Different virulence levels of the species of Sporothrix in a murine model

I. Arrillaga-Moncrieff1, J. Capilla2, E. Mayayo1, R. Marimon2, M. Mariné3, J. Genè3, J. Cano2 and J. Guarro3

1) Pathology Unit and 2) Microbiology Unit, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Tarragona, Spain

Abstract

A comparative study on the experimental pathogenicity of five species of Sporothrix of clinical interest, Sporothrix albicans, Sporothrix brasiliensis, Sporothrix globosa, Sporothrix mexicana, and Sporothrix schenckii sensu stricto, was performed using an immunocompetent murine model. Two strains of each species and two levels of inoculum for each strain (2 × 10^7 and 2 × 10^4 conidia/animal) were tested by intravenous inoculation of mice (ten per group). Mortality was caused by the low inoculum of one strain of S. brasiliensis only, and the high inocula of S. brasiliensis and S. schenckii strains. Other inocula and other species tested did not kill any of the experimental animals. Tissue burden studies showed fungal spread to kidneys, lungs, spleen, brain, and testicles. S. brasiliensis was recovered extensively from all of the studied organs, and S. schenckii and S. globosa were recovered in lower amounts. Histopathological studies revealed differences in the lesions, which ranged from local inflammation with a low number of fungal cells at the injection site in mice infected with S. globosa, to massive infiltration of fungal cells in organs of those infected with S. brasiliensis. Our findings showed that S. brasiliensis and S. schenckii were the most virulent species, and suggest that lesional mechanisms could be species-specific.

Keywords: Murine model, Sporothrix brasiliensis, Sporothrix schenckii, sporotrichosis, virulence

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Corresponding author and reprint requests: J. Guarro, Unitat de Microbiologia, Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Carrer Sant Llorenç 21, 43201 Reus, Tarragona, Spain
E-mail: josep.guarro@urv.cat

Introduction

Sporotrichosis is a chronic subcutaneous disease caused by the dimorphic fungus Sporothrix schenckii, characterized by the development of lymphatic nodules in humans and in some animals [1]. The fungus is found in habitats that are rich in organic matter and in regions with a warm and humid climate [2–4]. The infection is caused by traumatic inoculation of mycelia or conidia from the soil, wood, or plants [5]. Other studies have demonstrated varying mechanisms of infection, such as inhalation, insect bites, scratches by wild or domestic animals, and direct contact with exudates of feline lesions [4,6–10]. S. schenckii had been considered to be a well-defined species, with no significant intraspecific variations, but recent molecular studies have shown that this fungus is a complex of phylogenetic species with different geographical distributions [4,11]. In addition to S. schenckii sensu stricto, three new species, characterized phenotypically, have been proposed to be in the complex: Sporothrix brasiliensis and Sporothrix globosa are associated with human infections, and Sporothrix mexicana has until now only been identified among isolates of environmental origin [12].

Different models, including subcutaneous and intravenous infection in mice [13,14] and subcutaneous infection in hamsters [15], have been used in virulence studies on sporotrichosis. The use of animal models has demonstrated differences in virulence between isolates from environmental and clinical sources as well as differences between isolates of similar origin. Following the proposal of three new species of Sporothrix, it is not known whether the different degrees of severity attributed to S. schenckii correspond to different species of the complex or whether they are caused by different isolates of the same species. We demonstrated previously that the species of the complex showed different antifungal susceptibilities [16]. In the present study, we compared the virulence of different species of the complex in a murine model of disseminated infections.

Materials and Methods

Strains and inocula preparation
Ten isolates of Sporothrix spp. were used in the study, two isolates corresponding to each of the following species:
Sporothrix albicans, S. brasiliensis, S. globosa, S. mexicana, and S. schenckii (Table 1). The isolates were stored on cornmeal agar (30 g of corn, 15 g of agar, 1 L of tap water) slants covered with paraffin oil, and prior to the study they were cultured on potato dextrose agar (PDA) for 5–8 days at 30°C. The inocula were prepared by flooding the surface of the agar plate with sterile saline, scraping the sporulating mycelium with a culture loop, and drawing up the resultant suspension with a sterile Pasteur pipette. The suspensions were then filtered once through sterile gauze to remove hyphae. The conidia suspensions were transferred to 200 mL of potato dextrose broth and incubated in an orbital shaker (150 rpm) at 30°C for 4 days, filtered once through sterile gauze, and centrifuged at 325 g for 10 min. The resulting pellet was washed three times in 0.9% saline by centrifugation (325 g for 10 min), and the conidia concentration was adjusted after counting with a haemocytometer. The viability of these inocula was verified by plating dilutions of the suspension on PDA plates.

Animals
Six-week-old OF–1 male mice (ten per group) (Charles River, Crifa SA, Barcelona, Spain), weighing 28–30 g, were used. Animals were housed five per cage in standard boxes with corncob bedding and free access to food and water. Conditions were approved by the Animal Welfare Committee of the Faculty of Medicine of the University Rovira i Virgili.

Mortality study
Twenty groups of animals were established, one for each isolate and each of the two inocula. Infection was established intravenously with 200 μL of inoculum via the lateral tail vein. For each isolate, two inocula were prepared, i.e. 2 × 10⁷ conidia/animal (high) and 2 × 10⁴ conidia/animal (low), with the exception of S. brasiliensis 1, for which a high inoculum of 1.2 × 10⁸ conidia/animal was prepared. Mortality was recorded daily for 40 days.

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Reference no.</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporothrix schenckii</td>
<td>1</td>
<td>UTHSC 99-173</td>
<td>Biopsy tissue, hand, USA</td>
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<td></td>
<td>2</td>
<td>UTHSC 01-2137</td>
<td>Skin ulcer, USA</td>
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<td>1</td>
<td>CBS 120339</td>
<td>Skin lesion, Brazil</td>
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<tr>
<td></td>
<td>2</td>
<td>IPEC 16919</td>
<td>Skin lesion, Brazil</td>
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<td>1</td>
<td>CBS 120340</td>
<td>Face lesion, Spain</td>
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<tr>
<td></td>
<td>2</td>
<td>MCCCL 220082</td>
<td>Unknown, India</td>
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<td>1</td>
<td>CBS 120341</td>
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<tr>
<td></td>
<td>2</td>
<td>CBS 120342</td>
<td>Carnation leaves, Mexico</td>
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<td>CBS 302.73</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>KMU 4217</td>
<td>Wheat, China</td>
</tr>
</tbody>
</table>

Tissue burden study
Three groups of animals (ten per group) were infected intravenously with 2 × 10⁷ conidia/animal of S. schenckii 1, S. brasiliensis 2, or S. globosa 1, respectively. Twenty days post-infection, the mice were killed by CO₂ anoxia, and spleen, lungs, liver, testicles, kidneys and brain were removed aseptically. Approximately half of each organ was weighed, and homogenized in 0.9% saline; ten-fold dilutions were then placed into PDA for CFU determination.

Histopathology study
Half of each organ, plus skin from the tail, was fixed with 10% buffered formalin. Samples were dehydrated, paraffin-embedded, and sliced into 2-μm sections, which were stained with haematoxylin and eosin, periodic acid–Schiff or Grocott methamine silver, and examined in a blinded fashion by light microscopy.

Statistical analysis
Survival was compared using a two-tailed log-rank test. Organ burden data were log₁₀ transformed and compared by the two-tailed Mann–Whitney U-test, using GraphPad Prism 5 for Windows. p-Values ≤0.05 were considered to be significant.

Results

Mortality
The results show a clear correlation between the size of the inoculum and the mortality rate. When the low inocula were tested, only S. brasiliensis 1 caused the death of all of the mice between days 30 and 37; mice infected with the other isolates were alive at the end of the experiment. All of the animals infected with the high inocula of S. albicans, S. globosa and S. mexicana survived to the end of the experiment, while all of the animals infected with the high inocula of S. brasiliensis and S. schenckii died between days 9 and 23, with no significant differences (p >0.05) (Fig. 1).

Tissue burden
The tissue burdens of the different organs studied were consistent with the results of the survival study. Animals infected with S. brasiliensis or S. schenckii showed the highest fungal loads, and those infected with S. globosa were significantly lower (p <0.001 in all of the organs) (Fig. 2). The liver and testicles were the organs most affected in animals infected with S. schenckii. Fungal burden was significantly higher in animals infected with S. brasiliensis than in those infected with S. schenckii in all studied organs (p 0.043 to <0.001).
Interestingly, *S. brasiliensis* showed marked brain tropism (7.31 ± 0.64 CFU/g), whereas this organ was minimally affected by *S. schenckii* or *S. globosa* (3.84 ± 2.10 CFU/g and 0.74 ± 1.33 CFU/g, respectively). Fifty per cent of the animals infected with *S. globosa* cleared the infection in all of the organs.

**Histopathology**

During the experimental period, mice infected with *S. brasiliensis* or *S. schenckii* showed cutaneous lesions of the tail (Fig. 3a) and orchitis 10 days post-inoculation, whereas those infected with the other three species did not show any apparent lesion with either of the two inocula. Macroscopically, only animals infected with *S. schenckii* showed lesions in internal organs, consisting of abundant but not confluent white nodules, approximately 1 mm in diameter, randomly distributed on parenchyma of liver and spleen (Fig. 3b). An increase in testicular volume was observed, but changes were not noted macroscopically in other organs. Microscopically, all of the organs of mice challenged with *S. schenckii* showed granulomas with a necrotic centre (Fig. 4a), with mature round and immature oval and cigar-shaped fungal cells (Fig. 4b,c). Inside the tissue vessels, some fungal thrombi were present without observable infarction. Although granulomatous lesions affected the paratesticular soft tissue, the testicles did not show any inflammatory response. No lesions were found in brain or meninges. The organs of mice infected with *S. brasiliensis* showed necrotic areas, lacking inflammatory cells, with substitution of tissue by massive infiltration of fungal cells, mostly located in the Kupffer cells (Fig. 4d–f). Paratesticular and meningeal tissues showed granulomatous lesions, with some fungal cells located in the centre of the granuloma.

No inflammation or fungal cells were observed in the organs of mice infected with *S. globosa*. The tails of these mice showed a subcutaneous inflammation with scattered fungal cigar-shaped cells.

**Discussion**

Different studies using animal models have shown differences in virulence between *Sporothrix* isolates, but the causes remain unclear. Studies evaluating the pigmentation, capacity for growth at 37°C and the clinical or environmental origin
of the strains have shown a correlation with virulence. Mesa-Arango et al. [4] found that environmental isolates generally showed higher virulence than isolates from clinical sources, independently of geographical origin. By contrast, Dixon et al. [13] studied clinical and environmental isolates from the largest US epidemic of sporotrichosis, and concluded that isolates with pigmented conidia and that grew at 37°C were equivalently virulent in a murine model, independently of their origin. Our results agree with these findings, as the

S. globosa strains included in our study showed no pigmented conidia, did not grow at 37°C, and were avirulent in our murine model. More recently, it has been demonstrated that isolates from cutaneous infections are more virulent than isolates from fixed infections in a systemic murine model [14,17]. Comparisons with previous studies are difficult to make, as it not known which of the currently accepted species of Sporothrix are being referred to. Our results showed

**FIG. 3.** Ulcerated lesion on tail (a) and presence of nodules in liver (b) of OF-1 mice infected intravenously with $2 \times 10^7$ conidia/animal of Sporothrix schenckii 1.

**FIG. 4.** Histopathological findings in liver of OF-1 mice 20 days after intravenous infection with $2 \times 10^7$ conidia of Sporothrix spp. (a–c) Fungal cells and granulomatous lesions after infection with Sporothrix schenckii 1 (haematoxylin and eosin). (d, e) Massive infiltration of Sporothrix brasiliensis 2 fungal cells replacing hepatocytes. Arrows indicating conidia in the kupffer cells (haematoxylin and eosin). (f) Fungal cells of Sporothrix brasiliensis 2 on liver (periodic acid–Schiff). Magnification ×25 (a, d) and ×400 (b, c, e, f).
significant differences in the degree of virulence between species: *S. brasiliensis* was clearly the most virulent species in terms of mortality, tissue burden, and tissue damage, followed by *S. schenckii* and then *S. globosa*. *S. mexicana* and *S. albicans* showed low or no virulence in our animal model.

Skin, lungs, spleen and testicles have been reported as major organs affected in experimental systemic or subcutaneous infection [14,18]. Our study demonstrated that *S. brasiliensis*, *S. schenckii* and, to a lesser extent, *S. globosa* are able to spread to other organs, such as liver, kidneys, and brain. Interestingly, fungal forms of *S. schenckii* were located at the centre of liver granulomas, and *S. brasiliensis* conidia were found extensively in the Kupffer cells, showing similarity to *Histoplasma capsulatum* infections [19].

Now that differences are known to exist between the different species of the *S. schenckii* complex in their virulence and response to antifungals [16], it is important to differentiate between these species in the clinical setting.

This is crucial for the correct treatment of such infections and for a better understanding of their epidemiology. This task will be greatly facilitated by the use of recently described molecular and phenotypic markers [12].

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**Transparency Declaration**

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**References**


