Susceptibilities to antifungal agents of *Sporothrix albicans*, *S. brasiliensis* and *S. luriei* of the *S. schenckii* complex identified in Brazil


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Abstract

We studied 40 strains of the species complex formerly classified as the single species *Sporothrix schenckii* to identify new species within this complex and evaluate their antifungal susceptibility profiles. Based on phenotypic tests (ability to grow at 37 °C, colony diameters, pigmentation of the colonies, as well as assimilation of sucrose and raffinose) and molecular assays (amplification of a fragment of the calmodulin gene) here we report the identification of *S. albicans*, *S. brasiliensis*, *S. luriei*, and *S. schenckii*; two of them were detected as itraconazole-resistant strains.

Sporotrichosis is a subcutaneous mycosis affecting humans and animals caused by *Sporothrix schenckii*. It has a worldwide distribution, especially in tropical and subtropical areas of Latin America, where endemic areas have been recognized (1, 3, 11, 12). Recently, Marimon et al. (8, 10) proposed that *Sporothrix schenckii* is a complex encompassing six cryptic species that had been previously identified by others (12). In this context, variation in the antifungal susceptibility profiles among these new species was hypothesized. The aim of this study was to explore a collection of 40 isolates formerly classified as *Sporothrix schenckii* in order to identify new species and evaluate their susceptibility to antifungal agents.

The isolates were from cases of human (n = 31) and animal (n = 9) sporotrichosis diagnosed in the hinterlands of Rio Grande do Sul (Brazil) and were maintained in the Department of Microbiology of the UFSM, Santa Maria, Brazil. Among the human-derived strains, 18 (58.06%) were from fixed cutaneous sporotrichosis and 13 (41.9%) were from lymphocutaneous form of the mycosis. Of the strains isolated from animals (n=9), eight were from cats and one (*S. luriei*) was isolated from a dog with sporotrichosis. As proposed by Marimon et al. (8, 10), the
phenotypic tests included: the (in)ability to grow at 37 °C on Potato dextrose agar (PDA; HiMedia, Mumbay, India); differing colony diameters (mm) after 21 days of incubation at 30°C on PDA, the pigmentation of the colonies on corn meal agar (CMA), and the assimilation of sucrose and raffinose. The susceptibility tests were conducted according the procedures proposed by the M38-A2 technique (2). For molecular identification, a fragment of the calmodulin gene (CAL) was amplified from genomic DNA using degenerate primers: CL1 (5′-GA(GA)T(AT)CAAAGGAGCTTTCTC-3′) and CL2A (5′-TTTTGCATCATGACTTGGAC-3′) (8). DNA sequencing was performed on the purified amplicons using a MegaBace 500 automatic sequencer. The sequences were aligned against sequences available in GenBank with ClustalX (version 1.8) followed by manual adjustments with a text editor. The phylogenetic analysis was performed with the MEGA (Molecular Evolutionary Genetic Analysis) software version 4.0 (17).

Based on recently proposed procedures (8, 10), we phenotypically identified four species: *Sporothrix schenckii* (n = 37), *S. brasiliensis* (n = 1), *S. luriei* (n = 1) and *S. albicans* (n = 1). The phenotypic identification was easy to perform and was concordant with the molecular findings. The molecular sequencing confirmed 37 isolates as *Sporothrix schenckii*. The GenBank search also revealed three new species: 1) strain PG1 (*Sporothrix brasiliensis*) (GenBank accession HQ404315), 2) strain PG2 (*Sporothrix schenckii var. luriei*) (GenBank accession HQ404316), and 3) strain PG3 (*Sporothrix albicans*) (GenBank accession HQ404317), all showing high bootstrap support values (Figure 1) and corresponding with the species identified in the phenotypic tests. Based on studies of Marimon et al (8) and reinforced by Romeo et al (13), only the sequencing of highly informative genetic loci such as calmodulin-encoding gene is, at present, useful for elucidating relationships and differentiating
among cryptic species of the *S. schenckii* complex. The new species, *S. brasiliensis*, *S. luriei* and *S. albicans*, were respectively isolated from feline, canine and feline sporotrichosis. To date, *S. brasiliensis* has only been associated with human sporotrichosis (8). Table 1 describes the susceptibility parameters, highlighting terbinafine as the most active drug. By restricting the comparisons to studies employing the same susceptibility tests, our results are in accordance with those of Marimon et al. (9) and Silveira et al. (15). Terbinafine, ketoconazole and amphotericin B showed the best activities, while fluconazole (MIC range 32 to 128 µg/ml) and caspofungin (MIC range 8 to 32 µg/ml) were less active. The results of our tests with itraconazole against *S. brasiliensis* and *S. albicans* agreed with the results reported by Marimon et al. (9) but, in general, our *S. schenckii* strains were more susceptible. *S. schenckii* has been reported to show a high MIC to itraconazole by several authors (5, 6, 9). Although breakpoints have not been established for *S. schenckii*, the document M38-A3 (2) suggests that, for analytical purposes, an MIC $\geq 4.0$ µg/ml for itraconazole may be considered resistant for some filamentous fungi. In keeping with this finding the itraconazole-resistant strains (*S. albicans*, *S. luriei*) showed cross-resistance with all other azoles. Kohler et al. (6) and Meinerz et al. (11), prior to the studies of Marimon et al. (8), reported that isolates from animals were more resistant to itraconazole than isolates from humans. This observation was supported by our results because, among nine strains from animals, two showed itraconazole resistance, and among 31 strains from human cases of sporotrichosis, no one showed itraconazole-resistance. In addition, our results indicated the presence the greater proportion of itraconazole-resistant species in animal sporotrichosis (2/9) than in human sporotrichosis (0/31). Although Mann-Whitney test did not show differences between the two groups, the geometric mean showed that in general *S. schenckii* animal-derived isolates were more susceptible than human-
derived isolates. However, when the new *Sporothrix* species (*S. albicans*, *S. brasiliensis* and *S. luriei*) were included the animal-derived strains showed lesser sensibility to azoles than human-derived strains. The MIC values for amphotericin B and terbinafine were similar for both groups of strains. Due to the low number of animal isolates included here, these observations require further studies.

Finally, our findings emphasize two main points: i) *S. luriei* had a remarkable azole resistance, reported here for the first time; ii) the recent studies focusing on the susceptibility of the former *S. schenckii* (7, 15) or the new *Sporothrix* species (8, 9) included strains from different countries. However, here we included only strains isolated in the central region of Rio Grande do Sul State. Even in this limited area, we found a varied susceptibility profile to antifungal agents and detected four of the six new *Sporothrix* species. Therefore, our findings reinforce the importance of identifying *Sporothrix* spp. isolates as proposed by Marimon et al. (8) and of evaluating their susceptibility pattern(s) to better determine the best therapeutic option for each case of sporotrichosis.

**Conflict of Interest:**

The authors have no conflict of interest related to this report.

**References**


2. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing for filamentous fungi; approved


Figure 1. Evolutionary relationship of 15 taxa (linearized).

The evolutionary history was inferred using the neighbor-joining method (14). The optimal tree is shown, with the sum of branch lengths = 0.63953762. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (4). The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood Method (16) and are in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 265 positions in the final dataset. Phylogenetic analyses were conducted in MEGA software version 4.0 (17).
Table 1 – Parameters* of susceptibilities of the new Sporothrix species to antifungal agents.

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Human isolates (n=31)</th>
<th></th>
<th></th>
<th>Animal isolates (n=9)</th>
<th>S. schenckii (n=6)</th>
<th>S. brasiliensis</th>
<th>S. albicans</th>
<th>S. luriei</th>
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<td></td>
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<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>GM</td>
<td>Range</td>
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<td>0.06-2</td>
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<td>6.39</td>
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</table>

ITZ (itraconazole), KTZ (ketoconazole), MCZ (miconazole), V CZ (voriconazole), FLZ (fluconazole), AMB (amphotericin B), CAS (caspofungin).

GM = geometric mean

*all values in µg/ml