Characterization of virulence profile, protein secretion and immunogenicity of different *Sporothrix schenckii sensu stricto* isolates compared with *S. globosa* and *S. brasiliensis* species

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Keywords: *Sporothrix schenckii* complex, virulence, sporotrichosis, antigens, western blotting, protein secretion, immunogenicity

A comparative study about protein secretion, immunogenicity and virulence was performed in order to characterize and to compare eight *Sporothrix schenckii sensu stricto* isolates. For virulence characterization, a murine model, based on survival assay and CFU counting was used. *S. brasiliensis* and *S. globosa*, a highly virulent and a non-virulent isolates, respectively were used as external controls. Exoantigen profiles showed different secreted molecules; the 46- and 60-kDa molecules were commonly secreted by all three species. The *S. schenckii* s. str. isolates could be classified as non-virulent or presenting low, medium or high virulence, based on survival times after infection and recovery of viable fungi. The humoral response profiles of mice infected with *S. schenckii* s. str., *S. globosa* and *S. brasiliensis* were heterogeneous; five virulent isolates (*S. schenckii* s. str., n = 4 and *S. brasiliensis*, n = 1) had in common the recognition of the 60-kDa molecule by their respective antisera, suggesting that this antigen may be involved in virulence. Furthermore, the 110-kDa molecule was secreted and recognized by antisera from four virulent isolates (*S. schenckii* s. str., n = 3 and *S. brasiliensis*, n = 1), so there is a possibility that this molecule is also related to virulence. Our findings reveal different degrees of virulence in *S. schenckii* s. str. isolates and suggest the correlation of protein secretion and immunogenicity with virulence of *S. schenckii* complex. These findings provide new insights into the pathogenesis of *S. schenckii* s. str. and improve the knowledge about immunogenicity and protein profiles in *S. schenckii* complex.

Introduction

The genus Sporothrix consists of a rich-species taxon of Ascomycete, which vary according to the ecological niche, frequency, distribution and virulence.1-4 Some of these fungi present potential to survival in mammalian hosts being able to cause damage in multiple species of animals including humans. Since 1898 when the etiological agent of sporotrichosis, *Sporothrix schenckii sensu lato* (s.l.), was described,5,6 many studies have demonstrated high genetic diversity among different clinical isolates.7-9 Marimon et al. (2006)10 demonstrated that the fungus was actually a complex of cryptic species,10 afterward separated into different phylogenetic species11,12 including *S. schenckii* s. str., *S. brasiliensis*, *S. globosa*, *S. mexicana* and *S. luriei*. In addition, *S. pallida* (formerly *S. albicans*) is a non-pathogenic species closely related to *S. schenckii*.13 Sporotrichosis is the most common subcutaneous fungal infection found in South America and is usually acquired by traumatic inoculation of saprophytic fungal propagules into subcutaneous tissue, where this thermomorphic fungus changes its morphology to the parasitic yeast form.14,15 In Brazil, the incidence of sporotrichosis is on rise over the last two decades and it has been attributed to zoonotic outbreaks in cats.16-19 Patients may present several different clinical forms ranging from fixed cutaneous and lymphocutaneous to disseminated form.20 Disseminated sporotrichosis is the most severe infection that has been reported with increasing frequency in recent years mainly in immunosuppressed patients.21-26

Many studies have been conducted to better understand the pathogenesis of sporotrichosis in experimental infection. The decrease in cellular immune response27 and IL-1 and TNF-α levels28 between the fourth and sixth week post-infection were reported, thereby providing the colonization of some organs of infected mice by the fungus.29 The IL-4 interleukin is
predominantly produced in the fifth week, suggesting the prevalence of humoral immune response in the advanced infection.  

On the other hand, other studies showed the differences in *Sporothrix* virulence and the clinical presentation of the disease, geographic origin, genotype and thermotolerance. The time of cultivation of yeast and mycelial cells of *S. schenckii* induces changes in cell wall composition, causing a decrease of pathogenic power. Some comparative studies were conducted correlating the route of experimental infection or clinical manifestation in humans with the virulence degree of different isolates. Recently, Arrillaga-Moncrieff et al. showed different degrees of virulence among *S. schenckii* s. str., *S. brasiliensis* and *S. globosa* species.

The aim of this study was to characterize the virulence of eight *S. schenckii* s. str. isolates compared with *S. brasiliensis* and *S. globosa* isolates by the combination of data from CFU assays, survival times and immunoblot assays. Also, the protein secretion of these different isolates will be correlated with humoral response of infected mice in order to find possible antigenic molecules that could be proposed as a possible marker of virulence.

**Results**

Phenotypic and genotypic characterization of *Sporothrix* spp isolates. The macroscopic and microscopic morphologies of the *S. brasiliensis*, *S. globosa* and *S. schenckii* s. str. isolates identified herein were very similar to the corresponding reference type strains CBS 120339, CBS 120340 and CBS 359.36, respectively. The only discrepancy that we found was for the isolate Ss51, which presented similarity to the physiological profile of *S. brasiliensis*, once it did not assimilate sucrose; nevertheless it was identified as *S. schenckii* s. str. by phylogenetic analysis (described below). Due to this divergence, the phylogenetic analysis was considered to be more consistent for identification of *Sporothrix* species.

The amplified CAL genes and ITS region yielded DNA fragments of approximately 800bp and 620bp in size, respectively. BLAST searches using CAL and ITS region sequences as a query revealed that seven Brazilian isolates (Ss16, Ss39, Ss40, Ss47, Ss51, Ss126, Ss141 and Ss143) had a high level (99–100%) of similarity with *S. schenckii* reference strains. The exception was Ss51, which had 96% similarity with *S. brasiliensis* IPEC 17920, AM116888. Ss06 (CBS 132922) had high similarity with the type strain of *S. globosa* (99%, AM116908) and Ss54 (CBS 132990) with *S. brasiliensis* (100%, AM116899).

Similarity of CAL gene and ITS region among isolates. The CAL and ITS sequences used as reference for phylogenetic analysis were selected based on sequences deposited by De Beer et al., Kawasaki et al., Watanabe et al., Aghayeva et al., Marimon et al., Gujari et al., De Meyer et al., Madrid et al., Romeo et al. and Rodrigues et al. Both sequences, ITS and CAL loci of the same reference isolate, were not always available, thus, the data were analyzed separately.

The 55 sequences used in CAL data set (Fig. 1A) were distributed into six main groups, all of them identified in previous studies. Clades 1–3 and 6 (*S. brasiliensis*, *S. schenckii*, *S. globosa* and *S. luriei*, respectively) are largely composed of pathogens of mammalians whereas groups 4 and 5 (*S. mexicana* and *S. pallida*, respectively) are mainly recovered from environmental samples.

The 39 sequences used in ITS data set (Fig. 1B) were distributed into 13 main groups, comprising the same four clinical clades described in the CAL data set, as well as the close related environmental *Sporothrix* species complex and *Ophiostoma stenoceras* isolates.

The eight isolates (Ss16, Ss39, Ss40, Ss47, Ss51, Ss126, Ss141 and Ss143) clustered significantly into the *S. schenckii* clade in both analyses. The phylogenetic analysis showed that Ss51 co-clustered in the *S. schenckii* s. str. clade (reference strain CBS 359.36) in both analyses, confirming its identification as *S. schenckii* s. str. despite physiological characteristics that were more similar to *S. brasiliensis* as described above.

The Brazilian isolate Ss06 (CBS 132922) clustered with the *S. globosa* type strain (CBS 120340) described by Marimon et al., while the feline isolate Ss54 (CBS 132990) clustered with the *S. brasiliensis* reference strains.

Survival of BALB/c mice following inoculation with *Sporothrix* spp yeast cells. Different times of survival were observed among animals infected with *S. schenckii* s. str. isolates. The respective survival curves are shown in Figure 2. All the animals infected with the Ss39, Ss47 and Ss143 isolates survived until the end of the experiment as observed for animals infected with Ss06 (CBS 132922) (*S. globosa*, control group of non-virulence profile). The isolates Ss16, Ss51 and Ss126 caused the death of all of the mice between fourth and sixth week and Ss141 isolate killed the infected animals at fourteenth week. There were no significant differences (*p* > 0.05) among Ss16, Ss51, Ss126 and Ss141 but they were statistically different from Ss40 and Ss54 (CBS 132990) (*S. brasiliensis*, control group of high virulence profile) which killed mice in the second week of infection (*p* < 0.05). All controls survived until the end of the experiment (data not shown).

Number of viable *Sporothrix* spp cells recovered from organs. Fungal tissue burden results are shown in Figure 3. All virulent isolates colonized lungs and spleen. The fungal loads found in spleen and lungs of mice infected with *S. brasiliensis* (Ss54, CBS 132990) were significantly higher (*p* < 0.05) than that of *S. schenckii* s. str. and *S. globosa* (Ss06, CBS 132922). The fungal loads of spleen and lungs of Ss16, Ss40 and Ss51 were significantly different of Ss47 and Ss126. Interestingly, Ss16 showed remarkable difference of colonization of liver, being significantly higher than Ss54 (CBS 132990) (*S. brasiliensis*) and other *S. schenckii* s. str. isolates and *S. globosa* (*p* < 0.05). *S. globosa* were not recovered from any evaluated organs. Taken together the data of survival and CFU (colony forming units) analysis, we classified the virulence degree of each isolate of *S. schenckii* s. str. based on the profiles of control isolates (Ss54, CBS 132990, and Ss06, CBS 132922). These data showed that *S. schenckii* s. str. isolates present different degrees of virulence. Thus, Ss40 and Ss126 isolates were considered more virulent among *S. schenckii* s. str. isolates, followed by Ss16 and Ss51 as medium virulence, Ss47 and Ss141 as low virulence and Ss39 and Ss143 as non-virulent. No fungal load was observed in organs from
molecules were commonly produced, 46-kDa was immunogenic only for Ss16 and 35-kDa was not recognized by any antisera. Interestingly, all isolates that secreted the 110-kDa molecule had animals that received sterile PBS (data not shown).

Protein/glycoprotein profile of exoantigens of S. schenckii s. str., S. brasiliensis and S. globosa. The protein profiles of exoantigens obtained from S. schenckii s. str., S. brasiliensis and S. globosa isolates were heterogeneous (Fig. 4A and Table 1). Figure 4A shows the molecules secreted by each isolate. The 60- and 46-kDa molecules were secreted by all isolates, including S. brasiliensis and S. globosa isolates, and 35-kDa, 80-kDa and 90-kDa molecules were secreted by most of them (Fig. 4A). As shown in Figure 4, there was no association between the profile of the secreted molecules and the fungal species or origin.

Western blot: antigen recognition by immune sera. The molecules present in the exoantigens obtained from each isolate and recognized by the respectively antisera from infected mice are shown in Figure 4B and described in Table 1. In spite of the 35-kDa and 46-kDa molecules were commonly produced, 46-kDa was immunogenic only for Ss16 and 35-kDa was not recognized by any antisera. Interestingly, all isolates that secreted the 110-kDa molecule, had

Figure 1. Molecular phylogenetic tree generated in this study by Maximum Likelihood based on Kimura 2- parameter (K2P + G) for CAL data set (A) and Tamura 3-parameter (T92 + I) for ITS1/2 + 5.8s data set (B) of S. schenckii complex species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Bootstrap support values > 70% are indicated in bold). GenBank accession numbers are indicated next to strain code. Strains used in this study are indicated in bold type.

Figure 2. Survival of mice infected with Sporothrix spp. p < 0.05 for Ss54 (CBS 132990) and Ss40 vs. other isolates; p < 0.05 for Ss16, Ss51, Ss126 and Ss141 vs. other isolates and p < 0.05 for Ss143, Ss47, Ss39 and Ss06 (CBS 132922) vs. other isolates.
it recognized by the respective antiserum, including \textit{S. brasiliensis} isolate. All these isolates presented some pathogenic power. On the other hand, 90-kDa molecule was secreted by six \textit{S. schenckii} isolates, \textit{S. globosa} (Ss06) and by \textit{S. brasiliensis} (Ss54), but only five antisera were able to recognize this molecule, one of them belongs to the non-virulent isolate (Ss143). The 60-kDa molecule was secreted by all isolates and was recognized by four antisera from mice infected with isolates of \textit{S. schenckii s. str.} that presented some virulence power (Ss16, Ss40, Ss126 and Ss141) and \textit{S. brasiliensis} isolate (Ss54, CBS 132990). This molecule was not recognized by antisera from non-virulent isolates (Ss143 and Ss39) and \textit{S. globosa} isolate (Ss06, CBS 132922). Exceptionally, Ss51, a medium virulent isolate, had no molecule recognized by antisera from mice.

**Discussion**

Recently, several authors proposed that \textit{Sporothrix schenckii} is indeed a complex of different species that are phylogenetically related and fungi belonging to this genus share typical phenotypic characteristics: \textit{S. brasiliensis}, \textit{S. globosa}, \textit{S. mexicana}, \textit{S. schenckii}, \textit{S. luriei}, \textit{S. pallida}, \textit{S. humicola}, \textit{S. styloides}, \textit{S. lignivora}, \textit{S. brunneoviolacea} and \textit{S. dimorphospora}.\textsuperscript{11,12,41,42} Some studies have investigated the protein secretion profile of \textit{Sporothrix},\textsuperscript{43,44} the genetic variability,\textsuperscript{11,12,41,42} as well as virulence of some new species.\textsuperscript{2,3} This is the first study which evaluates the protein secretion of \textit{S. brasiliensis}, \textit{S. globosa} and \textit{S. schenckii s. str.} and connects the humoral immune response to virulence of these species.

The taxonomy was accessed by phenotyping and genotyping assays, using the key features for \textit{Sporothrix} species differentiation previously proposed by Marimon et al.\textsuperscript{11,12} and partial sequencing of the \textit{CAL} gene and \textit{ITS} region. We, thus, identified one isolate of \textit{S. brasiliensis} (Ss54, CBS 132990) and one isolate of \textit{S. globosa} (Ss06, CBS 132922), as well as eight isolates of \textit{S. schenckii} (Ss16, Ss39, Ss40, Ss47, Ss51, Ss126, Ss141 and Ss143). The characteristics of these species are in agreement with those proposed by Marimon et al.,\textsuperscript{11} except for Ss51, which was identified as \textit{S. schenckii} by phylogenetic analysis but did not assimilate sucrose, a key physiological characteristic of \textit{S. brasiliensis} species. The results of our phylogenetic analysis of the \textit{CAL} locus are in agreement with those obtained by others.\textsuperscript{11-13} The phylogenetic relationship using ITS sequence was established among our clinical isolates and some new environmental species recently proposed in the literature belonging to the genus \textit{Sporothrix}. \textit{Sporothrix schenckii s. str.} formed a well-supported cluster close related to its sister taxa \textit{S. brasiliensis}, \textit{S. globosa} and

![Figure 3](image-url)

*Figure 3.* Colony count (CFU/g) comparison among different strains of \textit{S. schenckii s. str.}, and \textit{S. brasiliensis} or \textit{S. globosa} in spleen (A), lungs (B) and liver (C) obtained from infected BALB/c mice. Wide bars represent the mean CFU/g of tissue recovered from five mice and thin bars represent the standard deviation. *p < 0.05.
isolates (Ss16, Ss40, Ss47 and Ss126) could colonize lungs and spleen and part of them (Ss16, Ss40 and Ss51) were able to disseminate and colonize liver but in low intensity which was similar to *S. brasiliensis* (Ss54, CBS 132990) colonization. The exception was only Ss16, which fungal loads of liver were significantly higher than Ss54. Based on control group profiles of CFU and survival analysis, Ss40 and Ss126 were classified as high virulence, followed by Ss16 and Ss51 as medium virulence, Ss47 and Ss141 as low virulence and Ss143 and Ss39 as non-virulent isolates. Although Ss40 and Ss126 isolates were considered as high virulent among *S. schenckii s. str.* isolates, they were less virulent than *S. brasiliensis* (Ss54, CBS 132990) isolate. In general, the virulence profiles of isolates obtained by CFU and survival assay.
were in agreement, except for Ss40, Ss47 and Ss141. The Ss40 isolate induced medium fungal loads of all the analyzed organs but the higher inoculum of yeast cells it could induce high mortality as S. brasiliensis (Ss54, CBS 132990). High fungal loads of Ss47 of lungs and spleen were recovered but all infected mice survived to the end of the survival experiment. Taken together, these data show that Ss47 isolate is able to infect mice but these animals, over a long time, can resolve the infection. Ss141 isolate had the opposite behavior, being unable to colonize any analyzed organ, but was able to cause infection when a high inoculum was employed, killing infected animals. These data suggest that a high inoculum of this isolate and a longer infection time is necessary for the consolidation of infection.

In our previous study about virulence factors of Sporothrix spp.,9 Ss126 (S. schenckii s. str.) showed proteinase, caseinase, gelatinase, urease and DNase activities. On the other hand, Ss54 (CBS 132990) showed DNase and urease activity.9 Taken together, the data of virulence factors9 and virulence profile (current study), Ss54 expressed less virulence factors9 than Ss126, but it is more virulent; showing that the mechanisms of pathogenesis are much more complex, involving these virulence factors and others molecules for evasion of immune system.

We found that the 60- and 46-kDa molecules were secreted by all of the isolates, including S. brasiliensis and S. globosa isolates, and thus probably represent important components that are common to all of the studied species. However, the 60-kDa molecule was recognized by five different antisera (one anti-S. brasiliensis and four anti-S. schenckii sera), and the 46-kDa molecule was recognized by only one antiserum (anti-S. schenckii). All isolates that had the 60-kDa molecule recognized by antisera from infected mice could kill them. Ss51 (S. schenckii) isolate was able to kill infected animals, but no molecules were recognized by antiserum from them. This isolate secreted the 60-kDa molecule, but the molecule was not recognized by the corresponding antiserum; however it was able to kill infected mice. One hypothesis that may explain this behavior could be related to possible post-translational modifications in the 60-kDa molecule among different isolates and thus its antigenicity varies, with some isoforms not eliciting a humoral response in infected mice. On the other hand, sera from mice infected with non-virulent isolates did not recognize the 60-kDa molecule. These data suggest that the 60-kDa molecule may have an influence on the virulence of the isolate that is independent of the species. Further studies are required to investigate the association between this molecule and fungal virulence. The 60-kDa molecule is a possible candidate for being the immunodominant molecule in the S. schenckii complex, although, future studies should be conducted to confirm this hypothesis.

Notwithstanding, the 90-kDa molecule was secreted by eight isolates, only five antiseras recognized it. Moreover, the antiserum from Ss143 (non-virulent isolate) recognized this molecule, therefore, this molecule should not be crucial in the pathogenesis. The 110-kDa molecule was secreted by four isolates, and was recognized by their respective antiseras. All of these isolates were virulent, so there is a possibility that this molecule is also related to mechanism of virulence.

It is important to know the virulence profile and immunogenicity of the S. schenckii complex for understanding the pathogenesis of sporotrichosis. This is the first study that shows different degrees of virulence among S. schenckii s. str. isolates and a common immunogenic molecule of the S. schenckii complex secreted by most of virulent isolates of S. schenckii and S. brasiliensis. These data bring new insights into the pathogenesis of S. schenckii complex.

Table 1. Analysis of Sporothrix spp antigens

| Isolate            | Molecules (kDa) secreted and recognized (bold) by antiseras |
|--------------------|-------------------------------------------------------------|
| Ss54 (CBS 132990)  | 110, 90, 80, 60, 46, 38, 35, 28                             |
| Ss16               | 110, 90, 80, 60, 46, 38, 35, 30, 28                         |
| Ss40               | 110, 90, 80, 60, 50, 46, 35                                 |
| Ss51               | 90, 80, 60, 48, 46, 38, 35                                  |
| Ss47               | 110, 90, 80, 75, 60, 50, 46, 35                              |
| Ss126              | 160, 90, 80, 60, 46, 35, 28                                 |
| Ss141              | 160, 120, 90, 75, 60, 46, 35                                |
| Ss143              | 120, 80, 60, 46, 38                                         |
| Ss39               | 120, 80, 60, 46, 38                                         |
| Ss06 (CBS 132922)  | 160, 120, 90, 80, 60, 46, 38, 30, 28                        |

Combined analysis of SDS-PAGE and western blot results.

Characterization of Sporothrix species isolates. The main characteristics of representative Brazilian isolates of S. brasiliensis (Ss54, CBS 132990), S. globosa (Ss06, CBS 132922), and S. schenckii s. str. (Ss16, Ss39, Ss40, Ss47, Ss51, Ss126, Ss141 and Ss143) used in the present study are shown in Table 2.

We evaluated the fungal growth in potato dextrose agar medium (PDA) at different temperatures (30, 35 and 37°C) by colony diameter measurements and carbohydrate (dextrose, sucrose, raffinose, and ribitol) assimilation according to the phenotyping characterization proposed by Marimon et al.11,12 The characteristics of each isolate were used as taxonomic parameters and applied to a dichotomous key of species in the S. schenckii complex. For phenotyping characterization, we used several S. schenckii complex as reference strains which were previously described by Marimon et al.11,12 including S. brasiliensis CBS 120339 and IPEC 16919, S. globosa CBS 120340 and FMR 8595 and S. schenckii CBS 359.36 and UTHSC 99-173.

For molecular characterization, the calmodulin (CAL) gene fragment was amplified directly from genomic DNA using the primers CL1 and CL2A.45 In addition, the ITS region was sequenced using primers ITS1 and ITS4, which amplify the 18S, ITS1, 5.8S, ITS2 and 28S46 in order to verify if these isolates were similar to other environmental Sporothrix isolates described by De Meyer et al.41 and Madrid et al.42

Amplified products were gel purified with the Wizard SV Gel and PCR Clean-Up System (Promega Corp.), following
the manufacturer’s instructions. DNA samples were completely sequenced with a MegaBACE 1000 DNA Sequencer (Amersham) using the DYEnamic ET Dye Terminator Kit (with Thermo Sequenase II DNA Polymerase). The fragments were sequenced on both strands to increase the quality of sequence data (phred > 30). Sequence alignment was performed using the ClustalW algorithm implemented with BioEdit software; retrieved alignments were manually corrected to avoid mispaired bases. Nucleotide sequences were exported as FASTA file for BLAST search (www.ncbi.nlm.nih.gov/BLAST).

**Phylogenetics analysis.** Calmodulin sequences (n = 55) from other isolates of *S. schenckii* (n = 25), *S. brasiliensis* (n = 8), *S. mexicana* (n = 5), *S. globosa* (n = 12), *S. pallida* (n = 4) and *S. luriei* (n = 1), as well as ITS sequences (n = 39) from *S. schenckii* (n = 15), *S. brasiliensis* (n = 4), *S. mexicana* (n = 1), *S. globosa* (n = 4), *S. pallida* (n = 1), *S. luriei* (n = 1), *S. infitala* (n = 1), *S. dimorphophora* (n = 2), *S. stylices* (n = 2), *S. humicola* (n = 2), *S. brunneoviolacea* (n = 2), *S. lignivora* (n = 2) and *Ophiostoma stenoceras* (n = 2) were included in the present study as reference strains for the phylogenetic analysis. These sequences were previously deposited at GenBank (www.ncbi.nlm.nih.gov/genbank) and described by De Beer et al., Kawasaki et al., Watanabe et al., Aghayeva et al., Marimon et al., Gujari et al., De Meyer et al., Madrid et al., Romeo et al. and Rodrigues et al. Evolutionary analyses were conducted in MEGA5 with Maximum Likelihood method. Evolutionary distances were computed using the Kimura 2-parameter method with a discrete Gamma distribution for the CAL data set and Tamura 3-parameter method for ITS data set, with 1,000 bootstrap replicates.

**Exoantigens preparation.** Exoantigens from mycelial phase of *S. schenckii* s. str., *S. globosa* and *S. brasiliensis* isolates were obtained as previously described using Sabouraud medium (Difco). Protein content was determined according to the Bradford method.

**Experimental infection in mice.** Preparation of Sporothrix spp yeast cells for inoculum. Our methods were based on those described by Carlos et al. Each isolate was initially grown in brain-heart-infusion agar slants (BHI) (Difco) until complete reversion to the yeast phase. Then, the total growth of three slants was transferred to 300 ml of BHI broth and incubated at 37°C for 7 d in a rotatory shaker with constant agitation (100 rpm/min). Yeast cultures were washed three times with phosphate-buffered saline (PBS), centrifuged at 700 × g for 15 min, and finally suspended in sterile PBS. The yeast cells concentration was adjusted to 1 × 10⁹ or 5 × 10⁶ after counting with a hemocytometer. The viability of these inocula was verified by plating dilutions of the suspension on BHI plates.

**Virulence assays.** Animals. A total of 165 male, 6–8 weeks old BALB/c mice, were purchased from Federal University of São Paulo (UNIFESP) for virulence assays of which 55 mice were used for CFU assay and 110 mice were used for survival assay. Mice were housed in temperature-controlled rooms at 23–25°C with ad libitum access to food and water. All the procedures were approved by UNIFESP Ethics Committee and are in accordance with the National Institutes of Health Animal and Care Guidelines.

**Mice survival assay.** Male BALB/c mice (n = 110) were divided into 11 groups of ten mice each (one group for each fungal strain and one negative control group). The mice were anesthetized with 0.2 mg/kg xilazine and 20 mg/kg ketamine and inoculated i.v. with 5 × 10⁶ fungal yeast cells/animal. Control groups were injected with 100 μl of PBS. Mice were observed daily over 31 weeks, and deaths were recorded daily.

**CFU analysis and sera obtaining.** Male BALB/c mice (n = 55) were divided into 11 groups of 5 mice per group (one group for each fungal strain and one negative control group). The mice were anesthetized with 0.2 mg/kg xilazine and 20 mg/kg ketamine and inoculated intravenously (i.v.) with 1 × 10⁶ yeast cells/animal. Animals from negative control group received 100 μl of sterile PBS. On the fifth week after infection, the animals were sacrificed by CO₂ anesthesia, and the spleen, lungs and liver were aseptically removed. The organs were separated, weighed and homogenized in sterile PBS using a tissue grinder. Samples (100 μl) of each homogenate were seeded on Petri dishes containing BHI agar and incubated at 37°C. Colonies were counted.

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### Table 2. Characteristics of *Sporothrix* spp isolates

| Isolate | Species | Geographic origin | Source | GenBank accession number |
|---------|---------|-------------------|--------|--------------------------|
| Ss54 (CBS 132990) | *S. brasiliensis* | Rio Grande do Sul (South) | Cat | JQ041903 JN885580 |
| Ss06 (CBS 132922) | *S. globosa* | Minas Gerais (Southeast) | Human infection (LC) | JF811336 JN885574 |
| Ss16 | *S. schenckii* | Piauí (Northeast) | Human infection (LC) | JQ041898 JN885575 |
| Ss39 | *S. schenckii* | Paraná (South) | Human infection (FC) | JQ041899 JN885576 |
| Ss40 | *S. schenckii* | Ceará (Northeast) | Human infection (FC) | JQ041900 JN885577 |
| Ss47 | *S. schenckii* | Goiás (Midwest) | Human infection (LC) | JQ041901 JN885578 |
| Ss51 | *S. schenckii* | Pará (North) | Human infection (FC) | JQ041902 JN885579 |
| Ss126 | *S. schenckii* | São Paulo (Southeast) | Human infection (FC) | JQ041904 JN885581 |
| Ss141 | *S. schenckii* | Distrito Federal (Midwest) | Human infection (LC) | JQ041905 JN885582 |
| Ss143 | *S. schenckii* | Pará (North) | Human infection (LC) | JQ041906 JN885583 |

Isolates belonging to the *S. schenckii* complex included in the study, according to Brazilian geographic origins, clinical sources and GenBank accession numbers. FC, fixed cutaneous sporotrichosis; LC, lymphocutaneous sporotrichosis; CAL, calmodulin; ITS, internal transcribed spacer.
from the seventh day until the fifteenth day. The results were expressed as colony-forming units (CFU)/g tissue. Sera from mice were collected and kept at –20°C for western blot assay.

Statistical analysis. For CFU assays, comparisons between groups were analyzed by analysis of variance (ANOVA) followed by post-hoc Tukey, with significance assumed to be p < 0.05. For survival assays, data were analyzed by Kaplan-Meier survival plots followed by log-rank test. Data with a p value < 0.05 or less were considered to be significant.34

SDS-PAGE and western blot assay. Exoantigens (2 μg of protein) were analyzed by SDS-PAGE using 10% gels, as described by Laemmli et al. The SDS-PAGE was stained with silver stain, and the relative molecular weights of the fractions were estimated using standard broad range molecular weight markers (Benchmark Invitrogen). For western blot assay, 5 μg per slot of protein were applied into SDS-PAGE and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.) (0.45-μm pore size) in a transblotting chamber (Biorad MiniPROTEAN Tetra Cell, Bio-Rad Laboratories, Inc.) using Tris-glycine-methanol buffer (pH 8.3)56 for 16 h at 14 mA at 4°C. The membranes were incubated in PBS containing 1% bovine serum albumin for 4 h at 37°C. The membrane was then cut lengthwise into 0.5-cm strips, and each strip was placed in a separate tray and incubated with 1:50 dilution (in PBS-Tween 20, 0.005%) of different sera for 1 h at room temperature (RT). The assay was done with sera from all of the mice from all of the experimental groups (serum vs. exoantigen from the same isolate). The membrane strips were washed three times in PBS-Tween 20, 0.005%) and then incubated for 1 h at RT with a 1:1,000 dilution of peroxidase-conjugated IgG goat anti-mouse IgG (Sigma-Aldrich Co.). After additional washes, the membrane strips were incubated with a substrate that consisted of 5 mg of DAB (3,3'-diaminobenzidine), 50 ml of 1 × PBS and 90 μl of hydrogen peroxide. After color development, the membrane strips were washed with distilled water and dried. Table 1 presents a combined analysis of SDS-PAGE and western blot results.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
This study was supported by FAPESP (2009/54024-2), G.F.F. (2011/01628-8) and A.M.R. (2011/07350-1) are fellows of FAPESP.

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