UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

Tese de Doutorado

CARACTERIZAÇÃO DE ISOLADOS DO COMPLEXO *SPOROTHRIX SCHENCKII* PROVENIENTES DE DIFERENTES ESTADOS BRASILEIROS

Cheila Denise Ottonelli Stopiglia

Orientadora: Maria Lúcia Scroferneker

Porto Alegre
2013

CIP - Catalogação na Publicação

Ottonelli Stopiglia, Cheila Denise
Caracterização de isolados do complexo Sporothrix schenckii provenientes de diferentes estados brasileiros / Cheila Denise Ottonelli Stopiglia. -- 2013.
191 f.

Orientadora: Maria Lúcia Scroferneker.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Medicina: Ciências Médicas, Porto Alegre, BR-RS, 2013.

1. complexo Sporothrix schenckii. 2. identificação molecular. 3. atividade enzimática. 4. sensibilidade in vitro aos antifúngicos. 5. leveduras killer. I. Scroferneker, Maria Lúcia, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

Tese de Doutorado

CARACTERIZAÇÃO DE ISOLADOS DO COMPLEXO *SPOROTHRIX SCHENCKII* PROVENIENTES DE DIFERENTES ESTADOS BRASILEIROS

Tese de Doutorado apresentada ao PPG em Medicina: Ciências Médicas como pré-requisito obrigatório para obtenção do grau de Doutor

Cheila Denise Ottonelli Stopiglia

Orientadora: Maria Lúcia Scroferneker

Porto Alegre
2013

“Jamais considere seus estudos como uma obrigação, mas como uma oportunidade invejável (...) para seu próprio prazer pessoal e para proveito da comunidade à qual seu futuro trabalho pertencer”

Albert Einstein

AGRADECIMENTOS

À minha querida orientadora, **Maria Lúcia Scroferneker**, pelos ensinamentos acadêmicos, apoio, amizade, confiança, incentivo e troca de experiência. Obrigada pelas conversas e pelos longos conselhos sobre a vida.

À professora **Patricia Valente** pelos ensinamentos, pela amizade, disponibilidade e colaboração fundamental no desenvolvimento desse trabalho.

Aos colegas dos laboratórios 210A e 210B do ICBS pelos cafezinhos maravilhosos, pela amizade, convivência, companheirismo e descontração.

Às minhas colegas de pós-graduação **Cibele Magagnin**, **Daiane Heidrich** e **Tatiane Daboit** pelo companheirismo, amizade, conversas, risadas, troca de conhecimentos e pela disponibilidade em me ajudarem a realizar este trabalho.

Às bolsistas de Iniciação Científica que trabalharam arduamente para a realização dos experimentos: **Julia Sorrentino**, **Fabiane Vieira, Andressa Mondadori**, **Lucia Meirelles**, **Suelen Vigolo**, **Karine Alves** e **Madeline Machado**. Muitíssimo obrigada!!!

Aos colegas **Maurício Castrillón** e **Sandra Mendes** pela ajuda e orientações fornecidas nos experimentos relacionados à Biologia Molecular.

Às minhas amigas e companheiras de longa data, **Ana Paula Santin**, **Cristiana Reimann**, **Daniela Cappa**, **Daiane Marchese**, **Francieli Rockenbach**, **Simone Cargnin**, **Dirleane Rossato**, **Fernanda Rossato**, **Michele Zeifert**, **Ana Paula Bottega**, **Anelise Schmitz** e **Denise Przylynski.** Obrigada pela amizade, apoio, conselhos e pela torcida durante as etapas desta Tese.

Às amigas **Mariana Carissimi** e **Thaís de Souza** pelos ensinamentos durante a minha iniciação na pesquisa.

Aos professores **Paulo Ivo de Bittencourt Júnior**, **João Henrique Kanan**, **Mercedes Geimba**, **Gertrudes Corção**, **Carlos Eugênio Silva** e **João Antonio Pegas Henriques** pela autorização do uso de equipamentos dos seus laboratórios e pelos empréstimos de reagentes.

Aos colegas dos laboratórios do ICBS pela acolhida, trocas de ideias e reagentes.

Aos professores **Gilsane Von Poser**, **Amélia Henriques**, **Adriane Belló**, **Alex Sander Araujo, Carmen Fortes, Fabrício Collares** e **Susana Samuel,** bem como as colegas **Damiana Vianna**, **Rafaela Marin**, **Júlia Fontoura**, **Gabriela Meirelles**, **Érika Macêdo** e **Flávia Lamb** pela confiança e parceria na realização dos trabalhos.

À querida **Sonia Santos** pelo auxílio na revisão da tese.

À UFRGS pela oportunidade de ensino e a CAPES pela bolsa de estudos.

Aos meus amados pais, **Antonia** e **Luiz**, que sempre estiveram presentes me ensinando a cultivar os verdadeiros valores da vida. Obrigada por toda a dedicação, amor e incentivo.

Às minhas irmãs **Leila** e **Luciara** e ao meu cunhado **Roberto** pelo apoio, paciência e confiança e por vibrarem com as minhas conquistas.

Aos meus sobrinhos **Eduarda** e **Pedro Henrique**, pelo simples fato de existirem e tornarem os meus dias mais alegres.

Ao meu esposo **Daniel**, meu amigo, companheiro, cúmplice e confidente. Por ter vindo "pro que der e vier comigo".

Ao meu **Deus** e pelo seu amor que excede a todo entendimento.

Muitíssimo obrigada!!!!

SUMÁRIO

RESUMO

O complexo *Sporothrix schenckii* reúne espécies etiologicamente relacionadas à esporotricose, uma micose que pode acometer seres humanos e animais. Foi realizada a identificação fenotípica e molecular de 85 isolados provenientes de quatro estados brasileiros (Minas Gerais, Rio de Janeiro, Rio Grande do Sul e São Paulo). Foram pesquisadas a produção de enzimas, o perfil de inibição por leveduras killer, a suscetibilidade aos antifúngicos comerciais e aos extratos de plantas. Os isolados foram identificados como *S. schenckii*, *S. brasiliensis* e *S. globosa*, com predomínio de *S. schenckii*. Houve discordância de 37,7% entre a identificação das espécies do complexo *S. schenckii* utilizando metodologias fenotípicas e genotípicas. Entre os antifúngicos testados, a terbinafina foi o fármaco mais ativo; seguido por cetoconazol e itraconazol, enquanto fluconazol e voriconazol foram os menos ativos. Cinco isolados fúngicos foram detectados como resistentes ao itraconazol, sendo um *S. globosa* e quatro *S. schenckii*. Não houve diferença nos perfis de suscetibilidade aos antifúngicos entre as espécies do complexo *Sporothrix schenckii*. Entre os extratos de origem vegetal, o mais ativo foi o proveniente de *Pterocaulon polystachyum*, mostrando que o uso popular das plantas reforça a importância de pesquisas etnofarmacológicas, abrindo a possibilidade de encontrar novos agentes antifúngicos clinicamente eficazes. Doze das 18 leveduras *killer* avaliadas apresentaram atividade frente a todos os isolados do complexo *S. schenckii* estudados. No entanto, não houve diferença na suscetibilidade as toxinas entre as espécies de *Sporothrix*. Todos os isolados produziram desoxiribonuclease, urease e proteinase. Atividade fosfolipase e esterase foi detectada em 83 (97,6%) e 80 (94,1%), respectivamente, dos isolados testados. Todas as amostras do complexo *S. schenckii* produziram, pelo menos, quatro das enzimas avaliadas, e 78 (91,8%) dos isolados produziram todas as enzimas analisadas no estudo. No entanto, não foi possível diferenciar as espécies de

Sporothrix baseado no perfil enzimático. Entre as enzimas extracelulares avaliadas nos isolados do complexo *S. schenckii*, desoxiribonuclease e esterase foram produzidas em maior quantidade, podendo vir a ser um fator de virulência. Além disso, o caldo Sabouraud dextrose mostrou potencial para ser usado na avaliação *in vitro* da atividade antifúngica frente ao complexo *S. schenckii*.

Palavras-chave: complexo *Sporothrix schenckii*; *S. brasiliensis*; *S. globosa*; identificação molecular; atividade enzimática; leveduras *killer*; *Pterocaulon;* sensibilidade *in vitro* aos antifúngicos.

ABSTRACT

The *Sporothrix schenckii* complex combines species etiologically related to sporotrichosis, a mycosis which can affect humans and animals. The phenotypic and genotypic identification of 85 strains from four Brazilian States (Minas Gerais, Rio de Janeiro, Rio Grande do Sul and São Paulo) was performed. The enzymatic production, profile of inhibition by killer yeasts, susceptibility to marketed antifungal and to plant extracts were surveyed. The isolates were identified as *S. schenckii*, *S. brasiliensis* and *S. globosa*, with the predominance of *S. schenckii*. There was 37.7% disagreement regarding the species classification using phenotypic and genotypic methodologies. Among the tested antifungals, terbinafine was the most active drug, followed by ketoconazole and itraconazole, while fluconazole and voriconazole were the least active ones. Five isolates - one *S. globosa* and four *S. schenckii* - were resistent to itraconazole. There was no difference as to the profiles of the susceptibility to the antifungal agents among the *Sporothrix* species. The most active vegetal extract was from *Pterocaulon polystachyum*, showing that the popular use of these plants reinforces the importance of ethnopharmacological researches, with the possibility of finding new clinically effective antifungal agents. Twelve out of the 18 evaluated killer yeasts showed activity against all the tested strains of the *S. schenckii* complex. However, there was no difference in susceptibility to the toxins among the *Sporothrix* species complex. All the isolates were desoxiribonuclease, urease and proteinase positive. Phospholipase and esterase activities were detected in 83 (97.6%) and 80 (94.1%), respectively, among the isolates evaluated. All the *S. schenckii* complex strains produced at least four of the evaluated enzymes, and 78 (91.8%) of the isolates produced all the enzymes analyzed in the study. However, it is not possible to differentiate the *Sporothrix* species based on their enzymatic profile. Among the extracellular enzymes evaluated in the *S. schenckii* complex isolates, desoxiribonuclease and esterase were

the most prominent ones, and their production may be a virulence factor. Furthermore, the Sabouraud dextrose broth showed potential to be used in the *in vitro* evaluation of the antifungal activity against the *S. schenckii* complex.

Keywords: *Sporothrix schenckii* complex; *S. brasiliensis*; *S. globosa*; molecular identification; enzymatic activity; killer yeasts; *Pterocaulon*; *in vitro* antifungal activity.

LISTA DE ABREVIATURAS

AIDS	Síndrome da imunodeficiência adquirida
CIM	Concentração inibitória mínima
CMA	Agar farinha de milho
CD4	Grupamento de diferenciação 4
DNA	Ácido Desoxirribonucleico
DNase	Desoxiribonuclease
ELISA	*Enzyme-linked immunosorbent assay*
HE	Hematoxilina e eosina
HIV	Vírus da imunodeficiência humana
ITS	*Internal transcribed spacer*
PAS	Ácido periódico de Schiff
PDA	Agar batata dextrose

LISTA DE FIGURAS

1. INTRODUÇÃO

A esporotricose é causada por um grupo de fungos dimórficos denominado complexo *Sporothrix schenckii*. Esta micose é uma infecção subaguda ou crônica que acomete o homem e uma grande variedade de animais como: gatos, cães, equinos, bovinos, camelos, golfinhos, porcos, ratos, entre outros [1,2]. Geralmente apresenta-se na forma de nódulos, que se ulceram e supuram, seguindo o trajeto de um tronco linfático ou na forma de uma placa cutânea verrucosa. Em pacientes imunodeprimidos pode desenvolver-se a forma disseminada desta micose, com lesões pulmonares, ósseas, articulares e do sistema nervoso central [3].

A esporotricose apresenta ampla distribuição mundial, ocorrendo com maior frequência em regiões de clima tropical, subtropical e temperado [4]. Atualmente predomina no México, América Central, Japão e no sul dos continentes africano e americano [5]. Na América do Sul, a maioria dos casos descritos é do Brasil [6], sendo a micose subcutânea de maior incidência no estado do Rio Grande do Sul [7].

O tratamento de escolha para esporotricose cutânea é a quimioterapia sistêmica com iodeto de potássio [8]. Nas formas cutâneas disseminadas, linfocutâneas recidivantes e extracutâneas, a anfotericina B é o fármaco mais efetivo. Porém, a frequência de intolerância ao iodo e a alta toxicidade da anfotericina B, muitas vezes, representam fatores que limitam o seu uso [8,9]. Nas últimas décadas, aumentou o emprego de derivados azólicos, dentre eles: o cetoconazol, o itraconazol e o fluconazol, como alternativa terapêutica aos esquemas clássicos, sendo o itraconazol o mais efetivo deles [10]. Por outro lado, a terbinafina, em virtude da sua elevada atividade *in vitro* e *in vivo*, está sendo utilizada no tratamento de diversas infecções fúngicas. No entanto, a experiência clínica da terbinafina, no tratamento da esporotricose, ainda é restrita [11-13].

Os frequentes relatos da resistência *in vitro* de *S. schenckii* à anfotericina B e aos derivados azólicos, bem como os problemas relacionados à toxicidade desses fármacos, apontam para a necessidade de mais estudos sobre a suscetibilidade deste patógeno aos

antifúngicos disponíveis, além da busca por novos fármacos para o tratamento da esporotricose [14,15].

A caracterização da variabilidade genética de isolados clínicos e ambientais de fungos potencialmente patogênicos ao homem é importante nos estudos de epidemiologia. Vários estudos, utilizando diferentes ferramentas moleculares, mostram que *S. schenckii* apresenta variabilidade genética, mas as tentativas de agrupá-los de acordo com a origem geográfica ou forma clínica ainda não são conclusivas [16,17].

Baseado em estudos fenotípicos e da variabilidade do gene da calmodulina foi proposto o complexo *Sporothrix schenckii*, o qual compreende atualmente as espécies: *S. albicans*, *S. brasiliensis*, *S. globosa*, *S. schenckii stricto sensu*, *S. mexicana* e *S. luriei* – de importância clínica; e *S. brunneoviolacea*, *S. dimorphospora* e *S. pallida* – isolados ambientais [18-21].

Embora estudo de Marimon e colaboradores (2007) [20] tenha agrupado todos os isolados brasileiros em uma única espécie, *S. brasiliensis*, estudos realizados com isolados brasileiros provenientes dos estados do Rio de Janeiro e Rio Grande do Sul identificaram, também, *S. mexicana*, *S. globosa*, *S. luriei*, *S. albicans* e *S. schenckii* [15,22,23]. Assim, estudos com um grupo maior de isolados originários de diferentes estados brasileiros são necessários para caracterizar os isolados do complexo *S. schenckii* no Brasil.

Além disso, é necessário o desenvolvimento de novas ferramentas de tipagem que sejam mais baratas e de uso fácil em laboratórios minimamente equipados. Entre essas ferramentas, a biotipagem por leveduras killer vem sendo cada vez mais utilizada [24].

Por outro lado, os fatores de virulência de *S. schenckii* ainda não estão bem elucidados, mas alguns deles podem estar relacionados à termotolerância, a produção de enzimas e de polissacarídeos extracelulares [25]. Nas últimas décadas, as enzimas dos

patógenos fúngicos têm merecido atenção pela sua importância na patogenicidade e como possível alvo para confecção de inibidores sintéticos que possam tratar as micoses [26].

Nesse contexto, este trabalho vem atender a uma necessidade de caracterização de isolados do complexo *S. schenckii* provenientes de quatro estados brasileiros: Minas Gerais, Rio Grande do Sul, Rio de Janeiro e São Paulo, realizando a identificação molecular, além de analisar suas características fenotípicas, como a produção enzimática, a suscetibilidade a agentes antifúngicos, bem como a sensibilidade a toxinas *killer* e a extratos de origem vegetal.

2. REVISÃO DA LITERATURA

Para a presente revisão bibliográfica foram buscados artigos publicados indexados no PUBMED e EMBASE/MEDLINE através das seguintes palavras-chave: "*Sporothrix"* ou "*Sporothrix schenckii*" ou "*Sporothrix schenckii* complex" ou "sporotrichosis" e "antifungal activity" e "enzymatic activity" ou "enzymes" e "yeast killer" e "*Pterocaulon*". Textos completos foram lidos para possível inclusão. Além disso, foram consultados livros e artigos que haviam sido coletados em outras buscas realizadas anteriormente.

2.1. Aspectos históricos

Em 1898 Benjamin Schenck publicou o primeiro caso de esporotricose com o isolamento do fungo, o qual denominou *Sporotricha*. O fungo foi isolado de lesões no dedo indicador e antebraço de um paciente de 36 anos do sexo masculino, do Hospital John Hopkins, em Baltimore, Estados Unidos [27]. O isolado foi enviado ao fitopatologista Erwin Smith que o identificou como do gênero *Sporotrichum*. Dois anos depois, Hektoen e Perkins comunicaram um segundo isolamento do fungo que denominaram *Sporotrichum schenckii*. O microrganismo foi isolado de um menino com ferimento no dedo, em Chicago, Estados Unidos [28]. Em 1903, De Beurmann e Ramond isolaram o fungo, pela primeira vez, na França e nominaram-no como *Sporotrichum beurmanni*, por ser considerado, por eles, diferente do isolado por Schenck. Neste mesmo ano, Sabouraud sugeriu o uso de iodeto de potássio para o tratamento da esporotricose [29].

Em 1910, Matruchot redescreveu o fungo como *Sporotrichum schenckii*. Em 1921, os agentes da esporotricose isolados nos Estados Unidos e na França, foram considerados idênticos. Finalmente, em 1963, Carmichael propôs como nome correto do fungo *Sporothrix schenckii* [30].

No Brasil, Lutz e Splendore descreveram, em 1907, os primeiros relatos de esporotricose em seres humanos e ratos em São Paulo, além de cultivarem *in vitro* a fase

parasitária do patógeno [31]. No Rio Grande do Sul, descreveu-se o primeiro caso em dois cães no ano de 1964 [32].

2.2. Epidemiologia

S. schenckii é amplamente distribuído na natureza e tem sido isolado do solo, de plantas (espinhos, folhas secas, frutas, roseiras e madeira), além de água, algas, insetos, aranhas, moscas, poeira, animais marinhos e matéria orgânica em decomposição. Ocupações como jardinagem, manejo rural, agricultura, construção civil estão relacionadas à maior predisposição a infecção [33-35].

A história natural da esporotricose vem mudando gradualmente em frequência, modo de transmissão e distribuição demográfica e geográfica. É possível que fatores ambientais, além do aumento da urbanização e do aperfeiçoamento de diagnósticos expliquem, em parte, as alterações no perfil da doença. Além disso, como a esporotricose não é uma doença de notificação na maioria dos países, há pouca informação sobre a incidência e os dados conhecidos são os gerados pelas publicações de casos [36].

No início do século passado, a esporotricose era uma doença comum na França, que diminuiu após duas décadas e hoje aparece esporadicamente na Europa. Em 2009, um caso autóctone foi relatado na França, e em 2008, outro foi relatado na Itália, porém estes casos são raros e isolados [37,38]. Apesar de ter sido descrita nos cinco continentes, a esporotricose tem uma maior prevalência nas áreas tropicais e zonas temperadas, todavia com diferentes condições climáticas.

As principais áreas de endemicidade estão localizadas no México, América Central, Japão e no sul dos continentes africano e americano [5]. Nos Estados Unidos, até 1932, havia menos de 200 casos de esporotricose reportados quando a incidência aumentou chegando-se a estimativa de 100 pacientes internados com esporotricose sistêmica [29]. Neste país, a maior

epidemia de esporotricose ocorreu em 1988 envolvendo 84 casos em 15 estados [39]. Na Guatemala há registro de surtos epidêmicos relacionados à limpeza de pescado e no Uruguai com a caça ao tatu, chegando até a 88% dos casos [40]. No Peru existe uma área hiperendêmica ao sul das montanhas andinas, com uma incidência anual estimada em 48 a 60 casos por 100 000 habitantes em Abancay [41].

A falta de infraestrutura para o diagnóstico laboratorial em muitos países não permite um conhecimento preciso da distribuição da doença. Estudos apontam que em regiões como o planalto da China, Laos e Vietnã, as quais apresentam condições favoráveis para o crescimento do gênero *Sporothrix*, a prevalência dos casos deve ser muito maior [36].

A ocorrência de esporotricose em animais e sua transmissão ao ser humano têm sido relatadas em diversos países. Contudo, em nenhum lugar a doença assumiu proporções epidêmicas, envolvendo pessoas e gatos, como no estado do Rio de Janeiro, Brasil. Entre 1987 e 1997 foram registrados apenas 13 casos humanos de esporotricose no Instituto de Pesquisa Evandro Chagas (IPEC) do Rio de Janeiro. No período de apenas 2 anos (1998 e 2000) foram descritos 66 casos humanos, 117 casos em felinos e 7 casos em cães [42]. Desde então, o número de casos aumentou constantemente, atingindo um total de 2200 casos de esporotricose humana e 3244 gatos com cultura positiva para *S. schenckii*, no período entre 1998 e 2009 [43]. Os autores relataram que 85% dos cães e 83% dos pacientes tiveram contato com gatos com esporotricose, e 56% deste último relatado mordidas de gato ou arranhões [44]. No estado de São Paulo, registrou-se um aumento dos casos de esporotricose felina a partir de 1989 [45]. Já no Rio Grande do Sul, nos últimos anos, houve um aumento no número de casos de esporotricose em felinos no litoral sul do estado, particularmente no município de Rio Grande [2].

No Rio Grande do Sul, uma análise retrospectiva do período de 1967 a 2002 mostrou 304 casos de esporotricose diagnosticados em Porto Alegre em pacientes oriundos de

diferentes regiões do estado [7]. Já na região central do estado do Rio Grande do Sul, entre 1988 e 1997, foram observados 31 casos de esporotricose registrados no Hospital Universitário de Santa Maria. Na mesma cidade, um estudo retrospectivo realizado entre 1957 e 1997 registrou 342 casos de esporotricose em humanos. Apesar de uma diminuição da incidência da esporotricose nas últimas décadas, esta patologia continua sendo a mais frequente micose subcutânea humana no Rio Grande do Sul [6].

A esporotricose pode acometer indivíduos independentemente do gênero e idade [46]. Adultos representam a maior proporção de casos nas áreas endêmicas, exceto no Peru, onde uma predominância marcante (60%) de indivíduos com menos de 15 anos tem sido descrita [7,41,47-49]. No que se refere a gênero, não há prevalência uniforme de homens ou mulheres acometidos por esporotricose em áreas endêmicas. Estudos realizados na Colômbia e no estado do Rio Grande do Sul, Brasil, mostraram uma maior prevalência em homens envolvidos na agricultura, bem como outras atividades de alto risco [7,47,48]. O oposto é observado no Japão e na Índia, onde pacientes do gênero feminino são igualmente ou mais afetados do que pacientes do gênero masculino, o que se deve ao grande número de mulheres que trabalham na agricultura nestes países [49-52]. No estado brasileiro do Rio de Janeiro, também houve um predomínio em mulheres, as quais estavam envolvidas no trabalho doméstico e no cuidado de gatos com esporotricose [44,53]. Deste modo, o risco da esporotricose é determinado, principalmente, pela possibilidade de contato com o agente etiológico. Este risco, independentemente de raça, idade ou sexo, está particularmente relacionado com as atividades e trabalho das pessoas [7].

Assim, a esporotricose é considerada uma doença ocupacional caracterizada como verdadeira ergodermatose que acomete horticultores, sementeiros, chacareiros, jardineiros e floristas, que se infectam através de espinhos, talos de plantas, madeira, flores, palha, ou ainda, de tosadores, tratadores de animais e veterinários, os quais são vítimas ocasionais de

arranhaduras e mordeduras [54]. Alcoolismo e diabetes também têm sido descritos como fatores de risco. Imunossupressão, independentemente da causa, predispõe para esporotricose disseminada ou doença sistêmica [4].

2.3. Etiologia

A esporotricose é causada por um grupo de fungos dimórficos que compreendem o complexo *S. schenckii*. Tais microrganismos pertencem à divisão *Ascomycota*, classe *Pyrenomycetos*, ordem *Ophiostomatales*, família *Ophiostomataceae* e gênero *Sporothrix* [1,55]. Não há consenso sobre a forma teleomórfica deste fungo, apesar da espécie *Ophiostoma stenoceras* já ter sido postulada como tal [35].

Estudos realizados por Marimon e colaboradores (2007) [20] com base nas características macroscópicas, assimilação de sacarose e rafinose, capacidade para crescer a 37°C, e o sequenciamento do gene da calmodulina, descreveram quatro novas espécies do complexo *Sporothrix*: (i) *S. globosa*, um fungo com distribuição universal [56], (ii) *S. brasiliensis*, espécie relacionada com a epidemia zoonótica de esporotricose no Rio de Janeiro, Brasil [20]; (iii) *S. mexicana*, limitada ao México [20], e (iv) *S. luriei*, anteriormente denominada *S. schenckii* var. *luriei*, isolada pela primeira vez na África do Sul [21]. A Figura 1 apresenta uma chave para diferenciar espécies dentro do complexo *S. schenckii*.

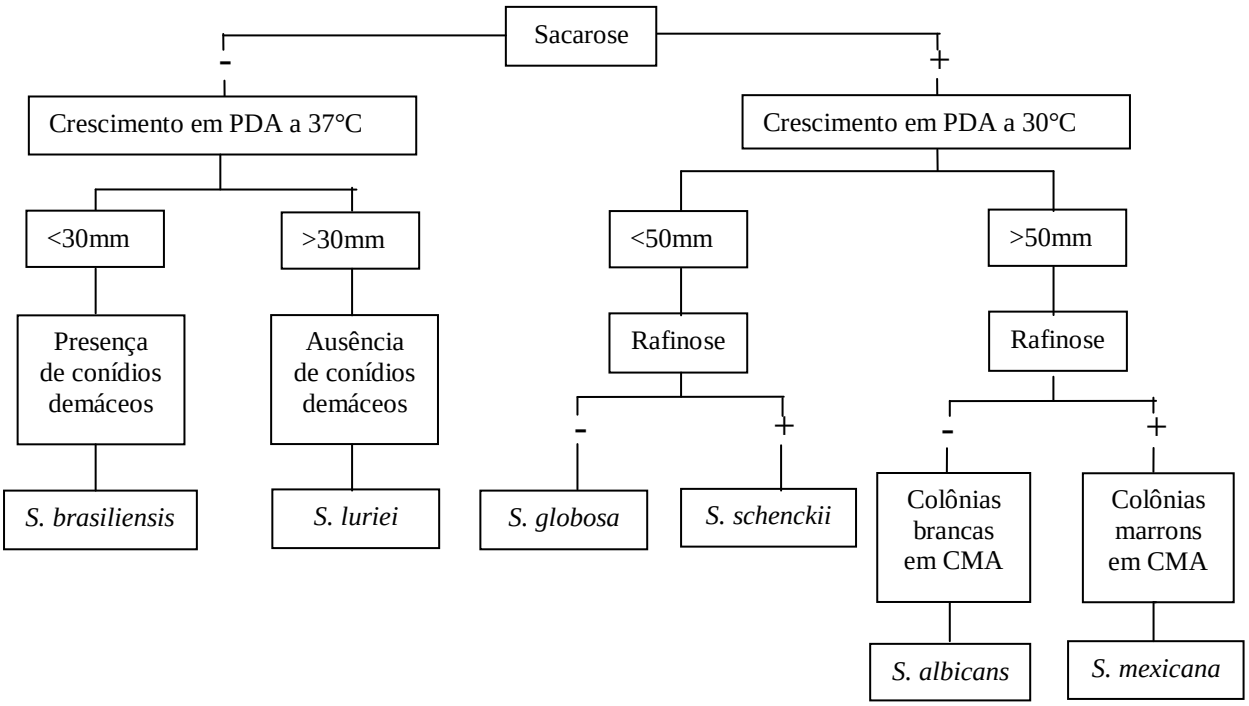

Figura 1: Chave de identificação para as espécies de *Sporothrix* de interesse clínico, com base em provas morfológicas e fenotípicas descritas por Marimon et al. (2007) [20]. PDA - agar batata dextrose; CMA - agar farinha de milho. Adaptado de Barros et al. (2011) [36].

Posterior análise filogenética baseada no sequenciamento de regiões da β-tubulina e de DNA ribossomal de *Sporothrix pallida*, *Sporothrix nivea*, e *Sporothrix albicans* revelaram uma semelhança genética notavelmente elevada, e portanto, as duas últimas espécies foram sinonimizadas com *S. pallida*, a primeira das três espécies a ser descrita [18]. No entanto, esta espécie só foi isolada de amostras do meio ambiente, embora tenha sido encontrada, algumas vezes, como parte da microbiota intestinal de alguns insetos [57]. Além disso, recente estudo realizado em modelo murino confirmou que esta espécie não é patogênica [58].

Recentemente, Madrid e colaboradores (2010) [19] sugeriram a inclusão de dois novos membros no complexo *S. schenckii*. Os isolados foram obtidos de amostras de solo coletadas na Espanha e nos Estados Unidos. A primeira espécie foi denominada *Sporothrix brunneoviolaceae* e é caracterizada fenotipicamente pela produção de pigmento difusível em

cultura e pela presença de blastoconídeos laterais, pigmentados e globosos. Já a segunda espécie, denominada *Sporothrix dimorphospora*, carece de pigmentos difusíveis em cultura e produz blastoconídeos laterais, subglobosos a obovóides e pigmentados.

Embora Marimon e colabores (2007) [20] tenham proposto apenas um ramo para os isolados brasileiros, recentemente foi descrita variabilidade genotípica entre isolados humanos e animais em um surto epidêmico no estado do Rio de Janeiro [59]. Além disso, estudo realizado com isolados de diferentes regiões brasileiras demonstrou alta variabilidade genotípica [60], que corrobora com outros estudos, os quais identificaram *S. mexicana*, *S. globosa, S. luriei*, *S. albicans* e *S. schenckii*, além de *S. brasiliensis* em isolados oriundos dos estados do Rio de Janeiro e Rio Grande do Sul [15,22]. No entanto, não foi encontrada correlação com a origem geográfica ou forma clínica da doença [16].

2.4. Morfologia

Por ser um fungo dimórfico, *S. schenckii* apresenta duas fases morfológicas no seu ciclo de vida, a fase miceliana (saprofítica) e a fase leveduriforme (parasitária). Em temperatura ambiente ou em condições *in vitro* a 25ºC, o agente tem rápido crescimento, apresentando-se como uma colônia inicialmente branco-amarelada, sedosa, membranosa, às vezes com micélio aéreo, posteriormente tornando-se pregueada e escurecida. Ao microscópio, são observadas hifas finas, septadas, ramificadas, medindo 1-2µm de diâmetro com conidióforos alongados, simpodiais, contendo ápices entumecidos, frutificando conídios hialinos, elípticos ou ovais, dispostos em rosetas. Na sua forma parasitária ou quando cultivado a 37ºC, a colônia adota forma leveduriforme, de coloração creme ou cinza. Microscopicamente são observadas células leveduriformes, pequenas (2 a 6 µm de diâmetro), ovais ou no formato de "charuto", com brotamento simples ou múltiplo [29,61] (Figura 2).

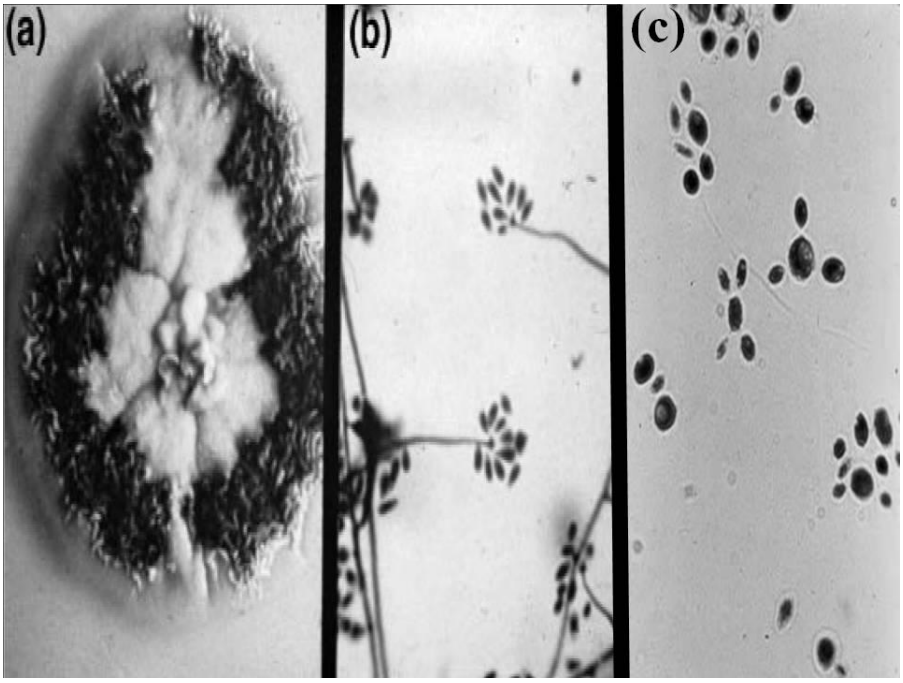

Figura 2: Características macroscópicas (a) e microcópicas (b) da fase filamentosa e microscópicas da fase leveduriforme (c) de *Sporothrix schenckii*. Adaptado de Araujo et al. (2001) [62] e Zaitz et al. (2004) [63].

2.5. Esporotricose

A esporotricose é uma infecção fúngica subaguda ou crônica causada pelo fungo *S. schenckii*. A infecção ocorre como consequência da inoculação traumática do microrganismo na pele e é geralmente associada à linfangite. Além disso, a inalação do conídio também foi postulada como via de infecção [62].

A esporotricose é, na maioria das vezes, uma infecção benigna limitada à pele e ao tecido celular subcutâneo, podendo disseminar-se para ossos e órgãos internos. Em alguns casos a doença pode ser primariamente sistêmica, tendo início pulmonar. Recentemente, as formas cutânea disseminada e extracutânea têm sido descritas com mais frequência em pacientes imunocomprometidos, principalmente, aqueles portadores do vírus da

imunodeficiência humana (HIV), com alcoolismo crônico, diabetes, e pacientes que fizeram uso de corticóides por um longo período [64,65].

A epidemia da síndrome da imunodeficiência adquirida (AIDS) tem levado a um aumento da prevalência da esporotricose, além da ocorrência de formas atípicas da micose [66]. Embora, a esporotricose não seja considerada uma infecção oportunista comum para estes pacientes, alguns autores descrevem que pacientes soropositivos e com contagem de células T CD4+ menor que 200/mm^3 constituem um fator crescente de risco para aquisição da forma disseminada e extracutânea desta patologia [67]. Assim, a esporotricose vem sendo considerada como uma micose emergente em pacientes HIV positivos [68]. Nestes pacientes, quando ocorre a disseminação, são sintomas bastante comuns a febre, perda de peso, fadiga, artrite e pneumonia, além de uma variedade de manifestações cutâneas não específicas. No curso da infecção, o *S. schenckii* pode atingir as articulações, observando-se quadro de osteoartrite, o qual é relativamente frequente [69,70].

2.5.1. Manifestações clínicas

A esporotricose apresenta-se sob várias formas clínicas: linfocutânea, cutânea fixa, cutânea disseminada e extracutânea. Não se sabe, com precisão, o que determina cada uma delas. No entanto, alguns fatores podem contribuir na patogênese desta doença, como a quantidade de inóculo, a profundidade da inoculação traumática, a tolerância térmica da cepa e o estado imunológico do hospedeiro [71].

2.5.1.1. Forma linfocutânea

A forma clínica de esporotricose mais frequente (cerca de 80%) é a linfocutânea [35]. Após a inoculação traumática de conídios na pele ou no tecido subcutâneo, surge uma pequena lesão, seguida pelo surgimento de uma pápula endurecida que aparece entre 7 e 30

dias. Essa lesão vai aumentando vagarosamente, com o aparecimento de nódulos, geralmente ulcerativos. Os sintomas sistêmicos são fracos ou ausentes. Como a doença é de curso progressivo, nódulos indolores similares vão formando uma cadeia ao longo dos vasos linfáticos, que podem ulcerar-se, permanecendo próximos à lesão inicial ou aparecerem em regiões mais distantes. No adulto, essa forma é mais comum nos membros e, na criança, na face. As lesões podem cicatrizar depois de várias semanas ou se tornarem crônicas se não tratadas. Essa cadeia de nódulos que segue o trajeto linfático, surgindo após o cancro inicial, e persistindo mesmo após a cicatrização deste, constitui uma imagem clássica da esporotricose. Estas lesões típicas desta patologia, conhecidas como lesões esporotricóides, também são observadas em micobacterioses atípicas e na leishmaniose [72].

É importante realizar o diagnóstico diferencial dessa forma clínica com a leishmaniose cutânea, infecções atípicas por micobactéria, tularemia, nocardiose linfocutânea e outras infecções fúngicas de inoculação primária [62].

2.5.1.2. Forma cutânea fixa

Nesta forma da esporotricose a lesão é restrita ao sítio de inoculação. Ela é caracterizada por placas endurecidas ou verrucosas e, ocasionalmente, úlceras, na maioria das vezes localizadas na face, pescoço, tronco e pernas. As lesões podem apresentar remissão espontânea, mas podem recidivar e persistirem por anos, se não forem tratadas [73]. O diagnóstico diferencial dessa forma clínica da esporotricose deve incluir a sífilis, leishmaniose, paracoccidioidomicose, cromoblastomicose, blastomicose, tuberculose verrucosa, infecção atípica por micobactérias, lepra e tuberculose cutânea [62].

2.5.1.3. Forma cutânea disseminada

Essa forma da esporotricose está geralmente associada a algum tipo de imunodepressão e patologias associadas. Alguns fatores como AIDS, idade avançada, alcoolismo, diabetes, síndrome de *Cushing*, corticoterapia prolongada, nefropatias e outras são condições predisponentes ao aparecimento dessa forma clínica. Após inoculação traumática, ocorre a disseminação por via hematogênica, com lesões inicialmente subcutâneas, que após semanas ou mesmo meses, podem ulcerar-se. As lesões são muito variadas e podem ser similares as da tuberculose cutânea, com depressão central e bordas elevadas. Outras lesões são do tipo ulcerosas, crateriformes e úlcero-crostosas. Lesões eritemato-papulosas, formando pequenas placas parcialmente atróficas e grandes placas vegetantes, também podem ocorrer [72].

2.5.1.4. Forma extracutânea

A forma extracutânea é de difícil diagnóstico clínico e tratamento [72] e está associada a quadros de imunocomprometimento [74,75], embora existam relatos em pacientes imunocompetentes [76,77]. Os sintomas da forma extracutânea estão relacionados com o órgão/tecido envolvido.

2.5.1.4.1. Esporotricose osteoarticular

Depois da pele, o tecido ósseo é o mais frequentemente envolvido. A infecção osteoarticular pode derivar da disseminação do patógeno a partir da inoculação cutânea e/ou da disseminação hematogênica a partir dos pulmões [75,78]. Os ossos mais afetados são tíbia, ossos pequenos das mãos, rádio, ulna e ossos do crânio e da face [72]. As lesões podem variar de pequenos granulomas a grandes lesões gomosas, com intensa destruição óssea, semelhantes a osteomielite [79]. O diagnóstico geralmente é tardio [75].

2.5.1.4.2. Meningite causada por *S. schenckii*

A meningite causada por *S. schenckii é* uma das mais graves complicações causada por esse patógeno. A maior parte dos casos está associada com portadores do vírus HIV [80]. O diagnóstico é difícil de ser estabelecido, pois o resultado da cultura de líquor geralmente é negativo [75]. Assim, faz-se necessário que sejam realizados estudos sorológicos para o diagnóstico dessa forma clínica da esporotricose [81].

O diagnóstico diferencial deve ser amplo, incluindo tuberculose, criptococose e histoplasmose [82]. Além disso, as opções de tratamento são limitadas [75].

2.5.1.4.3. Esporotricose pulmonar

É a forma mais rara. A apresentação clínica é semelhante à da tuberculose, e o diagnóstico muitas vezes é tardio, devido à raridade do comprometimento pulmonar. Padrões radiológicos incluem doença cavitária, traqueobrônquica, linfonodomegalias, e lesões nodulares [83]. Alguns dos sintomas mais comuns são febre, perda de peso, tosse crônica, fadiga e anorexia. O diagnóstico é geralmente tardio, levando o paciente à morte pela infecção ou pela severidade da doença pulmonar [84,85]. Como diagnóstico diferencial são incluídas outras infecções fúngicas como histoplasmose, blastomicose, coccidioidomicose e sarcoidose [86].

2.6. Diagnóstico da esporotricose

A esporotricose pode ser diagnosticada através da correlação clínica, epidemiológica e de dados laboratoriais. A análise laboratorial para o diagnóstico desta micose é realizada, principalmente, pelo isolamento e identificação do fungo a partir de material puncionado de lesões, pus ou biópsia. No caso de infecções disseminadas, outras amostras, tais como saliva,

urina, sangue, líquidos sinovial e cefalorraquidiano podem ser analisados, de acordo com os órgãos afetados [36].

2.6.1. Exame Micológico Direto e Cultura

O exame micológico direto é geralmente realizado com hidróxido de potássio, mas também podem ser utilizadas colorações como Gram ou Giemsa a fim de observar as células parasitárias leveduriformes. Estas leveduras são pequenas (2-6 µm de diâmetro), escassas e visualizadas em apenas 50% das espécimes coletadas de seres humanos. No entanto, quando o mesmo teste é realizado com amostras colhidas de gatos infectados, devido à elevada carga de fungos nesses animais, as células de levedura podem ser facilmente encontradas [4,36].

Porém, o padrão-ouro para o diagnóstico da esporotricose é o isolamento e identificação do agente etiológico em cultura. O isolamento de *S. schenckii* é facilmente obtido após a propagação de espécimes clínicos em agar Sabouraud com cloranfenicol ou em meio com cicloheximida. A colônia é inicialmente hialina e após alguns dias pode desenvolver pigmentação escura. As características microscópicas são conidióforos com conídios elípticos em forma de flor de margarida ou crisântemo. O cultivo demanda entre 10 e 14 dias para propiciar ou afastar o diagnóstico etiológico. Estas características determinam a correta identificação do gênero. No entanto, a identificação em nível de espécie requer o uso de técnicas moleculares, com o sequenciamento do gene da calmodulina [4,36,54].

2.6.2. Exame Histopatológico

Embora *S. schenckii* possa ser visualizado no tecido com a clássica coloração hematoxilina e eosina (HE), as técnicas de metenamina de Gomori ou Grocott ou, ainda, do ácido periódico de Schiff (PAS) podem ser empregadas para aumentar a detecção fúngica. No entanto, em seres humanos, as células de *S. schenckii* são difíceis de serem visualizadas,

devido à escassez de leveduras nas lesões [54]. Assim, a sensibilidade do exame histopatológico para detecção de formas fúngicas varia entre 5 e 90% [87].

A esporotricose geralmente provoca reação inflamatória granulosa na derme e tecido subcutâneo, com frequência acompanhada por microabscesso e fibrose. A epiderme hiperplásica, com ou sem ulceração, e o característico cancro esporotricótico estão presentes em mais de 80% dos casos. O cancro esporotricótico (granuloma supurativo) consiste em um microabscesso central com neutrófilos e uma área com necrose, na qual predominam os linfócitos e os macrófagos, rodeados por células plasmáticas, fibroblastos, e vasos sanguíneos. Corpos asteróides podem ser observados no centro do granuloma em aproximadamente 20% dos casos, mas eles não são patognomônicos da doença. Acredita-se que os corpos asteróides estejam relacionados com o mecanismo de resistência da levedura. Eles encontram-se rodeados por imunoglobulinas, caracterizando o fenômeno de Splendore-Hoeppli [4,87].

2.6.3. Teste intradérmico

O teste cutâneo da esporotriquina consiste na aplicação intradérmica de antígenos obtidos através da cultura filtrada das formas filamentosa ou leveduriforme de *S. schenckii*, submetendo, então, à leitura da eventual lesão presente, após decorridas 48 horas [54,88]. Esta reação é normalmente positiva em cerca 90% dos casos confirmados de esporotricose, mas também pode indicar infecção anterior com o fungo [51]. Pode se apresentar como negativa nas formas cutâneas disseminadas ou extracutâneas. Trata-se de exame complementar útil para a exclusão da suspeita clínica, já que a negatividade afasta tal diagnóstico. A positividade é perene, embora possa, também, retratar infecção com outros agentes fúngicos [54]. Apesar do uso atual do teste intradérmico com a esporotriquina em vários estudos em todo o mundo, o antígeno adotado nestes testes carece de padronização. Estas variações na produção do antígeno podem levar a diferenças nos resultados [36].

2.6.4. Detecção de anticorpos

Várias metodologias têm sido descritas para o diagnóstico imunológico da esporotricose com base na detecção de anticorpos no soro de pacientes infectados, como aglutinação, imunodifusão dupla e imunoeletroforese. No entanto, estas técnicas apresentam baixa sensibilidade em casos de esporotricose cutânea [89,90].

Técnicas imunológicas mais sensíveis começaram a ser padronizadas para sua utilização no sorodiagnóstico da esporotricose, como *enzyme-linked immunosorbent assay* (ELISA) utilizando antígenos purificados da parede celular da forma leveduriforme [82] ou extrato bruto de cultivo da forma filamentosa de *S. schenckii* [91]. Ambas as técnicas apresentam valores satisfatórios de especificidade e sensibilidade.

É importante salientar que os resultados de todos os testes de detecção de anticorpos fornecem um diagnóstico presuntivo da esporotricose e requerem correlação clínica e epidemiológica para uma avaliação precisa e determinação do diagnóstico final [36].

2.7. Caracterização molecular

Diversos estudos mostram que *S. schenckii* apresenta alta variabilidade genética e as tentativas de agrupá-los de acordo com a origem geográfica ou forma clínica são contraditórias. Suzuki e colaboradores (1988) [92] analisaram o perfil de restrição do DNA mitocondrial de isolados de *S. schenckii*, variantes desta espécie e de algumas espécies do gênero *Ceratocystis*, observando um único perfil de restrição entre todos os isolados analisados. No entanto, em trabalho posterior, isolados clínicos e ambientais de diferentes origens geográficas de *S. schenckii* foram comparados, demonstrando a existência de grupos geneticamente distintos entre os isolados ambientais [92]. Mesa-Arango e colaboradores (2002) [94] estudaram isolados do México, Guatemala e Colômbia e observaram agrupamento genético relacionado à origem geográfica, bem como Liu et al. (2003) [95], com

isolados do Japão, China e América. Em 2006, Kong et al. (2006) [96], utilizando os *primers* OPBG01 e OPBG14 para amplificar o DNA genômico de quinze isolados, observaram agrupamento por similaridade genética de acordo com a forma clínica da doença. Em estudo semelhante, Arenas e colaboradores (2007) [97] correlacionaram os padrões obtidos com as formas clínicas da esporotricose em isolados do México. Zhang e colaboradores (2006) [98] avaliaram o polimorfismo das regiões *internal transcribed spacer* (ITS) de isolados de *S. schenckii* oriundos da China de isolados do México e obtiveram padrões genotípicos distintos correlacionados a origem geográfica e a forma clínica da esporotricose. Galhardo et al. (2008) [99] avaliaram a diversidade genética pela análise do polimorfismo de regiões microssatélite e sequenciamento dos espaçadores ITS de isolados de *S. schenckii* obtidos de surto de esporotricose no Rio de Janeiro, demonstrando a existência de nove subtipos, porém sem relação com a forma clínica da doença.

Marimon e colaboradores (2006) [17] estudaram isolados da Europa, América do Sul e África do Sul através do sequenciamento dos genes da quitina sintase, β-tubulina e calmodulina. A partir disso, descreveram três grandes clades: o primeiro com todos os isolados da Europa, o segundo com isolados da América do Sul e África do Sul e o terceiro com apenas isolados brasileiros. Neste mesmo trabalho é sugerido pela primeira vez que a espécie *S. schenckii* seja formada por um complexo de espécies filogeneticamente relacionadas, já que as populações deste fungo podem estar em processo de divergência evolutiva. Em seguida, este mesmo grupo propõe novas espécies: *S. brasiliensis*, *S. albicans*, *S. globosa* e *S. mexicana* baseando-se em estudos fenotípicos e da variabilidade do gene da calmodulina [20] e posteriormente, recomendam que *S. schenckii* var. *luriei* seja uma nova espécie e não uma variedade denominando-a *S. luriei* [21]. Estudos posteriores sugeriram a inclusão de outras espécies ao complexo *S. schenckii*: *S. brunneoviolacea*, *S. dimorphospora* e *S. pallida*, todas de origem ambiental [18,19].

Embora o estudo de Marimon e colaboradores (2007) [20] tenha agrupado todos os isolados brasileiros em um único ramo e em uma única espécie, *S. brasiliensis*, neste mesmo estudo foram observados seis genótipos para os isolados do estado do Rio de Janeiro. Semelhantemente, Galhardo et al. (2008) [99] encontrou nove subtipos em isolados de um surto de esporotricose neste mesmo estado, evidenciando a alta variabilidade genética deste fungo, sem haver correlação com a forma clínica da doença. Assim, parece não ser conclusiva nenhuma especiação baseada na correlação geográfica ou na forma clínica da esporotricose.

Estudo recente propôs a diferenciação das espécies *S. brasiliensis*, *S. globosa*, *S. mexicana* e *S. schenckii* utilizando PCR-*fingerprinting* com o *primer* universal T3B. Esta metodologia mostrou-se simples, confiável, rápida e barata e pode tornar-se um sistema de identificação de rotina para laboratórios de micologia clínica [100].

2.8. Tratamento

O número de fármacos disponíveis para o tratamento de infecções fúngicas é, ainda hoje, limitado. Medicamentos diferentes são utilizados para o tratamento da esporotricose, incluindo iodeto de potássio, itraconazol, terbinafina, fluconazol e anfotericina B. A escolha é baseada na condição clínica do indivíduo, na extensão das lesões cutâneas, na avaliação das interações medicamentosas, nos eventos adversos, e no envolvimento sistêmico [35].

Historicamente definido como tratamento de escolha para as formas cutâneas e linfocutâneas da esporotricose humana, a solução saturada de iodeto de potássio ainda hoje é bastante utilizada, principalmente pelo baixo custo. O mecanismo preciso pelo qual o iodeto de potássio age sobre o fungo ainda hoje é desconhecido. Não há um consenso se este atua diretamente sobre as células leveduriformes ou age através de estímulo ao sistema imune do hospedeiro. Os inconvenientes associados ao uso de iodeto de potássio estão relacionados ao esquema posológico fracionado em muitas ingestões diárias e efeitos adversos tais como:

diarréia, vômitos, náuseas, acidose metabólica, hipertiroidismo, hipotiroidismo, dermatite herpetiforme e iododerma [9].

Nas últimas décadas aumentou o emprego de derivados azólicos como o cetoconazol, o itraconazol e o fluconazol como alternativa terapêutica aos esquemas clássicos [101]. Itraconazol é geralmente eficaz nas formas cutânea fixa e linfocutânea da doença. Já o fluconazol é menos eficaz do que itraconazol e deve ser utilizado somente se o paciente não tolerar o itraconazol ou apresentar interações medicamentosas com este medicamento [75]. Cetoconazol não tem apresentado uma boa resposta clínica, além de ocasionar uma maior toxicidade [102].

Nas formas cutâneas disseminadas, linfocutâneas recidivantes e extracutâneas, a anfotericina B é o fármaco mais efetivo [101]. O medicamento é administrado por via intravenosa e seu uso está associado a efeitos adversos significantes, tais como febre e calafrios durante a administração e, com maior gravidade, relacionando-se à nefrotoxicidade em até 30% dos pacientes com quadro de insuficiência renal aguda [62,103,104].

A terbinafina, em virtude da sua elevada atividade *in vitro* e *in vivo*, está sendo utilizada no tratamento de diversas infecções fúngicas [10]. Quando administrada por via oral, sofre rápida absorção e distribuição na pele, unhas e tecido adiposo [105]. Estudos têm mostrado que este fármaco é tão eficaz e bem tolerado quanto o itraconazol no tratamento de esporotricose cutânea [106].

Nas últimas décadas, as investigações sobre novos antifúngicos têm se concentrado em melhorar antigas formulações, tornando-as menos tóxicas, além de desenvolver novos fármacos a partir das estruturas já conhecidas. Neste contexto, surgem o voriconazol, o ravuconazol e o posaconazol, sendo mais potentes e com amplo espectro de ação sobre leveduras e fungos filamentosos [107]. No entanto, para o patógeno *S. schenckii* foi verificada apenas atividade *in vitro* e a significância clínica permanece desconhecida [108].

Outra nova classe de antifúngicos é representada pelas equinocandinas. Até o momento, três equinocandinas destacam-se pela importância clínica já evidenciada: caspofungina, anidulafungina e micafungina [109]. No entanto, para *S. schenckii* ainda não há relatos de experiência clínica, embora estes fármacos apresentem atividade *in vitro* [110-112].

Além do tratamento com antifúngicos, em alguns casos, pode ser indicada a termoterapia, que consiste em submeter à região afetada a uma temperatura acima de 42°C por 30 minutos, duas vezes ao dia. Esta técnica pode ser utilizada isoladamente ou combinada com antifúngicos [113]. Já na doença osteoarticular, a abordagem cirúrgica também pode ser indicada [114].

2.9. Avaliação da suscetibilidade aos antifúngicos

Infecções fúngicas estão associadas, muitas vezes, a tratamentos difíceis e longos, visto que os organismos fúngicos multiplicam-se mais lentamente e, em alguns casos, infectam tecidos ou órgãos que apresentam dificuldades para o acesso dos fármacos antifúngicos, resultando em concentrações abaixo das faixas terapêuticas estabelecidas. Além disso, os fungos também desenvolvem mecanismos de resistência aos fármacos resultando em falhas terapêuticas, as quais estão associadas ao aumento na prevalência de morbidade e mortalidade dos pacientes acometidos por micoses [107].

Considerando esses agravos na evolução das doenças fúngicas humanas, tornou-se imprescindível o aperfeiçoamento das metodologias laboratoriais para determinação das suscetibilidades *in vitro* dos diferentes patógenos frente aos agentes antifúngicos disponíveis para uso clínico. A possibilidade de predizer um insucesso terapêutico denota a importância da correlação entre um ensaio de suscetibilidade e a resposta clínica. Estes testes também podem ser utilizados para descoberta de novos fármacos e estudos epidemiológicos [115].

A partir da aprovação do protocolo M38-A em 2002 pelo *National Committee for Clinical Laboratory Standards*, tornou-se disponível a metodologia para os testes de suscetibilidade *in vitro* para alguns fungos filamentosos patogênicos, incluindo *S. schenckii* [116]. Mais recentemente, o *Clinical and Laboratory Standards Institute* (anteriormente denominado *National Committee for Clinical Laboratory Standards*) publicou o protocolo M38-A2, com atualizações sobre os ensaios de suscetibilidade aos antifúngicos. No entanto, ainda não foram estabelecidos os pontos de corte para o fungo *S. schenckii*. Baseado em estudos com outros fungos foi postulado que, para fins analíticos, microrganismos com concentrações inibitórias mínimas (CIMs) menores ou iguais a 1µg/ml podem ser classificados como suscetível; CIM = 2µg/ml como intermediário e CIM ≥ 4µg/ml como resistente. Porém, os pontos de corte com relevância comprovada ainda precisam ser padronizados pelas agências reguladoras [117].

A partir da publicação destes protocolos, ampliou-se o conhecimento sobre o perfil de suscetibilidade de *S. schenckii* aos antifúngicos utilizados na terapêutica desta micose. Estudos mostram que isolados obtidos de diferentes hospedeiros (humanos e animais), espécies do complexo *S. schenckii* e localizações geográficas apresentam significativas variações quanto à virulência e suscetibilidades aos antifúngicos tanto *in vitro* quanto *in vivo* [14,15,118].

No entanto, estes ensaios ainda são muito trabalhosos e de elevado custo para serem realizados na prática clínica. Assim, são necessárias adaptações ou novas ferramentas que permitam determinar a sensibilidade dos patógenos fúngicos frente aos agentes antifúngicos na prática clínica.

2.9.1. Substâncias alternativas no tratamento da esporotricose

O tratamento de infecções fúngicas é dificultado pelo número reduzido de antifúngicos disponíveis, além da resistência a estes agentes. Assim, estudos científicos são necessários para desenvolver novos fármacos com propriedades antifúngicas. Embora a maioria dos antimicrobianos em uso clínico tenha sido obtido a partir de microrganismos, um renovado interesse em antimicrobianos a partir de plantas surgiu nos últimos vinte anos. Na verdade, as plantas e os seus derivados têm uma longa e relevante história como potenciais fontes de medicamentos [119].

Com raras exceções, a micologia humana e veterinária possuem os mesmos patógenos e, portanto, a experiência tradicional ou popular no tratamento de micoses nos animais (tratamento etnoveterinário) é considerada, hoje em dia, uma valiosa ferramenta para a descoberta de novos fármacos antifúngicos [119]. De fato, um estudo etnoveterinário realizado por Avancini (2002) [120] indicou que "quitoco" (*Pterocaulon* spp.) foi eficaz para tratar doenças de pele popularmente diagnosticadas como micoses, tanto em humanos como em animais.

O gênero *Pterocaulon* pertence a família *Asteraceae*, a qual compreende cerca de 1100 gêneros com aproximadamente 23000 espécies [121]. Este gênero é amplamente distribuído no sul e no norte da América e da Austrália e está relacionado a propriedades medicinais [122].

Extratos metanólicos brutos e frações hexano, diclorometano e metanol de *Pterocaulon alopecuroides*, *Pterocaulon balansae* e *Pterocaulon polystachyum* mostraram atividade antifúngica *in vitro* frente a leveduras e alguns fungos filamentosos [123]. Mais recentemente, foi demonstrada a atividade antifúngica do extrato metanólico bruto de *P. alopecuroides* frente à *Fonsecaea pedrosoi* e *Fonsecaea compacta*, agentes etiológicos da cromoblastomicose, uma micose subcutânea de difícil tratamento [119].

2.10. Fatores de virulência

Vários mecanismos estão envolvidos nos processos que permitem que os patógenos sejam capazes de aderir a uma superfície do hospedeiro e colonizá-la, de sobreviver e multiplicar-se em ambientes desfavoráveis, além de enganar a resposta imune do hospedeiro. Estes mecanismos são conhecidos como fatores de virulência [13].

Os fatores de virulência de *S. schenckii* não estão bem elucidados, mas alguns deles podem estar relacionados à termotolerância, a produção de enzimas e de polissacarídeos extracelulares [25]. Nas últimas décadas, as enzimas dos patógenos fúngicos têm merecido atenção pela sua importância na patogenicidade e como possível alvo para confecção de inibidores sintéticos que possam tratar as micoses [26].

Outros fatores de virulência relacionados à parede celular têm sido descritos. A melanina, presente na parede celular do fungo, é considerada um importante fator associado à virulência de *S. schenckii* [124]. Madrid e colaboradores (2010) [125] mostraram que isolados produtores de melanina tem maior capacidade invasiva do que os que não produzem este pigmento. Outros estudos sugerem que a melanina produzida pelo fungo pode protegê-lo contra a fagocitose e morte por monócitos, macrófagos e proteinases extracelulares, além de remover os radicais livres [126,127].

Recentemente foi descrita uma glicoproteína que confere proteção aos animais infectados experimentalmente com *S. schenckii*, além de inibir a ligação com a fibronectina e a laminina [128]. Além disso, foram caracterizadas outras glicoproteínas que participariam na adesão do patógeno a células epiteliais [129].

Diferentes estudos utilizando modelo animal já foram realizados visando comparar a virulência dos diferentes isolados de *S. schenckii*. Em um deles, cepas isoladas de lesões cutâneas e subcutâneas foram incapazes de levar os animais infectados à morte, enquanto as isoladas de esporotricose disseminada causaram a morte dos mesmos. Além disso, os animais

inoculados com cepas de esporotricose disseminada apresentaram um início mais precoce da doença e lesões mais graves do que os inoculados com cepas de esporotricose linfocutânea e de esporotricose cutânea fixa, respectivamente [96]. Em outro modelo murino, Fernandes e colaboradores (1999) [130] observaram que conídios isolados de culturas micelianas após 10 ou 12 dias de cultivo são menos virulentos que conídios isolados após 4 ou 7 dias de cultivo. Posteriormente, observaram que a forma de levedura apresentou maior grau de infectividade e maior resistência aos macrófagos do que a micelial. Além disso, a análise da composição de carboidratos da parede celular revelou a presença de manose na superfície dos conídios menos virulentos, sugerindo que variações na expressão de componentes de superfície poderiam estar relacionadas a virulência deste patógeno [131].

2.11. Leveduras *Killer*

Leveduras com fenótipo micocinogênico secretam proteínas ou glicoproteínas (toxinas *killer* ou micocinas) que são inibitórias para células sensíveis de outros microrganismos. Os isolados micocinogênicos são imunes ao efeito de sua própria toxina, mas podem ser suscetíveis a toxinas produzidas por outras leveduras micocinogênicas [132]. O primeiro uso do sistema *killer* de leveduras para discriminação de microrganismos foi com isolados de *Candida albicans,* provando ser uma ferramenta epidemiológica de grande valor na identificação de casos presumidos de infecção fúngica nosocomial [133]. Desde então, o sistema *killer* foi aplicado na diferenciação de outros fungos patogênicos e/ou de importância industrial, como *Candida glabrata, Candida kefyr, Candida parapsilosis, Candida tropicalis, Cryptococcus neoformans, Saccharomyces cerevisiae, Pseudallescheria boydii, Penicillium camembertii* e *Aspergillus niger* [24,134-139]. Há relatos da inibição de quatro isolados de *S. schenckii* nas formas miceliana e leveduriforme por toxinas produzidas por leveduras [140]. A

ampliação do número de amostras de *S. schenckii* testadas é necessária para se avaliar a utilização das toxinas *killer* na biotipagem deste fungo.

Além da biotipagem de microrganismos patogênicos, as toxinas produzidas por estas leveduras estão sendo testadas como novos agentes antifúngicos em pesquisas clínicas [141]. Sua atividade, comparada aos convencionais antifúngicos azólicos e poliênicos, apresentou melhores resultados em concentrações menores que a usada com os fármacos [137,142]. Testes histopatológicos em fígado e pulmão humanos com cepas de *Paracoccidioides brasiliensis* têm demonstrado que uma toxina produzida a partir de *Pichia anomala* causa uma significativa redução da infecção, principalmente quando associada a antifúngicos convencionais [141]. A mesma toxina está sendo testada na profilaxia da pneumocistose em pacientes imunocomprometidos [143].

3. OBJETIVOS

3.1. Objetivo Geral

Determinar a variabilidade genética, o perfil enzimático, a suscetibilidade a antifúngicos e a sensibilidade a toxinas *killer* dos isolados do complexo *S. schenckii* oriundos de quatro estados brasileiros: Minas Gerais, Rio Grande do Sul, Rio de Janeiro e São Paulo.

3.2. Objetivos específicos

a) Identificar fenotípica e genotipicamente os isolados dentro das espécies do complexo *S. schenckii*;

b) Determinar os perfis genéticos para o conhecimento da epidemiologia molecular dos isolados;

c) Desenvolver ferramentas de biotipagem do complexo *S. schenckii*, utilizando 18 leveduras com potencial *killer*;

d) Avaliar a produção das enzimas esterase, proteinase, urease, desoxiribonuclease (DNase) e fosfolipase como fator de virulência dos isolados de diferentes origens geográficas e espécies do complexo *S. schenckii*;

e) Analisar o perfil de suscetibilidade do complexo *S. schenckii* frente a antifúngicos comercialmente disponíveis e a extratos de origem vegetal;

f) Avaliar a viabilidade do meio de cultura líquido Sabouraud dextrose para os ensaios *in vitro* de sensibilidade a antifúngicos.

4. REFERÊNCIAS

[1] Crothers SL, White SD, Ihrke PJ, Affolter VK. Sporotrichosis: a retrospective evaluation of 23 cases seen in northern California (1987-2007). Vet Dermatol. 2009;20(4):249-59.

[2] Nobre MO, Meireles MCA, Caetano DT, Faé F, Cordeiro JMC, Meireles RM, et al. Esporotricose zoonótica na região sul do Rio Grande do Sul (Brasil) e revisão da literatura brasileira. Rev Bras Cien Vet. 2002;9(1):36-41.

[3] Silva-Vergara ML, Maneira FRZ, Oliveira RM, Santos CTB, Etchebehere M, Adad SJ. Multifocal sporotrichosis with meningeal involvement in a patient with AIDS. Med Mycol. 2005;43(2):187-90.

[4] Vásquez-del-Mercado E, Arenas R, Padilla-Desgarenes C. Sporotrichosis. Clin Dermatol. 2012;30(4):437-43.

[5] Torres-Rodríguez JM, Palácio-Hernanz A, Negroni-Briz R, Pereiro-Miguens M. Esporotricosis In: Torres-Rodríguez JM, Palácio-Hernanz A, Negroni-Briz R, Pereiro-Miguens M, editores. Micología Médica, Barcelona: Marron S.A.; 1993. P. 157-66.

[6] Lopes JO, Alves SH, Mari CR, Brum LM, Westphalen JB, Alternmann MJ, et al. Epidemiologia da esporotricose na região central do Rio Grande do Sul. Rev Soc Bras Med Trop. 1999;32(5):541-5.

[7] da Rosa ACM, Scroferneker ML, Vettorato R, Gervini RL, Vettorato G, Weber A. Epidemiology of sporotrichosis: a study of 304 cases in Brazil. J Am Acad Dermatol. 2005;52(3):451-9.

[8] Coskun B, Saral Y, Akpolat N, Ataseven A, Cicek D. Sporotrichosis successfully treated with terbinafine and potassio iodide: case report and review of the literature. Mycopathologia. 2004;158(1):53-6.

[9] Sterling JB, Heymann WR. Potassium iodide in dermatology: a 19th century drug for the 21st century – Uses, pharmacology, adverse effects, and contraindications. J Am Acad Dermatol. 2000;43(4):691-7.

[10] Kohler LM, Hamdana JS, Ferrari TCA. Successful treatment of a disseminated *Sporothrix schenckii* infection and *in vitro* analysis for antifungal susceptibility testing. Diagn Microbiol Infect Dis. 2007;58(1):117-20.

[11] Heidrich D, Stopiglia CD, Senter L, Vetoratto G, Valente P, Scroferneker ML. Successful treatment of terbinafine in a case of sporotrichosis. An Bras Dermatol. 2011;86(4 Suppl 1):S182-5.

[12] Meinerz ARM, Nascente PS, Schuch LFD, Cleff MB, Santin R, Brum CS, et al. Suscetibilidade *in vitro* de isolados de *Sporothrix schenckii* frente à terbinafina e itraconazol. Rev Soc Bras Med Trop. 2007;40(1):60-2.

[13] Köhler GA, Brenot A, Haas-Stapleton E, Agabian N, Deva R, Nigam S. Phospholipase A2 and phospholipase B activities in fungi. Biochim Biophys Acta. 2006;1761(11):1391-9.

[14] Silveira CP, Torres-Rodríguez JM, Alvarado-Ramírez E, Murciano-Gonzalo F, Dolande M, Panizo M, et al. MICs and minimum fungicidal concentrations of amphotericin B, itraconazole, posaconazole and terbinafine in *Sporothrix schenckii*. J Med Microbiol. 2009;58(Pt 12):1607–10.

[15] Oliveira DC, Lopes PG, Spader TB, Mahl CD, Tronco-Alves GR, Lara VM, et al. Antifungal susceptibilities of *Sporothrix albicans*, *S. brasiliensis*, and *S. luriei* of the *S. schenckii* complex identified in Brazil. J Clin Microbiol. 2011;49(8):3047-9.

[16] Neyra E, Fonteyne P, Swinne D, Fauche F, Bustamante B, Nolard N. Epidemiology of human sporotrichosis investigated by amplified fragment length polymorphism. J Clin Microbiol. 2005;43(3):1348-52.

[17] Marimon R, Gené J, Cano J, Trilles L, dos Santos Lazéra M, Guarro J. Molecular phylogeny of *Sporothrix schenckii*. J Clin Microbiol. 2006;44(9):3251-6.

[18] de Meyer EM, de Beer ZW, Summerbell RC, Moharram AM, de Hoog GS, Vismer HF, et al. Taxonomy and phylogeny of new wood and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. Mycologia. 2008;100(4):647–61.

[19] Madrid H, Gené J, Cano J, Silvera C, Guarro J. *Sporothrix brunneoviolacea* and *Sporothrix dimorphospora*, two new members of the *Ophiostoma stenoceras-Sporothrix schenckii* complex. Mycologia. 2010;102(5):1193-203.

[20] Marimon R, Cano J, Gené J, Sutton DA, Kawasaki M, Guarro J. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. J Clin Microbiol. 2007;45:3198–206.

[21] Marimon R, Gené J, Cano J, Guarro J. *Sporothrix luriei*: a rare fungus from clinical origin. Med Mycol. 2008;46(6):621-5.

[22] Oliveira MM, Almeida-Paes R, Muniz MM, Gutierrez-Galhardo MC, Zancope-Oliveira RM. Phenotypic and molecular identification of *Sporothrix* isolates from an epidemic area of sporotrichosis in Brazil. Mycopathologia. 2011;172(4):257-67.

[23] Rodrigues AM, de Hoog S, de Camargo ZP. Emergence of pathogenicity in the *Sporothrix schenckii* complex. Med Mycol. In press 2012.

[24] Fuentefria AM, Faganello J, Pazzini F, Schrank A, Valente P, Vainstein M. Typing and patterns of cellular morphological alterations in *Cryptococcus neoformans* and *Cryptococcus gattii* isolates exposed to a panel of killer yeasts. Med Mycol. 2007;45(6):503-12.

[25] Hogan LH, Klein BS, Levitz SM. Virulence factors of medically important fungi. Clin Microbiol Rev. 1996;9(4):469-88.

[26] Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev. 2003;67(3):400-28.

[27] Schenck BR. On refractory subcutaneous abscesses caused by a fungus possibly related to the *Sporotricha*. Bulletin of Johns Hopkins Hospital.1898;9:286-90.

[28] Hektoen L, Perkins CF. Refractory subcutaneous caused by *Sporothrix schenckii*. A new pathogenic fungus. J Exp Med. 1900;5(1):77– 89.

[29] Kwon-Chung K, Bennett J. Sporotrichosis. In: Kwon-Chung K, Bennett J, editores. Medical mycology, Philadelphia: Lea&Febiger; 1992. P. 707– 29.

[30] Antunes T. Efeito do itraconazol e da terbinafina no tratamento da esporotricose cutânea experimental [Dissertação]. Pelotas(RS): Universidade Federal de Pelotas; 2004.

[31] Lutz A, Splendore A. Sobre uma micose observada em homens e ratos. Rev Med São Paulo. 1907;21:433–50.

[32] Londero AT, Castro RM, Fischman, O. Two cases of sporotrichosis in dog in Brazil. Sabouraudia.1964;3(4):273-4.

[33] Campbell I. Esporotricose. In: Zaitz D, editor. Compêndio de micologia médica. Rio de Janeiro: Editora Médica Científica; 1988. P.123-37.

[34] Donadell KW, Werlang K, Diaz RW, Carvalhaes OJ, Rubén A. Esporotricose – revisão. An Bras Dermatol. 1993;68(1):45-62.

[35] Lopes-Bezerra LM, Schubach A, Costa RO. *Sporothrix schenckii* and sporotrichosis. An Acad Bras Cienc. 2006;78(2):293-308.

[36] Barros MBL, Paes RA, Schubach AO. *Sporothrix schenckii* and sporotrichosis. Clin Microbiol Rev. 2011;24(4):633–54.

[37] Criseo G, Malara G, Romeo O, Puglisi Guerra A. Lymphocutaneous sporotrichosis in an immunocompetent patient: a case report from extreme southern Italy. Mycopathologia. 2008;166(3):159–62.

[38] Magand F, Perrot JL, Cambazard F, Raberin MH, Labeille B. Sporotrichose cutanée autochtone française. Ann Dermatol Venereol. 2009;136:273–5.

[39] Dixon DM, Duncan RA, Hurd NJ. Use of a mouse model to evaluate clinical and environmental isolates of *Sporothrix* spp. from the largest U.S. epidemic of sporotrichosis. J Clin Microbiol. 1992;30(4):951-4.

[40] Gezuele E, da Rosa D. Importance of the sporotrichosis asteroid body for the rapid diagnosis of sporotrichosis. Rev Iberoam Micol. 2005;22(3):147-50.

[41] Pappas P, Tellez I, Deep A, Nolasco D, Holgado H, Bustamante B. Sporotrichosis in Peru: description of an area of hiperendemicity. Clin Infect Dis. 2000;30(1):65-70.

[42] Barros MBL, Schubach TMP, Galhardo MCG, Schubach AO, Monteiro PCF, Reis RS, et al. Sporotrichosis: an emergent zoonosis in Rio de Janeiro. Mem Inst Osw Cruz. 2001;96(6):777-9.

[43] Barros MBL, Schubach TP, Coll JO, Gremião ID,Wanke B, Schubach A. Esporotricose: a evolução e os desafios de uma epidemia. Rev Panam Salud Publica. 2010;27(6):455–60.

[44] Schubach A, Barros MB, Wanke B. Epidemic sporotrichosis. Curr Opin Infect Dis. 2008;21(2):129–33.

[45] Fleury RN, Taborda PR, Grupta AK, Fujita MS, Rosa PS, Weckwerth AC, et al. Zoonotic sporotrichosis. Transmission to humans by infected domestic cat scratching: report of four cases in São Paulo, Brazil. Int J Dermatol. 2001;40(5):318-22.

[46] Bonifaz A, Vázquez-González D, Perusquía-Ortiz AM. Subcutaneous mycoses: chromoblastomycosis, sporotrichosis and mycetoma. J Dtsch Dermatol Ges. 2010;8(8):619-27.

[47] Alves SH, Boettcher CS, Oliveira DC, Tronco-Alves GR, Sgaria MA, Thadeu P, et al. *Sporothrix schenckii* associated with armadillo hunting in Southern Brazil: epidemiological and antifungal susceptibility profiles. Rev Soc Bras Med Trop. 2010;43(5):523–5.

[48] Rubio G, Sanchez G, Porras L, Alvarado Z. Esporotricosis: prevalencia, perfil clinico y epidemiologico en un centro de referencia em Colombia. Rev Iberoam Micol. 2010;27(2):75–9.

[49] Takenaka M, Sato S, Nishimoto K. Survey of 155 sporotrichosis cases examined in Nagasaki Prefecture from 1951 to 2007. Nippon Ishinkin Gakkai Zasshi. 2009;50(2):101-8.

[50] Ghosh A, Chacrabarti A, Sharma VK, Singh K, Singh A. Sporotrichosis in Himachal Pradesh (North India). Trans R Soc Trop Med Hyg. 1999;93(1):41-5.

[51] Itoh M, Okamoto S, Kariya H. Survey of 200 cases of sporotrichosis. Dermatologica. 1986;172(4):209-13.

[52] Kariya H, Iwatsu T. Statistical survey of 100 cases of sporotrichosis. J Dermatol. 1979;6(4):211-7.

[53] Barros MBL, Schubach AO, Schubach TM, Wanke B, Lambert-Passos SR. An epidemic of sporotrichosis in Rio de Janeiro, Brazil: epidemiological aspects of a series of cases. Epidemiol Infect. 2008;136(9):1192–6.

[54] Larsson CE. Esporotricose. Braz J Vet Res Anim Sci. 2011;48(3):250-9.

[55] Guarro J, Gené J, Stchigel AM. Developments in fungal taxonomy. Clin Microbiol Rev. 1999;12(3):454–500.

[56] Madrid H, Cano J, Gené J, Bonifaz A, Toriello C, Guarro J. *Sporothrix globosa*, a pathogenic fungus with widespread geographical distribution. Rev Iberoam Micol. 2009;26(3):218–222.

[57] Prillinger H, König H. The intestinal yeasts. In: König H, Varma A, editores. Intestinal microorganisms of termites and other invertebrates. Heidelber: Springer Verlag; 2006. P. 328.

[58] Arrillaga-Moncrieff I, Capilla J, Mayayo E, Marimon R, Mariné M, Gené J, et al. Different virulence levels of the species of *Sporothrix* in a murine model. Clin Microbiol Infect. 2009;15(7):651–5.

[59] Reis RS, Almeida-Paes R, Muniz M de M, Tavares PM, Monteiro PC, Schubach TM, et al. Molecular characterisation of *Sporothrix schenckii* isolates from humans and cats involved in the sporotrichosis epidemic in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2009;104(5):769-74.

[60] Fernandes GF, dos Santos PO, do Amaral CC, Sasaki AA, Godoy-Martinez P, de Camargo ZP. Characteristics of 151 Brazilian *Sporothrix schenckii* isolates from 5 different geographic regions of Brazil: a forgotten and re-emergent pathogen. The Open Mycol J. 2009;3(1):48-58.

[61] Lacaz CS, Porto E, Martins JECM. Esporotricose e outras micoses gomosas. In: Micologia Médica. São Paulo: Sarvier; 1991. P. 233-47.

[62] Araujo T, Marques AC, Kerdel F. Sporotrichosis. Intern J Dermatol. 2001;40:737-42.

[63] Zaitz C, Ruiz LRB, Souza VM. Atlas de micologia médica: diagnóstico laboratorial. 2ª ed. Rio de Janeiro(RJ): MEDSI Editora; 2004.

[64] Carvalho MT, Castro AP, Baby C, Werner B, Filus Neto J, Queiroz-Telles F. Disseminated cutaneous sporotrichosis in a patient with AIDS: report of a case. Rev Soc Bras Med Trop. 2002;35(6):655-9.

[65] Howell SJ, Toohey JS. Sporotrichal arthritis in south central Kansas. Clin Orthop. 1998;346(1):207-14.

[66] Ware AJ, Cockerell CJ, Skiest DJ, Kussman HM. Disseminated sporotrichosis with extensive cutaneous involvement in a patient with AIDS. J Am Acad Dermatol. 1999; 40(2 Pt 2):350-5.

[67] Losman JA, Cavanaugh K. Cases from the Osler Medical Service at John Hopkins University. Diagnosis: *P. carinii* pneumonia and primary pulmonary sporotrichosis. Am J Med. 2004;117(5):353-6.

[68] Durden FM, Elewski B. Fungal infections in HIV-Infected patients. Sem Cut Med Sug.1997;16(3):200-12.

[69] Al-Tawfiq JA, Wools KK. Disseminated sporotrichosis and *Sporothrix schenckii* fungemia as the initial presentation of human immunodeficiency virus infection. Clin Inf Dis. 1998;26(6):1403-6.

[70] Neto RJ, Machado AA, Castro G, Quaglio ASS, Martinez R. Acquired immunodeficiency syndrome presenting as disseminated cutaneous sporotrichosis. Rev Soc Bras Med Trop. 1999;32(1):57-61.

[71] Dixon DM, Salkin IF, Duncan RA, et al. Isolation and characterization of *Sporothrix schenkii* from clinical and environmental sources associated with the largest U.S. epidemic of sporotrichosis. J Clin Microbiol. 1991;29(6):1106-13.

[72] Campbell IF, Zaitz C. Esporotricose. In: Sidrim JJC, Rocha MFG, editores. Micologia médica à luz de autores contemporâneos. Rio de Janeiro: Guanabara Koogan; 2004. P.177-88.

[73] Shiraishi H, Gomi H, Kawada A, Matsuo I, Hiruma M. Solitary sporotrichosis lasting for 10 years. Dermatol. 1999;198(1):100-1.

[74] Bustamante B, Campos PE. Endemic sporotrichosis. Curr Opin Infect Dis. 2001; 14(2):145-9.

[75] Kauffman CA, Hajjeh R, Chapman SW. Practice guidelines for the management of patients with sporotrichosis. Clin Inf Dis. 2000;30(4):684-7.

[76] Arechavala A, Orduna T, Maiolo E, Mujica MT, Fernández M, Negroni R. Esporotricosis diseminada, con compromiso cutáneo y visceral. Rev Patol Trop. 2011; 40(1):73-84.

[77] Patange V, Cesani F, Phillpott J, Villanueva-Meyer J. Three-phase bone and Ga-67 scintigraphy in disseminated sporotrichosis. Clin Nuclear Med. 1995;20(10):909-12.

[78] Morris-Jones R. Sporotrichosis. Clin Dermatol. 2002; 27(6):427-31.

[79] Kumar R, van der Smissen E, Jorizzo J. Systemic sporotrichosis with osteomyelitis. J Can Assoc Radiol. 1984;35(1):83–4.

[80] Rotz LD, Slater LN, Wrack MF, Boyd AL, Scott EN, Greefield RA. Disseminated sporotrichosis with meningitis in a patient with AIDS. Infect Dis Clin Pract. 1996;5(9): 566-8.

[81] Scott EN, Kauffman L, Brown AC, Muchmore HG. Serologic studies in the diagnosis and management of meningitis due to *Sporothrix schenckii*. New Engl J Med 1987;317(15):935-40.

[82] Bernardes-Engemann AR, Costa RC, Miguens BR, Penha CV, Neves E, Pereira BA, et al. Development of an enzyme-linked immunosorbent assay for the serodiagnosis of several clinical forms of sporotrichosis. Med Mycol. 2005;43(6):487–93.

[83] Palomino J, Saeed O, Daroca P, Lasky J. A 47-year-old man with cough, dyspnea, and an abnormal chest radiograph. Chest. 2009;135(3):872–5.

[84] Pluss JL, Opal SM. Pulmonary sporotrichosis: review of treatment and outcome. Medicine (Baltimore) 1986;65(3):143–53.

[85] Singhai M, Rawat V, Verma P, Jha PK, Shree D, Goyal R, et al. Primary pulmonary sporotrichosis in a sub-Himalayan patient. J Lab Physicians. 2012;4(1):48-9.

[86] Ramirez J, Byrd RPJ, Roy TM. Chronic cavitary pulmonary sporotrichosis: efficacy of oral itraconazole. J Ky Med Assoc. 1998;96(3):103–5.

[87] Quintella LP, Passos SR, de Miranda LH, Cuzzi T, Barros MB, Francesconi-do-Vale AC, et al. Proposal of a histopathological predictive rule for the differential diagnosis between American tegumentary leishmaniasis and sporotrichosis skin lesions. Br J Dermatol. 2012;167(4):837-46.

[88] Sanchez-Aleman MA, Araiza J, Bonifaz A. Isolation and characterization of wild *Sporothrix schenkii* strains and investigation of sporototrichin reactors. Gac Med Mex. 2004;140(5):507–12.

[89] Albornoz MB, Villanueva E, Torres ED. Application of immunoprecipitation techniques to the diagnosis of cutaneous and extracutaneous forms of sporotrichosis. Mycopathologia. 1984;85(3):177–83.

[90] Rippon, J. Sporotrichosis. In: Rippon, editor. Medical mycology - the pathogenic fungi and the pathogenic actinomycetes. 3rd ed. W. B. Philadelphia: Saunders Company; 1988. P. 325–52.

[91] Almeida-Paes R, Pimenta MA, Pizzini CV, Monteiro PC, Peralta JM, Nosanchuk JD, et al. Use of mycelial-phase *Sporothrix schenckii* exoantigens in an enzyme-linked immunosorbent assay for diagnosis of sporotrichosis by antibody detection. Clin Vaccine Immunol. 2007;14(3):244-9.

[92] Suzuki K, Kawasaki M, Ishizaki H. Analysis of restriction profiles of mitochondrial DNA from *Sporothrix schenckii* and related fungi. Mycopathologia. 1988;103(3):147-51.

[93] Ishizaki H. Mitochondrial DNA analysis of *Sporothrix schenckii*. Nihon Ishinkin Gakkai Zasshi. 2003;44(3):155-7.

[94] Mesa-Arango AC, Del Rocío Reyes-Montes M, Pérez-Mejía A, Navarro-Barranco H, Souza V, Zúñiga G, et al. Phenotyping and genotyping of *Sporothrix schenckii* isolates according to geographic origin and clinical form of sporotrichosis. J Clin Microbiol. 2002;40(8):3004-11.

[95] Liu X, Lian C, Jin L, An L, Yang G, Lin X. Characterization of *Sporothrix schenckii* by random amplification of polymorphic DNA assay. Chin Med J (Engl). 2003;116(2):239-42.

[96] Kong X, Xiao T, Lin J, Wang Y, Chen HD. Relationships among genotypes, virulence and clinical forms of *Sporothrix schenckii* infection. Clin Microbiol Infect. 2006;12(11):1077-81.

[97] Arenas R, Miller D, Campos-Macias P. Epidemiological data and molecular characterization (mtDNA) of *Sporothrix schenckii* in 13 cases from Mexico. Int J Dermatol. 2007;46(2):177-9.

[98] Zhang Z, Liu X, Yang G, Gao X, Jin L, An L. Genotyping of *Sporothrix schenckii* by analysis of ribosomal DNA regions. Mycoses. 2006;49(4):305-10.

[99] Galhardo MC, De Oliveira RM, Valle AC, Paes R de A, Silvatavares PM, Monzon A, et al. Molecular epidemiology and antifungal susceptibility patterns of *Sporothrix schenckii* isolates from a cat-transmitted epidemic of sporotrichosis in Rio de Janeiro, Brazil. Med Mycol. 2008;46(2):141-51.

[100] de Oliveira MM, Sampaio P, Almeida-Paes R, Pais C, Gutierrez-Galhardo MC, Zancope-Oliveira RM. Rapid identification of *Sporothrix* species by T3B fingerprinting. J Clin Microbiol. 2012;50(6):2159-62.

[101] Kohler LM, Monteiro PCF, Hahn RC, Hamdan JS. *In vitro* susceptibilities of isolates of *Sporothrix schenckii* to itraconazole and terbinafine. J Clin Microbiol. 2004; 42(9):4319-20.

[102] Kauffman CA, Bustamante B, Chapman SW, Pappas PG. Clinical practice guidelines for the management of sporotrichosis: 2007 update by the Infectious Diseases Society of America. Clin Infect Dis. 2007;45(10):1255–65.

[103] Deray G. Amphotericin B nephrotoxicity. J Antimicrob Chemother. 2002;49(Suppl. 1):37-41.

[104] Kauffman CA. Old and new therapies for sporotrichosis. Clin Infect Dis. 1995; 21(4):981–5.

[105] Rang HP, Dale MM, Ritter JM, Moore PK. Fármacos antifúngicos. In: Rang HP, Dale MM, Ritter JM, Moore PK, editores. Farmacologia. 5ª ed. Rio de Janeiro: Elsevier; 2004. P. 758-63.

[106] Francesconi G, Francesconi do Valle AC, Passos SL, de Lima Barros MB, de Almeida Paes R, Curi AL, et al. Comparative study of 250 mg/day terbinafine and 100 mg/day itraconazole for the treatment of cutaneous sporotrichosis. Mycopathologia. 2011;171(5):349-54.

[107] Neely MN, Ghannoum MA. The exciting future of antifungal therapy. Eur J Clin Microbiol Infect Dis. 2000;19(12): 887-914.

[108] Gutierrez-Galhardo MC, Zancopé-Oliveira RM, Monzón A, Rodriguez-Tudela JL, Cuenca-Estrella M. Antifungal susceptibility profile *in vitro* of *Sporothrix schenckii* in two growth phases and by two methods: microdilution and E-test. Mycoses. 2010;53(3):227-31.

[109] Chen SCA, Sorrell TC. Antifungal agents. The Med J Austr. 2007;187(7):404-9.

[110] Espinel-Ingroff A. Comparison of *in vitro* activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J Clin Microbiol. 1998;36(10):2950-6.

[111] Nakai T, Uno J, Ikeda F, Tawara S, Nishimura K, Miyaji M. *In vitro* antifungal activity of Micafungin (FK463) against dimorphic fungi: comparison of yeast-like and mycelial forms. Antimicrob Agents Chemother. 2003;47(4):1376-81.

[112] Ruíz-Cendoya M, Rodriguez MM, Mariné M, Pastor FJ, Guarro J. *In vitro* interactions of itraconazole and micafungin against clinically important filamentous fungi. Int J Antimicrob Agents. 2008;32(5):418-20.

[113] Badgwell Doherty C, Doherty SD, Rosen T. Thermotherapy in dermatologic infections. J Am Acad Dermatol. 2010;62(6):909-27.

[114] Köeter S, Jackson RW. Successful total knee arthroplasty in the presence of sporotrichal arthritis. Knee 2006;13(3):236-7.

[115] Rex JH, Pfaller MA, Walsh TJ, Chaturvedi V, Esoinel-Ingroff A, Ghannoum MA, et al. Antifungal susceptibility testing: practical aspects and current challenges. Clin Microbiol Rev.2001;14(4):643-58.

[116] National Committee for Clinical Laboratory Standards (NCCLS). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, approved standard. Vilanova (PA): NCCLS 2002: 221–7.

[117] Clinical and Laboratory Standards Institute. reference method for broth dilution antifungal susceptibility testing of filamentous fungi, approved standard. 2nd ed. Vilanova (PA): CLSI: 2008.

[118] Marimon R, Serena C, Gené J, Cano J, Guarro J. *In vitro* antifungal susceptibilities of five species of *Sporothrix*. Antimicrob Agents Chemother. 2008;52(2):732–4.

[119] Daboit TC, Stopiglia CD, von Poser GL, Scroferneker ML. Antifungal activity of *Pterocaulon alopecuroides* (*Asteraceae*) against chromoblastomycosis agents. Mycoses. 2010;53(3):246-50.

[120] Avancini CAM. Saneamento aplicado em saúde e produção animal: etnografia, triagem da atividade antibacteriana de plantas nativas do sul do Brasil [Dissertação]. Porto Alegre(RS): Universidade Federal do Rio Grande do Sul; 2002.

[121] Bremer K. *Astearaceae*: Cladistics and classification. Portland: Timber; 1994.

[122] Cabrera AL, Ragonese AM. Revisión del gênero *Pterocaulon* (*Compositae*). Buenos Aires: Darwiniana; 1978.

[123] Stein AC, Sortino M, Avancini C, Zacchino S, von Poser G. Ethnoveterinary medicine in the search for antimicrobial agents: antifungal activity of some species of *Pterocaulon* (*Asteraceae*). J Ethnopharmacol 2005;99(2):211–4.

[124] Nobre MO, Antunes TA, Meireles MCA, Ferreiro L. Production and evalution of albino mutants of *Sporothrix schenckii*. Acta Scientiae Veterinariae. 2004;32(2):119-23.

[125] Madrid IM, Xavier MO, Mattei AS, Fernandes CG, Guim TN, Santin R, et al. Role of melanin in the pathogenesis of cutaneous sporotrichosis. Microbes Infect. 2010;12(2):162-5.

[126] Davis BA. Sporotrichosis. Dermatol Clin. 1996;14(1):69-76.

[127] Romero-Martinez R, Wheeler M, Guerrero-Plata A, Rico G, Torres-Guerrero H. Biosynthesis and function of melanin in *Sporothrix schenckii*. Infect. Immun. 2000;68(6):3696-703.

[128] Nascimento RC. Caracterização da glicoproteína de 70kDa de *Sporothrix schenckii* e sua participação na regulação da resposta imune na esporotricose experimental [Tese]. São Paulo(SP): Universidade de São Paulo; 2008.

[129] Sandoval-Bernal G, Barbosa-Sabanero G, Shibayama M, Perez-Torres A, Tsutsumi V, Sabanero M. Cell wall glycoproteins participate in the adhesion of *Sporothrix schenckii* to epithelial cells. Mycopathologia. 2011;171(4):251-9.

[130] Fernandes KSS, Mathews HL, Lopes Bezerra LM. Differences in virulence of *Sporothrix schenckii* conidia related to culture conditions and cell wall components. J Med Microbiol. 1999;48(2):195-203.

[131] Fernandes KSS, Coelho ALJ, Lopes Bezerra LM, Barja-Fidalgo C. Virulence of *Sporothrix schenckii* conidia and yeast cells, and their susceptibility to nitric oxide. Immunol. 2000;101(4):563-9.

[132] Schmitt MJ, Breinig F. The viral killer system in yeast: from molecular biology to application. FEMS Microbiol Rev. 2002;26(3):257-76.

[133] Polonelli L, Archibusacci C, Sestito M, Morace G. Killer system: a simple method for differentiating *Candida albicans* strains. J Clin Microbiol. 1983;17(5):774–80.

[134] Buzzini P, Martini A. Differential growth inhibition as a tool to increase the discriminating power of killer toxin sensitivity in fingerprint of yeasts. FEMS Microbiol Lett. 2000;193(1):31-6.

[135] Buzzini P, Martini A. Discrimination between *Candida albicans* and other pathogenic species of the genus *Candida* by their differential sensitivities to toxins of a panel of killer yeasts. J Clin Microbiol. 2001;39(9):3362-4.

[136] Buzzini P, Berardinelli S, Turchetti B, Cardinali G, Martini A. Fingerprinting of yeasts at the strain level by differential sensitivity responses to a panel of selected killer toxins. Syst Appl Microbiol. 2003;26(3):466-70.

[137] Buzzini P, Turchetti B, Martini A. Assessment of discriminatory power of three different fingerprinting methods based on killer toxin sensitivity for the differentiation of *Saccharomyces cerevisiae* strains. J Appl Microbiol. 2004;96(5):1194-1201.

[138] Fanti F, Conti S, Campani L, Morace G, Dettori G, Polonelli L. Studies on the epidemiology of *Aspergillus fumigatus* infections in a university hospital. Eur J Epidemiol. 1989;5(1):8-14.

[139] Polonelli L, Dettori G, Cattel C, Morace G. Biotyping of mycelial fungus cultures by the killer system. Eur J Epidemiol. 1987;3(3):237–42.

[140] Polonelli L, Conti S, Campani L, Morace G, Fanti F. Yeast killer toxins and dimorphism. J Clin Microbiol. 1989;27(6):1423-5.

[141] Travassos LR, Silva LS, Rodrigues EG, Conti S, Salati A, Magliani W, et al. Therapeutic activity of a killer peptide against experimental paracoccidioidomycosis. J Antimicrob Chemother.2004;54(5):956–8.

[142] Weiler F, Schmitt MJ. Zygocin, a secreted antifungal toxin of the yeast *Zygosaccharomyces bailii*, and its effect on sensitive fungal cells. FEMS Yeast Res. 2003;3(1):69-76.

[143] Séguy N, Polonelli L, Dei-Cas E, Cailliez J. Effect of a killer toxin of *Pichia anomala* to *Pneumocystis*: perspectives in the control of pneumocystosis. FEMS Immunol Med Microbiol. 1998;22(1-2):145-9.

5. RESULTADOS E DISCUSSÃO

Os resultados e discussão dessa tese estão apresentados na forma de capítulos apresentando os manuscritos redigidos segundo as normas das revistas nas quais foram submetidos ou publicados.

5.1. Capítulo I

ANTIFUNGAL SUSCEPTIBILITIES AND IDENTIFICATION OF SPECIES OF THE *SPOROTHRIX SCHENCKII* COMPLEX ISOLATED IN BRAZIL

Submetido ao periódico:

Medical Mycology.

Antifungal susceptibilities and identification of species of the *Sporothrix schenckii* complex isolated in Brazil

Cheila Denise Ottonelli Stopiglia[a], Cibele Massotti Magagnin[a], Mauricio Ramírez Castrillón[b], Sandra Denise Camargo Mendes[b,c], Daiane Heidrich[a], Patricia Valente[d], Maria Lúcia Scroferneker[a,d*]

[a]Programa de Pós-Graduação em Medicina: Ciências Médicas, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2400 – 2º andar – CEP: 90035-003, Porto Alegre – RS, Brazil.

[b]Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, prédios 43421/43431 - setor IV - Campus do Vale – caixa postal 15005 - CEP 91501-970, Porto Alegre – RS , Brazil.

[c]Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina, Laboratório de Análises de Vinhos e Derivados, Estação Experimental de Videira, Rua João Zardo s/nº, Campo Experimental, CEP 89560-000, Videira, SC, Brazil.

[d]Departamento de Microbiologia, Imunologia e Parasitologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 – CEP 90050-170, Porto Alegre – RS, Brazil.

*Correspondence and reprints: Maria Lúcia Scroferneker, Departamento de Microbiologia, Imunologia e Parasitologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 sala 210, CEP: 90050-170, Porto Alegre - RS, Brasil. Tel. (+55) 51-33083934 Fax. (+55) 51-33083121 (e-mail: scrofern@ufrgs.br)

Abstract

Sporotrichosis is a subacute or chronic mycosis present worldwide that is caused by the dimorphic fungus *Sporothrix schenckii*, which is in fact a species complex. We studied 85 strains isolated in Brazil aiming their identification and evaluation of their antifungal susceptibility profiles. Based on phenotypic tests (microscopic features, ability to grow at 30ºC and 37°C, colony diameters, as well as assimilation of sucrose and raffinose) and molecular assays (amplification of a fragment of the calmodulin gene), the strains were identified as *S. schenckii*, *S. brasiliensis* and *S. globosa*, with the predominance de *S. schenckii*. There was 37.7% disagreement between the species classification using phenotypic and genotypic methodologies. In general, terbinafine was the most active drug, followed by ketoconazole and itraconazole, while fluconazole and voriconazole were less active. Five isolates were detected as itraconazole-resistant strains, being one *S. globosa* and four *S. schenckii*. There was no difference in susceptibility profiles to the antifungal agents among the *Sporothrix* species.

Keywords: *Sporothrix*; sporotrichosis; antifungal agents.

1. Introduction

Sporothrix schenckii is the etiologic agent of sporotrichosis, a subacute or chronic mycosis that can affect humans and animals [1]. Although sporotrichosis has been reported throughout the world, endemic areas are usually considered to be Latin America, South Africa, India, and Japan [2]. In Brazil, it has been described primarily in São Paulo and Rio Grande do Sul, mostly in males engaged in activities which would expose them to the etiologic agent [3-5] and in Rio de Janeiro, with cases involving the transmission of the etiologic agent from infected cats [6].

DNA techniques were used to examine clinical and environmental isolates of *S. schenckii* since 1988 [7]. Recent molecular studies have assessed the genetic diversity of *S. schenckii*, demonstrating that different clusters of *S. schenckii* strains are related to different geographical origins [6,8-9]. Moreover, other studies recognized the existence of new species with clinical importance: *Sporothrix brasiliensis*, *Sporothrix globosa*, *Sporothrix luriei*, and *Sporothrix mexicana*, through physiological and molecular studies, and an identification key for the *S. schenckii* species complex was proposed [10,11]. Posterior studies have suggested the inclusion of other species: *S. brunneoviolacea*, *S. dimorphospora* and *S. pallida*, all of environmental origins [12,13].

The treatment of choice for sporotrichosis while still at the skin lesion stage is systemic chemotherapy with potassium iodide [14]. In its disseminated cutaneous forms, and with recurrent, extracutaneous and lymphocutaneous infections, amphotericin B has been used with different therapeutic results [14,15]. Intolerance to iodine and the high toxicity of amphotericin B are however factors that often limit their use. In the last few decades, there has been an increase in the use of azole derivates, such as ketoconazole, itraconazole and fluconazole, as therapeutic alternatives to the classic schemes, and itraconazole came as the

most effective of them [16]. Another option is voriconazole, a new promising triazole antifungal agent for the treatment of mycoses caused by a broad spectrum of fungal pathogens [17]. On the other hand, by virtue of its excellent *in vitro* and *in vivo* activity, terbinafine has been used in various fungal infections [18-20].

The aim of this study was to characterize, at the species level, *Sporothrix* strains isolated in Brazil using the newer methods of taxonomic analysis, and to evaluate their antifungal susceptibility profiles.

2. Materials and methods

2.1. Fungal isolates

Eighty-five isolates of *S. schenckii* from different Brazilian states (Minas Gerais (n=15), Rio de Janeiro (n=20), São Paulo (n=19) and Rio Grande do Sul (n=31)) were examined in this study. All strains were previously identified as *S. schenckii* by direct examination and cultivation in routine laboratory analysis. Furthermore, the type strains *S. brasiliensis* FMR 8309 (CBS 120339, IPEC 16490), *S. globosa* FMR 8600 (CBS 120340), and *S. schenckii* FMR 6618 (CBS 359.36) were also included and used as reference strains.

2.2. Phenotypic identification

The isolates were identified phenotypically according to Marimon et al. [11]. The microscopic features were determined from slide cultures made on cornmeal agar after 10 to 12 days of incubation at 30 °C. Coverslips were mounted in lactic acid and examined under a

light microscope. The growth rate at 30°C of all the isolates included in the study was determined on potato dextrose agar (PDA). The petri dishes were centrally inoculated with pieces of the fungus that were approximately 1 mm in diameter, placed upside down. The colony diameters were measured after 21 days of incubation. The mean of the diameters was determined. To check growth at 37°C, the strains were grown on petri dishes with PDA and incubated for 3 weeks. Carbohydrate assimilation tests were performed using freshly prepared yeast nitrogen base (YNB) medium and tested for sucrose and raffinose. Cultures on YNB supplemented with glucose were used as positive control for growth and YNB without carbohydrates was used as a negative control. Experiments were performed at least three times on different days and, in case of discordant results, repeated two additional times.

2.3. Molecular identification

For molecular analysis, total genomic DNA was isolated using Power Soil DNA Isolation Kit (Mobio Laboratories). Partial sequencing of the nuclear calmodulin (CAL) gene was performed with primers CL1 (5'-GA(GA)T(AT)CAAGGAGGCCTTCTC-´3) and CL2A (5'-TTTTTGCATCATGAGTTGGAC-´3), as described by Arechavala et al. [15]. Briefly, the PCR mix consisted of 1x buffer PCR (Invitrogen), 3 mM magnesium chloride, 0.2 mM deoxynucleoside triphosphate mix, 10 pmol of each primer, 1.0 U Taq DNA polymerase (Invitrogen) and 12 ng fungal DNA. Samples were amplified using the following cycling parameters: one initial cycle of 4 min at 94°C followed by thirty-five cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C, and a single extension cycle at 72°C for 7 min.

The PCR product was purified using the Invisorb Fragment Clean-Up Kit (Invitek), and sequenced in the ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems), according to

the manufacturer's instructions. The sequence was assembled and compared with sequences reported in GenBank using the basic local alignment search tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Antifungal activity

The antifungal susceptibility test was developed according to the technique of broth microdilution recommended by the M38-A2 protocol of the Clinical Laboratory Standards Institute [21]. Six antifungal drugs commercially available for the treatment of sporotrichosis were used: ketoconazole, fluconazole, voriconazole, itraconazole, terbinafine and amphotericin B. The culture medium used was 1640-RPMI buffered at pH 7.0 with 165 mM of morpholinepropanesulfonic acid (MOPS).

The determination of the minimum inhibitory concentration (MIC) was performed visually by comparison with the growth in the drug free control. The MIC was defined as the lowest concentration of drug able to inhibit completely the fungal growth for itraconazole, voriconazole and amphotericin B, 80% of the fungal growth for terbinafine and 50% of the growth for fluconazole and ketoconazole.

In order to determine the minimum fungicidal concentration (MFC), 10 µL aliquots from the wells that showed 100% of growth inhibition were spread on Sabouraud dextrose agar. Plates were incubated at 28ºC for 72 h. The MFC was defined as the lowest drug concentration that yielded three or fewer colonies (i.e. 99% of the inoculum was killed) [22]. All the experiments were conducted in triplicate.

2.5. Statistical analysis

The statistical analysis was performed with nonparametric tests Kruskal Wallis and Bonferroni multiple comparison test using the software R version 3.0.0.

3. Results

From the Brazilian isolates identified after partial sequencing of the CAL gene, 22 (25.9%) were *S. brasiliensis*, 3 *S. globosa* (3.5%), and 60 (70.6%) *S. schenckii*. In the Brazilian states of Rio Grande do Sul and Rio de Janeiro predominated *S. schenckii*, followed by *S. brasiliensis*, and two *S. globosa* isolates were found in Rio Grande do Sul, while in São Paulo all isolates were identified as *S. schenckii*. Minas Gerais had isolates of *S. brasiliensis*, *S. schenckii*, and one isolate identified as *S. globosa* (Table 1).

Of the 85 strains phenotypically characterized, 14 isolates (16.5%) showed inconclusive or ambiguous results, and 18 (21.2%) were misidentified using the phenotypic methods in comparison to the molecular identification. Overall, there was 37.7% disagreement between the species classification using phenotypic and genotypic methodologies. Among the isolates misidentified phenotypically, twelve were characterized phenotypically as *S. mexicana* and identified genotypically as *S. schenckii*, five isolates were characterized phenotypically as *S. brasiliensis* and identified genotypically as *S. schenckii*, and one isolate was characterized phenotypically as *S. schenckii* and identified genotypically as *S. brasiliensis*.

Table 1: Antifungal susceptibility of the *Sporothrix schenckii* complex isolates according to the geographic origin.

Origin[c]	Species	Similarity	Antifungal agents											
			Amphotericin B		Ketoconazole		Fluconazole		Itraconazole		Voriconazole		Terbinafine	
			MIC[a]	MFC[b]	MIC[a]	MFC[b]	MIC[a]	MFC[b]	MIC[a]	MFC[b]	MIC[a]	MFC[b]	MIC[a]	MFC[b]
Brazil, Minas Gerais state (n = 15)														
CV	*S. brasiliensis*	99%[f]	2	2	0.50	2	128	>128	0.50	1	16	>16	0.03	0.50
EM-1	*S. schenckii*	96%[g]	2	4	0.06	8	128	>128	0.25	1	8	>16	0.12	1
GM-1	*S. schenckii*	97%[g]	1	1	0.12	4	64	>128	0.50	1	4	>16	0.50	2
JA-1	*S. schenckii*	97%[g]	2	4	0.06	1	32	>128	0.25	16	4	>16	0.03	0.25
JAT-6	*S. brasiliensis*	99%[f]	0.5	2	0.12	1	64	>128	0.25	16	4	>16	0.06	0.25
MYC-1	*S. brasiliensis*	99%[f]	1	1	0.25	8	128	>128	0.50	4	16	>16	0.25	1
MYC-3	*S. schenckii*	97%[g]	1	1	1	8	32	>128	2	16	8	>16	0.06	1
MYC-5	*S. schenckii*	96%[g]	2	4	0.5	16	64	>128	0.50	16	8	>16	0.12	1
164	*S. globosa*	97%[h]	0.25	2	0.12	2	32	>128	16	16	16	>16	0.01	0.25
357	*S. brasiliensis*	99%[f]	1	2	0.25	2	128	>128	0.50	0.50	4	>16	0.12	0.25
15572	*S. brasiliensis*	99%[f]	1	8	0.25	8	64	>128	0.50	16	4	>16	0.06	0.50
16042	*S. brasiliensis*	100%[f]	1	4	0.25	8	64	>128	0.25	16	8	>16	0.06	0.12
17943	*S. brasiliensis*	99%[f]	2	4	1	16	32	>128	0.50	16	16	>16	0.12	4
201679 ATCC	*S. schenckii*	97%[g]	1	1	0.5	4	128	>128	0.25	0.50	16	>16	0.06	0.12
201681 ATCC	*S. brasiliensis*	99%[f]	0.5	2	0.12	2	64	>128	0.25	1	4	>16	0.12	0.50
Range			0.2-2	1-8	0.06-1	1->16	32->128	>128	0.2-16	0.5->16	4-16	>16	0.01-0.5	0.1-0.5
Geometric mean			1.0	2.2	0.20	4.1	67.0	>128	0.50	4.4	8.0	>16	0.08	0.50
Brazil, Rio de Janeiro state (n = 20)														
51[e]	*S. brasiliensis*	99%[f]	2	2	0.06	2	64	>128	0.50	16	16	>16	0.12	0.50
52	*S. schenckii*	97%[g]	2	4	0.12	1	64	>128	0.25	8	8	>16	0.01	1
53	*S. brasiliensis*	99%[f]	2	2	0.50	2	128	>128	0.50	1	8	>16	0.03	0.50
54	*S. schenckii*	96%[g]	2	4	0.12	4	128	>128	0.25	16	8	>16	0.06	2
55	*S. schenckii*	96%[g]	1	1	0.25	2	128	>128	0.50	8	16	>16	0.12	1
56[e]	*S. schenckii*	97%[g]	2	4	0.03	2	64	>128	16	16	8	>16	0.06	1
57	*S. brasiliensis*	99%[f]	1	1	0.03	0.25	16	>128	0.25	16	8	>16	0.01	0.25
58	*S. brasiliensis*	99%[f]	1	1	0.25	16	64	>128	0.50	16	8	>16	0.50	1
59	*S. brasiliensis*	99%[f]	1	2	0.25	16	64	>128	0.50	16	8	>16	0.03	0.12
60	*S. schenckii*	97%[g]	1	1	0.25	4	64	>128	0.50	16	8	>16	0.06	2
61	*S. brasiliensis*	99%[f]	1	2	0.50	4	32	>128	0.50	16	8	>16	0.01	1
62	*S. brasiliensis*	98%[f]	1	2	0.50	16	32	>128	2	16	8	>16	0.12	16
63	*S. brasiliensis*	98%[f]	1	2	0.12	8	64	>128	0.50	16	8	>16	0.06	1
64	*S. brasiliensis*	99%[f]	0.5	2	0.03	1	32	>128	1	16	2	>16	0.01	1
65	*S. schenckii*	98%[g]	1	2	0.03	4	32	>128	1	16	8	>16	0.06	4
66	*S. schenckii*	97%[g]	0.5	2	0.12	16	16	>128	0.50	16	8	>16	0.03	0.50

67	*S. schenckii*	99%[g]	1	2	0.06	0.25	32	>128	1	16	8	>16	0.06	1
68	*S. schenckii*	97%[g]	1	8	0.03	8	64	>128	0.25	16	8	>16	0.06	1
69	*S. schenckii*	97%[g]	1	2	0.12	2	64	>128	0.25	16	4	>16	0.0625	0.5
70	*S. schenckii*	97%[g]	2	2	0.06	8	128	>128	0.50	16	8	>16	0.01	0.50
Range			0.5-2	1-8	0.03-0.50	0.2->16	16-128	>128	0.2-16	1->16	2-16	>16	>16	0.1-16
Geometric mean			1.1	2.1	0.1	3.2	53.8	>128	0.3	13.0	8.4	>16	>16	0.9
Brazil, Rio Grande do Sul state (n = 31)														
01	*S. brasiliensis*	99%[f]	1	1	0.06	16	32	>128	0.25	16	8	>16	0.06	1
02	*S. schenckii*	94%[g]	0.03	0.50	0.25	4	64	>128	1	16	8	>16	0.25	1
05	*S. schenckii*	99%[g]	2	4	0.03	2	32	>128	0.25	0.50	8	>16	0.06	0.50
07	*S. schenckii*	97%[g]	2	8	0.25	2	64	>128	0.25	4	16	>16	0.25	0.50
08	*S. schenckii*	97%[g]	1	2	0.03	2	16	>128	0.25	16	16	>16	0.25	1
09	*S. schenckii*	97%[g]	2	2	0.12	2	64	>128	2	16	8	>16	0.12	0.50
11	*S. schenckii*	97%[g]	0.50	1	0.06	4	32	>128	0.25	16	16	>16	0.06	0.25
12	*S. schenckii*	97%[g]	2	2	0.12	4	64	>128	0.25	16	8	>16	0.12	0.25
13	*S. brasiliensis*	96%[f]	0.25	1	0.06	4	128	>128	0.12	16	1	>16	0.06	0.25
14	*S. schenckii*	97%[g]	2	2	0.03	2	64	>128	0.12	16	16	>16	0.06	0.25
16	*S. schenckii*	97%[g]	1	1	0.06	8	64	>128	0.25	16	16	>16	0.06	4
19	*S. schenckii*	97%[g]	1	4	0.06	4	64	>128	0.06	4	16	>16	0.50	4
20	*S. schenckii*	96%[g]	2	4	0.06	2	64	>128	1	16	16	>16	0.06	0.12
22	*S. schenckii*	95%[g]	1	1	0.03	2	32	>128	0.12	16	8	>16	0.03	0.50
23	*S. schenckii*	97%[g]	1	4	0.03	0.50	128	>128	0.50	8	16	>16	0.06	0.50
24	*S. schenckii*	97%[g]	0.50	1	0.03	0.12	32	>128	0.25	0.50	16	>16	0.12	2
25[d]	*S. schenckii*	96%[g]	0.50	2	0.12	2	8	>128	0.25	16	2	>16	0.03	0.50
26	*S. schenckii*	97%[g]	1	1	0.06	2	32	>128	0.50	16	8	>16	0.06	0.50
3222876	*S. brasiliensis*	99%[f]	4	8	0.12	4	32	>128	0.12	1	2	>16	0.12	0.50
4674294	*S. schenckii*	97%[g]	1	1	0.25	2	128	>128	0.25	1	16	>16	0.25	2
4900941	*S. globosa*	99%[h]	0.50	0.50	0.03	0.06	16	>128	0.06	0.50	8	>16	0.03	0.50
I	*S. schenckii*	97%[g]	0.50	2	1	16	64	>128	8	16	16	>16	0.25	4
II	*S. schenckii*	97%[g]	2	2	0.12	8	32	>128	4	16	4	>16	0.03	0.25
III	*S. schenckii*	97%[g]	0.50	2	0.25	8	64	>128	1	16	2	>16	0.03	2
IV	*S. globosa*	99%[h]	2	2	0.50	2	128	>128	0.25	16	8	>16	0.03	0.25
V	*S. brasiliensis*	99%[f]	1	8	0.12	8	64	>128	0.06	4	4	>16	0.03	0.12
VI	*S. brasiliensis*	99%[f]	1	4	0.12	0.50	64	>128	0.12	16	16	>16	0.12	2
F32	*S. schenckii*	97%[g]	2	2	0.12	1	128	>128	0.25	8	4	>16	0.06	0.25
MLS	*S. schenckii*	97%[g]	1	2	0.12	0.50	64	>128	0.50	16	16	>16	0.12	0.50
UCS 31	*S. schenckii*	97%[g]	1	2	0.25	4	32	>128	0.25	2	8	>16	0.12	0.50
UCS 193/09	*S. schenckii*	97%[g]	2	2	0.25	4	64	>128	0.25	4	8	>16	0.01	0.03
Range			0.03-4	0.5-8	0.03-1	0.06->16	8-128	>128	0.06-8	0.5->16	1-16	>16	0.01-0.5	0.03-4
Geometric mean			1.0	2.0	0.1	2.3	50.0	>128	0.3	7.1	8.2	>16	0.08	0.6
Brazil, São Paulo state (n = 19)														
237	*S. schenckii*	97%[g]	1	8	0.03	16	64	>128	0.03	0.50	8	>16	0.06	0.50
329	*S. schenckii*	94%[g]	1	2	0.12	2	64	>128	0.50	16	8	>16	0.03	1

339	*S. schenckii*	97%[g]	1	2	0.25	0.25	64	>128	1	16	8	>16	0.01	0.01
424	*S. schenckii*	97%[g]	1	2	0.25	2	128	>128	1	16	4	>16	0.12	0.25
432	*S. schenckii*	97%[g]	1	4	0.50	8	64	>128	0.50	16	16	>16	0.01	0.03
440	*S. schenckii*	97%[g]	0.25	2	0.03	0.25	64	>128	0.12	16	2	>16	0.01	0.25
441	*S. schenckii*	96%[g]	2	2	0.06	0.25	64	>128	0.50	16	4	>16	0.12	0.25
444	*S. schenckii*	97%[g]	2	2	0.06	4	64	>128	0.50	2	8	>16	0.06	0.25
450	*S. schenckii*	96%[g]	2	8	0.12	1	64	>128	0.12	1	4	>16	0.01	1
478	*S. schenckii*	97%[g]	2	8	0.03	4	64	>128	0.50	16	8	>16	0.01	0.25
576	*S. schenckii*	94%[g]	1	1	0.03	1	64	>128	0.25	0.50	8	>16	0.01	1
579	*S. schenckii*	97%[g]	0.50	2	0.06	4	128	>128	0.50	2	8	>16	0.03	0.50
611	*S. schenckii*	99%[g]	2	4	0.03	0.25	64	>128	0.06	16	16	>16	0.06	0.03
794	*S. schenckii*	95%[g]	1	8	0.12	1	64	>128	0.25	16	16	>16	0.03	0.06
805	*S. schenckii*	99%[g]	1	4	0.50	1	64	>128	0.50	16	16	>16	0.01	0.01
810	*S. schenckii*	96%[g]	2	4	0.12	0.50	64	>128	0.50	8	8	>16	0.25	2
853	*S. schenckii*	99%[g]	0.50	1	0.03	0.12	32	>128	0.06	0.12	16	>16	0.01	0.01
864	*S. schenckii*	95%[g]	1	2	0.12	2	64	>128	0.25	16	16	>16	0.03	0.50
SN	*S. schenckii*	97%[g]	0.50	1	0.03	0.12	32	>128	16	16	8	>16	0.01	0.01
Range			0.2-2	1-8	0.03-0.5	0.1-16	32-128	>128	0.03-16	0.1->16	2-16	>16	0.01-0.2	0.01-2
Geometric mean			1.0	2.7	0.1	1.0	64.0	>128	0.3	5.7	8.3	>16	0.03	0.2
Brazil, all states of the study (n = 85)														
Range			0.03-4	0.5-8	0.03-1	0.06->16	8-128	>128	0.03-16	0.1->16	1-16	>16	0.01-0.5	0.01-16
Geometric mean			1.0	2.2	0.1	2.3	56.0	>128	0.4	7.1	8.2	>16	0.06	0.5
Type strains														
8309 FMR	*S. brasiliensis*	99%[f]	1	2	0.12	4	32	>128	0.50	16	2	>16	0.06	1
8600 FMR	*S. globosa*	98%[h]	4	8	0.25	1	128	>128	2	16	16	>16	0.06	0.25
6618 FMR	*S. schenckii*	99%[g]	0.50	2	0.06	1	128	>128	1	16	8	>16	0.12	8

a: minimum inhibitory concentration (µg/mL); b: minimum fungicidal concentration (µg/mL); c: clinical isolates; d: animal isolates; e: environmental isolates; f: *S. brasiliensis* FMR 8309 (CBS 120339, IPEC 16490) - GenBank accession AM116899; g: *S. schenckii* FMR 6618 (CBS 359.36) - GenBank accession AM117437; h: *S. globosa* FMR 8600 (CBS 120340) - GenBank accession AM116908.

Table 1 shows the antifungal susceptibility profile of the *Sporothrix schenckii* complex isolates according to the geographic origin. Terbinafine, ketoconazole and itraconazole showed the lowest MIC ranges (0.01 to 0.5 µg/mL; 0.03 to 1.0 µg/mL and 0.03 to 16.0 µg/mL, respectively), while fluconazole and voriconazole showed higher MIC ranges (8 to >128 µg/mL and 1 to 16 µg/mL), and had no fungicidal activity at the concentrations evaluated (MFC: >128 µg/mL and >16 µg/mL, respectively). Amphotericin B MICs were only one to two dilutions lower than the corresponding MFCs in all strains evaluated, while concerning terbinafine, MICs were two to four dilutions lower than the corresponding MFCs for all strains evaluated. Even though, terbinafine had a lower MFC geometric mean than amphotericin B. Ketoconazole and itraconazole showed the largest MFC range (0.06 to >16 µg/mL and 0.1 to >16 µg/mL, respectively). The Brazilian isolates showed similar susceptibility profiles to the antifungal agents, with the exception of the isolates from Minas Gerais and São Paulo, which presented, respectively, higher MICs and lower MFCs for ketoconazole ($p<0.05$).

Table 2 shows the minimum inhibitory concentration and minimum fungicidal concentration obtained for the *Sporothrix* species. There were no significant statistical differences among the species of the *Sporothrix schenckii* complex evaluated ($p>0.05$).

Table 2: Activities of antifungal drugs against isolates beloging to species of the *Sporothrix schenckii* complex.

Antifungal agents	**Species**					
	S. brasiliensis (n=23)		*S. globosa* (n=4)		*S. schenckii* (n=61)	
Amphotericin B	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Geometric mean	1.03	2.26	1.00	2.00	1.06	2.24
Range	0.2-4.0	1.0-8.0	0.2-4.0	0.5-8.0	0.03-2.0	0.5-8.0
Ketoconazole						
Geometric mean	0.16	3.88	0.15	0.70	0.10	2.01
Range	0.03-1.0	0.25-16.0	0.03-0.50	0.06-2.0	0.03-1.0	0.12->16.0
Fluconazole						
Geometric mean	56.7	>128.0	53.8	>128.0	57.7	>128.0
Range	16.0-128.0	>128.0	16.0-128.0	>128.0	8.0-128.0	>128.0
Itraconazole						
Geometric mean	0.36	7.53	0.83	6.72	0.42	7.38
Range	0.06-2.0	0.5->16.0	0.06-16.0	0.5->16.0	0.03-16.0	0.12->16.0
Voriconazole						
Geometric mean	6.10	16.0	11.3	16.0	8.46	>16.0
Range	1.0-16.0	>16.0	8->16.0	>16.0	2.0-16.0	>16.0
Terbinafine						
Geometric mean	0.06	0.61	0.03	0.30	0.05	0.45
Range	0.01-0.50	0.12-16.0	0.01-0.06	0.25-0.50	0.01-0.50	0.01-8.0

MIC: minimum inhibitory concentration (µg/mL); MFC: minimum fungicidal concentration (µg/mL).

4. Discussion

Recently, Marimon et al. [10,11] demonstrated that clinical isolates of *S. schenckii* constitute a complex of several cryptic species. Although they identified all the Brazilian isolates as *S. brasiliensis*, other study performed in Rio de Janeiro identified 83.4% of the isolates as *S. brasiliensis*, 6.0% as *S. schenckii*, and 0.5% as *S. mexicana* [23]. In our study, only 25.9% of the isolates were *S. brasiliensis*, with the predominance of *S. schenckii* (70.6%) and occurrence of three (3.5%) *S. globosa* strains. A study performed with isolates from Rio Grande do Sul, Brazil, reported 92.5% of *S. schenckii* isolates and 2.5% of *S. brasiliensis*, with the *S. brasiliensis* strains isolated from felines [24]. A recent study with Brazilian isolates from Rio de Janeiro, São Paulo, Paraná, Espírito Santo, Pará, Minas Gerais, Goiás and Ceará states identified 50% of the isolates as *S.*

brasiliensis, 39.3% as *S. schenckii* and 10.7% as *S. globosa* [25]. However, this study had a high number of isolates from Rio de Janeiro, where strains of *S. brasiliensis* are associated with the sporotrichosis epidemics [23,26].

Interestingly, in our study, in Rio de Janeiro predominated *S. schenckii* (11 isolates), followed by *S. brasiliensis* (9 isolates). As our samples were obtained randomly, it is probable that we were not able to get a sample truly representative of the state, obtaining more isolates of *S. schenckii* than *S. brasiliensis*, and disagreeing with other authors [23,26]. Similarly, in our study all isolates from São Paulo were identified as *S. schenckii*, although *S. brasiliensis* has also been reported elsewhere in this state [25].

The state of Minas Gerais and Rio Grande do Sul had isolates of *S. brasiliensis*, *S. schenckii*, and *S. globosa*. The species *S. globosa* is widespread globally, having been found in the UK, Spain, Italy, China, Japan, the USA, India, Mexico, Guatemala, and Colombia [11,27]. In Brazil, this species has been reported few times before. The presently reported *S. globosa* strains represent the first isolation of this pathogen in Rio Grande do Sul, and the third in Minas Gerais [23,28]. The other *S. globosa* isolates in Brazil are from Goiás, Ceará and Rio de Janeiro, which indicates that other species beyond *S. brasiliensis* and *S. schenckii* are also contributing to the persistence of this disease in Brazil [23].

Oliveira et al. [23] had 10.1% of strains with inconclusive or ambiguous identification results. We obtained a high level (37.7%) of disagreement between the species classification using phenotypic and genotypic methodologies. This was also reported by other authors [23,29], and emphasizes the importance of using genotypic methods to identify species in this complex. Thus, the high percentage of isolates that showed inconclusive results and disagreement between the

species classification using the phenotypic and genotypic methodologies showed the importance of using genotypic methods to identify species in this complex.

In this study, the antifungal terbinafine presented low MICs and MFCs, agreeing with other studies [24,30,31]. In the clinical, the cure has been observed in 51 (92.7%) patients with cutaneous sporotrichosis in treatment with this antifungal agent [32]. These results support the therapeutic use of terbinafine as a first option for the treatment of sporotrichosis [33].

Ketoconazole, also showed low MICs, being similar to the other studies with *S. brasiliensis* and *S. schenckii* strains from Brazil [24,31]. However, our results showed MFCs similar to the MICs obtained by Marimon et al. [31] for isolates of *S. globosa* originated from different geographical sources.

In general, the antifungal itraconazole had the third lowest geometric mean MIC in our work. McGinnis et al. [17] have demonstrated good activity of this antifungal in terms of MIC and MFC values, which is in agreement with our results. However, other studies have reported a high MIC and MFC for itraconazole against the *S. schenckii* complex [31,34-36]. Although breakpoints have not been established for this fungus, the document M38-A2 suggests that, for analytical purposes, a MIC $\geq$ 4.0 μg/mL for itraconazole may be considered resistant for some filamentous fungi. In this study, we obtained the proportion 5/85 (5.9%) of *in vitro* itraconazole-resistant strains in human sporotrichosis, agreeing with the reported efficacy in the clinical setting, which showed the cure in over 92% of cases of cutaneous sporotrichosis treated with itraconazole [32,37]. Two itraconazole-resistant strains were from Rio Grande do Sul and the other three were from different Brazilian states. Although Minas Gerais and Rio de Janeiro have shown only one resistant isolate each, these states had the highest geometric means for MIC and MFC, respectively. Among

itraconazole-resistant strains, one was *S. globosa* and four were *S. schenckii*. Itraconazole-resistance has already been reported in the literature [31].

According to literature [17,30,36], amphotericin B also presents a wide susceptibility range, indicating that antifungal susceptibilities are strain-dependent. Some isolates of this fungus show primary resistance to this polyene [38]. For this reason, in severe sporotrichosis, if amphotericin B is the treatment of choice, the MIC of the specific isolate should be determined, as failure of treatment could be associated with the lower susceptibility of the *S. schenckii* complex to this antifungal agent [17].

Voriconazole and fluconazole presented high MICs and MFCs, coinciding with the results of several other authors [31,34,39-41]. The broad *in vitro* resistance to fluconazole in clinical isolates of the *S. schenckii* complex suggests an intrinsic resistance of the species to this drug. This is an interesting topic for further study, because fluconazole is considered the second-line treatment for sporotrichosis [42]. In spite of the poor activity that this drug has shown in this and other studies, its clinical efficacy has been estimated to be 71% for cases of lymphocutaneous infection [43]. Unfortunately, for voriconazole there are no publications available referring to the treatment in experimental models or in clinical cases of sporotrichosis.

Our study showed that there were no significant antifungal susceptibilities differences among the species of the *Sporothrix* complex, disagreeing with the results reported by Marimon et al. [31]. Nevertheless, despite the few *S. globosa* isolates, our isolates were obtained from Brazil, while Marimon et al. [31] evaluated strains from different countries. Therefore, the difference in the susceptibility profiles to antifungal agents could be related to the geographic origin of the isolates.

Acknowledgements

The authors thank the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and *Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul* (FAPERGS) for financial support and scholarships.

5. References

[1] Dixon DM, Salkin IF, Duncan RA, et al. Isolation and characterization of *Sporothrix schenckii* from clinical and environmental sources associated with the largest U.S. epidemic of sporotrichosis. J Clin Microbiol 1991; 29: 1106-1113.

[2] Queiroz-Telles F, Nucci M, Colombo AL, Tobón A, Restrepo A. Mycoses of implantation in Latin America: an overview of epidemiology, clinical manifestations, diagnosis and treatment. Med Mycol 2011; 49: 225-236.

[3] da Rosa AC, Scroferneker ML, Vettorato R, Gervini RL, Vettorato G, Weber A. Epidemiology of sporotrichosis: a study of 304 cases in Brazil. J Am Acad Dermatol 2005; 52: 451-459.

[4] Landell MF, Stopiglia CD, Billodre RG, et al. Evaluation of the origin of a sample of *Sporothrix schenckii* that caused contamination of a researcher in Southern Brazil. Mycopathologia 2011; 171: 203-207.

[5] Sampaio SAP, Lacaz CS, Almeida F. Clinical aspects of sporotrichosis in SP. Analysis of 235 cases. Rev Hosp Clín 1954; 1: 391- 402 [in Portuguese].

[6] Galhardo MC, de Oliveira RM, Valle AC, et al. Molecular epidemiology and antifungal susceptibility patterns of *Sporothrix schenckii* isolates from a cat-transmitted epidemic of sporotrichosis in Rio de Janeiro, Brazil. Med Mycol 2008; 46: 141-151.

[7] Cooper CR, Breslin BJ, Dixon DM, Salkin IF. DNA typing of isolates associated with the 1988 sporotrichosis epidemic. J Clin Microbiol 1992; 30: 1631-1635.

[8] Marimon R, Gené J, Cano J, Trilles L, dos Santos Lazera M, Guarro J. Molecular phylogeny of *Sporothrix schenckii*. J Clin Microbiol 2006; 44: 3251-3256.

[9] O'Reilly LC, Altman SA. Macrorestriction analysis of clinical and environmental isolates of *Sporothrix schenckii*. J Clin Microbiol 2006; 44: 2547-2552.

[10] Marimon R, Gené J, Cano J, Guarro J. *Sporothrix luriei*: a rare fungus from clinical origin. Med Mycol 2008; 46: 621-625.

[11] Marimon R, Cano J, Gené J, Sutton DA, Kawasaki M, Guarro J. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. J Clin Microbiol 2007; 45: 3198–3206.

[12] de Meyer EM, de Beer ZW, Summerbell RC, et al. Taxonomy and phylogeny of new wood- and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex. Mycologia 2008; 100: 647–661.

[13] Madrid H, Gené J, Cano J, Silvera C, Guarro J. *Sporothrix brunneoviolacea* and *Sporothrix dimorphospora*, two new members of the *Ophiostoma stenoceras-Sporothrix schenckii* complex. Mycologia 2010; 102: 1193-1203.

[14] Coskun B, Saral Y, Akpolat N, Ataseven A, Cicek D. Sporotrichosis successfully treated with terbinafine and potassio iodide: case report and review of the literature. Mycopathologia 2004; 158: 53-56.

[15] Arechavala A, Orduna T, Maiolo E, Mujica MT, Fernández M, Negroni R. Disseminated sporotrichosis with cutaneous and visceral. Rev Patol Trop 2011; 40: 73-84 [in Spanish].

[16] Kohler LM, Hamdan JS, Ferrari TC. Successful treatment of a disseminated *Sporothrix schenckii* infection and *in vitro* analysis for antifungal susceptibility testing. Diagn Microbiol Infect Dis 2007; 58: 117-120.

[17] McGinnis MR, Nordoff N, Li RK, Pasarell L, Warnock DW. *Sporothrix schenckii* sensitivity to voriconazole, itraconazole and amphotericin B. Med Mycol 2001; 39: 369-371.

[18] Meinerz ARM, Nascente PS, Schuch LFD, et al. *In vitro* susceptibility of isolates of *Sporothrix schenckii* to terbinafine and itraconazole. Rev Soc Bras Med Trop 2007; 40: 60-62 [in Portuguese].

[19] Heidrich D, Stopiglia CDO, Senter L, Vetoratto G, Valente P, Scroferneker ML. Successful treatment of terbinafine in a case of sporotrichosis. An Bras Dermatol 2011; 86: S182-185.

[20] Kohler LM, Soares BM, de Assis Santos D, Da Silva Barros ME, Hamdan JS. *In vitro* susceptibilities of isolates of *Sporothrix schenckii* to amphotericin B, itraconazole, and terbinafine: comparison of yeast and mycelial forms. Can J Microbiol 2006; 52: 843-847.

[21] Clinical and Laboratory Standards Institute (CLSI). Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, approved standard – Second Edition. CLSI, Vilanova, 2008.

[22] Espinel-Ingroff A, Fothergill A, Meter J, Rinaldi MG, Walsh TJ. Testing conditions for determination of minimum fungicidal concentrations to new and established antifungal agents for *Aspergillus* spp.: NCCLS collaborative study. J Clin Microbiol 2002; 40: 3204–3208.

[23] Oliveira MM, Almeida-Paes R, Muniz MM, Gutierrez-Galhardo MC, Zancope-Oliveira RM. Phenotypic and molecular identification of *Sporothrix* isolates from an epidemic area of sporotrichosis in Brazil. Mycopathologia 2011; 172: 257-267.

[24] Oliveira DC, Lopes PG, Spader TB, et al. Antifungal susceptibilities of *Sporothrix albicans*, *S. brasiliensis*, and *S. luriei* of the *S. schenckii* complex identified in Brazil. J Clin Microbiol 2011; 49: 3047-3049.

[25] Rodrigues AM, de Hoog S, de Camargo ZP. Emergence of pathogenicity in the *Sporothrix schenckii* complex. Med Mycol 2012 In press.

[26] Schubach A, Barros MB, Wanke B. Epidemic sporotrichosis. Curr Opin Infect Dis 2008; 21: 129–133.

[27] Madrid H, Cano J, Gené J, Bonifaz A, Toriello C, Guarro J. *Sporothrix globosa*, a pathogenic fungus with widespread geographical distribution. Rev Iberoam Micol 2009; 26: 218-222.

[28] Oliveira MME, Almeida-Paes R, Muniz MM, Barros MBL, Gutierrez-Galhardo MC, Zancopé-Oliveira RM. Sporotrichosis caused by *Sporothrix globosa* in Rio de Janeiro, Brazil: case report. Mycopathologia 2010; 169: 359–363.

[29] de Oliveira MM, Sampaio P, Almeida-Paes R, Pais C, Gutierrez-Galhardo MC, Zancope-Oliveira RM. Rapid identification of *Sporothrix* species by T3B fingerprinting. J Clin Microbiol 2012; 50: 2159-2162.

[30] Alvarado-Rodriguez E, Torres-Rodriguez JM. *In vitro* susceptibility of *Sporothrix schenckii* to six antifungal agents determined using three different methods. Antimicrob Agents Chemother 2007; 51: 2420-2423.

[31] Marimon R, Serena C, Gene J, Cano J. *In vitro* antifungal susceptibilities of five species of *Sporothrix*. Antimicrob Agents Chemother 2008; 52: 732-734.

[32] Francesconi G, Francesconi do Valle AC, Passos SL, et al. Comparative study of 250 mg/day terbinafine and 100 mg/day itraconazole for the treatment of cutaneous sporotrichosis. Mycopathologia 2011; 171: 349-354.

[33] Chapman SW, Pappas P, Kauffman C, et al. Comparative evaluation of the efficacy and safety of two doses of terbinafine (500 and 1000 mg day(-1)) in the treatment of cutaneous or lymphocutaneous sporotrichosis. Mycoses 2004; 47: 62–68.

[34] Gutierrez-Galhardo MC, Zancopé-Oliveira RM, Monzón A, Rodriguez-Tudela JL, Cuenca-Estrella M. Antifungal susceptibility profile *in vitro* of *Sporothrix schenckii* in two growth phases and by two methods: microdilution and E-test. Mycoses 2010; 53: 227-231.

[35] Kohler LM, Monteiro PCF, Hahn RC, Hamdan JS. *In vitro* susceptibilities of isolates of *Sporothrix schenckii* to itraconazole and terbinafine. J Clin Microbiol 2004; 42: 4319–4320.

[36] Silveira CP, Torres-Rodríguez JM, Alvarado-Ramírez E, et al. MICs and minimum fungicidal concentrations of amphotericin B, itraconazole, posaconazole and terbinafine in *Sporothrix schenckii*. J Med Microbiol 2009; 58: 1607-1610.

[37] de Lima Barros MB, Schubach AO, de Vasconcellos Carvalhaes de Oliveira R, Martins EB, Teixeira JL, Wanke B. Treatment of cutaneous sporotrichosis with itraconazole-study of 645 patients. Clin Infect Dis 2011; 52: 200-206.

[38] Ellis D. Amphotericin B: spectrum and resistance. J Antimicrob Chemother 2002; 49: 7-10.

[39] Chryssanthou E, Cuenca-Estrella M. Comparison of the antifungal susceptibility testing subcommittee of the European Committee on antibiotic susceptibility testing proposed standard and

the E-test with the NCCLS broth microdilution method for voriconazole and caspofungin susceptibility testing of yeast species. J Clin Microbiol 2002; 40: 3841–3844.

[40] Trilles L, Fernández-Torres B, Dos Santos Lazéra M, et al. *In vitro* antifungal susceptibilities of *Sporothrix schenckii* in two growth phases. Antimicrob Agents Chemother 2005; 49: 3952–3954.

[41] Morera-López Y, Torres-Rodríguez JM, Jiménez-Cabello T. *In vitro* susceptibility of fungal and yeast clinical isolates to itraconazole and voriconazole. Rev Iberoam Micol 2005; 22: 105-109.

[42] Kauffman CA, Pappas PG, McKinsey DS, et al. Treatment of lymphocutaneous and visceral sporotrichosis with fluconazole. Clin Infect Dis 1996; 22: 46–50.

[43] Diaz M, Negroni R, Montero-Gei F, et al. A pan-American 5-year study of fluconazole therapy for deep mycoses in the immunocompetent host. Clin Infect Dis 1992; 14: 68–76.

5.2. Capítulo II

SECRETION OF EXTRACELLULAR ENZYMES BY *SPOROTHRIX SCHENCKII* COMPLEX BRAZILIAN ISOLATES

Submetido ao periódico:

Canadian Journal of Microbiology.

Secretion of extracellular enzymes by *Sporothrix schenckii* complex Brazilian isolates

Cheila Denise Ottonelli Stopiglia[a], Daiane Heidrich[a], Cibele Massotti Magagnin[a], Karine de Oliveira Alves[a], Suelen Vigolo[a], Lucia Collares Meirelles[a], Patricia Valente[b], Maria Lúcia Scroferneker[a,b*]

[a]Programa de Pós-Graduação em Medicina: Ciências Médicas, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2400 – 2º andar – CEP: 90035-003, Porto Alegre – RS, Brazil.

[b]Departamento de Microbiologia, Imunologia e Parasitologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 – CEP 90050-170, Porto Alegre – RS, Brazil.

*Correspondence and reprints: Maria Lúcia Scroferneker, Departamento de Microbiologia, Imunologia e Parasitologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 sala 210, CEP: 90050-170, Porto Alegre - RS, Brasil. Tel. (+55) 51-33083934 Fax. (+55) 51-33083121 (e-mail: scrofern@ufrgs.br)

Abstract

The *Sporothrix schenckii* complex is the etiologic agent of sporotrichosis. Pathogenicity of fungi is connected with their ability to easily penetrate the host tissues, survive in the infected host organism and use the elements of the host tissues as nutrients. The present study was aimed at determining the *in vitro* phospholipase, esterase, urease, desoxiribonuclease (DNase), and protease activities of 85 *S. schenckii* complex isolates from Brazilian states. All the isolates were DNase, urease and proteinase positive. The enzymes DNase and esterase had the most intense enzymatic activities, with most of the isolates showing mild or strong Pz indexes. Phospholipase and esterase activities were detected in 83 (97.6%) and 80 (94.1%), respectively, of the isolates in this study. All the *S. schenckii* complex strains produced at least four of the evaluated enzymes, and 78 (91.8%) of the isolates produced all the enzymes analyzed in the study. However, it is not possible to differentiate the *Sporothrix* species based on their enzymatic profiling. Among the extracellular enzymes evaluated from members of the *S. schenckii* complex, DNase and esterase were the most prominent, and their production may be a virulence factor when the yeast parasitizes the human body.

Keywords: *Sporothrix schenckii* complex, sporotrichosis, enzymatic activity, phenotypic characterization.

1. Introduction

Sporotrichosis, a subacute or chronic mycosis which can affect humans and animals is caused by a group of species belonging to the *Sporothrix schenckii* complex (Dixon et al. 1991, Marimon et al. 2007). This mycosis predominates in tropical and temperate zones, and the main endemic areas are found in Japan, India, South Africa, Mexico, Peru, Uruguay, and Brazil (Kusuhara et al. 1988, Pappas et al. 2000), being the most common and cosmopolitan subcutaneous mycosis (Gutierrez-Galhardo et al. 2010).

Recent studies revealed high genetic variability among isolates that were morphologically identified as *S. schenckii*, which has led to the introduction of new species (Marimon et al. 2006, Marimon et al., 2007). Medically relevant *Sporothrix* species now include *S. brasiliensis*, *S. schenckii*, *S. globosa*, *S. mexicana* and *S. luriei*, which can be identified through physiological and molecular studies (Marimon et al. 2007, Marimon et al. 2008).

The secretion of enzymes to the extracellular environment might be an important adaptive mechanism during the life cycle of fungi (Monod and Borg-Von-Zepelin, 2002). The first studies of fungal enzymatic activities aimed at establishing the role of enzymes in fungal pathogenicity, as well as their capacity to induce inflammatory reactions in the host (Rippon 1982). Extracellular enzymes, have been considered to be important virulence factors in fungi, facilitating the adherence, tissue penetration and invasion of the host, furthermore they could also participate in infection by eliminating some mechanisms of the immune defense and/or helping in nutrient assimilation, thus causing injuries to the human host (Birch et al. 2004, Blanco et al. 2002, da Silva et al. 2005, Rementeria et al. 2005, Schaller et al. 2005). However, these determinants of pathogenicity remain largely undescribed for the *S. schenckii* complex. So, the purpose of this study was to establish enzymatic profiles of the *S. schenckii* complex through the evaluation of the *in vitro* production of

esterase, desoxiribonuclease (DNase), phospholipase, urease and proteinase by a collection of 85 clinical, environmental and animal isolates from different Brazilian states.

2. Materials and methods

2.1.Fungal isolates

Eighty-five isolates of the *S. schenckii* complex from different Brazilian states (Minas Gerais (n=15), Rio de Janeiro (n=20), São Paulo (n=19) and Rio Grande do Sul (n=31)) were examined in this study. All strains were previously identified according to Marimon et al. (2007). Furthermore, *Sporothrix brasiliensis* FMR 8309 (CBS 120339), *Sporothrix globosa* FMR 8600 (CBS 120340) and *Sporothrix schenckii* FMR 6618 (CBS 359.36) were also included and used as reference strains.

Candida albicans ATCC 10231, *Malassezia furfur* IMTSP 225, *Nocardia brasiliensis* IMTSP 739 and *Staphylococcus aureus* ATCC 25923 were utilized as positive controls in the enzyme assays.

2.2.Enzyme assays

The *S. schenckii* complex strains were subcultured onto potato dextrose agar (PDA) at 35°C for 7 days. The surface was gently scraped with a sterile bent glass after flooding with sterile saline solution. Standard suspensions of *S. schenckii* complex were prepared by a spectrophotometric method as described by Pfaller et al. (1994) in sterile saline solution to a density of approximately 10^6 CFU/mL, and aliquots of 0.1mL were spotted onto Petri dishes containing the specific medium. The assays were conducted in triplicate.

The phospholipase assay was determined according to the method described by Price et al. (1982). The culture medium consisted of peptone 1%, glucose 1%, sodium chloride 5.73%, calcium

chloride 0.05% and agar 2%, in distilled water. The medium was autoclaved and then cooled to approximately 50°C before the addition of sterile egg yolk at a final concentration of 4%. The plates were incubated at 35°C for 21 days. Precipitation around the colonies attested phospholipase production. *C. albicans* ATCC 10231 was used as positive control.

The proteinase assay was carried as described by Rüchel et al. (1982). The proteinase medium consisted of yeast carbon base 1.17% (Difco), and bovine serum albumin fraction V (Sigma) 0.2 %, plus 2.5 mL of Protovit (Roche) in distilled water. The isolates were inoculated on the plates and incubated at 35ºC for 7 days. To facilitate the visualization of degradation halos, the plates were stained with starch black 1.0% in 20% acetic acid solution and the excess stain was washed away with a 10% acetic acid solution. *C. albicans* ATCC 10231 was used as positive control.

The esterase activity was evaluated as previously described by Muhsin et al. (1997). The test medium consisted of peptone 1%, sodium chloride 0.5%, calcium chloride 0.01% and agar 2 %, in distilled water. Tween 80 was autoclaved separately and added as a substrate at a final concentration of 1%. After inoculation, the plates were incubated at 35°C for 7 days. Formation of a precipitate around the colonies demonstrated esterase production. *M. furfur* IMTSP 225 was used as positive control.

For DNase assay the isolates were inoculated on Petri dishes containing DNase agar test (Oxoid) and incubated at 35ºC for 13 days. The test was considered positive when a degradation halo around the colony could be visualized following the addition of 5 N hydrochloric acid. *S. aureus* ATCC 25923 was utilized as positive control (Lopez-Martinez et al. 1994).

The urease activity was determined according to the method proposed by Christensen (1946) and Seeliger (1956). The strains were added onto specific medium consisted of peptone 0.1%, glucose 0.1%, sodium chloride 0.5%, potassium dihydrogen phosphate 0.08%, disodium

phosphate 0.12%, and phenol red 0.0012%, in 95mL distilled water, adjusted to pH 7.0. The medium was autoclaved and then cooled to approximately 50°C, followed by the aseptic introduction of 5 mL of sterile 40% urea solution. After inoculation, the plates were incubated at 35ºC for 3 days. They were considered positive when the medium became pinkish after incubation. *N. brasiliensis* IMTSP 739 was utilized as positive control.

The activity of all enzymes, except urease, was expressed according to the Pz index, i.e., colony diameter/total diameter of the colony plus the precipitation halo. The following ranges of activity were established according to the Pz index: strong, Pz < 0.40; mild, Pz = 0.40 – 0.69; weak, Pz = 0.70 – 0.99; and negative, Pz = 1 (Koga-Ito et al. 2006). Moreover, we determined the geometric mean of Pz index of each *S. schenckii* complex species and the clinical, environmental and animal isolates.

The enzymatic profiles were compared among the *S. schenckii* species complex, as well as among the different Brazilian states using the Kruskal-Wallis test in the SPSS package (SPSS v17.0 for Windows); a P –value ≤ 0.05 was considered significant.

2.3. Statistical analysis

The statistical analysis was performed with nonparametric tests Kruskal Wallis and Bonferroni multiple comparison test using the software R version 3.0.0.

3. Results

Of the 85 isolates of *S. schenckii* complex from Brazilian states tested, all were DNase, urease and proteinase positive. Phospholipase activity was detected in 83 (97.6%) of the strains tested. The two phospholipase negative strains were from São Paulo state. Esterase activity was detected in 80 (94.1%) of the isolates in the study, with two isolates from Minas Gerais state and three from Rio de Janeiro state not able to produce this enzyme.

Table 1 shows the distribution of Pz values among the 85 isolates of *Sporothrix schenckii* complex in the four Brazilian states. The enzymes DNase and esterase had the most intense enzymatic activities, with most of the isolates showing mild or strong Pz indexes. One hundred percent of the isolates presented mild or strong DNase Pz indexes, and 77.6% presented this range of Pz for esterase. Concerning phospholipase, no strain showed strong Pz index, with 60.0% of the isolates presenting mild Pz values. Finally, most strains showed weak proteinase Pz indexes. All the *S. schenckii* complex strains produced at least four of the evaluated enzymes, and 78 (91.8%) of the isolates produced all the enzymes analyzed in the study.

Statistically significant differences in phospholipase activities were observed between the isolates from the Brazilian states of Rio Grande do Sul and São Paulo. In the Rio Grande do Sul state, mild phospholipase production was observed in 74.2% and weak production in 25.8% of the isolates, respectively, while in São Paulo 10.5% of the São Paulo isolates did not produce phospholipase, 57.9% showed weak production, and 31.6% showed mild production of this enzyme (Table 1).

The proteinase activity was detected in all isolates analyzed in this study and a statistically significant difference was observed between the Brazilian states of Rio Grande do Sul and Rio de Janeiro. Both states showed the majority of isolates with weak Pz (95% Rio de Janeiro and 90.3% Rio Grande do Sul), however one isolate from Rio de Janeiro showed strong Pz, while three isolates

from Rio Grande do Sul showed mild Pz. The other enzymes showed no differences between the four states analyzed. Esterase activity was detected in 94.1% of the *S schenckii* complex isolates in the present study, being two isolates from Minas Gerais state and three from Rio de Janeiro state not able to produce this enzyme (Table 1).

Table 1: Pz value distribution among 85 isolates of *Sporothrix schenckii* complex in the four Brazilian states.

Pz index[a]	Strong	Mild	Weak	Negative
DNase				
MG (n=15)	3 (20%)[b]	12 (80%)	-	-
RJ (n=20)	7 (35%)	13 (65%)	-	-
RS (n=31)	4 (12.9%)	27 (87.1%)	-	-
SP (n=19)	1 (5.3%)	18 (94.7%)	-	-
Total (n=85)	15 (17.6%)	70 (82.4%)	-	-
Esterase				
MG (n=15)	1 (6.7%)	11 (73.3%)	1 (6.7%)	2 (13.3%)
RJ (n=20)	1 (5.0%)	16 (80.0%)	-	3 (15.0%)
RS (n=31)	2 (6.4%)	20 (64.5%)	9 (29.0%)	-
SP (n=19)	-	15 (78.9%)	4 (21.1%)	-
Total (n=85)	4 (4.7%)	62 (72.9%)	14 (16.5%)	5 (5.9%)
Phospholipase				
MG (n=15)	-	8 (53.3%)	7 (46.7%)	-
RJ (n=20)	-	14 (70.0%)	6 (30.0%)	-
RS (n=31)*	-	23 (74.2%)	8 (25.8%)	-
SP (n=19)*	-	6 (31.6%)	11 (57.9%)	2 (10.5%)
Total (n=85)	-	51 (60.0%)	32 (37.7%)	2 (2.3%)
Proteinase				
MG (n=15)	-	-	15 (100%)	-
RJ (n=20)*	1 (5.0%)	-	19 (95%)	-
RS (n=31)*	-	3 (9.7%)	28 (90.3%)	-
SP (n=19)	-	-	19 (100%)	-
Total (n=85)	1 (1.2%)	3 (3.5%)	81 (95.3%)	-

[a]Pz index: colony diameter/total diameter of the colony plus the precipitation halo; Strong, Pz < 0.40; mild, Pz = 0.40 – 0.69; weak, Pz = 0.70 – 0.99; and negative, Pz = 1; MG: Minas Gerais, RJ: Rio de Janeiro; RS: Rio Grande do Sul; SP: São Paulo; [b]Results expressed in number of isolates (percentual); * P –value ≤ 0.05.

Table 2 shows the geometric means obtained for the clinical, environmental and animal isolates. The environmental isolates of *S. schenckii* complex had similar profiles to the ones from clinical origins, while the animal isolates showed Pz indexes lower than the environmental and clinical strains.

Table 2: Enzymatic activity of clinical, environmental and animal isolates of *Sporothrix schenckii* complex from Brazilian states.

Enzyme	**Brazil**		
	Animal isolates (n=1)	**Environmental isolates (n=2)**	**Clinical isolates (n=82)**
DNase	0.64	0.46	0.46
Esterase	0.71	0.56	0.62
Phospholipase	0.78	0.62	0.69
Proteinase	0.86	0.81	0.82

* Values obtained by geometric mean of the Pz values.

When comparing the Pz index between the species of *S. schenckii* complex, no significant difference was found (Table 3). Most of the isolates of *S. brasiliensis*, *S. schenckii* and *S. globosa* isolates showed mild Pz indexes for the enzymes DNase and esterase and weak production of proteinase. Concerning phospholipase activity, most of the *S. brasiliensis* and *S. schenckii* isolates showed mild Pz indexes, while most of the *S. globosa* isolates showed weak Pz index. Three *S. brasiliensis* isolates and two *S. schenckii* isolates did not produce esterase, as well as other two *S. schenckii* isolates did not produce phospholipase.

Table 3: Enzymatic profile of the species of *Sporothrix schenckii* complex from Brazilian states and reference strains.

Enzyme	*S. brasiliensis* (n=23)	*S. globosa* (n=4)	*S. schenckii* (n=61)
DNase			
Strong	9 (39.1%)	1 (25%)	6 (9.8%)
Mild	14 (60.9%)	3 (75%)	55 (90.2%)
Esterase			
Strong	1 (4.3%)	-	2 (3.3%)
Mild	15 (65.2%)	3 (75%)	46 (75.4%)
Weak	4 (17.4%)	1 (25%)	11 (18.0%)
Negative	3 (13.0%)	-	2 (3.3%)
Phospholipase			
Mild	15 (65.2%)	1 (25%)	35 (57.4%)
Weak	8 (34.8%)	3 (75%)	24 (39.3%)
Negative	-	-	2 (3.3%)
Proteinase			
Strong	1 (4.3%)	-	-
Mild	-	1 (25%)	2 (3.3%)
Weak	22 (95.7%)	3 (75%)	59 (96.7%)

*Values obtained by Pz index: colony diameter/total diameter of the colony plus the precipitation halo; Strong, Pz < 0.40; mild, Pz = 0.40 – 0.69; weak, Pz = 0.70 – 0.99; and negative, Pz = 1.

The reference strains showed weak Pz index to phospholipase and proteinase activity. *S. brasiliensis* FMR 8309 showed mild and weak Pz indexes to DNase and esterase, respectively; while *S. schenckii* FMR 6618 obtained strong and mild Pz indexes to DNase and esterase, respectively and *S. globosa* FMR 8600 showed mild Pz indexes to this two enzymes.

4. Discussion

Pathogenicity in infections caused by fungi is connected with their ability to survive in the infected organism, the use of nutrients present in the host organism and the properties which allow them to penetrate tissues (Samdani and Ayub 2005). Therefore many authors strongly stress the co-occurrence of pathogenic properties with the ability to produce various enzymes, including extracellular ones (Ciebiada-Adamiec et al. 2010, da Silva et al. 2005, Schaller et al. 2005).

In general, the lipases, including phospholipases and esterases, are enzymes that hydrolyze phospholipids to fatty acids. This enzymes have an important function in fungal infection as they hydrolyze fat and provide fatty acid residues for fungal nutrition, mainly in the subcutis and cuts (Aktas et al. 2002, Ciebiada-Adamiec et al. 2010). The use of esterase activity for differentiating pathogenic fungal genera is well reported in the literature, especially concerning dermatophytes and *Candida* species (Aktas et al. 2002, Nobre and Viegas 1972, Slifkin and Cumbie 1996). Greater lipase activity was also frequently verified on isolates from chronic mycotic infections (Monod and Borg-Von-Zepelin 2002). Esterase activity was detected in 94.1% of the *S. schenckii* complex isolates in the present study, results similar to those found in other studies with *C. albicans* (Kumar et al. 2006, Yucesoy and Marol 2003). Tosun et al. (2012) observed esterase activity in 33.3% (14 out of 42) of *C. parapsilosis* isolates from blood, urine, oral swab, skin, ear, and endotracheal aspirate, while Nobre and Viegas (1972) demonstrated variation in enzyme secretion according to the dermatophyte species. They found that *Trychophyton mentagrophytes* and *Microsporum canis* were high esterase producers, while *T. rubrum* and *T. tonsurans* were low esterase producers. More recently, Palmeira et al. (2010) reported that high levels of esterase activity in the fungus *Fonsecaea pedrosoi* were related to adherence to mammalian cells.

Extracellular phospholipases are considered virulence factors for many pathogenic bacteria, protozoa and fungi such as *Clostridium* species, *Listeria monocytogenes*, *Pseudomonas* species, *S. aureus*, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Entamoeba histolytica*, *Fusarium* species, *C. albicans* and *Paracoccidioides brasiliensis* (Ghannoum 2000, Ibrahim et al. 1995, Ishida et al. 2012, Soares et al. 2010). Furthermore, a study correlated the production of phospholipase by *C. albicans* with its pathogenic nature, demonstrating that isolates with a high pathogenic potential (high level of adhesion to oral epithelial cells and greater pathogenicity for mice) had higher phospholipasic activity than yeasts with a low pathogenic potential (Barret-Bee et al. 1985). In

addition, *C. albicans* blood isolates have shown greater *in vitro* phospholipasic activity than oral isolates from healthy patients (Ibrahim et al. 1995) and *Malassezia sympodialis* strains isolated from patients with pityriasis versicolor had significantly higher phospholipasic activity than those isolated from healthy individuals (Pini and Faggi 2011). Similarly, it has been shown that isolates of *Cryptococcus neoformans* taken from infections in AIDS patients show a higher level of phospholipasic activity than isolates taken from from bird droppings (Vidotto et al. 1998). In our study, 83 out of 85 isolates (97.7%) showed phospholipase activity, most with mild Pz values (Table 1).

The ability of *S. schenckii* complex to produce proteinase was demonstrated for all isolates in this study. The proteinases have been found to digest biologically important proteins involved in invasion such as collagen and elastin (Rechnitzer et al. 1992) and modulate the immune response by digesting the cell surface markers, receptors, complements and immunoglobulins (Mintz et al. 1993). These enzymes are capable of causing tissue necrosis and promoting localized lesions during infection, besides being related with the ability of the *S. schenckii* to invade tissues of the host after the conidia were introduced through even a small trauma (da Rosa et al. 2009). It has been determined that 24 to 48 hours is the lapsed time between the conidia invasion and the transformation to the yeast-like form in the host. This lapsed time is coincidental with the expression of proteolytic activity observed already at day two in cultures (da Rosa et al. 2009, Hempel and Goodman 1975). Proteinases are also important virulence factors in *Candida* species and relate with disseminated infections (de Bernardis et al. 1999, Schaller et al. 2000). Furthermore, strains with higher proteolytic activity were shown to be more virulent (Naglik et al. 2003).

The urease is considered an important factor in many pathogenic microorganisms, as *Proteus mirabilis* and *Helicobacter*, and one of the major virulence factors of in *C. neoformans* (Cox et al. 2000). In an experimental model of cryptococcosis, the urease produced during

hematogenous dissemination facilitated the invasion of the central nervous system by increasing the presence of yeast cells in the capillary bed of the mouse brain (Olszewski et al. 2004). In the our study, all strains of *S. schenckii* produced urease, in agreement with other studies with *S. schenckii* and dematiaceous fungi (Ghosh et al. 2002, Mendoza et al. 2005, Souza et al. 2008).

Most literature reports generally focus on only one enzymatic activity and its possible effect on virulence. However very few studies investigated more than one enzyme activity in the same strains. In our study, all *S. schenckii* complex strains produced, at least, four enzymes, and 78 (91.8%) isolates tested, produced all the enzymes evaluated. Shimizu et al. (1996) investigated the ability of different *Candida* species to produce hyaluronidase, chondroitin sulphatase, protease and phospholipase to assess whether they could be related to *Candida* pathogenicity. They found that apart from *C. albicans*, in the other *Candida* species tested, none of them produced the four enzymes. Kantarcioglu and Yücel (2002) showed 56 of 60 *C. albicans* strains and 2 of 4 *C. kefyr* strains to produce both phospholipase and protease - the other strains observed lacked the capacity to produce one of the enzymes assayed.

We observed that the environmental isolates of *S. schenckii* complex had similar profiles as the ones from clinical origin, in accordance with other works, in which the production of enzymes, as protease, phospholipase and urease, exhibited no difference in clinical and environmental isolates of *C. neoformans* and *C. gattii* (Torres-Rodríguez et al. 2008, Vellasamy et al. 2009). Considering that many other non-pathogenic fungi produce extracellular enzymes and that *S. schenckii* complex are also free-living organisms, it seems that fungi, in their environmental niche, may have developed adaptive tools, such as extracellular enzyme production, to protect themselves from soil amoebas, and other biotic factors, as well as to obtain nutrients from the environment. These protective mechanisms enhance survival in the environment but produce disease in the mammalian host (Sánchez and Colom 2010). In this study, the animal isolates produced lower levels of the

evaluated enzymes. However, our results need to be validated using more isolates. Animal and human isolates of *S. schenckii* have different susceptibility profiles to antifungals (Oliveira et al. 2011), furthermore species of the *S. schenckii* complex vary in virulence and antifungal sensibility (Arrillaga-Moncrieff et al. 2009, Dixon et al. 1992, Marimon et al. 2008, Mesa-Arango et al. 2002).

A study with *C. neoformans* strains isolated from patients in different geographic locations showed differences between isolates from different countries in some of the enzymes analyzed (Vidotto et al. 2006). In this work, there was no difference in the enzymatic profile of isolates from Argentina and four Brazilian states. However, these countries are very close geographically which may have contributed to the similar enzyme profiles. However, it is necessary to increase the number of isolates to confirm this hypothesis.

A comparative study on the experimental pathogenicity of five species of *Sporothrix* of clinical interest an murine model showed that *S. brasiliensis* and *S. schenckii* were the most virulent species, and suggested that lesional mechanisms could be species-specific (Arrillaga-Moncrieff et al. 2009). However, in this study, no differences were found in the enzymatic profiles of *S. schenckii* complex, corroborating with other studies which analyzed urease and gelatinase activities (Marimon et al. 2007). Hence it is not possible to differentiate the *Sporothrix* species based on the enzymatic profiling.

Regarding the results of our study, we conclude that members of the *S. schenckii* complex produce extracellular enzymes: DNase, esterase, phospholipase, proteinase, and urease, and that the enzymes DNase and esterase had the most intense enzymatic activities which may be a virulence factor when the yeast parasitizes the human body. However, further investigations should continue on the enzymatic activity of the *S. schenckii* complex isolated from animal and environmental sources, as well as different clinical presentations and geographical origin, to clarify their contribution to fungal virulence associated with sporotrichosis.

5. Acknowledgements

The authors are grateful to *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES), *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and *Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul* (FAPERGS), for their financial support.

6. References

Aktas, E., Yigit, N., and Ayyildiz, A. 2002. Esterase activity in various *Candida* species. J. Int. Med. Res. **30**:322-324.

Arrillaga-Moncrieff, I., Capilla, J., Mayayo, E., Marimon, R., Mariné, M., Gené, J., et al. 2009. Different virulence levels of the species of *Sporothrix* in a murine model. Clin. Microbiol. Infect. **15**(7): 651-655. doi: 10.1111/j.1469-0691.2009.02824.x

Barret-Bee, K., Hayes, K.Y., Wilson, R.G., and Ryley, J.F. 1985. A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J. Gen. Microbiol. **131**(5): 1217–1222.

Birch, M., Denning, D.W., and Robson, G.D. 2004. Comparison of extracellular phospholipase activities in clinical and environmental *Aspergillus fumigatus* isolates. Med. Mycol. **42**(1): 81-86.

Blanco, J.L., Hontecillas, R., Bouza, E., Blanco, I., Pelaez, T., Muñoz, P., et al. 2002. Correlation between the elastase activity index and invasiveness of clinical isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **40**(5): 1811-1813.

Christensen, W.B. 1946. Urea decomposition as a means of differentiating *Proteus* and Paracolon cultures from each other and from *Salmonella* and *Shigella* types. J. Bact. **52**(4): 461-466.

Ciebiada-Adamiec, A., Małafiej, E., and Ciebiada, I. 2010. Inhibitory effect of nicotinamide on enzymatic activity of selected fungal strains causing skin infection. Mycoses. **53**(3): 204-207.

Cox, G.M., Mukherjee, J., Cole, G.T., Casadevall, A., and Perfect, J.R. 2000. Urease as a virulence factor in experimental cryptococcosis. Infect. Immun. **68**(2): 443-448.

da Rosa, D., Gezuele, E., Calegari, L., and Goñi, F. 2009. Excretion-secretion products and proteases from live *Sporothrix schenckii* yeast phase: immunological detection and cleavage of human IgG. Rev. Inst. Med. Trop. S. Paulo. **51**(1): 1-7.

da Silva, B.C., Auler, M.E., Ruiz, L.S., Gandra, R.F., dos Santos, J.I., Paula, C.R., et al. 2005. *Trichophyton rubrum* isolated from AIDS and human immunodeficiency virus-infected patients in São Paulo, Brazil: antifungal susceptibility and extracellular enzyme production. Chemotherapy. **51**(1): 21-26.

de Bernardis, F., Mondello, F., San Milan, R., Ponton, J., and Cassone, A. 1999. Biotyping and virulence properties of skin isolates of *Candida parapsilosis*. J. Clin. Microbiol. **37**(11): 3481–3486.

Dixon, D.M., Duncan, R.A., and Hurd, N.J. 1992. Use of a mouse model to evaluate clinical and environmental isolates of *Sporothrix* spp. from the largest U.S. epidemic of sporotrichosis. J. Clin. Microbiol. **30**(4): 951-954.

Dixon, D.M., Salkin, I.F., Duncan, R.A., Hurd, N.J., Haines, J.H., and Kemna, M.E. 1991. Isolation and characterization of *Sporothrix schenckii* from clinical and environmental sources associated with the largest U.S. epidemic of sporotrichosis. J. Clin. Microbiol. **29**(6): 1106-1113.

Ghannoum, M.A. Potential role of phospholipases in virulence and fungal pathogenesis. 2000. Clin. Microbiol. Rev. **13**(1): 122-143.

Ghosh, A., Maity, P.K., Hemashettar, B.M., Sharma, V.K., and Chakrabarti, A. 2002. Physiological characters of *Sporothrix schenckii* isolates. Mycoses. **45**(11-12): 449-454.

Gutierrez-Galhardo, M.C., do Valle, A.C., Fraga, B.L., Schubach, A.O., Hoagland, B.R., Monteiro, P.C., et al. 2010. Disseminated sporotrichosis as a manifestation of immune reconstitution inflammatory syndrome. Mycoses. **53**(1): 78-80. doi: 10.1111/j.1439-0507.2008.01655.x.

Hempel, H. and Goodman, N.L. 1975. Rapid conversion of *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Sporothrix schenckii* in tissue culture. J. Clin. Microbiol. **1**(5): 420-424.

Ibrahim, A.S., Mirbod, F., Filler, S.G., Banno, Y., Cole, G.T., Kitajima, Y., et al. 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect. Immun. **63**(5): 1993–1998.

Ishida, K., Alviano, D.S., Silva, B.G., Guerra, C.R., Costa, A.S., Nucci, M., et al. 2012. Negative correlation between phospholipase and esterase activity produced by *Fusarium* isolates. Braz. J. Med. Biol. Res. **45**(5):411-416.

Kantarcioglu, A.S., and Yücel, A. 2002. Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. Mycoses. **45**(5-6): 160-165.

Koga-Ito, C.Y., Lyon, J.P., Vidotto, V., and de Resende, M.A. 2006. Virulence factors and antifungal susceptibility of *Candida albicans* isolates from oral candidosis patients and control individuals. Mycopathologia. **161**(4): 219-223.

Kumar, C.P., Menon, T., Sundararajan, T., Nalini, S., Thirunarayan, M.A., Rajasekaran, S., et al. 2006. Esterase activity of *Candida* species isolated from immunocompromised hosts. Rev. Iberoam. Micol. **23**(2):101-103 .

Kusuhara, M., Hachisuka, H., and Sasai, Y., 1988. Statistical survey of 150 cases with sporotrichosis. Mycopathologia. **102**(2): 129–133.

Lopez-Martinez, R., Manzano-Gayosso, P., Mier, T., Mendez-Tovar, L.J., and Hernández, F. 1994. Exoenzimas de dermatofitos aislados de tiñas agudas y crónicas. Rev. Lat. Amer. Microbiol. **36**: 17-20.

Marimon, R., Cano, J., Gené , J., Sutton, D. A., Kawasaki, M., and Guarro, J. 2007. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. J. Clin. Microbiol. **45**(10): 3198–3206.

Marimon, R., Gené, J., Cano, J., Trilles, L., dos Santos Lazéra, M., and Guarro J. Molecular phylogeny of *Sporothrix schenckii*. 2006. J. Clin. Microbiol. **44**(9): 3251-3256.

Marimon, R., Serena, C., Gené, J., Cano, J., and Guarro, J. 2008. *In vitro* antifungal susceptibilities of five species of *Sporothrix*. Antimicrob. Agents Chemother. **52**(2): 732-734.

Mendoza, M., Alvarado, P., Díaz de Torres, E., Lucena, L., and de Albornoz, M.C. 2005. Physiological comportment and in vivo sensitivity of *Sporothrix schenckii* isolates maintained for 18 years by two preservation methods. Rev. Iberoam. Micol. **22**(3): 151-156.

Mesa-Arango, A.C., Del Rocío Reyes-Montes, M., Pérez-Mejía, A., Navarro-Barranco, H., Souza, V., Zúñiga, G., et al. 2002. Phenotyping and genotyping of *Sporothrix schenckii* isolates according to geographic origin and clinical form of Sporotrichosis. J. Clin. Microbiol. **40**(8): 3004-3011.

Mintz, C.S., Miller, R.D., Gutgsell, N.S., and Malek, T. 1993. *Legionella pneumophila* protease inactivates interleukin-2 and cleaves CD4 on human T cells. Infect. Immun. **61**(8): 3416-3421.

Monod, M., and Borg-Von-Zepelin, M. 2002. Secreted proteinases and other virulence mechanisms of *Candida albicans*. Chem. Immunol. **81**: 114-128.

Muhsin, T.M., Aubaid, A.H., and Al-Duboon, A.H. 1997. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. Mycoses. **40**(11-12): 465–469.

Naglik, J.R., Challacombe, S.J., and Hube, B. 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol. Mol. Biol. Rev. **67**(3): 400-428.

Nobre, G., and Viegas, M.M.P. 1972. Lipolytic activity of dermatophytes. Mycopathologia. **46**: 319-323.

Oliveira, D.C., Lopes, P.G., Spader, T.B., Mahl, C.D., Tronco-Alves, G.R., Lara, V.M., et al. 2011. Antifungal susceptibilities of *Sporothrix albicans*, *S. brasiliensis*, and *S. luriei* of the *S. schenckii* complex identified in Brazil. J. Clin. Microbiol. **49**(8):3047-3049. doi: 10.1128/JCM.00255-11.

Olszewski, M.A., Noverr, M.C., Chen, G.H., Toews, G.B., Cox, G.M., Perfect, J.R., and Huffnagle, G.B. 2004. Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. Am. J. Pathol. **164**(5): 1761-1771.

Palmeira, V.F., Kneipp, L.F., Alviano, C.S., and dos Santos, A.L. 2010. Phospholipase and esterase production by clinical strains of *Fonsecaea pedrosoi* and their interactions with epithelial cells. Mycopathologia. **170**(1):31-37.

Pappas, P.G., Tellez, I., Deep, A.E., Nolasco, D., Holgado, W., and Bustamante, B. 2000. Sporotrichosis in Peru: description of an area of hyperendemicity. Clin. Infect. Dis. **30**(1): 65–70.

Pfaller, M.A., Buschelman, B., Bale, M.J., Lancaster, M., Espinel-Ingroff, A., Rex, J.H., et al. 1994. Multicenter comparison of a colorimetric microdilution broth method with the reference macrodilution method for *in vitro* susceptibility testing of yeast isolates. Diagn. Microbiol. Infect. Dis. **19**(1): 9-13.

Pini, G., and Faggi, E. 2011. Extracellular phospholipase activity of *Malassezia* strains isolated from individuals with and without dermatological disease. Rev. Iberoam. Micol. **28**(4): 179-182.

Price, M.F., Wilkinson, I.D., and Gentry, L.O. 1982. Plate method for detection of phospholipase in *Candida albicans*. Sabouraudia. **20**: 7–14.

Rechnitzer, C., Williams, A., Wright, J.B., Dowsett, A.B., Milman, N. and Fitzgeorge, R.B. 1992. Demonstration of the intracellular production of tissue-destructive protease by *Legionella pneumophila* multiplying within guinea-pig and human alveolar macrophages. J. Gen. Microbiol. **138**: 1671-1677.

Rementeria, A., López-Molina, N., Ludwig, A. Vivanco, A.B., Bikandi, J., Pontón, J., et al. 2005. Genes and molecules involved in *Aspergillus fumigatus* virulence. Rev. Iberoamer. Micol., **22**(1): 1-23.

Rippon, J.W. 1982. Medical mycology: the pathogenic fungi and the pathogenic actinomycetes. Philadelphia, W.B. Saunders. pp. 277-302.

Rüchel, R., Tegeler, R., and Trost, M.A. 1982. A comparison of secretory proteinases from different strains of *Candida albicans*. Sabouraudia. **20**(3): 233–234.

Samdani, A.J., and Ayub, J. 2005. Dermatophyte growth and degradation of human stratum corneum *in vitro* (pathogenesis of dermatophytosis). Med. Coll. Abbottabad. **17**(4): 19–21.

Sánchez, M., and Colom, F. 2010. Extracellular DNase activity of *Cryptococcus neoformans* and *Cryptococcus gattii*. Rev. Iberoam. Micol. **27**(1):10-13. doi: 10.1016/j.riam.2009.11.004.

Schaller, M., Borelli, C., Korting, H.C., and Hube, B. 2005. Hydrolytic enzymes as virulence factors of *Candida albicans*. Mycoses. **48**(6): 365-377.

Schaller, M., Schackert, C., Korting, H. C., Januschke, E. and Hube, B. 2000. Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infection of human epidermis. J. Invest Dermatol. **114**(4): 712–717.

Shimizu, M.T., Almeida, N.Q., Fantinato, V., and Unterkircher, C.S. 1996. Studies on hyaluronidase, chondroitin sulphatase, proteinase and phospholipase secreted by *Candida* species. Mycoses. **39**(5-6): 161–167.

Seeliger, H.P.R. 1956. Use of a urease test for the screening and identification of cryptococci. J. Bact. **72**: 127-131.

Slifkin, M., Cumbie, R. 1996. Evaluation of the Tween 80 opacity test in the identification of dermatophytes. Med. Microbiol. Lett. **5**:401-407.

Soares, D.A., de Andrade, R.V., Silva, S.S., Bocca, A.L., Soares Felipe, S.M., and Petrofeza, S. 2010. Extracellular *Paracoccidioides brasiliensis* phospholipase B involvement in alveolar macrophage interaction. BMC Microbiol. **10**: 241. doi: 10.1186/1471-2180-10-241.

Souza, T.F., Scroferneker, M.L., Costa, J.M., Carissimi, M. and Corbellini, V.A. 2008. Secretion of five extracellular enzymes by strains of chromoblastomycosis agents. Rev.Inst. Med. Trop. S. Paulo. **50**(5): 269-272.

Torres-Rodríguez, J.M., Alvarado-Ramírez, E., and Gutiérrez-Gallego, R. 2008. Urease activity in *Cryptococcus neoformans* and *Cryptococcus gattii*. Rev. Iberoam. Micol. **25**(1): 27-31.

Tosun, I., Akyuz, Z., Guler, N.C., Gulmez, D., Bayramoglu, G., Kaklikkaya, N., et al. 2012. Distribution, virulence attributes and antifungal susceptibility patterns of *Candida parapsilosis* complex strains isolated from clinical samples. Med Mycol. *In press*.

Vellasamy, K.M., Vasu, C., Puthucheary, S.D. and Vadivelu, J. 2009. Comparative analysis of extracellular enzymes and virulence exhibited by *Burkholderia pseudomallei* from different sources. Microb. Pathog. **47**(3): 111-117. doi: 10.1016/j.micpath.2009.06.003.

Vidotto, V., Ito-Kuwa, S., Nakamura, K., Aoki, S., Melhem, M., Fukushima, K., et al. 2006. Extracellular enzymatic activities in *Cryptococcus neoformans* strains isolated from AIDS patients in different countries. Rev. Iberoam. Micol. **23**(4):216-20.

Vidotto, V., Leone, R., Sinicco, A., Ito-kuwa, S., and Criseo, G. 1998. Comparison of phospholipase production in *Cryptococcus neoformans* isolates from AIDS patients and bird droppings. Mycopathologia. **142**(2):71–6.

Yucesoy, M., and Marol, S. 2003. Determination of esterase activity of *Candida* varieties. Mikrobiyol. Bul. **37**(1):59-63.

5.3. Capítulo III

SUSCEPTIBILITY OF SPECIES WITHIN THE *SPOROTHRIX SCHENCKII* COMPLEX TO A PANEL OF KILLER YEASTS

Aceito no periódico:

Journal of Basic Microbiology.

Susceptibility of species within the *Sporothrix schenckii* complex to a panel of killer yeasts.

Cheila Denise Ottonelli Stopiglia[a,b], Daiane Heidrich[a], Julia Medeiros Sorrentino[b], Fabiane Jamono Vieira[b], Melissa Fontes Landell[c], Patrícia Valente[b], Maria Lúcia Scroferneker[a,b*]

[a]Programa de Pós-Graduação em Medicina: Ciências Médicas, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2400 – 2º andar – CEP: 90035-003, Porto Alegre – RS, Brazil.

[b]Departamento de Microbiologia, Imunologia e Parasitologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 – CEP 90050-170, Porto Alegre – RS, Brazil.

[c]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul , Av. Bento Gonçalves, 9500, prédios 43421/43431 - setor IV - Campus do Vale – caixa postal 15005 - CEP 91501-970, Porto Alegre – RS , Brazil.

[*] Correspondence and reprints: Maria Lúcia Scroferneker, Departamento de Microbiologia, Imunologia e Parasitologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 sala 210, CEP: 90050-170, Porto Alegre - RS, Brasil. Tel. (+55) 51-33083934 Fax. (+55) 51-33083121 (e-mail: scrofern@ufrgs.br)

Abstract

The *Sporothrix schenckii* complex is the etiologic agent of sporotrichosis, a subacute or chronic mycosis which can affect humans and animals. Killer yeasts have been used in the medical field for development of novel antimycotics and biotyping of pathogenic fungi. The action of eighteen killer yeasts on the growth of eighty-eight characterized *S. schenckii*, *S. globosa* and *S. brasiliensis* isolates was evaluated. Killer studies were performed on Petri dishes containing cheese black starch agar. The yeasts *Candida catenulata* (QU26, QU31, QU127, LV102); *Trichosporon faecale* (QU100); *Trichosporon japonicum* (QU139); *Kluyveromyces lactis* (QU30, QU99, QU73); *Kazachstania unispora* (QU49), *Trichosporon insectorum* (QU89) and *Kluyveromyces marxianus* (QU103) showed activity against all strains of the *S. schenckii* complex tested. Observation by optical microscopy of the fungal structures of *S. brasiliensis* 61 within the inhibition haloes around the colonies of the killer yeasts QU100, QU139 and LV102 showed that there was no conidiation, but there was hyphal proliferation. The toxins were fungistatic against *S. brasiliensis* 61. There was no difference in susceptibility to the toxins among the *S. schenckii* species complex. Further investigations are necessary to clearly establish the mechanism of action of the toxins.

Keywords: killer toxin; yeasts; *Sporothrix*

1. Introduction

Sporothrix schenckii, now considered as a complex of species, is the etiologic agent of sporotrichosis, a subacute or chronic mycosis which can affect humans and animals [1]. This mycosis predominates in tropical and temperate zones, with the main endemic areas found in Japan, India, South Africa, Mexico, Peru, Uruguay, and Brazil [2,3], and it is the subcutaneous mycosis of highest incidence in the state of Rio Grande do Sul [4]. The standard treatment of cutaneous-lymphatic sporotrichosis is potassium iodide. Other drugs commonly used are itraconazole for the treatment of cutaneous-lymphatic and systemic sporotrichosis, whereas visceral and disseminated forms have classically been treated with amphotericin B. Although these drugs are generally effective, the long duration of therapy and the toxicity of these drugs make it necessary to explore new alternatives for the treatment of severe infections [5].

Some yeasts possess antagonistic property (killer activity) towards moulds and other yeasts by producing killer toxins [6]. This property has been used to control contaminating wild yeasts in food and pathogenic fungi in plants [7,8]. In the medical field, these yeasts have been used in the development of novel antimycotics for the treatment of human and animal fungal infections [9-11] and for the biotyping of pathogenic yeasts and yeast-like fungi [12,13]. The action of killer yeasts against *S. schenckii* has already been reported in the literature [14,15], but this once considered a single species is presently recognized as a species complex. Thus, we wondered if the species within this complex had similar susceptibilities to yeast killer toxins.

Other studies have demonstrated the killer activity against yeasts and filamentous fungi. The yeasts *Mrakia frigida* and *Wickerhamomyces anomalus* were found to be able to produce killer toxins against a yeast (*Metschnikowia bicuspidata* WCY) pathogenic for crabs [16,17]. A killer toxin, secreted by the yeast *W. anomalus*, induced a significant decrease in *Pneumocystis carinii in vitro* attachment and inhibited the parasite infectivity in mice [18]. *Aureobasidium pullulans* and

other environmental yeasts showed killer activity against *Candida* spp. [19], and the killer toxin wicaltin was even more effective than other frequently used antifungals (clotrimazole and miconazole) against *C. albicans* [20]. The toxin of *Pichia membranifaciens* proved to be active against filamentous fungi, although with a lower efficiency [21].These selected examples demonstrate the potential of yeast killer toxins to inhibit pathogenic fungi.

Previous studies have shown that yeasts obtained from raw milk and cheese produce killer toxins against *Cryptococcus neoformans* and *Cryptococcus gattii*, and are promising for the development of new antifungal agents [13]. The aim of this study was to evaluate the action of eighteen of these previously tested killer yeasts on the growth of eighty-eight isolates of the *S. schenckii* species complex. In addition, we evaluated the effect of the killer toxins on the cellular morphology of the sensitive cells by means of optical microscopy.

2. Materials and methods

2.1. Strains

Eighty-six clinical, environmental and animal isolates belonging to the *Sporothrix schenckii* complex, identified according Marimon et al. (2007) [1], were used in this study. The samples were isolated from Argentina and different states of Brazil: Rio Grande do Sul, Rio de Janeiro, Minas Gerais and São Paulo. Moreover, two reference isolates from *American Type Culture Collection* - ATCC (201679 and 201681) were also used. The killer panel was composed by 18 strains selected from a previous screening of 44 non-*Saccharomyces* killer strains obtained from raw milk (LV) and cheese (QU), in Brazil. These killer yeasts were selected due to their capability to grow and express the toxin at temperatures up to 37ºC [13].

2.2. Killer assay

S. schenckii strains were subcultured onto potato dextrose agar (PDA) at 35 °C for 7 days. The surface was gently scraped with a sterile bent glass after flooding with sterile saline solution. Standard suspensions of *S. schenckii* were prepared by a spectrophotometric method as described by Pfaller et al. [22] in sterile saline solution to a density of approximately 10^6 CFU/mL, and aliquots of 1mL were placed on the Petri dishes containing cheese black starch agar (Minas cheese 33%, glucose 2%, peptone 1%, agar 1.5% and black starch 0.003%). Inoculum suspensions (10^8 CFU/mL) of 48 h grown cells of potential killer yeasts were prepared in the same medium, point-inoculated over the *S. schenckii* inoculum and incubated at 25°C for 4 days. The killer activity was considered positive if there was an evident zone of inhibition around the inoculum of the killer strain, surrounded by a crown of dark-blue stained cells, which was measured with a caliper. Measurements were obtained from the subtraction of the diameter of yeast colonies from the measures of the diameters of the zone of inhibition plus yeast colony. The values obtained were grouped on the following scale: A: < 1 mm; B: 1 - 10 mm; C: 10.1 - 20 mm; D: 20.1 - 30 mm; E: 30.1 - 35 mm. Moreover, for each killer yeast, we determined the mean halo size, using the geometric mean of the inhibition zones obtained against all isolates of the *S. schenckii* complex. All the experiments were conducted in triplicate.

2.3. Determination of fungicidal or fungistatic activity

The fungicidal or fungistatic activity was evaluated with the isolate *S. brasiliensis* 61 against the killer yeasts QU100, QU139 and LV102, using a methodology adapted from Espinel-Ingroff et al. [23]. The zone within the inhibition halo was gently scraped with a bacteriological loop, the contents were transferred to tubes containing Sabouraud dextrose broth and incubated at 25°C for

10 days. After this period, fungal growth was confirmed by the turbidity of the growth medium, and by observation of hyphae by optical microscopy.

2.4. Optical microscopy of cells within the inhibition halo

For the observation of morphological alterations, *S. brasiliensis* 61 was used as sensitive strain and *Trichosporon faecale* - QU100, *Trichosporon japonicum* - QU139 and *Candida catenulata* - LV102 as killer yeasts. After 4 days of action of the killer toxins, the inhibition haloes were lightly scraped with a bacteriological loop, mounted between lamina and coverslip with the use of lugol, and observed using optical microscopy. *S. brasiliensis* 61 grown without the killer yeasts was used as control.

3. Results

Of the 18 yeasts tested, two (*Yarrowia lipolytica* QU11 and *Candida parapsilosis* QU134) had no activity against isolates of the *S. schenckii* complex, and four (*C. catenulata* QU18, *Kluyveromyces lactis* QU37, and *C. parapsilosis* LV02 and LV36) lost activity after one year. The remainders were selected for evaluation of the growth inhibition against isolates of the *S. schenckii* complex (Table 1). Among these, the ones that showed larger inhibition haloes were *Kazachstania unispora* QU49 (mean halo size 19.0mm), *T. faecale* QU100 (mean halo size 18.6mm), *T. japonicum* QU139 (mean halo size 18.5mm), *K. lactis* QU99 (mean halo size 18.5mm) and *Trichosporon insectorum* QU89 (mean halo size 17.5mm). Most inhibition haloes were up to 20.0mm (scale B and C), with the species in the *S. schenckii* complex showing similar susceptibility profiles. *S. brasiliensis* 61 was the most susceptible to the killer yeasts, especially to QU49 and QU139, and was thus chosen for a more detailed evaluation of the susceptibility to the killer toxins, while *S. schenckii* 193/09, 181683 and 4674294 were the least susceptible. *S. brasiliensis* strain

ATCC 201681 had inhibition haloes in the scale ranges B and C, while *S. schenckii* strain ATCC 201679 showed 83.3% of the inhibition haloes in the B and C scales, and 8.3% in both the A and D scales (Table 1). Figure 1 shows the inhibition zone around a strain of the *S. schenckii* complex against four yeasts: three with killer activity and one with no activity.

Table 1: Evaluation of the growth inhibition of *Sporothrix schenckii* complex isolates against killer yeasts.

	Killer yeasts[a]											
Fungal strain	**QU 26**	**QU 30**	**QU 31**	**QU 49**	**QU 73**	**QU 89**	**QU 99**	**QU 100**	**QU 103**	**QU 127**	**QU 139**	**LV 102**
S. brasiliensis[b] (n=21)	B-D[c]	B-D	A-C	B-E	B-D	B-D	B-D	B-D	B-D	B-C	B-E	B-D
Environmental isolates (n=1)	D	D	C	C	C	C	C	C	D	C	D	D
ATCC 201681	B	B	B	C	B	C	C	C	B	C	C	C
S. globosa[b] (n=1)	C	B	B	D	B	B	B	B	B	C	B	B
S. schenckii												
Clinical isolates (n=61)	A-D	A-D	A-D	B-D	A-D	B-D	B-D	B-D	A-D	B-D	B-D	B-D
Animal isolates (n=1)	D	C	C	D	C	C	D	C	C	D	C	D
Environmental isolates (n=1)	C	C	C	C	C	B	C	C	C	C	C	C
ATCC 201679	B	C	C	C	B	B	B	C	A	B	D	C
All strains (n=88)	A-D	A-D	A-D	B-E	A-D	B-D	B-D	B-D	A-D	B-D	B-E	B-D
Mean halo size[d] (n=88)	14.1	12.9	11.9	19.0	13.5	17.5	18.5	18.6	14.9	13.0	18.5	14.5

[a] Killer yeasts: C*andida catenulata* (QU26, QU31, QU127, LV102); *Trichosporon faecale* (QU100); *Trichosporon japonicum* (QU139); *Kluyveromyces lactis* (QU30,QU99, QU73); *Kazachstania unispora* (QU 49), *Trichosporon insectorum* (QU 89) and *Kluyveromyces marxianus* (QU103); [b]Clinical isolates. A: < 1 mm; B: 1 - 10 mm; C: 10.1 - 20 mm; D: 20.1 - 30 mm; E: 30.1 - 35 mm.
[c] The inhibition haloes measurements were obtained subtracting the diameter of yeast colonies from the measures of the diameters of the zone of inhibition plus yeast colonies.
[d] The mean halo size was determined calculating the geometric mean of the inhibition haloes obtained with all isolates of the *S. schenckii* complex for each killer yeast strain.

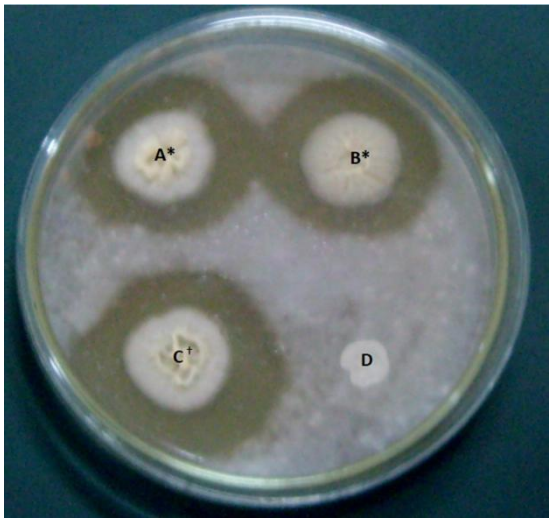

Figure 1: Inhibition zones around *S. brasiliensis* 61 against killer yeasts *Trichosporon faecale* QU100 (A), *Candida catenulata* LV102 (B) and *Kluyveromyces marxianus* QU103 (C), and a yeast with no activity: *Candida parapsilosis* QU134 (D).* scale C and † scale D.

When the cells of the isolate *S. brasiliensis* 61 were scrapped from the inhibition haloes and transferred to fresh medium, growth could be observed, suggesting that the toxins have a fungistatic mode of action, at least against this isolate. This was confirmed by the data from optical microscopy of cells within the inhibition halo, which revealed that the cells of *S. brasiliensis* presented an alteration in the growth pattern when compared to control cells. Specifically, after four days of incubation we noted spore germination but the hyphae did not form conidia, whereas the conidiation was observed in the same *S. brasiliensis* isolate unexposed to killer yeasts (control) (Fig. 2).

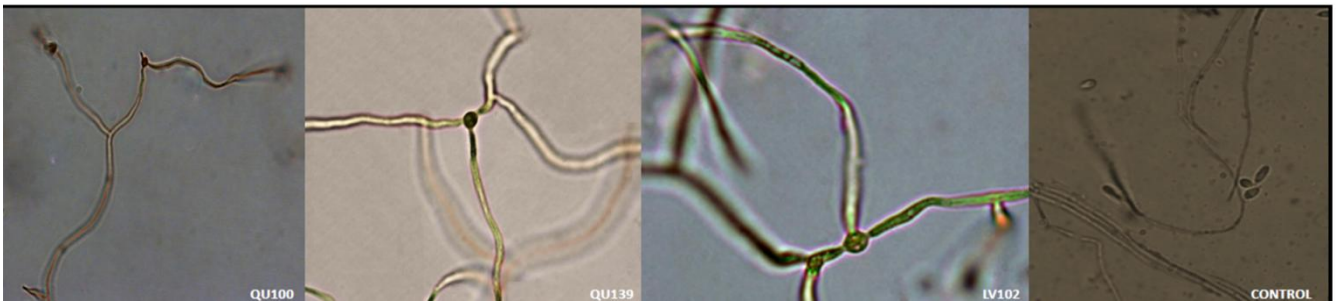

Figure 2: Optical microscopy (1000X) of cells within the inhibition halo of *S. brasiliensis* 61 exposed to the following killer yeasts: *Trichosporon faecale* QU100, *Trichosporon japonicum* QU139 and *Candida catenulata* LV102. Control: *S. brasiliensis* 61 not exposed to killer yeasts, showing conidiation.

4. Discussion

S. schenckii was long considered a single taxon, although great genetic variation within this species has been described. Associating phenotypic and genotypic features, Marimon et al. [1] recognized three new species, *S. brasiliensis*, *S. globosa* and *S. mexicana*, and proposed an identification key for these *Sporothrix* species [24]. These species vary in virulence and antifungal sensitivity [25-27], furthermore animal and human isolates have different susceptibility profiles to antifungals [28]. Although Fuentefria et al. observed differences between *C. neoformans* and *C. gattii* against a panel of killer yeasts obtained from raw milk and cheese [13], in our study those same killer yeasts were not able to differentiate the species within the *S. schenckii* complex, nor there was correlation between sensitivity and origin of the isolates (clinical, animal and environmental). De Oliveira et al. reported that some species of the *S. schenckii* complex are too closely related to show phenotypic differences [24].

After its initial discovery in *Saccharomyces cerevisiae* [6], the killer phenotype has been found in yeasts belonging to the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Schwanniomyces*, *Torulopsis*,

Trichosporon, *Williopsis* and *Zygosaccharomyces* [9,21,29]. Various mechanisms of action have been suggested for the different killer toxins. *S. cerevisiae* K1 and K2 toxins exhibit their lethal effect on sensitive cells by disrupting the cytoplasmic membrane function [10]; the K28 toxin has no ionophoric effect, but it inhibits both nuclear DNA synthesis and the budding cycle [30]. *Filobasidium capsuligenum* FC-1 toxin releases cellular components through pores formed in the cytoplasmic membrane [31], whereas HM-1, from *Williopsis mrakii*, kills sensitive cells presumably by interfering with β-(1→3)-glucan synthesis [32].

In spite of this diversity, most killer toxins are proteins or glycoproteins that kill sensitive cells via a two-step mode of action. During the first step, the killer toxin binds a receptor site on the cell wall of its sensitive target. In the second step, which has been far less characterized, the killer toxin is assumed to interact with receptors on the cell membrane and to kill the sensitive cells via different mechanisms [33].

The panel of killer yeasts used in the present study was the same tested by Fuentefria et al. [13] against *C. neoformans* and *C. gattii*. These killer yeasts showed fungicidal activity against the *Cryptococcus* isolates, and it was suggested that they act by interrupting cell cycle development [13]. However, our results showed fungistatic activity, with hyphal proliferation and lack of conidiation, at least for *S. brasiliensis* 61.

Asexual sporulation (conidiation) is the main mechanism of dispersion of higher fungi. Conidiation is a highly successful reproductive process by which large numbers of spores can be produced from a single colony through mitosis [34]. Regulation of conidiation-specific genes in filamentous fungi is controlled by a G protein-mediated signaling pathway that senses environmental conditions and regulates the switch between vegetative growth and conidiation [35]. In *Aspergillus nidulans* the activation of this G protein-mediated pathway results in blockage of conidiation and activation of vegetative growth [36]. However, the entomopathogenic fungus

Beauveria bassiana was shown to behave differently than the other fungi. Upon deletion of a gene related to the regulation of conidiation, reduction of conidia production was observed [37], whereas conidiation is completely blocked in other fungi [38,39]. Further investigations are necessary to evaluate if the mechanism of action of the killer toxins against the *S. schenckii* complex may involve the G protein signaling pathway, blocking the switch between vegetative growth and conidiation.

Growth alterations due to the action of yeast killer toxins have already been reported in the literature. Polonelli et al. [14] showed that the killer toxins produced by *Wickerhamomyces canadensis*, *Barnettozyma californica* (syn. *Pichia dimennae*), *Meyerozyma guilliermondii* and *W. anomalus* reduced the number of expected colonies of *S. schenckii* isolates in both the mycelial and yeast forms. Microscopic examination of the growth of *S. schenckii* on the plates inoculated with the mycelial and yeast inocula revealed that both of them reverted to an intermediate, apparently uniform type of growth characterized by conidial germination and formation of germ tube-like structures. De Lima et al. [40] reported that yeasts isolated from tropical fruits provided a significant reduction in the mycelial growth and conidial germination of *Colletotrichum gloeosporioides*. *Penicillium digitatum* showed growth alterations when challenged with *W. anomalus*: hyphae appeared wilted, folded, coiled, and had granulation when compared with the thin, elongated, well-extended mycelial growth in the control [8]. Furthermore, collapsed hyphae with loss of intracellular content were observed, as has been previously reported when it was challenged with *Meyerozyma guillermondii* [41].

Recent studies attempted to identify killer peptides, engineered from an anti-idiotypic recombinant antibody that mimics the activity of a wide-spectrum antimicrobial yeast killer toxin targeting cell-wall receptors. The *in vitro* and *in vivo* antifungal and immunomodulatory activities of these killer peptides may represent the prototypes of novel antifungal vaccines and anti-infective

drugs characterized by different mechanisms of action [42-44]. Our study highlights killer toxins that could be used for the development of new anti-idiotypic antibodies for the treatment of sporotrichosis, following the protocols established in the above mentioned reports.

The killer yeasts analyzed in this study have been previously evaluated against other yeasts, including *C. neoformans* and *C. gattii* [13,45]. The strains *C. catenulata* QU31 and LV102 showed a higher percentage (40.9% and 48.9%, respectively) of haloes ≥10.1mm (scales C, D or E) against the *S. schenckii* complex than against the *Cryptococcus* species (17% and 35%, respectively), while *C. catenulata* QU127 obtained a lesser percentage (35.2% against *Sporothrix* spp. and 58% against *Cryptococcus* spp.). *T. faecale* QU100 (mean halo size 18.6mm) showed inhibition haloes classified as C or D against approximately 74% of the *S. schenckii* isolates, the same percentage of *Cryptococcus* strains that were inhibited by this killer strain in the previous study [13]. Finally, *T. insectorum* QU89 (syn. CBS10423, NRRL Y-48122) produced one of the largest inhibition zones in the present study (mean halo size 17.5mm), while it only weakly inhibited 96 *C. neoformans* isolates and clinically relevant *Candida* species, and did not inhibit *Y. lipolytica* and *C. gattii* isolates [45]. These comparisons corroborate the knowledge that the killer phenotype is a strain-associated trait, and is also dependent on the sensitive microorganism chosen as target.

The killer activity of yeasts is dependent on several factors, such as medium, pH, salinity and temperature [46]. Initially, the killer activity assays were performed on Sabouraud dextrose agar, however we observed that the yeasts reduced their killer activity over time (data not shown). To circumvent this situation, we developed the cheese black starch agar, to maintain the original nutrients of the substrata from which the yeasts were isolated (cheese and milk). According to Wloch-Salamon et al. [47], toxin production has evolved as a competitive strategy under conditions where resources are abundant and growth is allowed. Killers may have an advantage in productive habitats, because more resources are available for the production of toxins and the costs of

production may be reduced [48], especially under conditions where killing occurs only at high toxin production rates [49]. The medium formulated in the present study was able to maintain activity of 63% of the killer yeasts during the period of one year.

In summary, we demonstrated that a large panel of strains belonging to the *S. schenckii* complex is susceptible to yeast killer toxins, making these toxins potentially useful for the development of new antifungal agents. Further investigations are necessary to clearly establish the mechanism of action of the toxins, including the isolation of these substances, potentially indicating new targets for the treatment of sporotrichosis.

5. Acknowledgements

The authors thank the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and *Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul* (FAPERGS) for financial support.

6. Conflict of Interest Statement: The authors report no conflict of interest.

7. References

[1] Marimon, R., Cano, J., Gené , J., Sutton, D. A., et al., 2007. *Sporothrix brasiliensis, S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. J. Clin. Microbiol., **45**, 3198–3206.

[2] Kusuhara, M., Hachisuka, H., Sasai, Y., 1988. Statistical survey of 150 cases with sporotrichosis. Mycopathologia., **102**, 129–133.

[3] Pappas, P.G., Tellez, I., Deep, A.E., Nolasco, D., et al., 2000. Sporotrichosis in Peru: description of an area of hyperendemicity. Clin. Infect. Dis., **30**, 65–70.

[4] da Rosa, A. C., Scroferneker, M. L., Vettorato, R., Gervini, R. L., et al., 2005. Epidemiology of sporotrichosis: a study of 304 cases in Brazil. J. Am. Acad. Dermatol., **52,** 451-459.

[5] Kauffman, C. A., 1995. Old and new therapies for sporotrichosis. Clin. Infect. Dis., **21**, 981–985.

[6] Bevan, E. A., Makower, M., 1963. The physiological basis of the killer character in yeast. Proc. Int. Congr. Genet., **1**, 202-203.

[7] Palpacelli, V., Ciani, M., Rosini, G., 1991. Activity of different killer yeasts on strains of yeast species undesirable in the food industry. FEMS Microbiol. Lett., **84**, 75-78.

[8] Platania, C., Restuccia, C., Muccilli, S., Cirvilleri, G., 2012. Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on *Tarocco orange* fruits (*Citrus sinensis*). Food Microbiol., **30**(1), 219-225.

[9] Hodgson, V.J., Button, D., Walker, G.M., 1995. Anti-*Candida* activity of a novel killer toxin from the yeast *Williopsis mrakii*. Microbiology., **141**, 2003-2012.

[10] Schmitt, M.J., Breinig, F., 2002. The viral killer system in yeast: from molecular biology to application. FEMS Microbiol. Rev., **26**, 257-276.

[11] Seguy, N., Polonelli, L., Dei-Cas, E., Cailliez, J.C., 1998. Effect of a killer toxin of *Pichia anomala* to *Pneumocystis*. Perspectives in the control of pneumocystosis. FEMS Immunol. Med. Microbiol., **22**, 145-149.

[12] Buzzini, P., Martini, A., 2001. Large-scale screening of selected *Candida maltosa*, *Debaryomyces hansenii* and *Pichia anomala* killer toxin activity against pathogenic yeasts. Med. Mycol., **39**, 479-482.

[13] Fuentefria, A.M., Faganello, J., Pazzini, F., Schrank, A., et al., 2007. Typing and patterns of cellular morphological alterations in *Cryptococcus neoformans* and *Cryptococcus gattii* isolates exposed to a panel of killer yeasts. Med. Mycol., **45**(6), 503-512.

[14] Polonelli, L., Conti, S., Campani, L., Morace, G., et al., 1989. Yeast killer toxins and dimorphism. J. Clin. Microbiol., **27**(6), 1423-1425.

[15] Polonelli, L., Dettori, G., Cattel, C., Morace, G., 1987. Biotyping of micelial fungus cultures by the killer system. Eur. J. Epidemiol., **3**(3), 237-242.

[16] Hua, M.X., Chi, Z., Liu, G.L., Buzdar, M.A., et al., 2010. Production of a novel and cold-active killer toxin by *Mrakia frigida* 2E00797 isolated from sea sediment in Antarctica. Extremophiles., **14**(6), 515-521.

[17] Wang, X., Chi, Z., Yue, L., Li, J., et al., 2007. A marine killer yeast against the pathogenic yeast strain in crab (*Portunus trituberculatus*) and an optimization of the toxin production. Microbiol. Res., **162,** 77-85.

[18] Cailliez, J.C., Seguy, N., Aliouat, E.M., Polonelli, L., et al., 1994. The yeast killer phenomenon: a hypothetical way to control *Pneumocystis carinii* pneumonia. Med. Hypo., **43,**167–171.

[19] Tan, H.W., Tay, S.T., 2011. Anti-*Candida* activity and biofilm inhibitory effects of secreted products of tropical environmental yeasts. Trop. Biomed., **28**(1), 175–180.

[20] Theisen, S., Molkenau, E., Schmitt, M.J., 2000. Wicaltin, a new protein toxin secreted by the yeast *Williopsis californica* and its broad-spectrum antimycotic potential. J. Microbiol. Biotechnol., **10**(4), 547-550.

[21] Santos, A., San Mauro, M., Bravo, E., Marquina, D., 2009. PMKT2, a new killer toxin from *Pichia membranifaciens,* and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. Microbiology., **155**(Pt 2), 624-634.

[22] Pfaller, M. A, Buschelman, B., Bale, M. J., Lancaster, M., et al., 1994. Multicenter comparison of a colorimetric microdilution broth method with the reference macrodilution method for *in vitro* susceptibility testing of yeast isolates. Diagn. Microbiol. Infect. Dis., **19**, 9-13.

[23] Espinel-Ingroff, A., Fothergill, A., Meter, J., Rinaldi, M. G.,et al., 2002. Testing conditions for determination of minimum fungicidal concentrations to new and established antifungal agents for *Aspergillus* spp.: NCCLS collaborative study. J. Clin. Microbiol., **40,** 3204–3208.

[24] de Oliveira, M.M., Sampaio, P., Almeida-Paes, R., Pais, C., et al., 2012. Rapid identification of *Sporothrix* species by T3B PCR fingerprinting. J. Clin. Microbiol., **50**(6), 2159-2162.

[25] Arrillaga-Moncrieff, I., Capilla, J., Mayayo, E., Marimon, R., et al., 2009. Different virulence levels of the species of *Sporothrix* in a murine model. Clin. Microbiol. Infect., **15**(7), 651-655.

[26] Dixon, D.M., Duncan, R.A., Hurd, N.J., 1992. Use of a mouse model to evaluate clinical and environmental isolates of *Sporothrix* spp. from the largest U.S. epidemic of sporotrichosis. J. Clin. Microbiol., **30**(4), 951-954.

[27] Marimon, R., Serena, C., Gené, J., Cano, J., et al., 2008. *In vitro* antifungal susceptibilities of five species of *Sporothrix*. Antimicrob. Agents Chemother., **52**(2), 732-734.

[28] Oliveira, D.C., Lopes, P.G., Spader, T.B., Mahl, C.D., et al., 2011. Antifungal susceptibilities of *Sporothrix albicans*, *S. brasiliensis*, and *S. luriei* of the *S. schenckii* complex identified in Brazil. J. Clin. Microbiol., **49**(8):3047-3049.

[29] Chen, W. B., Han, Y. F., Jong, S. C., Chang, S. C., 2000. Isolation, purification, and characterization of a killer protein from *Schwanniomyces occidentalis*. Appl. Environ. Microbiol., **66**, 5348-5352.

[30] Schmitt, M.J., Klavehn, P., Wang, J., Schonig, I., et al., 1996. Cell cycle studies on the mode of action of yeast K28 killer toxin. Microbiology., **142**, 2655–2662.

[31] Keszthelyi, A., Ohkusu, M., Takeo, K., Pfeiffer, I., et al., 2006. Characterisation of the anticryptococcal effect of the FC-1 toxin produced by *Filobasidium capsuligenum*. Mycoses., **49**(3), 176-183.

[32] Takasuka, T., Komiyama, T., Furuichi, Y., Watanabe, T., 1995. Cell wall synthesis specific cytocidal effect of *Hansenula mrakii* toxin-1 on *Saccharomyces cerevisiae*. Cell Mol. Biol. Res., **41**, 575–581.

[33] de Ingeniis, J., Raffaelli, N., Ciani, M., Mannazzu, I., 2009. *Pichia anomala* DBVPG 3003 secretes a ubiquitin-like protein that has antimicrobial activity. Appl. Environ. Microbiol., **75**(4), 1129-1134.

[34] Adams, T.H., Wieser, J.K., Yu, J.H., 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiol. Mol. Biol. Rev., **62**, 35–54.

[35] Bölker, M., 1998. Sex and crime: heterotrimeric G proteins in fungal mating and pathogenesis. Fungal Genet. Biol., 25, 143–156.

[36] Yu, J.H., Wieser, J., Adams, T.H., 1996. The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. EMBO J., **15**, 5184–5190.

[37] Fang, W., Pei, Y., Bidochka, M.J., 2007. A regulator of a G protein signalling (RGS) gene, cag8, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation, virulence and hydrophobin synthesis. Microbiology., **153**, 1017–1025.

[38] Fang, W., Scully, L.R., Zhang, L., Pei, Y., et al., 2008. Implication of a regulator of G protein signalling (BbRGS1) in conidiation and conidial thermotolerance of the insect pathogenic fungus *Beauveria bassiana*. FEMS Microbiol. Lett., **279**, 146–156.

[39] Segers, G.C., Regier, J.C., Nuss, D.L., 2004. Evidence for a role of the regulator of G-protein signaling protein CPRGS-1 in got subunit CPG-1-mediated regulation of fungal virulence, conidiation, and hydrophobin synthesis in the chestnut blight fungus *Cryphonectria parasitica*. Eukaryot. Cell., **3**, 1454–1463.

[40] de Lima, J.R., Gonçalves, L.R.B., Brandão, L.R., Rosa, C.A., et al., 2012. Isolation, identification and activity *in vitro* of killer yeasts against *Colletotrichum gloeosporioides* isolated from tropical fruits. J. Basic Microbiol., doi: 10.1002/jobm.201200049.

[41] Wisniewski, M., Biles, C., Droby, S., 1991. The use of the yeast *Pichia guillermondii* as a biocontrol agent: characterization of attachment to *Botrytis cinerea*. In: Wilson, C., Chalutz, E. (Eds.), Biological control of postharvest diseases of fruits and vegetables. US Department of Agriculture, Agricultural Research Service, Shepherdstown WV Workshop, 12 e 14 September 1990.

[42] Kabir, M.E., Karim, N., Krishnaswamy, S., Selvakumar, D., et al., 2011. Peptide derived from anti-idiotypic single-chain antibody is a potent antifungal agent compared to its parent fungicide HM-1 killer toxin peptide. Appl. Microbiol. Biotechnol., **92**(6):1151-1160.

[43] Magliani, W., Conti, S., Ciociola, T., Giovati, L., et al., 2011. Killer peptide: a novel paradigm of antimicrobial, antiviral and immunomodulatory auto-delivering drugs. Future Med. Chem., **3**(9):1209-1231.

[44] Polonelli, L., Magliani, W., Ciociola, T., Giovati, L., et al., 2011. From *Pichia anomala* killer toxin through killer antibodies to killer peptides for a comprehensive anti-infective strategy. Antonie Van Leeuwenhoek., **99**(1):35-41.

[45] Fuentefria, A.M., Suh, S.O., Landell, M.F., Faganello, J., et al., 2008. *Trichosporon insectorum* sp. nov., a new anamorphic basidiomycetous killer yeast. Mycol. Res., **112**(Pt 1):93-99.

[46] Lopes, C.A., Sangorrín, M.P., 2010. Optimization of killer assays for yeast selection protocols. Rev. Argent. Microbiol., **42**(4), 298-306.

[47] Wloch-Salamon, D.M., Gerla, D., Hoekstra, R.F., de Visser, J.A., 2008. Effect of dispersal and nutrient availability on the competitive ability of toxin-producing yeast. Proc. Biol. Sci., **275**(1634), 535-41.

[48] Starmer, W. T., Schmedicke, R. A., Lachance, M. A., 2003. The origin of the cactus–yeast community. FEMS Yeast Res., **3**, 441–448.

[49] Brown, S. P., Le Chat, L., De Paepe, M., Taddei, F., 2006. Ecology of microbial invasions: amplification allows virus carriers to invade more rapidly when rare. Curr. Biol., **16**, 2048–2052.

5.4. Capítulo IV

ANTIFUNGAL ACTIVITY OF *PTEROCAULON SPECIES* (*ASTERACEAE*) AGAINST *SPOROTHRIX SCHENCKII*

Publicado no periódico:

Journal de Mycologie Médicale, v. 21, p. 169-172, 2011.

Antifungal activity of *Pterocaulon* species (*Asteraceae*) against *Sporothrix schenckii*

Activité antifongique de *Pterocaulon* sp. contre *Sporothrix schenckii*

C.D.O. Stopiglia[a], D. da Rocha Vianna[b], G. de Carvalho Meirelles[b],

H. Teixeira[b], G.L. von Poser[b], M.L. Scroferneker[a,c,*]

[a] Programa de Pós Graduação em Medicina: Ciências Médicas, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos nº 2400, 2º andar, CEP 90035-003, Porto Alegre, RS, Brazil.

[b]Programa de Pós Graduação em Ciências Farmacêuticas, Universidade Federal do Rio Grande do Sul, Av. Ipiranga nº 2752, 1º andar, CEP 90610-000, Porto Alegre, RS, Brazil.

[c]Departamento de Microbiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite nº 500, CEP 90050-170, Porto Alegre, RS, Brazil.

*** Corresponding author:** E-mail address: scrofern@ufrgs.br (M.L. Scroferneker).

Summary

Plants of the genus *Pterocaulon* (*Asteraceae*) are popularly used in the treatment of skin diseases caused by fungi and bacteria. The aim of this work was to investigate the *in vitro* activity of the crude methanolic extracts obtained from the aerial parts of *Pterocaulon polystachyum*, *P. balansae*, *P. lorentzii*, *P. lanatum*, and *P. cordobense* against 24 *Sporothrix schenckii* clinical isolates and determine the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC). MIC were performed by the broth microdilution method according guidelines recommended by Clinical and Laboratory Standards Institute for filamentous fungi and MFC were determined for transference of aliquots of the well that showed 100% of growth inhibition into tubes with culture medium. The extract from *P. polystachyum* was the most active sample, presenting MIC range of 156 and 312 µg/mL. The popular use of these plants corroborates the importance of ethnopharmacological surveys and opens the possibility for finding new clinically effective antifungal agents.

Keywords: *Pterocaulon*; *Asteraceae*; Antifungal activity; Sporotrichosis; *Sporothrix schenckii*.

Résumé

Les plantes du genre *Pterocaulon* (*Asteraceae*) sont couramment utilisées dans le traitement des maladies de peau causées par des champignons et des bactéries. Le but de ce travail était d'étudier l'activité *in vitro* des extraits méthanoliques bruts obtenus à partir des parties aériennes de *Pterocaulon polystachyum, P. balansae, P. lorentzii, P. lanatum,* et *P. cordobense* contre 24 isolats de *Sporothrix schenckii* ainsi que d'en déterminer la concentration minimale inhibitrice (CMI) et la concentration minimale fongicide (CMF). Les CMI ont été réalisées par la méthode de microdilution selon les lignes directrices recommandées par *Clinical and Laboratory Standards Institute* pour les champignons filamenteux et les CMF ont été déterminées pour le transfert des parties aliquotes du puits qui a montré 100% d'inhibition de la croissance dans des tubes de milieu de culture. L'extrait de *P. polystachyum* a été l'échantillon le plus actif avec une gamme CMI de 156 à 312 µg/mL. L'usage populaire de ces plantes corrobore l'importance des enquêtes ethnopharmacologiques et ouvre la possibilité de trouver de nouveaux agents antifongiques cliniquement efficaces.

Mots Clés: *Pterocaulon*; Activité antifongique; *Asteraceae*; Sporotrichose; *Sporothrix schenckii*.

Introduction

Treatment of invasive fungal infections is not always successful due to the limited number of available antifungal agents and resistance to these antimicrobials among fungal pathogens [9]. Therefore, substantial research studies in the field of antifungal compounds are necessary to develop new prototype antimicrobial agents to avert this situation.

Sporothrix schenckii is the etiological agent of sporotrichosis [6] a mycosis that can affect humans and other animals. This mycosis is acquired by traumatic inoculation of contaminated soil, plants, and organic matter. The lesions are usually limited to the skin, subcutaneous tissue, and surrounding lymph vessels. Sporotrichosis predominates in tropical and temperate zones, and Brazil is one of main endemic area, being the most common subcutaneous mycosis occurring in the state of Rio Grande do Sul, in the south of Brazil [4].

Although most antibiotics in clinical use have been obtained from microorganisms, the interest in plant antimicrobials has emerged in the last few decades. Plants and plant-derived compounds have a long clinically history as sources of potential chemotherapeutic agents [7]. The investigation of plants used by traditional medicine is a strategy for finding alternative antimicrobial agents [17].

An ethnoveterinary study indicated that some *Pterocaulon* species were useful in treating humans and animals skin diseases. In another study, three *Pterocaulon* species native to southern Brazil showed antifungal properties against opportunistic pathogenic yeasts and filamentous fungi, including dermatophytes [14,15]. Besides that, extract of *Pterocaulon alopecuroides* presented a broad spectrum of activity against a panel of chromoblastomycosis agents [5]. In another study, the amebicidal activity of extract of *P. polystachyum* was demonstrated [11].

The broad spectrum of action against some pathogenic fungi shown by extracts of *P. alopecuroides*, *P. balansae*, and *P. polystachyum* [15] makes it important to perform assays aimed at evaluating the antifungal activity against other fungal pathogens such as sporothrichosis agents. Thus, the aim of this work was to test the methanolic extracts of five *Pterocaulon* species against 24 *S. schenckii* clinical isolates determining the minimum inhibitory concentration (MIC) and minimal fungicidal concentration (MFC).

Materials and methods

Plant material

Plant material was collected in the southern region of Brazil in January, 2007 and were identified by N. Matzembacker (PPGBotânica, Universidade Federal do Rio Grande do Sul, Brazil). Vouchers were deposited in the herbariumof the Universidade Federal do Rio Grande do Sul (ICN) (*Pterocaulon balansae*: ICN 59572; *P. cordobense*: ICN 140001; *P. lanatum* ICN 140002; *P. lorentzii*: ICN 140005; *P. polystachyum*: ICN 140011).

Extract preparation

The crude methanolic extracts of *P. polystachyum*, *P. balansae*, *P. lorentzii*, *P. lanatum*, and *P. cordobense* were prepared with 50 g of the drug, using a drug: solvent ratio = 1:10 (w/v) by maceration. The extract was evaporated to dryness under reduced pressure at 45 °C affording 9-15%.

Microorganisms

For the antifungal evaluation, 24 clinical isolates of sporotrichosis agents from the Pathogenic Fungi Laboratory (Department of Microbiology of the Institute of Basic Health Sciences of Universidade Federal do Rio Grande do Sul, Brazil) were used: *S. schenckii* 20, 339, 424, 441, 478, 611, 794, 13534, 13845, 16498, 1099-18, 1400, s175, SN, STT, UFSM (02, 08, 09, 23, 26), and Santa Casa (I, II, III, F32).

Antifungal susceptibility testing

Antifungal susceptibility assays were performed by the broth microdilution method according to guidelines recommended by Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi - M38-A2 [3]. Strains were subcultured onto potato dextrose agar (PDA/Difco, Becton, Dickinson and Company, USA) at 35 ºC for 7 days. The surface was gently scraped with a sterile bent glass after flooding with sterile saline solution. The standard suspensions were adjusted by UV-visible spectrophotometry (Spectrum Instruments Co., Shanghai, China) to show transmittance at 530 nm of 80-82%. Adjusted suspensions were diluted in RPMI-MOPS (1:50) to obtain a final inoculum of 10^4 CFU/mL, and 100 µL of the fungal suspensions were added to each microdilution well containing 100 µL of the extracts (2x). The final concentration ranges of the extracts were 156 to 5000 µg/mL. Itraconazole (Janssen Pharmaceutica N. V., Beerse, Belgium) was used as a positive control. The stock solution (1600 µg/mL) was prepared in dimethyl sulfoxide (DMSO; Vetec) and other solutions were diluted in RPMI-MOPS for obtaining the final concentrations ranges of 0.0313 to 16 µg/mL. Plates were incubated at 35 °C for 46-50 hours, and the MIC was determined visually by comparison with the extract-free growth control well. The

MIC was defined as the lowest concentration of the antifungal agent that produced no visible fungal growth. Controls without the extract (growth control), without microorganisms (sterility control), and with the solvent were included. In order to determine the minimum fungicidal concentration (MFC), aliquots of 100 µL of the well that showed 100% of growth inhibition were seeded into culture tubes with 2 mL of Sabouraud dextrose broth medium (Difco, Becton, Dickinson and Company, USA). The tubes were incubated at 35 ºC for 10 days to determine fungal growth. The MFC was the minimal concentration that prevented fungal growth. All the experiments were conducted in triplicate [16].

Results

The probability of detecting antifungal activity in plants is higher when they have reported ethnopharmacological uses. This approach is useful in guiding the discovery of antifungal plants that are traditionally used for infections in which the pathological expression is evident, such as in superficial infections [17]. The increasing data regarding the *in vitro* efficacy of plants of genus *Pterocaulon* serve as an excellent example. The plants used in traditional medicine to treat mycoses demonstrated antifungal activity against a range of fungal pathogens [5,14,15]. In this study, the crude methanolic extracts of five species of *Pterocaulon* were screened for antifungal activity against 24 *Sporothrix schenckii* strains. Results showed (Table 1) that all fungi tested were inhibited by the different extracts (MIC values between 156 and 1250 µg/mL and MFC values between 312 and 5000 µg/mL). Among the tested extracts, the *P. polystachyum* extract presented the best results, with MIC ranging from 156 to 312 µg/mL and MFC ranging from 312 to 1250 µg/mL. The remaining extracts presents MIC in more than 75% of the tested isolates at a concentration of 625 µg/mL and an MFC of greater than or equal to 625 µg/mL for all evaluated isolates. The *P.*

cordobense extract proved to be the least active extract, with MIC ranging from 625 to 1250 µg/mL and MFC ranging from 1250 to 2500 µg/mL. The *P. lanatum* extract presented the highest MIC range between 156 and 1250 µg/mL, while the *P. balansae* extract presented the highest MFC range between 625 and 5000 µg/mL.

Table 1: Amount and percentage of isolated susceptible *S. schenckii* to extracts of *Pterocaulon*, based on the minimum inhibitory concentration and minimum fungicidal concentration.

Extracts		**Concentrations (µg/mL)**					
		156(%)	**312(%)**	**625(%)**	**1250(%)**	**2500(%)**	**5000(%)**
P. polystachyum	MIC	14 (58.3)	10 (41.7)	-	-	-	-
	MFC	-	1 (4.2)	10 (41.7)	13 (54.1)	-	-
P. lorentzii	MIC	-	1 (4.2)	20 (83.3)	3 (12.5)	-	-
	MFC	-	-	-	19 (79.2)	5 (20.8)	-
P. lanatum	MIC	1 (4.2)	-	22 (91.6)	1 (4.2)	-	-
	MFC	-	-	1 (4.2)	16 (66.7)	7 (29.1)	-
P. cordobense	MIC	-	-	19 (79.2)	5 (20.8)	-	-
	MFC	-	-	-	15 (62.5)	8 (33.3)	1 (4.2)
P. balansae	MIC	-	4 (16.6)	19 (79.2)	1 (4.2)	-	-
	MFC	-	-	1 (4.2)	17 (70.8)	8 (33.3)	1 (4.2)

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; —: 0 (0%).

However, 21% of *S. schenckii* isolates analyzed, which were inhibited by the five extracts, were not inhibited by the therapy standard that showed MIC greater than 8 µg/mL for itraconazole (Table 2). Moreover, it proved to be impossible to determine the MFC of 70% of these isolates due to the high concentration of drug.

Table 2: Amount and percentage of susceptible isolated of *S. schenckii* to the itraconazole, based on the minimum inhibitory concentration and minimum fungicidal concentration.

	Concentrations (µg/mL)						
	0.50	**1**	**2**	**4**	**8**	**16**	**>16**
MIC	9 (37.4)	8 (33.2)	1 (4.2)	1 (4.2)	1 (4.2)	2 (8.4)	2 (8.4)
MFC	-	-	-	1 (4.2)	1 (4.2)	5 (20.8)	17 (70.8)

MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; -: 0 (0%).

Discussion

The crude methanolic extracts are mixtures of multiple components, active and non-active compounds. Thus, the identified range of MIC must be interpreted in the appropriate context [18]. There is no validated criteria for the MIC end points for in vitro testing of plant extracts. However, Aligiannis et al. (2001) [1] proposed classification for plant materials based on MIC results as follows: strong inhibitors - MIC up to 500 µg/mL; moderate inhibitors - MIC between 600 and 1500 µg/mL and weak inhibitors — MIC above 1600 µg/mL. Based on the above classification, *P. polystachyum* showed strong activity against all tested microorganisms and the other extracts showed moderate inhibitory activity against the other tested microorganisms.

Interestingly, some strains of *S. schenckii* (21% of the isolates analyzed), which were inhibited by the five extracts, were not inhibited by the therapy standard that showed MIC greater than 8 µg/mL for itraconazole (Table 2), indicating a probable clinical resistance [8]. Moreover, all the extracts of *Pterocaulon* presented fungicidal activity in the concentrations analyzed, which could suggest a potential usefulness of this natural resource for the treatment of immunosupressed patients.

The observed antifungal activity could be due to the inhibition of the enzyme tyrosinase since this activity was recently reported in *P. alopecuroides* [2] this protein catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and the consequent oxidation of L-DOPA to dopaquinone [13]. The enzyme is widespread in many organisms and is involved in the formation of pigments such as melanins. Yeast cells can also produce melanin *in vitro* [10]. The reduction in the melanization process may be crucial for antifungal activity. *In vivo* the melanization of *S. schenckii* cells reduces their phagocytosis, which contributes to the development of infection [12].

Conclusion

P. polystachyum showed important antifungal activity against *Sporothrix schenckii*. These results are promising; it explains the use of these plants in folk medicine for the treatment of various diseases related to fungal infections. We have begun isolating and identifying the active compounds responsible for the antifungal effects from *Pterocaulon*.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

The authors are grateful to FAPERGS, CNPq, Propesq/UFRGS and CAPES for their financial support and to Dr. Nelson Matzembacker for the identification of the plant material.

References

[1] Aligiannis N, Kalpotzakis E, Mitaku S, et al. Composition and antimicrobial activity of the essential oils of two *Origanum* species. J Agric Food Chem 2001;49:4168-70.

[2] Chiari ME, Joray MB, Ruiz G, et al. Tyrosinase inhibitory activity of native plants from central Argentina: isolation of an active principle from *Lithrea molleoides*. Food Chem 2010;1:10-4.

[3] Clinical, Laboratory Standards Institute - CLSI. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, approved standard, . 2nd ed., Vilanova: CLSI; 2008.

[4] da Rosa AC, Scroferneker ML, Vettorato R, et al. Epidemiology of sporotrichosis: a study of 304 cases in Brazil. J Am Acad Dermatol 2005;52:451-9.

[5] Daboit TC, Stopiglia CDO, von Poser G, et al. Antifungal activity of *Pterocaulon alopecuroides* (*Asteraceae*) against chromoblastomycosis agents. Mycoses 2010;53:246-50.

[6] Dixon DM, Salkin IF, Duncan RA, et al. Isolation and characterization of *Sporothrix schenckii* from clinical and environmental sources associated with the largest U.S. epidemic of sporotrichosis. J Clin Microbiol 1991;29:1106-13.

[7] Duarte MCT, Figueira GM, Sartoratto A, et al. Anti-*Candida* activity of Brazilian medicinal plants. J Ethnopharmacol 2005;97:305-11.

[8] Espinel-Ingroff A, Diekema DJ, Fothergill A, et al. Wild-type MIC distributions and epidemiological cutoff values for the triazoles and six *Aspergillus* spp. for the CLSI broth microdilution method (M38-A2 document). J Clin Microbiol 2010;48:3251-7.

[9] Kontoyiannis DP, Lewis RE. Antifungal drug resistance in pathogenic fungi. Lancet 2002;359:1135-43.

[10] Morris-Jones R, Youngchim S, Gomez BL, et al. Synthesis of melanin-like pigments by *Sporothrix schenckii in vitro* and during mammalian infection. Infect Immun 2003;71:4026-33.

[11] Ródio C, Vianna DR, Kowalski KP, et al. *In vitro* evaluation of the amebicidal activity of *Pterocaulon polystachyum* (*Asteraceae*) against trophozoites of *Acanthamoeba castellanii*. Parasitol Res 2008;104:191-4.

[12] Romero-Martinez R, Wheeler M, Guerrero-Plata A, et al. Biosynthesis and functions of melanin in *Sporothrix schenckii*. Infect Immun 2000;68:3696-703.

[13] Schallreuter KU, Kothari S, Chavan B, et al. Regulation of melanogenesis-controversies and new concepts. Exp Dermatol 2008;17:395-404.

[14] Stein AC, Alvarez S, Avancini C, et al. Antifungal activity of some coumarins obtained from species of *Pterocaulon* (*Asteraceae*). J Ethnopharmacol 2006;107:95-8.

[15] Stein AC, Sortino M, Avancini C, et al. Ethnoveterinary medicine in the search for antimicrobial agents: antifungal activity of some species of *Pterocaulon* (*Asteraceae*). J Ethnopharmacol 2005;99:211-4.

[16] Stopiglia CD, Vieira FJ, Mondadori AG, et al. *In vitro* antifungal activity of dihydroxyacetone against causative agents of dermatomycosis. Mycopathologia 2011;171:267-71.

[17] Svetaz L, Zuljan F, Derita M, et al. Value of the ethnomedical information for the discovery of plants with antifungal properties. A survey among seven Latin American countries. J Ethnopharmacol 2010;127:137-58.

[18] Webster D, Taschereau P, Belland RJ, et al. Antifungal activity of medicinal plant extracts; preliminary screening studies. J Ethnopharmacol 2008;115:140-6.

5.5. Capítulo V

COMPARISON BETWEEN TWO CULTURE MEDIA FOR *IN VITRO* EVALUATION OF ANTIFUNGAL SUSCEPTIBILITY OF THE *SPOROTHRIX SCHENCKII* COMPLEX

Publicado no periódico:

Anais Brasileiros de Dermatologia, v. 87, p. 561–565, 2012.

Comparison between two culture media for *in vitro* evaluation of antifungal susceptibility of the *Sporothrix schenckii* complex

Comparação entre dois meios de cultura para avaliação *in vitro* da suscetibilidade a antifúngicos do complexo *Sporothrix schenckii*

Cheila Denise Ottonelli Stopiglia[1], Daiane Péres Marchese[2], Daiane Heidrich[3], Julia Medeiros Sorrentino[4], Fabiane Jamono Vieira[4], Maria Lúcia Scroferneker[5]

[1]Pharmacist-Biochemist – PhD Candidadate at the Post-Graduation Program in Medicine, Medical Sciences of the Universidade Federal do Rio Grande do Sul (UFRGS) - Porto Alegre (RS), Brazil.

[2]Pharmacist-Biochemist by the Universidade Federal do Rio Grande do Sul (UFRGS) - Porto Alegre (RS), Brazil.

[3]Pharmacist – Masters Candidate by the Post-Graduation Program in Medicine, Medical Sciences of the Universidade Federal do Rio Grande do Sul (UFRGS) - Porto Alegre (RS), Brazil.

[4]Student at the Pharmacy School of the Universidade Federal do Rio Grande do Sul (UFRGS) - Porto Alegre (RS), Brazil.

[5]Post-Doc - Associate Professor of the Microbiology, Immunology and Parasitology Department of the Universidade Federal do Rio Grande do Sul (UFRGS) - Porto Alegre (RS), Brazil.

** Work developed in the Human Pathogenic Fungi Laboratory of the Instituto de Ciências Básicas da Saúde of the Universidade Federal do Rio Grande do Sul - Porto Alegre (RS), Brazil.

Abstract

BACKGROUND: The standard methodology for determining the antifungal sensitivity against the *Sporothrix schenckii* complex recommends the use of the 1640 Roswell Park Memorial Institute culture medium (RPMI) buffered with morpholinepropanolsulfonic acid (MOPS). However, while this is a high-cost medium which requires a laborious implementation and sterilization by filtration, the Sabouraud dextrose broth is a low-cost medium, widely used in mycology, sterilized by autoclave. OBJECTIVE: To evaluate the performance of the Sabouraud dextrose broth culture medium as a substitute for the RPMI-MOPS in determining the antifungal sensitivity of *S. schenckii*. METHODS: Forty-eight clinical isolates were evaluated against five antifungal agents: itraconazole, ketoconazole, fluconazole, amphotericin B and terbinafine, using the method of broth microdilution advocated by the M38-A2 protocol of the Clinical and Laboratory Standards Institute. RESULTS: There were no significant differences between the Minimum Inhibitory Concentrations obtained in the two culture media for all the antifungals, with the exception of the amphotericin B. Regarding this drug, the Minimum Inhibitory Concentration range obtained were wider for the Sabouraud dextrose broth than for the Roswell Park Memorial Institute – morpholinepropanelsulfonic acid. CONCLUSIONS: The Sabouraud dextrose broth showed potential to be used in the *in vitro* evaluation of the *S. schenckii* complex antifungal activity.

Keywords: Antifungal agents; Microbial sensitivity tests; Mycoses; *Sporothrix*; Sporotrichosis

Resumo

FUNDAMENTOS: A metodologia padronizada para a determinação da sensibilidade aos antifúngicos frente ao complexo *Sporothrix schenckii* preconiza a utilização do meio de cultura *Roswell Park Memorial Institute* (RPMI) 1640 tamponado com ácido morfolinopropanosulfônico (MOPS). No entanto, este meio possui custo elevado, execução trabalhosa e esterilização por filtração. Já o caldo Sabouraud-dextrose é amplamente utilizado em micologia, de baixo custo e pode ser esterilizado por autoclavagem. OBJETIVO: Avaliar o desempenho do meio de cultura caldo Sabouraud-dextrose em substituição ao RPMI 1640-MOPS na determinação da sensibilidade de *S. schenckii* a antifúngicos. MÉTODO: Foram avaliados 48 isolados clínicos frente a cinco antifúngicos: itraconazol, cetoconazol, fluconazol, anfotericina B e terbinafina, utilizando a metodologia da microdiluição em caldo preconizada pelo protocolo M38-A2 do *Clinical and Laboratory Standards Institute*. RESULTADOS: Não houve diferenças significativas nas Concentrações Inibitórias Mínimas obtidas nos dois meios de cultura para todos os antifúngicos, com exceção da anfotericina B. Para este fármaco, foram obtidas faixas mais amplas de Concentrações Inibitórias Mínimas para caldo Sabouraud-dextrose do que para RPMI 1640-MOPS. CONCLUSÕES: O caldo Sabouraud-dextrose mostrou potencial para ser utilizado na avaliação *in vitro* da atividade antifúngica do complexo *S. schenckii*.

Palavras-chave: Antimicóticos; Esporotricose; Micoses; *Sporothrix*; Testes de sensibilidade microbiana

INTRODUCTION

Sporotrichosis is a subacute or chronic infection caused by dimorphic fungi of the genus *Sporothrix*. Nowadays, *Sporothrix schenckii* is considered as a complex of species composed of *S. brasiliensis*, *S. mexicana*, *S. globosa*, *S. schenckii stricto sensu* and *S. schenckii* var. *luriei*[1]. Sporotrichosis predominates in tropical and temperate zones and Brazil is one of main endemic areas[2]. An epidemic of sporotrichosis was reported in Rio de Janeiro in which cases of human infection were related to exposure to cats[3].

The most frequently observed clinical forms of sporotrichosis are the nodular-lymphangitic and the fixed ones, which usually affect immunecompetent individuals, unlike the disseminated cutaneous forms with or without visceral involvement, and the osteoarticular and the pulmonary forms, which are less frequent and affect patients with changes in their adaptive immunity mediated by cells such as in acquired immune deficiency syndrome (AIDS), lymphoma, cirrhosis, etc[4].

Systemic chemotherapy with potassium iodide or sodium iodide is the treatment of choice for the cutaneous lesions of sporotrichosis[5]. Amphotericin B has been used with different therapeutic results in the disseminated cutaneous, the recurrent lymphocutaneous and in the extracutaneous forms[4,5]. However, the frequency of intolerance to iodine and the high toxicity of amphotericin B may often represent limiting factors to their use. In the last few decades, there has been an increase in the use of azole derivates such as ketoconazole, itraconazole and fluconazole as a therapeutic alternative to the classic schemes, and itraconazole came as the most effective of them[6]. On the other hand, by virtue of its excellent *in vitro* and *in vivo* activity, terbinafine has been used in various fungal infections[7,8,9].

With the increase in the incidence of systemic fungal infections and with the growing number of antifungal agents, the interest in laboratory methods to guide the selection of antifungal therapy has also increased. In 2002, the M38-A norm of the Clinical and Laboratory Standards Institute (CLSI), a reference method for *in vitro* tests of broth microdilution for determination of the antifungal sensitivity of filamentous fungi, including *S. schenckii*[10].

This methodology recommends the use of the 1640 Roswell Park Memorial Institute (RPMI) culture medium buffered with morpholinepropanesulfonic acid (MOPS). However, while this is a high-cost medium which requires a laborious implementation, the use of buffer and sterilization by filtration, the Sabouraud dextrose broth is a low-cost medium, widely used in mycology, sterilized by autoclave. In addition, the RPMI broth has been described as an obstacle for the detection of isolates resistant to amphotericin B[11].

From the evidence above, the objective of this study was to evaluate the performance of the Sabouraud dextrose broth culture medium as a substitute for the 1640-RPMI culture medium buffered with MOPS to evaluation of the in vitro antifungal susceptibility of *Sporothrix schenckii* complex against the antifungals, with the purpose of cost reduction of the in vitro test, so that helpfully it can be widely used in clinical practices.

MATERIALS AND METHODS

Microorganisms

Forty-eight clinical isolates of *Sporothrix schenckii* complex were used, namely 13 *S. brasiliensis*, 22 *S. schenckii* and 13 *S. mexicana*.

Antifungal activity *in vitro*

The antifungal sensitivity test was developed according to the technique of broth microdilution advocated by the M38-A2 protocol of the Clinical and Laboratory Standards Institute[12]. Five antifungal drugs commercially available for the treatment of sporotrichosis were used: ketoconazole (Pharmaceutical Chemistry, Bayer, Barcelona, Spain), fluconazole (Sigma, St. Louis, MO, USA), itraconazole (Janssen-Cilag, São Paulo, Brazil), terbinafine (Novartis Research Institute, Vienna, Austria) and amphotericin B (Sigma-Aldrich Co.St . Louis, USA).

The culture media used were the Sabouraud dextrose broth (Difco Laboratories, USA) and the 1640-RPMI (Gibco-BRL, USA) buffered at pH 7.0 with 165mM of morpholinepropanesulfonic acid – MOPS (Sigma-Aldrich, Spain).

A stock solution of the antifungal agents was prepared in dimethyl sulfoxide (DMSO; Vetec, Brazil) and, subsequently, dilutions in both culture media were held to obtain final concentrations ranges of 0.25 to 128μg/ml for fluconazole, of 0.03 to 16μg/ml for itraconazole, ketoconazole, and amphotericin B and of 0.01 to 8 μg/ml for terbinafine.

The clinical isolates were cultured in potato dextrose agar (Biobrás S.A., Brazil) at 35°C for 7 days. After the growth, the spores were suspended in a 0.85% sterile saline solution, obtaining optical density from 0.09 to 0.13, standardized in the spectrophotometer at 530 nm. The suspension was diluted 1:50 on each of the culture media.

The test was carried out on sterile plates with 96 U-wells, where 100 μl of each concentration of the antifungal agent to be tested were added. Subsequently, aliquots of 100 μl of the inoculums at 1:50 dilution were inoculated into each of the wells. The final concentration of microorganisms reached 10^4 CFU/ml.

A free control of antifungal drugs (growth control) and a free control of microorganism (sterility control) were included in those tests. The plates were incubated at 35 °C, for 46-50 hours. The determination of the minimum inhibitory concentration (MIC) was performed visually by comparison with the growth of the drug free control. The MIC was defined as the lowest concentration of the drug able to inhibit the fungal growth completely for itraconazole and amphotericin B, 80% of the fungal growth for terbinafine and 50% of the growth for fluconazole and ketoconazole[12]. All analyzes were performed in triplicate.

The statistical analysis was assessed by the t Test from the SPSS program for Windows, version 17.0.

RESULTS

The resulting values of the in vitro susceptibility for the 48 isolates of *S. schenckii* complex are shown in table 1. The statistical analysis showed that the MICs obtained for amphotericin B were statistically different for the two utilized culture media ($p<0.05$). However, for the other antifungal agents, there were no significant differences between the MICs obtained in the two culture media.

Terbinafine was the most active of the drugs, followed by ketoconazole, itraconazole, amphotericin B and fluconazole.

Table 1: Comparison of the susceptibility *in vitro* of 48 isolates of the *S. schenckii* complex against five antifungal agents using two culture media.

	Itraconazole		Ketoconazole		Fluconazole		Terbinafine		Amphotericin B	
	SDB*	**RPMI†**	**SDB***	**RPMI†**	**SDB***	**RPMI†**	**SDB***	**RPMI†**	**SDB***	**RPMI†**
S. brasiliensis (n=13)										
Range	0.03-2.00	0.06-2.00	0.06-0.5	0.12-0.50	32->128	32->128	0.01-0.25	0.01-0.25	0.50-8.00	0.50-2.00
GM**	0.28	0.33	0.19	0.22	71.20	71.20	0.07	0.07	1.79	1.00
S. schenckii (n=22)										
Range	0.06-2.00	0.06-2.00	0.03-1.00	0.03-1.00	32->128	32->128	0.01-0.50	0.01-0.50	0.25-16.0	0.50-2.00
GM**	0.32	0.32	0.18	0.17	60.09	62.01	0.06	0.07	2.41	1.24
S. mexicana (n=13)										
Range	0.03-0.50	0.03-0.50	0.03-1.00	0.03-1.00	32->128	32->128	0.01-0.50	0.01-0.50	0.01-8.00	0.50-4.00
GM**	0.15	0.17	0.08	0.09	67.50	71.20	0.05	0.06	1.44	1.45
All strains (n=48)										
Range	0.03-2.00	0.03-2.00	0.03-1.00	0.03-1.00	32->128	32->128	0.01-0.50	0.01-0.50	0.01-16	0.50-4.00
GM**	0.25	0.27	0.15	0.15	64.93	66.83	0.06	0.07	1.94	1.22

*SDB: Sabouraud-dextrose broth; †RPMI: Roswell Park Memorial Institute broth buffered with 165mM of morpholinepropanesulfonic acid (MOPS).
**Geometric mean

DISCUSSION

The increase in the incidence of opportunistic fungal infections, as well as the use of invasive surgical procedures, and even the appearance of new diseases and immunosuppressive drugs, have aroused interest in the search of new alternatives to drugs for the treatment of these infections and, also, alternatives to methodologies for the analysis of the antifungal sensitivity[13].

Methods for the analysis of antifungal activity amongst laboratories, in order to determine the sensitivity of the antifungal agents, were recommended. The Clinical and Laboratory Standards Institute standardized the method of broth microdilution with the aim of analyzing the antifungal susceptibility of filamentous fungi in a consensual way to facilitate the interpretation of the results

by the laboratories. In addition, this methodology provides a basic pattern from which other methods may be developed[10].

The effect of the culture media upon the antifungal activity *in vitro* for filamentous fungi has shown contradictory results[14-16]. For example, using the method of microdilution to evaluate caspofungin against the species of *Aspergillus*, *Fusarium*, Arikan *et al.* (2000) obtained lower MICs with the Antibiotic Medium 3 (AM3) culture medium than with the RPMI[14]. In the same way, Llop *et al.* (1999) observed that the five culture media evaluated had influenced the MIC values of amphotericin B, ketoconazole and flucytosine for dematiaceos fungi[17]. However, Tortorano *et al.* (2000) and Manavathu *et al.* (2000), did not observe differences between AM3 and RPMI when comparing them to evaluate amphotericin B and itraconazole against *Aspergillus fumigatus*[18,19]. The only exception was for amphotericin B in peptone-yeast extract, which gave higher MICs than those obtained with RPMI and AM3. Fernández-Torres *et al.* (2006), demonstrated that the differences in MICs are influenced by the culture medium used and the microorganism analyzed[20].

In addition, the RPMI broth has been described as an obstacle for the detection of isolates resistant to amphotericin B[11]. However, failures to therapies with amphotericin B continue to be reported, with isolates showing, mostly, *in vitro* sensitivity to this drug[21,22]. The inadequacy of RPMI determined that in the document M27-A2 the CLSI suggested the evaluation of susceptibility using other means of culture such as the AM3[23].

Boff *et al.* (2008) showed that the AM3 media allowed better differentiation of susceptibility among groups of *Candida*[11]. The capacity of this media to generate wide ranges of MICs by the RPMI broth has been observed, but as it is a complex medium, without a completely known chemical composition, there are problems of reproducibility of the results when the same samples are tested with different batches of AM3[20].

Our results show that the maximum and minimum values, as well as the geometric mean (GM) of MICs were equal to or very similar between the two culture media for all the antifungal agents evaluated, with the exception of amphotericin B. For this, the range of MICs obtained for the Sabouraud dextrose broth (0.01 - 16 μg/ml) was wider than for the RPMI-MOPS (0.50 - 4 μg/ml), and the GM for the Sabouraud dextrose broth was superior to the GM for the RPMI-MOPS.

The study conducted by Espinel-Ingroff (1997) suggests that the 1640 RPMI medium does not offer the necessary conditions for the proper growth of *C. neoformans*, thus making the detection of resistance to amphotericin B difficult[24].

Throughout this work, terbinafine showed high activity against all isolates tested, which is in agreement with other studies[1,25]. However, the therapeutic potential of terbinafine was confirmed only for the cutaneous and lymphocutaneous sporotrichosis[9,26]. On the other hand, this drug did not demonstrate efficacy in the treatment of systemic sporotrichosis in murine model[27]. Studies *in vivo* are necessary to confirm this activity.

Ketoconazole was the second more active drug. However, the detection of *in vitro* susceptibility to this antifungal agent should be interpreted with caution because it does not often correlate with the therapeutic activity, mainly for the extracutaneous forms[28].

Despite the absence of comparative clinical studies (randomized, double-blind), itraconazole is considered the first choice in the treatment of cutaneous, lymphocutaneous and osteoarticular sporotrichosis[28]. Our results showed a good *in vitro* performance of this drug, which can be correlated with the therapeutic success of the treatment obtained in 94.6% of the cases of lymphocutaneous and fixed sporotrichosis reported in another study[29].

For fluconazole, the values are compatible with the results from other studies[1,9]. In spite of its low sensitivity profile, fluconazole is considered a possible therapeutic option in cases of lymphocutaneous or cutaneous sporotrichosis, when high doses should be used[30].

CONCLUSION

The results obtained indicate that the Sabouraud dextrose broth has the potential to be used in the assessment of the antifungal activity of the *Sporothrix schenckii* complex using the CLSI method of broth microdilution, since the results were very similar to the RPMI-MOPS results for all tested antifungal agents, with the exception of amphotericin B. For this drug, the MICs range obtained was wider for the Sabouraud dextrose broth than for the RPMI-MOPS. However, *in vivo* studies are necessary to assess the clinical correlation between these results, as well as the reproducibility with different batches of culture media.

Conflict of Interests: None.

Financial Support: The authors thank the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and the *Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul* (FAPERGS) for their financial support.

Mailing address: Cheila Denise Ottonelli Stopiglia - Rua Sarmento Leite, 500 - Laboratório 210 – Centro – CEP 90050-170 Porto Alegre, RS, Brazil. E-mail: cheila.dos@gmail.com/scrofern@ufrgs.br

REFERENCES

1. Marimon R, Serena C, Gené J, Cano J, Guarro J. *In vitro* antifungal susceptibilities of five species of *Sporothrix*. Antimicrob Agents Chemother. 2008;52:732-4.

2. Sidrim JJC, Rocha MFG. Esporotricose. In: Sidrim JJC, Rocha MFG, editores. Micologia médica à luz de autores contemporâneos. Rio de Janeiro: Guanabara Koogan; 2004. p.177-88.

3. Reis RS, Almeida-Paes R, Muniz MM, Tavares PMS; Monteiro PCF, Schubach TMP, et al. Molecular characterisation of *Sporothrix schenckii* isolates from humans and cats involved in the sporotrichosis epidemic in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2009;104:769-74.

4. Arechavala A, Orduna T, Maiolo E, Mujica MT, Fernanadez M, Negroni R. Esporotricosis diseminada, con compromiso cutáneo y visceral. Rev Patol Trop. 2011;40:73-84.

5. Coskun B, Saral Y, Akpolat N, Ataseven A, Cicek D. Sporotrichosis sucessfully treated with terbinafine and potassio iodide: case report and review of the literature. Mycopathologia. 2004;158:53-6.

6. Kohler LM, Hamdan JS, Ferrari TC. Successful treatment of a disseminated *Sporothrix schenckii* infection and *in vitro* analysis for antifungal susceptibility testing. Diagn Microbiol Infect Dis. 2007;58:117-20.

7. Meinerz ARM, Nascente PS, Schuch LFD, Cleff MB, Santin R, Brum CS, et al. Suscetibilidade *in vitro* de isolados de *Sporothrix schenckii* frente à terbinafina e itraconazol. Rev Soc Bras Med Trop. 2007;40:60-2.

8. Kohler LM, Soares BM, de Assis Santos D, Da Silva Barros ME, Hamdan JS. *In vitro* susceptibilities of isolates of *Sporothrix schenckii* to amphotericin B, itraconazole, and terbinafine: comparison of yeast and mycelial forms. Can J Microbiol. 2006;52:843-7.

9. Heidrich D, Stopiglia CDO, Senter L, Vetoratto G, Valente P, Scroferneker ML. Sucesso terapêutico da terbinafina em um caso de esporotricose. An Bras Dermatol. 2011;86:S182-5.

10. Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Moulds; Approved standard. CLSI document M38-A2. Wayne (PA): Clinical and Laboratory Standards Institute; 2002.

11. Boff E, Lopes PGM, Spader T, Scheid LA, Loreto E, Dal Forno NF, et al. Reavaliação da suscetibilidade de *Candida* à anfotericina B: estudo comparativo com isolados de três hospitais do Estado do Rio Grande do Sul. Rev Soc Bras Med Trop. 2008;41:36-40.

12. Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi, Approved Standard – Second Edition. Vilanova (PA): Clinical and Laboratory Standards Institute; 2008.

13. Lopes-Bezerra LM, Schubach A, Costa RO. *Sporothrix schenckii* and sporotrichosis. An Acad Bras Cienc. 2006;78:293-308.

14. Arikan S, Lozano-Chiu M, Paetznick V, Nangia S, Rex JH. Microdilution susceptibility testing of amphotericin B, itraconazole, and voriconazole against clinical isolates of *Aspergillus* and *Fusarium* species. J Clin Microbiol. 1999;37:3946-51.

15. Meletiadis J, Meis JFGM, Mouton JW, Verweij PE. Analysis of growth characteristics of filamentous fungi in different nutrient media. J Clin Microbiol. 2001; 39:478-84.

16. Pfaller MA, Messer SA, Karlsson A, Bolmström A. Evaluation of the Etest method for determining fluconazole susceptibilities of 402 clinical yeast isolates by using three different agar media. J Clin Microbiol. 1998;36:2586-9.

17. Llop C, Sala J, Riba MD, Guarro J. Antimicrobial susceptibility testing of dematiaceous filamentous fungi: effect of medium composition at different temperatures and times of reading. Mycopathologia. 1999;148:25-31.

18. Tortorano AM, Dannaoui E, Cogliati M, Piens MA, Rigoni AL, Grillot R, et al. Evaluation of different *in vitro* procedures for testing amphotericin B and itraconazole susceptibility of *Aspergillus fumigatus*. J Mycol Med. 2000;10:123-7.

19. Manavathu EK, Nune U, Kanuri K, Chandrasekar PH, Abraham OC. Effect of test medium on *in vitro* susceptibility testing results for *Aspergillus fumigatus*. Rev Iberoam Micol. 2000;17:107-10.

20. Fernández-Torres B, Carrillo-Muñoz A, Inza I, Guarro J. Effect of culture medium on the disk diffusion method for determining antifungal susceptibilities of dermatophytes. Antimicrob Agents Chemother. 2006;50:2222-4.

21. Hospenthal DR, Murray CK, Rinaldi MG. The role of antifungal susceptibility testing in the therapy of candidiasis. Diagn Microbiol Infect Dis. 2004;48:1153-60.

22. Nguyen MH, Clancy CJ, Yu VL, Yu YV, Morris AJ, Snydman DR, et al. Do *in vitro* susceptibility data predict the microbiologic response o amphotericin B? Results of a prospective study of patients with *Candida* fungemia. J Infect Dis. 1998;177:425-30.

23. Clinical and Laboratory Standards Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard. CLSI document M27-A2. Wayne (PA): Clinical and Laboratory Standards Institute; 2002.

24. Espinel-Ingroff A. Clinical relevance of antifungal resistance. Inf Dis Clin North Am.1997;11:929-44.

25. Silveira CP, Torres-Rodríguez JM, Alvarado-Ramírez E, Murciano-Gonzalo F, Dolande M, Panizo M, et al. MICs and minimum fungicidal concentrations of amphotericin B, itraconazole, posaconazole and terbinafine in *Sporothrix schenckii*. J Med Microbiol. 2009;58:1607-10.

26. Hay RJ. Therapeutic potential of terbinafine in subcutaneous and systemic mycoses. Br J Dermatol. 1999;141Suppl 56:36-40.

27. Kan VL, Bennett JE. Efficacies of four antifungal agents in experimental murine sporotrichosis. Antimicrob Agents Chemother. 1988;32:1619-23.

28. Bustamante B, Campos PE. Sporotrichosis: a forgotten disease in the drug research agenda. Expert Rev Anti Infect Ther. 2004;2:85-94.

29. de Lima Barros MB, Schubach AO, de Vasconcellos Carvalhaes de Oliveira R, Martins EB, Teixeira JL, Wanke B. Treatment of cutaneous sporotrichosis with itraconazole-study of 645 patients. Clin Infect Dis. 2011;52:200-6.

30. Kauffman CA, Bustamante B, Chapman SW, Pappas PG; Infectious Diseases Society of America. Clinical practice guidelines for the management of sporotrichosis: 2007 update by the Infectious Diseases Society of America. Clin Infect Dis. 2007;45:1255-65.

5.6. Capítulo VI

EVALUATION OF THE ORIGIN OF A SAMPLE OF *SPOROTHRIX SCHENCKII* THAT CAUSED CONTAMINATION OF A RESEARCHER IN SOUTHERN BRAZIL

Publicado no periódico:

Mycopathologia, v. 171, p. 203–207, 2011.

Evaluation of the origin of a sample of *Sporothrix schenckii* that caused contamination of a researcher in Southern Brazil

Melissa Fontes Landell[a,b], Cheila Denise Ottonelli Stopiglia[a,c], Raisa G. Billodre[a], Daiane Heidrich[a], Julia Medeiros Sorrentino[a], Marilene H. Vainstein[a,b], Maria Lúcia Scroferneker[a,c], Patricia Valente[a,b.]

[a]Departamento de Microbiologia, ICBS, Universidade Federal do Rio Grande do Sul , Porto Alegre – RS, Brazil.

[b]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul , Porto Alegre – RS , Brazil.

[c]Programa de Pós-Graduação em Medicina: Ciências Médicas, Universidade Federal do Rio Grande do Sul, Porto Alegre – RS, Brazil.

[*] Correspondence to: Patricia Valente, Departamento de Microbiologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 sala 158, Cep: 90.050-170, Porto Alegre, RS, Brasil. Tel. (+55) 51-33084196 Fax. (+55) 51-33083445 (e-mail: patricia.valente@ufrgs.br)

Abstract

We report a case of a researcher from a laboratory of Mycology in Rio Grande do Sul, Brazil that presented a clinical evidence of sporotrichosis. The researcher had an accident while manipulating the microculture slides of chromoblastomycosis agents and presented a clinical evidence of sporotrichosis. As the laboratory has some cultures of *Sporothrix schenckii*, it was suggested that it might be a laboratory contamination. In order to test this hypothesis, the genotypic characterization of the samples was performed by means of the random amplified polymorphic DNA (RAPD) analysis method. In addition, we evaluated the *in vitro* antifungal activity of four antifungal agents against the isolated fungus. The sample obtained from the researcher was not genetically similar to any of the samples kept in the laboratory and showed the minimum inhibitory concentrations of 0.5 μg/mL for itraconazole and ketoconazole, > 64μg/mL for fluconazole and 0.125 μg/mL for terbinafine. It is suggested that the contamination had an environmental origin.

Keywords: *Sporothrix schenckii*; RAPD fingerprinting; sporotrichosis; environmental contamination.

1. Introduction

Sporotrichosis in human beings and animals is caused by the cosmopolitan fungus *Sporothrix schenckii*. It is worldwide in distribution with focal areas of hyperendemicity in tropical and subtropical countries. So far, ecologic factors that have been associated with *S. schenckii* development and survival are mean temperature between 26°C and 28°C and presence of organic material such as decaying vegetation, algae, grass, sphagnum moss, and hay [1-5]. For this reason, sporotrichosis is considered to be acquired during outdoor leisure or occupational activities (e.g., farming, landscaping and gardening) that promote frequent and traumatic contact with plant material or soil, because *Sporothrix schenckii* gets inoculated in the skin even after minor injuries from a thorn or a wooden splinter [6-10]. Also, the transmission of feline sporotrichosis has been widely reported [11-13]. The most frequent clinical presentations are the cutaneous and subcutaneous forms with or without regional lymphatic involvement [3,14].

We report a case that happened in 2007, in which a researcher from a laboratory of Mycology in Rio Grande do Sul, Brazil, presented a clinical evidence of sporotrichosis. The researcher was manipulating the microculture slides of chromoblastomycosis agents in order to visualize them at the microscope when one of the slides slipped under his nail and caused a wound. This wound was immediately washed with soap, followed by an acetic acid solution (10%) and a solution of timerosal (0.1%). Before going home, the researcher went to a market, bought some vegetables and washed them out. After 21 days, an infection appeared in the wound and the researcher went to a reference center in order to be evaluated. Material from the infected wound was sampled for the isolation of the microorganism, according to routine methods (direct observation and cultivation in Mycosel and Sabouraud agar). The direct observation was negative, but a *S. schenckii* isolate was recovered in solid media. The researcher was treated with oral

potassium iodide for 3 months, and the lymph node swelling and finger nodule resolved after this treatment.

As the laboratory has some cultures of *S. schenckii*, it was suggested that the hypothesis of a laboratory contamination should be tested. Several molecular techniques have proved to be useful for identifying, typing and grouping *S. schenckii* [15-19]. The random amplified polymorphic DNA (RAPD) analysis is widely used for genotypic characterization of pathogenic fungi, including *S. schenckii*, as it detects subtle genotypic changes among close groups of isolates [20-23]. Hence, this study was aimed at determining the RAPD genotype of the *S. schenckii* strain isolated from the researcher and comparing it to the genotypes of other isolates from the laboratory. We also evaluated the sensitivity profile of the sample isolated from the researcher to the antifungal agents fluconazole, itraconazole, ketoconazole and terbinafine.

2. Materials and methods

A total of 15 clinical and environmental isolates of *S. schenckii* were studied, including the one obtained from the researcher (MLS) (Table 1). DNA was extracted and purified according to Ramos et al. [24]. The RAPD assay was performed according to Mesa-Arango et al. [23] with 100 pmol of each *primer* (*primers* OPG-10, OPG-14, and OPG-19 Prodimol Biotecnologia S.A., Brazil). Optimal amplification conditions for *S. schenckii* RAPD analyses were as follows: one initial cycle at 94ºC for 1 min, 45 cycles at 94°C for 1 min, 35°C for 1 min, 72°C for 1 min and a final extension cycle at 72ºC for 7 min. The polymorphic amplified patterns revealed by RAPD were examined by electrophoresis on a 2% agarose gel at 60 V for 4h and stained with gel red for visualization under UV light. Digital images of agarose gels were acquired with the GelDoc XR

System Software (Bio-Rad). RAPD assays were repeated independently three times with each *primer* with all the 15 isolates studied, with consistent gel patterns detected among the assays. Relationships among isolates were examined by analyzing the RAPD fingerprints of each isolate with BioNumerics software (Applied Maths, Kortrijk, Belgium). Tagged image files were first converted and normalized in relation to the external reference standards on the same gel, so as to allow the subsequent merging of data from different gels. The software was then used with the Pearson coefficient to calculate levels of similarity between fingerprints. Cluster analysis was performed by the unweighted pair group method with arithmetical averages (UPGMA).

Table 1: Origin of samples of *S. schenckii.*

Strains	Origin
450, 478, 576, 579, 611, 794, 237, 805, 339, 329, 853, 432	Micological Collection of School of Medicine of Universidade de São Paulo, Brazil
ATCC 201679	American Type Culture Collection (ATCC)
Santa Casa II 44107, MLS	Micological Service of Complexo Hospitalar Santa Casa de Porto Alegre, Brazil

Antifungal susceptibility assays were performed by the broth microdilution method according to guidelines recommended by Clinical and Laboratory Standards Institute (CLSI) for filamentous fungi (M38-A) [25]. RPMI 1,640 medium (Sigma, St. Louis, MO, USA) with L-glutamine, without sodium bicarbonate, buffered with 165 mM morpholinepropanesulfonic acid (MOPS; Sigma, St. Louis, MO, USA), pH 7.0 was used as a test medium. The final concentration ranges of the antifungal agents were 0.0312 to 16 µg/mL for itraconazole (DEG, Brazil) and ketoconazole (Galena Química e Farmacêutica, Brazil); 0.125 to 64 µg/mL for fluconazole (Pfizer, EUA), and 0.0156 to 8 µg/mL for terbinafine (Galena Química e Farmacêutica, Brazil).

Strains were cultivated onto potato dextrose agar (PDA/DIFCO, Becton, Dickinson and Company, USA) at 25ºC for 7 days. The surface was gently scraped with a sterile bent glass after flooding with sterile saline solution. The standard suspensions were adjusted by UV-visible spectrophotometry (Spectrum Instruments Co., Shangai, China) in order to show transmittance at 530 nm of 80-82%. Adjusted suspensions were diluted in RPMI (1:50) to obtain a final inoculum of 10^4 CFU/mL, and 100µl of the fungal suspensions were added to each microdilution well containing 100µl of the drugs (2X). Plates were incubated at 35ºC for 46-50 hours, and the minimum inhibitory concentration (MIC) was determined visually by comparison with the drug-free growth control well. The MIC was defined as the lowest concentration of the antifungal agent preventing 100% visible fungal growth for terbinafine and itraconazole and 50% of growth inhibition for the ketoconazole and fluconazole. All assays were performed in triplicate. The control strains *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were tested in parallel.

3. Results and discussion

As demonstrated in Fig. 1, the sample obtained from the researcher was not genetically similar to any of the *S. schenckii* samples kept in the laboratory. The *primers* OPG-10 and OPG-14 were not able to genotypically differentiate the isolates (data not shown). As it was not a contamination with a laboratory strain, it is suggested that the researcher has been contaminated by an environmental sample of *S. schenckii*. A probable source of this strain is the vegetable the researcher manipulated after the accident, although there is no way to prove this hypothesis. The *S. schenckii* sample may have used the previous injure acquired by the researcher at the laboratory as an entry for initiating the disease.

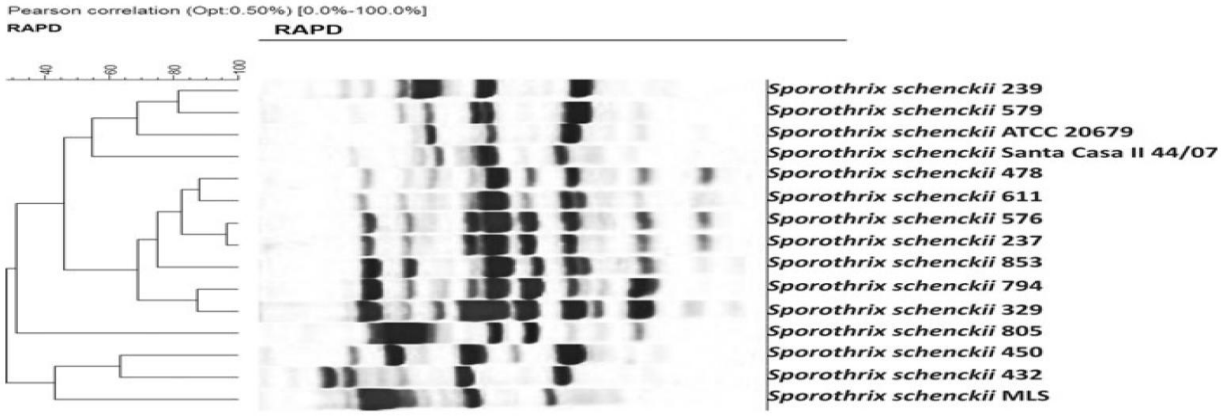

Figure 1: Dendrogram obtained after RAPD-PCR analysis of 15 isolates of *S. schenckii* with *primer* OPG-19 were grouped by means of the Pearson product moment correlation coefficient and UPGMA cluster analysis with Bionumerics software.

The researcher´s sample had the minimum inhibitory concentrations of 0.5 μg/mL for itraconazole and ketoconazole, 0.125 μg/mL for terbinafine and > 64μg/mL for fluconazole, characterizing that the strain is resistant to fluconazole. The *in vitro* resistance to fluconazole in clinical isolates of *S. schenckii* and other *Sporothrix* species has been reported by other authors [26-32], suggesting an intrinsic resistance of the species to this drug. This is an interesting topic for further study, because fluconazole is considered the second-line treatment for sporotrichosis [33]. In spite of the poor activity that this drug has shown in this and other studies, its clinical efficacy has been estimated to be 71% for cases of lymphocutaneous infection [34].

Laboratory contamination by *S. schenckii* has already been reported in the literature [35,36], but the laboratory personnel were mostly infected by laboratory strains or by environmental strains manipulated in the laboratory. This case illustrates that laboratory personnel are also at risk of acquiring sporotrichosis from environmental origin, with the fungus taking advantage of an already established trauma to cause infection. This differs from most case reports that describe the

inoculation of the fungus concomitantly to the trauma event. A result similar to ours was described by Mehta et al. [10], who isolated *S. schenckii* from soil and corn stalks in the vicinity of patients with cutaneous sporotrichosis and suggested that contamination of a previous wound with infected soil/material is probably more important for the clinical development of the disease than inoculation by trauma.

There are several risk factors associated with sporotrichosis, such as contact with animals, especially cats, jobs or recreational activities in close contact with soil or crop fields, living in houses made of raw woods and conditions associated with a lower socioeconomic status [37,38]. Occupational hazard due to *S. schenckii* manipulation in laboratories should be added to this list, thus care must be taken at all laboratory procedures, not only by using biological safety cabinets and appropriate EPIs, but also by careful manipulation of the glassware, including microscope slides. This glassware may act opening an entry by which pathogenic fungi can be inoculated.

Acknowledgements

M.F. Landell and C.D.O. Stopiglia received fellowships from *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES).

4. References

1. Rippon JW. Sporotrichosis. In: Rippon JW, editor. Medical Mycology: the pathogenic fungi and the pathogenic actinomycetes. 3rd ed. Philadelphia: WB Saunders; 1988. p. 325–52.
2. Pappas PG, Tellez I, Deep AE, Nolasco D, Holgado W, Bustamante B. Sporotrichosis in Peru: description of an area of hyperendemicity. Clin Infect Dis. 2000;30:65–70.
3. Bustamante B, Campos PE. Endemic sporotrichosis. Curr Opin Infect Dis. 2001;14:145–9.
4. Sampaio SAP, Rivitti EA. Dermatologia, 2nd ed. São Paulo: Artes Médicas; 2001. pp. 548–52.
5. Carvalho MT, de Castro AP, Baby C, Werner B, Filus Neto J, Queiroz-Telles F. Disseminated cutaneous sporotrichosis in a patient with AIDS: report of a case. Rev Soc Bras Med Trop. 2002;35:655–9.
6. Winn RE. A contemporary view of sporotrichosis. Curr Top Med Mycol. 1995;6:73–9.
7. Dooley DP, Bostic PS, Beckinus ML. Spook house sporotrichosis: a point source outbreak of sporotrichosis associated with hay bale props in a Halloween haunted house. Arch Intern Med. 1997;157:1885–7.
8. Barros MBL, Schubach TMP, Gutierrez-Galhardo MC, Monteiro PCF, Reis RS, Zancopé-Oliveira RM, Lazéra MS, Cuzzi-Maya TC, Blanco TM, Marzochi KBF, Wanke B, Valle ACF. Sporotrichosis: an emergent zoonosis in Rio de Janeiro. Mem Inst Oswaldo Cruz. 2001;96:777–9.
9. Da Rosa AC, Scroferneker ML, Vettorato R, Gervini RL, Vettorato G, Weber A. Epidemiology of sporotrichosis: a study of 304 cases in Brazil. J Am Acad Dermatol. 2005;52:451–9.
10. Mehta KI, Sharma NL, Kanga AK, Mahajan VK, Ranjan N. Isolation of *Sporothrix schenckii* from the environmental sources of cutaneous sporotrichosis patients in Himachal Pradesh, India: results of a pilot study. Mycoses. 2007;50:496–501.
11. Schubach TM, de Oliveira Schubach A, dos Reis RS, Cuzzi-Maya T, Blanco TC, Monteiro DF, Barros BM, Brustein R, Zancopé-Oliveira RM, Fialho Monteiro PC, Wanke B. *Sporothrix schenckii* isolated from domestic cats with and without sporotrichosis in Rio de Janeiro, Brazil. Mycopathologia. 2002;153:83–6.

12. Schubach TM, Schubach A, Okamoto T, Barros MB, Figueiredo FB, Cuzzi T, Fialho-Monteiro PC, Reis RS, Perez MA, Wanke B. Evaluation of an epidemic of sporotrichosis in cats: 347 cases (1998–2001). J Am Vet Med Assoc. 2004;224:1623–9.

13. Kovarik CL, Neyra E, Bustamante B. Evaluation of cats as the source of endemic sporotrichosis in Peru. Med Mycol. 2008;46:53–6.

14. Ramos e Silva M, Vasconcelos C, Carneiro S, Cestari T. Sporotrichosis. Clin Dermatol. 2007;25:181–7.

15. Ishizaki H, Kawasaki M, Aoki M, Wu S, Lin J, Kim J, Won Y-H, Calvo CR. Mitochondrial DNA Analysis of *Sporothrix schenckii* from China, Korea and Spain. Jpn J Med Mycol. 2004;45:23–5.

16. Marimon R, Gené J, Cano J, Trilles L, Lazéra MS, Guarro J. Molecular phylogeny of *Sporothrix schenckii*. J Clin Microbiol. 2006;44:3251–6.

17. O'Reilly LC, Altman SA. Macrorestriction analysis of clinical and environmental isolates of *Sporothrix schenckii*. J Clin Microbiol. 2006;44:2547–52.

18. Arenas R, Miller D, Campos-Macias P. Epidemiological data and molecular characterization (mtDNA) of *Sporothrix schenckii* in 13 cases from Mexico. Int J Dermatol. 2007;46:177–9.

19. Reis RS, Almeida-Paes R, Muniz MM. Tavares PMS; Monteiro PCF; Schubach TMP; Gutierrez-Galhardo MC; Zancopé-Oliveira RM. Molecular characterisation of *Sporothrix schenckii* isolates from humans and cats involved in the sporotrichosis epidemic in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2009;104:769–74.

20. Boekhout T, Belkum A. Variability of karyotypes and RAPD types in genetically related isolates of *Cryptococcus neoformans*. Curr Genet. 1997;32:203–8.

21. Melo AS, Almeida LP, Colombo AL, Briones MR. Evolutionary distances and identification of *Candida* species in clinical isolates by randomly amplified polymorphic DNA (RAPD). Mycopathologia. 1998;142:57–66.

22. Caligiorne RB, Resende MA, Paiva E, Azevedo V. Use of RAPD (random amplified polymorphic DNA) to analyse genetic diversity of dematiaceous fungal pathogens. Can J Microbiol. 1999;45:408–12.

23. Mesa-Arango AC, Reyes-Montes MR, Peres-María A, Navarro-Barranco H, Souza V, Zúñiga G, Toriello C. Phenotyping and genotyping of *Sporothrix schenckii* isolates according to geographic origin and clinical form of sporotrichosis. J Clin Microbiol. 2002;40:3004–11.

24. Ramos JP, Valente P, de Souza RA, Rosa CA, Leoncini O. Heteroduplex mobility assay of the D1/D2 region of the 26s rDNA for differentiation of *Saccharomyces* species. Lett Appl Microbiol. 2001;33:206–10.

25. National Committee for Clinical Laboratory Standards (NCCLS). Reference method for Broth Dilution Antifungal susceptibility testing of Filamentous Fungi, Approved Standard. Vilanova: NCCLS; 2002. pp. 221–227.

26. Espinel-Ingroff A, Rodríguez-Tudela JL, Martínez-Suárez JV. Comparison of two alternative microdilution procedures with the National Committee for Clinical Laboratory Standards reference macrodilution method M27-P for in vitro testing of fluconazole-resistant and -susceptible isolates of *Candida albicans*. J Clin Microbiol. 1995;33:3154–8.

27. Koç AN, Uksal Ü, Oymak O. Case report. Successfully treated subcutaneous infection with *Sporothrix schenckii* in Turkey. Mycoses. 2001;44:330–3.

28. Trilles L, Fernández-Torres B, Lazéra MS, Wanke B, Schubach AO, Paes RA, Inza I, Guarro J. *In vitro* antifungal susceptibilities of *Sporothrix schenckii* in two growth phases. Antimicrob Agents Chemother. 2005;49:3952–4.

29. Alvarado-Ramírez E, Torres-Rodríguez JM. *In vitro* susceptibility of *Sporothrix schenckii* to six antifungal agents determined using three different methods. Antimicrob Agents Chemother. 2007;51:2420–3.

30. Kohler LM, Hamdan JS, Ferrari TCA. Successful treatment of a disseminated *Sporothrix schenckii* infection and *in vitro* analysis for antifungal susceptibility testing. Diagn Microbiol Infect Dis. 2007;58:117–20.

31. Marimon R, Serena C, Gené J, Cano J, Guarro J. *In vitro* antifungal susceptibilities of five species of *Sporothrix*. Antimicrob Agents Chemother. 2008;52:732–4.

32. Gutierrez-Galhardo MC, Zancopé-Oliveira RM, Monzón A, Rodriguez-Tudela JL, Cuenca-Estrella M. Antifungal susceptibility profile in vitro of *Sporothrix schenckii* in two growth phases and by two methods: microdilution and E-test. Mycoses. 2010;53:227–31.

33. Kauffman CA, Pappas PG, McKinsey DS, Greenfield RA, Perfect JR, Cloud GA, Thomas CJ, Dismukes WE. Treatment of lymphocutaneous and visceral sporotrichosis with fluconazole. Clin Infect Dis. 1996;22:46–50.

34. Diaz M, Negroni R, Montero-Gei F, Castro LG, Sampaio SA, Borelli D, Restrepo A, Franco L, Bran JL, Arathoon EG. A pan-American 5-year study of fluconazole therapy for deep mycoses in the immunocompetent host. Clin Infect Dis. 1992;14:68–76.

35. Collins CH. Laboratory-acquired infections: history, incidence, causes and prevention. 2nd ed. London: Butterworths;1988.

36. Cooper CR, Dixon DM, Salkin IF. Laboratory-acquired sporotrichosis. J Med Vet Mycol. 1992;30:169–71.

37. Bonifaz A, Saul A, Paredes-Solis V, Fierro L, Rosales A, Palacios C, Araiza J. Sporotrichosis in childhood: clinical and therapeutic experience in 25 patients. Pediatr Dermatol. 2007;24:369–72.

38. Lyon GM, Zurita S, Casquero J, Holgado W, Guevara J, Brandt ME, Douglas S, Shutt K, Warnock DW, Hajjeh RA. Population-based surveillance and a case-control study of risk factors for endemic lymphocutaneous sporotrichosis in Peru. Clin Infect Dis. 2003;36:34–9.

5.7. Capítulo VII

SUCCESSFUL TREATMENT OF TERBINAFINE IN A CASE OF SPOROTRICHOSIS

Publicado no periódico:

Anais Brasileiros de Dermatologia, v. 86, p. S182-185, 2011.

Successful treatment of terbinafine in a case of sporotrichosis

Sucesso terapêutico da terbinafina em um caso de esporotricose

Daiane Heidrich[1], Cheila Denise Ottonelli Stopiglia[2], Luciana Senter[3], Gerson Vetoratto[4], Patricia Valente[5] and Maria Lúcia Scroferneker[6]

[1]Degree in Pharmacy. MA student of the Post-Graduate Program in Medicine/Medical Sciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

[2]Degree in Pharmacy, specializing in clinical analysis. PhD student in the Post-Graduate Program in Medicine/Medical Sciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

[3]MA in Agricultural and Environmental Microbiology. CAPES Fellow. PhD student in the Agricultural and Environmental Microbiology Graduate Program, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

[4]MD. Dermatologist at the Santa Casa de Misericórdia de Porto Alegre Hospital, Porto Alegre, Brazil.

[5]PhD in Biological Sciences (Genetics). Associate Professor, Department of Microbiology, Immunology and Parasitology, teaches in the Post-Graduate Programs of Agricultural and Environmental Microbiology and Cellular and Molecular Biology, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

[6]PhD in Biological Sciences (Microbiology and Immunology). Associate Professor, Department of Microbiology, teaches in the Post-Graduate Medicine/Medical Sciences Program and is Chairperson of the Medical Immunology Program, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

**Study undertaken at the Human Pathogenic Fungi Laboratory, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil.

Abstract

Sporotrichosis is a chronic subacute infection caused by fungi belonging to the *Sporothrix* complex. In the present clinical case, nasal sporotrichosis was treated with potassium iodide. This was unsuccessful, and the treatment was restarted with a combination of potassium iodide and itraconazole. This however resulted in a further recurrence of the infection. The mycological cultures were tested *in vitro* for antifungal activity to assist in treatment. Terbinafine, an antifungal drug, produced the best results and was therefore used for the rest of the treatment course, with no recurrence after two years of its completion. In addition, both cultures were compared using RAPD and different fragment patterns were observed. This indicated that the isolates were either different or indicated a microevolutionary process of this microorganism.

Keywords: antifungal agents; disk diffusion antimicrobial tests; microbial sensitivity tests; mycoses; *Sporothrix*; sporotrichosis.

Resumo

A esporotricose é uma infecção subaguda ou crônica, causada por fungos pertencentes ao complexo *Sporothrix*. Relato do caso: esporotricose de localização nasal foi tratada com iodeto de potássio e como não se obteve sucesso, reiniciou-se o tratamento com associação de iodeto de potássio e itraconazol. Porém, ocorreu nova recidiva. As culturas dos exames micológicos foram submetidas a ensaios de atividade antifúngica *in vitro* para auxiliar no tratamento. A terbinafina foi o antifúngico que apresentou melhores resultados, por isso, o tratamento foi reiniciado com este antifúngico e, após dois anos do término do mesmo, não recidivou. Adicionalmente, ambas as culturas foram comparadas por RAPD, obtendo padrões de fragmentos distintos, indicando que os isolados são diferentes ou demonstrando um processo microevolutivo do microrganismo.

Palavras-chave: antimicóticos; esporotricose; micoses; *Sporothrix*; testes de sensibilidade a antimicrobianos por disco-difusão; testes de sensibilidade microbiana.

INTRODUCTION

Sporotrichosis is a subacute or chronic infection caused by fungi belonging to the *Sporothrix* complex: *S. schenckii*, *S. brasiliensis*, *S. mexicana*, *S. globosa* and *S. albicans*. Sporotrichosis occurs all over the world, mainly in tropical and temperate climes. The highest number of cases of subcutaneous mycosis is found in the Brazilian state of Rio Grande do Sul[2]. The infection is normally caused by traumatic inoculation of the fungus into the skin or subcutaneous tissues, and can be either confined to the puncture point or reach the regional lymphatic system. It could even spread through the bloodstream causing systemic infection. Extracutaneous sporotrichosis is however a rare condition, affecting immunocompromised patients almost exclusively[3].

The treatment of choice for sporotrichosis while still at the skin lesion stage is systemic chemotherapy with potassium iodide[4]. In its disseminated cutaneous forms, and with recurrent, extracutaneous and lymphocutaneous infections, amphotericin B is the most effective drug. Intolerance to iodine and the high toxicity of amphotericin B are however factors that often limit their use[5]. Recent years have seen the increased use of azoles (including ketoconazole, itraconazole and fluconazole) as a therapeutic alternative to the "classic" schemes, with itraconazole the most effective[6, 7]. On the other hand, terbinafine, in view of its high activity *in vitro* and *in vivo*, is now being used to treat various fungal infections, although clinical experience with this drug for treating sporotrichosis is fairly limited[8].

In this study we report a case of localized nasal sporotrichosis in which the strains isolated in the mycological culture were tested *in vitro* for antifungal activity to assist treatment. Molecular markers were also employed to conduct comparative analysis of the isolates.

CASE REPORT

A 65-year-old diabetic craftswoman sought medical assistance at the Dermatology Department in August 2006, presenting a nasal nodular lesion after undergoing cauterization for a nasal polyp. Test results indicated a chronic pathological ulcerated inflammation with granulation tissue, but with no presence of fungal structures. Four days later a biopsy was performed of the site. Part of the material was subjected to mycological culture. Culture was done using Sabouraud agar incubated at 25 °C for seven days. Subsequently we conducted the phenotypic characterization in accordance with Marimon et al. (2007)[1], which identified the pathogenic fungus *Sporothrix schenckii*. Initial treatment consisted of twenty drops of potassium iodide (KI) three times a day for three months until the lesion was cured and treatment discontinued. Four months later, however, the patient presented again with the lesion, which was submitted again to mycological culture followed by phenotypic characterization. On that occasion the pathogen was identified as *Sporothrix* spp. A new treatment was started with KI (25 drops, 3 times a day) combined with itraconazole (200 mg/day). After 11 months of treatment the lesion appeared to have healed. The patient nevertheless returned to the hospital two months later with a new recurrence. A further mycological culture followed by phenotypic characterization confirmed the presence of *Sporothrix schenckii*. We then requested an antifungal activity test according to protocol M38-A2 of the Clinical and Laboratory Standards Institute (CLSI)[9]. We used two isolates, one before treatment (the first) and one after the two treatments (the second), without success. The antifungal agents assessed were itraconazole, ketoconazole, fluconazole and terbinafine, in concentrations of between 0.25 and 128 μg/mL for fluconazole, 0.01 and 8 μg/mL for terbinafine and 16 and 0.03 μg/mL for itraconazole and ketoconazole. In the tests we included an antifungal control (growth control) and a microorganism-free control (sterility control). Determination of the minimum inhibitory concentration (MIC) was done visually by comparison with the growth control. The MIC was defined as the lowest drug

concentration capable of completely inhibiting fungal growth for itraconazole and terbinafine and 50% growth for fluconazole and ketoconazole.

All tests were performed in triplicate. The profiles of antifungal activity *in vitro* for both strains of *S. schenckii* are shown in Table 1. The first isolate showed greater sensitivity to antifungal agents than the second, but both were more sensitive to terbinafine. As a result this drug was chosen to restart the treatment of the patient with a dosage of 500 mg/day for five months. Two years after the treatment ended no relapses were reported.

TABLE 1: Profile of the antifungal susceptibility to both strains of *S. schenckii*.

	MIC (µg/mL)	
Antifungals	**1st strain**	**2nd strain**
Itraconazole	0.25	16.0
Cetoconazole	0.25	2.0
Fluconazole	32.0	64.0
Terbinafine	0.03	0.12

The DNA of the isolates was extracted by the Power Soil DNA Isolation Kit (MO BIO Laboratories) and subjected to Random Amplified Polymorphic DNA (RAPD), using the *primers* OPG-10, OPG-14 and OPG-19 (Prodimol Biotechnology SA, Brazil), with the components and amplification conditions according to Landell et al[10]. Polymorphic amplified patterns were subjected to electrophoresis on 1% agarose gel at 60 V for 90 minutes, followed by transluminator visualization under ultraviolet light. The two strains showed different fragment patterns when the *primer* OPG-10 was used. However, with the *primer* OPG-19 we observed two bands of the same molecular weight. No fragment was observed when using *primer* OPG-14 (Figure 1).

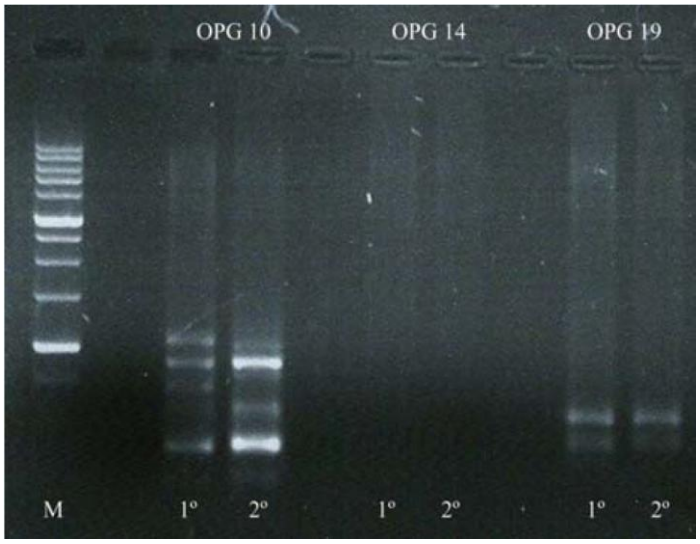

FIGURE 1: Fragments de DNA obtained by RAPD. Ladder molecular weight marker (M), the first and second strains amplified with OPG-10, the first and second with OPG-14 and the first and second with OPG-19.

DISCUSSION

Fungal infections are among the most common dermatological conditions affecting elderly people. A number of factors contribute to the increased prevalence of these infections in the elderly, including decreased skin barrier function, reduction of the functional capacity of the immune system of cells and the occurrence of diseases such as diabetes that inhibit scarring[11, 12].

In the dermis, and occasionally in the subcutaneous tissue, *S. schenckii* causes sporotrichosic nodules that may be confused with neoplasms[3]. The origin of the sporotrichosis in the present study case may be related to this or be a result of contamination after the cauterization of the patient´s nasal polyps, which would provide a gateway for the fungus to enter.

The absence of fungal structures in our pathological examination was due to the *S. schenckii* yeasts being fairly sparse and difficult to observe. In this respect they are rarely found on direct examination and require cultivation of the isolate to diagnose the disease[3]. Moreover, the mycological culture was needed because the presence of nodules tending to ulceration is a common feature of so-called sticky mycoses, which are mainly caused by *S. schenckii* and, less frequently, by other types of fungi such as *Aspergillus* spp [3].

The strains tested showed sensitivity profile to antifungal agents similar to the study of Marimon et al[13]. being more sensitive to terbinafine, followed by ketoconazole and itraconazole. In addition, they were resistant to fluconazole, as has been described by other authors[8,10,14]. The failure of therapy with itraconazole may be due to acquired resistance by the organism to the drug after treatment with this antifungal and/or even by the reduction of the production of gastric juice which often occurs in the elderly, reducing the bioavailability of the drug and thus its inability to reach the serum concentration needed to fight the fungus[9]. Thus, terbinafine is presented as an antifungal agent of choice for use in elderly patients compared with itraconazole, since terbinafine is not influenced by gastric pH and presents fewer interactions with other drugs[12].

The difference in the genotypic profile observed raises two assumptions: first, that the two strains are different, indicating that the fungal infection had been cured after treatment and that re-infection occurred, or (secondly) that the patient had been initially infected with two strains of *S. schenckii*. This hypothesis seems unlikely. Observing the genotypic profile obtained by the three *primers*, only with one of them (OPG-10) were small differences obtained. This leads us to venture the more probable hypothesis that the fungus could have undergone a microevolutionary process, with mutations occurring which caused sensitivity to antifungal agents to be reduced. Microevolution is an important factor during host colonization[15]. These mutations may have

occurred during the treatments, since 22 months elapsed between the first and second collection. In order to elucidate this question it would be necessary to do the RAPD with several other *primers* or to sequence hypervariable regions in the DNA of the isolates.

Few reports exist on the correlation between *in vitro* susceptibility and therapeutic response in the treatment of sporotrichosis[8]. In the above reported case the test for sensitivity to antifungal agents was very important for selecting the appropriate antifungal, indicating that terbinafine is indeed a viable alternative for treating sporotrichosis.

Conflict of interest: None

Financial funding: The authors are grateful for the financial support provided by the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and the *Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul* (FAPERGS).

Mailing address: Cheila Denise Ottonelli Stopiglia - Rua Sarmento Leite, 500 - Laboratório 210 – Centro – CEP 90050-170 Porto Alegre, RS, Brazil. E-mail: cheila.dos@gmail.com

REFERENCES

1. Marimon R, Cano J, Gené J, Sutton DA, Kawasaki M, Guarro J. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. J Clin Microbiol. 2007;45:3198-6.

2. da Rosa AC, Scroferneker ML, Vettorato R, Gervini RL, Vettorato G, Weber A. Epidemiology of sporotrichosis: a study of 304 cases in Brazil. J Am Acad Dermatol. 2005;52:451-9.

3. Lacaz CS, Porto E, Martins JEC, Heins-Vaccari EM, de Melo NT. Tratado de Micologia Médica. 9th ed. São Paulo: Sarvier; 2002. p. 479-497.

4. Coskun B, Saral Y, Akpolat N, Ataseven A, Cicek D. Sporotrichosis successfully treated with terbinafine and potassio iodide: case report and review of the literature. Mycopathologia. 2009;158:53-6.

5. Sterling JB, Heymann WR. Potassium iodide in dermatology: a 19th century drug for the 21st century - uses, pharmacology, adverse effects, and contraindications. J Am Acad Dermatol. 2000;43:691-7.

6. Meinerz ARM, Nascente PS, Schuch LFD, Cleff MB, Santin R, Brum CS, et al. Suscetibilidade *in vitro* de isolados de *Sporothrix schenckii* frente à terbinafina e itraconazol. Rev Soc Bras Med Trop. 2007;40:60-2.

7. Accioly Filho JW, Jacques CSC, Leverone AP, Oliveira JC, Mendonça IRSM, Azulay RD. Itraconazol no tratamento da esporotricose - Relato de 3 casos. An Bras Dermatol. 1993;68:117-9.

8. Kohler LM, Hamdana JS, Ferrari TCA. Successful treatment of a disseminated *Sporothrix schenckii* infection and *in vitro* analysis for antifungal susceptibility testing. Diagn Microbiol Infect Dis. 2007;58:117-20.

9. Clinical and Laboratory Standards Institute (CLSI). Reference method for Broth Dilution Antifungalsusceptibility testing of Filamentous Fungi, Approved Standard - 2nd ed. CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne. 2008;28:1-52.

10. Landell MF, Stopiglia CD, Billodre RG, Heidrich D, Sorrentino JM, Vainstein MH, et al. Evaluation of the origin of a sample of *Sporothrix schenckii* that caused contamination of a researcher in southern Brazil. Mycopathologia. 2011;171:203-7.

11. Saeed S, Sagatys E, Wasserman JR, Morgan MB. Geriatric Fungal Infections. In: Norman RA, editor. Diagnosis of aging skin diseases. London: Springer; 2008. p.123-150.

12. Hof H. Mycoses in the elderly. Eur J Clin Microbiol Infect Dis. 2010;29:5-13.

13. Marimon R, Serena C, Gené J, Cano J, Guarro J. *In vitro* antifungal susceptibilities of five species of *Sporothrix*. Antimicrob Agents Chemother. 2008;52:732-4.

14. Gutierrez-Galhardo MC, Zancopé-Oliveira RM, Monzón A, Rodriguez-Tudela JL, Cuenca-Estrella M. Antifungal susceptibility profile *in vitro* of *Sporothrix schenckii* in two growth phases and by two methods: microdilution and E-test. Mycoses. 2010;53:227-1.

15. Fleischhacker M, Pasligh J, Moran G, Ruhnke M. Longitudinal genotyping of *Candida dubliniensis* isolates reveals strain maintenance, microevolution, and the emergence of itraconazole resistance. J Clin Microbiol. 2010;48:1643-0.

6. CONSIDERAÇÕES FINAIS

Os isolados avaliados neste trabalho, provenientes de quatro estados brasileiros foram identificados, predominantemente, como *S. schenckii*. Nos estados do Rio Grande do Sul e do Rio de Janeiro, houve preponderância da espécie *S. schenckii*, seguida por *S. brasiliensis*, corroborando com outros estudos realizados no Rio Grande do Sul e contrariando relatos de isolados do Rio de Janeiro, os quais descrevem predomínio de *S. brasiliensis*. No estado de São Paulo, todos os isolados analisados foram identificados como *S. schenckii* e em Minas Gerais ocorreu o mesmo percentual de isolados das espécies *S. schenckii* e *S. brasiliensis*. Além disso, três isolados dos estados de Minas Gerais e do Rio Grande do Sul foram identificados como da espécie *S. globosa*, representando o quinto relato desta espécie no Brasil, o primeiro no Rio Grande do Sul e o terceiro em Minas Gerais.

Nossos resultados mostraram um alto índice de discordância (37,7%) entre os testes fenotípicos e genotípicos preconizados para a identificação das espécies do complexo *S. schenckii*, fato que já foi relatado por outros grupos de pesquisa. Assim, é necessária a padronização de novos testes fenotípicos para serem incluídos na chave de identificação deste complexo, visando uma menor taxa de erro e proporcionando a identificação em nível de espécie em laboratório clínico minimamente equipado.

Neste contexto, avaliamos o perfil enzimático e a sensibilidade a leveduras *killers* de três espécies do complexo *S. schenckii*. Infelizmente, nenhuma das leveduras e das enzimas avaliadas foi capaz de diferenciar os isolados em nível de espécie. No entanto, entre as dezoito leveduras com potencial *killer* avaliadas, doze apresentaram atividade frente a todos os isolados de *S. schenckii* testados, revelando a possibilidade destas toxinas serem base para o desenvolvimento de novos agentes antifúngicos.

A principal dificuldade no ensaio de leveduras *killer* frente ao complexo *S. schenckii* foi manter a atividade inibitória das leveduras com o passar do tempo. Para minimizar este problema,

desenvolvemos o “agar queijo”, o qual mantem os nutrientes originais do meio em que as leveduras foram isoladas: queijo e leite. Assim, conseguimos manter a atividade das leveduras por um longo tempo.

Nos ensaios *in vitro* de sensibilidade a antifúngicos constatamos que a terbinafina é o fármaco mais efetivo. A partir disso, uma paciente com esporotricose nasal não responsiva a iodeto de potássio e itraconazol passou a ser tratada com este antifúngico e obteve sucesso terapêutico. Após a terbinafina, os fármacos com melhores resultados nos ensaios *in vitro* foram cetoconazol e itraconazol, embora o último tenha apresentado resistência em 5,9% dos isolados. Já o fluconazol e o voriconazol foram os fármacos menos efetivos entre os avaliados. Não houve diferença nos perfis de suscetibilidade aos antifúngicos entre as espécies do complexo *Sporothrix schenckii* avaliadas.

Buscando antifúngicos mais eficazes e menos tóxicos para o tratamento da esporotricose, avaliamos o potencial de extratos metanólicos brutos obtidos de diferentes espécies do gênero *Pterocaulon*, planta com reconhecido uso popular para o tratamento de micoses. As cinco espécies avaliadas apresentaram atividade frente a todos os isolados de *S. schenckii* testados, validando o uso medicinal desta planta. O extrato de *P. polystachyum* foi o mais ativo dentre os avaliados, apresentando potencial para o desenvolvimento de novos agentes antifúngicos a partir do isolamento da(s) substância(s) responsável(is) pela atividade.

Nos ensaios de sensibilidade a antifúngicos buscamos alternativas para reduzir os custos do teste de microdiluição em caldo preconizado pelo *Clinical and Laboratory Standards Institute*, através da avaliação do potencial do caldo Sabouraud dextrose como substituto do meio RPMI-1640 tamponado com MOPS, o qual se mostrou adequado para todos os antifúngicos avaliados, com exceção da anfotericina B. No entanto, outros estudos tem mostrado que o meio RPMI-MOPS pode não ser adequado para detectar resistência *in vitro* para este fármaco. Portanto, são necessários

estudos de correlação *in vitro* e *in vivo* para concluir a viabilidade da substituição do meio RPMI-MOPS pelo caldo Sabouraud dextrose para a avaliação de *S. schenckii* frente à anfotericina B.

Também avaliamos a origem da esporotricose de uma pesquisadora do nosso laboratório, a qual suspeitávamos que fosse decorrente de uma contaminação laboratorial ocorrida com algum dos isolados da nossa micoteca. Felizmente, constatamos que a amostra não era geneticamente similar às do laboratório e, assim, a contaminação tem origem ambiental.

Todos os isolados avaliados foram capazes de secretar as enzimas urease, proteinase e desoxiribonuclease e a grande maioria deles produziu fosfolipase e esterase. Assim, a produção destas enzimas pode ser um fator de virulência quando *S. schenckii* parasita o corpo humano. No entanto, isolados ambientais apresentaram perfis enzimáticos semelhantes aos isolados clínicos, fato que precisa ser confirmado com um maior número de isolados.