Stopiglia et al. 2014	0.01	0.5	0.06	0.12	0.06
	Stopiglia et al. 2012	0.01	0.25	NI	NI	0.07
	Oliveira et al. 2011	0.25	0.25	NC	NC	NC
	Marimon et al. 2008	0.06	0.25	0.06	0.25	0.09
Amphotericin B	This study	0.5	4.0	2.0	4.0	2.17
	Brilhante et al. 2016	0.12	4	1.0	2.0	0.90
	Borba-Santos et al. 2015	NI	NI	NI	NI	1.4 / 1.2
	Rodrigues et al. 2014	1.0	8.0	4.0	4.0	NI
	Stopiglia et al. 2014	0.25	4.0	1.0	2.0	1.03
	Stopiglia et al. 2012	0.5	2.0	NI	NI	1.00
	Oliveira et al. 2011	0.5	0.5	NC	NC	NC
	Marimon et al. 2008	1.0	4.0	2.0	4.0	1.67

[a]All values expressed in mg/l.
[b]NI, not informed.
[c]NC, not calculated, due to the small number of strains.
[d]These authors divided their samples in two groups, comprising old (first presented value) and new (last presented value) *S. brasiliensis* strains.

those found by other authors, which also does not corroborate their findings. To the best of our knowledge, the pharmacodynamics and pharmacokinetics of terbinafine in sporotrichosis patients were not studied up to now, making it difficult to correlate *in vitro* and *in vivo* studies.

Terbinafine is an allylamine that possesses high *in vitro* activity not only against the *Sporothrix schenckii* complex but also against other fungi, such as dermatophytes,[30] *Syncephalastrum racemosum*,[31] *Arthrographis kalrae*,[32] agents of chromoblastomycosis,[33] and *Candida dubliniensis*,[34]

among others. In these fungi the MIC90 values are equal to or lower than 0.25 mg/l and elicit a better *in vitro* response than azole antifungal agents. Due to differences in chemical structure and mechanism of action between the azoles and allylamines, we believe that an *in vitro* comparison between the MICs of these two antifungal classes is not adequate. Nevertheless, the efficacy of terbinafine against *Sporothrix* spp., both *in vitro* and *in vivo*, cannot be denied.

A severe case of disseminated disease was difficult; the response to treatment was also investigated in patient 7 who also had advanced AIDS. In this patient, the depression of cellular immunity and reduced host defenses can account for her unresponsiveness. Deep sites of infection in AIDS patients such as the cerebrospinal fluid,[35] as noted by others, also contributes to more difficult management. This case also highlights the need for better therapeutic options, such as posaconazole, for severe sporotrichosis, as this and other studies demonstrate the poor activity of amphotericin B against *Sporothrix* spp.

We would also like to emphasize the important role of adjuvant therapy such as local destructive methods in keratotic or vegetative lesions. These methods improve skin penetration by antifungal agents and were employed in all patients in this study. The bioavailability of drug in tissues is not related to drug resistance and is an important consideration leading to therapeutic failure. The importance of cryosurgery as an inducer of immunologic response in the treatment of many skin lesions, including infectious diseases, is also well documented.[36]

There is no breakpoint values determined for *Sporothrix*.[19] Therefore, the strains were not categorized according to their susceptibility status, but it could be observed that two of three patients from the study initially treated with itraconazole 100 mg/day presented itraconazole MIC of 1.0 mg/l, which suggests susceptibility. The MIC values for itraconazole found in this study were higher than other previous publications in this field, which include strains from several Brazilian states and not only Rio de Janeiro. However, our results are comparable with some single studies. For instance, MIC50 and MIC90 values to itraconazole found by Rodrigues and collaborators[24] are equal to MIC50 and MIC90 values for itraconazole reported in this study.

A study[23] about antifungal susceptibilities of *S. brasiliensis*, *S. globosa*, and *S. schenckii* observed resistance to itraconazole, that is, MICs equal to or more than 4.0 mg/l, for *S. globosa* (one strain) and *S. schenckii* (four strains), while all *S. brasiliensis* strains presented MICs less than 4.0 mg/l, as we observed in our study. The management of refractory patients to itraconazole in this study was similar to cases reported elsewhere.[9,10] Refractory sporotrichosis due to *S. globosa* and *S. schenckii* need to be further studied, to check if these cases are caused by strains harboring resistance mechanisms against this azole agent.

The CLSI document for antifungal susceptibility of filamentous fungi that includes *Sporothrix* does not correlate yet with clinical course as it is for yeasts. Moreover, the CLSI document for antifungal susceptibility of yeasts emphasizes that it should not be used with the dimorphic fungi. During parasitism, all species of the *S. schenckii* complex assume a yeast morphology, and therefore the MIC determinations using the filamentous form of the fungus could bring a bias to the analysis. In fact, susceptibilities of *Sporothrix* to antifungals differ between morphotypes.[37] Since antifungal susceptibilities of the strains are important aspects of the therapeutic failure, more studies are necessary in order to improve the MIC determination of the *S. schenckii* complex.

Among the azoles, posaconazole was the most effective drug, with the lowest MIC90 and geometric mean. Three other studies also have shown the good *in vitro* efficacy of posaconazole to *S. brasiliensis*,[15, 21, 24] with no statistical differences between MICs. Posaconazole is a second generation of triazoles derived from itraconazole, it has a broad spectrum of activity over a great number of fungi, and has good penetration in bone and in the central nervous system.[35] However, only a few sporotrichosis cases have been treated with posaconazole and they did not it solely as the first drug choice.[35, 38] A mouse model of sporotrichosis showed a good response of posaconazole, despite the MIC of the strain used to inoculate the animals, within the 0.5–2.0 mg/l range.[39] Therefore, the good *in vitro* efficacy of posaconazole to *S. brasiliensis* support previously published data, encouraging clinical trials confirming its efficacy in human sporotrichosis and its use in the management of difficult cases of this mycotic infection. In patient 7, the use of posaconazole for 2 months coincided with fungal clearance from bimonthly CSF cultures and a sustained response for 10 months.

Voriconazole displayed the most variable MICs among the drugs tested. It is worth mentioning that in one case (patient 6), all strains had high MICs ($\geq$16 mg/l) and in another case (patient 7), all strains were more susceptible with MICs of 2.0 mg/l, thereby reinforcing a stable *Sporothrix* phenotype during prolonged infections. A mouse model of sporotrichosis also showed a lack of *in vivo* efficacy for voriconazole against *S. brasiliensis*.[40] These results coupled with the high MICs observed in this and other studies[14–16, 21] discourage the use of voriconazole in the treatment of sporotrichosis, especially when caused by *S. brasiliensis*.

Amphotericin B is used in the treatment of severe cases of sporotrichosis, like osteoarticular, pulmonary, meningeal, or systemic forms of this disease.[5] Only one patient of this

study, with systemic sporotrichosis, was treated with amphotericin B, making it difficult to assess the activity of this polyene agent against *S. brasiliensis* and the clinical response. This patient had a MIC of 2.0 mg/l for all of her strains, which was analytically interpreted as intermediate.[19] Despite the fact that MICs for amphotericin B in this study are higher than those published by other authors, our MIC50 and MIC90 values are similar to two studies retrieved in the literature search.[15,24]

Despite antifungal susceptibilities of the strains, there are other reasons for unresponsiveness to treatment that do not involve just this parameter but also drug diffusion and bioavailability, host factors such as an intrinsic higher metabolism through specific cytochrome P450 routes, or patient disturbance in drug absorption. The results of the present study suggest that unresponsiveness to sporotrichosis treatment could be multifactorial, and more studies are necessary to clarify the mechanisms that lead to therapeutic failure in this mycosis.

The results do not support *in vivo* development of resistance in human sporotrichosis to the six tested drugs, since MICs from different strains from the same patient present are equal or present a one to two dilution difference, which is inherent to the methodology. However, currently we do not know if a similar scenario occurs in feline sporotrichosis that is ongoing in Rio de Janeiro, Brazil. Pathogenesis of sporotrichosis in cats and humans are very different and the number of yeast cells present on feline lesions is high, contrary to what is observed in human sporotrichosis.[2] Moreover, ketoconazole dosages used in feline sporotrichosis[41] are much higher than those used in the past in the management of severe forms of human sporotrichosis.[42] These factors could promote the development of *in vivo* resistance to ketoconazole in cats infected with *S. brasiliensis*. The samples obtained here were isolated from patients living in the endemic area of zoonotic sporotrichosis with contact with cats, and their MICs do not suggest resistance to ketoconazole.

Resistance of *S. brasiliensis* to some antifungal drugs such as amphotericin B and terbinafine may be enhanced by melanins produced by the fungus.[43,44] The CLSI M38-A2 protocol does not detect efficiently the melanin-driven resistance to the antifungal drugs. Therefore, one explanation for the refractoriness of patients 4, 5, and 6, initially treated with terbinafine, and patient 7 treated with amphotericin B could be the increased melanization of the strains isolated from these cases. Studies are under way to investigate this hypothesis.

In conclusion, the results suggest that that acquisition of *in vivo* resistance during prolonged antifungal treatment is not the cause of the refractoriness of human sporotrichosis due to *S. brasiliensis*, and the *in vitro* antifungal susceptibility determined by the M38-A2 protocol does not successfully predict therapeutic failure in human sporotrichosis, especially for terbinafine. The isolates, collected from 2000 to 2013 at the Rio de Janeiro hyperepidemic area of zoonotic sporotrichosis, have similar susceptibility profiles with a previous study using strains from the same geographic area collected from 1998 to 2004, suggesting maintenance of the susceptibility profile of the human sporotrichosis cases. Other studies in this field are strongly encouraged for a better comprehension of the clinical, therapeutic, and virulence aspects of the species of the *S. schenckii* complex.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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