UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO

Avaliação do estresse oxidativo em *Sporothrix* spp.

Letícia Lazzarotto

Porto Alegre, julho de 2017.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO

Avaliação do estresse oxidativo em *Sporothrix* spp.

Letícia Lazzarotto

Trabalho de Conclusão de Curso

Profa. Dra. Maria Lúcia Scroferneker

Orientadora

Daiane Heidrich

Coorientadora

Porto Alegre, julho de 2017.

CIP - Catalogação na Publicação

Lazzarotto, Letícia
Avaliação do estresse oxidativo em Sporothrix spp. / Letícia Lazzarotto. -- 2017.
25 f.

Orientadora: Maria Lúcia Scroferneker.
Coorientadora: Daiane Heidrich.

Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Curso de Farmácia, Porto Alegre, BR-RS, 2017.

1. Sporothrix. 2. Esporotricose. 3. Estresse oxidativo. 4. Peróxido de hidrogênio. I. Scroferneker, Maria Lúcia, orient. II. Heidrich, Daiane, coorient. III. Título.

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

AGRADECIMENTOS

- A Profª. Maria Lúcia Scroferneker, pela oportunidade de estagiar no Laboratório de Fungos Patogênicos Humanos.
- À doutora e coorientadora Daiane Heidrich, que muito me ensinou.
- À mestranda Lidiane Bonapaz e colegas estagiárias, Amanda Ribeiro e Alessandra Hellwig.
- A todos os professores, pelo conhecimento transmitido.
- À Universidade Federal do Rio Grande do Sul pela oportunidade de cursar Farmácia.
- A Deus, que esteve comigo em todos os momentos, ajudando a superar as dificuldades.
- Aos meus pais, Edite e Paulo, com todo meu amor e gratidão, pelo incentivo e apoio incondicional.
- A todos os amigos, que de alguma forma, contribuíram para a minha formação.
- Em especial a minha querida e amada prima, Lediane Benetti, falecida em 2012, que apesar de não poder presenciar a concretização deste sonho, tenho certeza que ajudou e torceu durante toda a caminhada. A todos vocês, meu muito obrigada.

Letícia Lazzarotto

Este artigo foi elaborado segundo as normas da revista *Mycoses,* sendo estas apresentadas no Anexo 1.

Avaliação do estresse oxidativo em *Sporothrix* spp.

Letícia Lazzarotto[1], Daiane Heidrich[2], Alessandra Helena da Silva Hellwig[1], Maria Lúcia Scroferneker[3].

[1] Acadêmica da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul.

[2] Doutora em Medicina: Ciências Médicas da Universidade Federal do Rio Grande do Sul.

[3] Professora titular do Departamento de Microbiologia, Imunologia e Parasitologia do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul, RS, Brasil.

Título curto: Estresse oxidativo em *Sporothrix* spp.

Palavras-chave: *Sporothrix***,** esporotricose, estresse oxidativo, peróxido de hidrogênio.

Autor correspondente: Maria Lúcia Scroferneker, Departamento de Microbiologia, Imunologia e Parasitologia, ICBS, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, sala 325, CEP: 90050-170, Porto Alegre - RS, Brasil. Tel. (+55) 51-33083934 Fax. (+55) 51-33083121 (e-mail: scrofern@ufrgs.br).

RESUMO

Introdução: A esporotricose é uma micose subcutânea causada por espécies de fungos do gênero *Sporothrix*. Após a instalação da infecção, células fagocitárias do hospedeiro tentam debelar o patógeno invasor através da produção de radicais livres de oxigênio.

Objetivos: Comparar a capacidade de sobrevivência ao estresse oxidativo induzido por peróxido de hidrogênio (H_2O_2) de cinco espécies do gênero *Sporothrix*.

Métodos: Dez isolados (dois de cada espécie) foram submetidos ao tratamento com H_2O_2 10 mmol l^{-1}, sendo as alíquotas livres do reagente consideradas como controle de viabilidade. Realizou-se a contagem das colônias e determinou-se a taxa de sobrevivência por espécie.

Resultados: Diferenças (média ± desvio padrão) quanto à taxa de sobrevivência ao estresse oxidativo foram observadas quando *S. brasiliensis* (25,1% ± 2,8) foi comparada a *S. chilensis* (89,6% ± 13,6, p = 0, 043). As demais espécies apresentaram taxa de sobrevivência de 77,3% ± 2,9 para *S. schenckii*; 54,8% ± 25,2 para *S. mexicana*; 38,2% ± 18,9 para *S. globosa*.

Conclusões: A espécie *S. brasiliensis* mostrou maior sensibilidade à exposição ao peróxido de hidrogênio em comparação a *S. chilensis.* Dessa forma, *S. chilensis* mostrou maior adaptação ao estresse oxidativo induzido por peróxido de hidrogênio.

INTRODUÇÃO

A esporotricose é uma micose subcutânea de distribuição cosmopolita, que atinge humanos e animais especialmente em áreas tropicais e subtropicais[1], sendo a micose subcutânea de maior prevalência na América Latina[2]. Desde o final da década de 1990, esta micose vem se tornando um fenômeno epidemiológico no estado do Rio de Janeiro, Brasil, sendo que entre os anos de 1998 e 2009 foram descritos aproximadamente 2200 casos de esporotricose em humanos e 3244 em felinos[3]. É causada por espécies de fungos dimórficos do complexo *Sporothrix schenckii*: *S. brasiliensis, S. schenckii stricto sensu, S. globosa* e *S. lauriei*[4]. Além destas, espécies do complexo *Sporothrix pallida*[5] descritas como ambientais, S. *chilensis* e *S. mexicana*, já foram isoladas como causadoras de doenças em humanos[5-7].

A infecção ocorre pela inoculação traumática acidental do fungo na pele ou nos tecidos subcutâneos durante a manipulação de material contaminado, sendo o fungo comumente encontrado em solos e vegetais em decomposição[1]. A infecção pode ficar limitada ao ponto de inoculação ou então disseminar-se pelo tronco linfático, podendo atingir a corrente sanguínea[8]. Formas extracutânea, pulmonar e disseminada são raramente observadas, e estão associadas a hospedeiros imunocomprometidos[9].

Após a instalação da infecção, células fagocitárias do hospedeiro começam a produzir espécies reativas de oxigênio (EROs) como ânion superóxido ($O_2^{\cdot -}$), peróxido de hidrogênio (H_2O_2) ou radical hidroxila (OH•) em resposta ao patógeno, as quais são tóxicas para o mesmo, causando danos ao ácido desoxirribonucleico (ADN) e eventualmente, morte celular[10]. EROs são produzidas principalmente nas mitocôndrias pelo complexo enzimático oxidase nicotinamida adenina dinucleotídeo fosfato (NADPH) dependente, que gera radicais reativos livres após reações de transferência de elétrons[11]. Contudo, características intrínsecas dos microrganismos como capacidade de

adesão as células hospedeiras, secreção de enzimas hidrolíticas extracelulares e capacidade em montar respostas de evasão ao sistema imunológico garantem adaptação e sobrevivência dos patógenos dentro do hospedeiro[10].

O objetivo deste estudo foi comparar cinco espécies (*S. brasiliensis, S. schenckii stricto sensu, S. globosa, S. mexicana* e *S. chilensis*) quanto à capacidade de sobreviver ao estresse oxidativo induzido por peróxido de hidrogênio.

MATERIAIS E MÉTODOS

Microrganismos

Foram avaliados dez isolados de agentes da esporotricose, como apresentado na **Tabela 1**, obtidos da coleção de fungos do Laboratório de Fungos Patogênicos Humanos do Departamento de Microbiologia, Imunologia e Parasitologia da Universidade Federal do Rio Grande do Sul. As espécies foram identificadas em trabalho anterior por sequenciamento molecular da região do ADN pertencente ao gene da calmodulina, utilizando os *primers* CL1 e CL2A[12], com exceção dos isolados das espécies *S. mexicana* e *S. chilensis*, provenientes do Laboratório de Micologia Médica e Molecular do Departamento de Microbiologia, Imunologia e Parasitologia da Universidade Federal de São Paulo. As cepas foram inoculadas em ágar batata dextrose (Himedia, Mumbai, Índia) a 30º C. Após sete dias, as suspensões de conídios foram preparadas por raspagem através da superfície das colônias fúngicas utilizando alças de plástico estéreis e solução salina tamponada com fosfato (PBS 1X), pH 7,4. As suspensões foram filtradas com papel filtro, para separar hifas e conídios, e o filtrado foi lavado e ressuspenso em 1,0 mL de PBS 1X. As suspensões de inóculo foram padronizadas para a concentração de $2x10^4$ conídios/ mL usando câmara de Neubauer.

Teste de estresse oxidativo

A concentração de peróxido de hidrogênio utilizada neste estudo baseou-se em estudo publicado anteriormente[13] e em experimento utilizando uma cepa da espécie *S. schenckii stricto sensu*. Portanto, a concentração de H_2O_2 (10 mmol l^{-1}) foi baseada na concentração que levou a uma baixa sobrevivência, mas com pelo menos 25 colônias por placa, para diminuir o erro da estimativa[14]. Da mesma forma, o tempo de leitura (sétimo dia de incubação) foi escolhido de acordo com o menor tempo que resultou em contagem de colônias semelhantes entre intervalos.

As suspensões de inóculos expostas a H_2O_2 10 mmol l^{-1} foram incubadas durante uma hora a 37º C com agitação. Alíquotas de cada amostra que não foram expostas ao reagente de oxigênio foram utilizadas como controle de viabilidade e consideradas como 100% de sobrevivência para fins de comparação.

Após exposição a H_2O_2, as suspensões foram lavadas com PBS 1X, ressuspensas para volume inicial e diluídas 1:10. Para contagem de colônias, 100 µL de inóculo foram espalhados em placa de ágar Sabouraud dextrose e incubados durante sete dias a 30º C. Os testes foram realizados em triplicata.

As percentagens de sobrevivência entre as diferentes espécies foram submetidas à análise de variância (ANOVA) seguida do teste de Tukey. O SPSS versão 18 foi utilizado para todas as análises estatísticas realizadas, considerando $\alpha = 0,05$.

RESULTADOS

A **Fig.1** mostra as taxas de sobrevivência e o desvio padrão de cada espécie exposta ao peróxido de hidrogênio 10 mmol l^{-1}: 89,6% ± 13,6 para *S. chilensis*; 77,3% ± 2,9 para *S. schenckii*; 54,8% ± 25,2 para *S. mexicana*; 38,2% ± 18,9 para *S. globosa* e 25,1% ± 2,8 para *S. brasiliensis*. Diferenças foram observadas quando *S. brasiliensis* foi

comparado a *S. chilensis* (p = 0,043). Os isolados de *S. chilensis*, um clínico humano e outro de origem ambiental, apresentaram semelhantes taxas de sobrevivência ao peróxido de hidrogênio.

DISCUSSÃO

Este é o primeiro estudo que comparou a sobrevivência ao estresse oxidativo em diferentes espécies do gênero *Sporothrix*. Pouco se sabe sobre o processo de adaptação à explosão oxidativa neste gênero de fungos: Wang et al[15], expondo um isolado chinês de *S. schenckii* ao estresse por peróxido induziu a expressão da enzima catalase. Mais recentemente, Ortega et al[10] avaliou cepas de *S. brasiliensis* e *S. schenckii* quanto a resistência a H_2O_2 e menadiona, além de tentar explicar a maior resistência observada de *S. brasiliensis* em comparação a *S. schenckii*, apesar de não terem sido encontradas diferenças genéticas que pudessem indicar disparidade na resposta ao estresse oxidativo entre as duas espécies. Análises *in silico* acerca da resposta ao estresse oxidativo também foram realizadas, demonstrando a existência de enzimas antioxidantes como catalases, superóxido dismutases, glutationa peroxidases e tioredoxina peroxidases.

Sabe-se que fungos ambientais possuem eficácia na adaptação a ambientes hostis, tornando-se relativamente resistentes às injúrias químicas[16]. Contudo, os isolados de *S. chilensis*, um de origem ambiental e outro isolado clínico humano, apresentaram semelhantes taxas de sobrevivência ao H_2O_2, demonstrando que a origem de isolamento não exerceu influência na resposta ao estresse oxidativo. Além disso, *S. chilensis* pode ter desenvolvido defesas contra H_2O_2, apesar de não haver relatos na literatura sobre a adaptação deste fungo, descrito em 2016[5]. Supõe-se que enzimas anteriormente citadas

e presentes em outras espécies do gênero devem estar presentes também em *S. chilensis* e exercer função de proteção contra danos ao ADN e morte celular.

Dessa forma, a secreção de enzimas antioxidantes contra EROs juntamente com outros mecanismos de evasão ao sistema imunológico garantem adaptação e sobrevivência do patógeno dentro do hospedeiro. Estudos analisando os tipos e quantidades de enzimas antioxidantes presentes nas diferentes espécies de *Sporothrix* spp. e a influência destas na resposta ao estresse oxidativo podem ser úteis para avaliar a capacidade do microrganismo em ultrapassar a barreira imunológica e assim, poder traçar estratégias de potencialização imune, como demonstrado em *Aspergillus fumigatus*, em que o pré-tratamento de células polimorfonucleares com interleucina-15 ou fator de necrose tumoral, *in vitro*, levou a um aumento do estímulo oxidativo e, consequentemente, maior dano as hifas do patógeno[17].

Como conclusão, este estudo mostrou maior sensibilidade de *S. brasiliensis* ao estresse oxidativo em comparação a *S. chilensis*, indicando maior adaptação desta última espécie ao peróxido de hidrogênio.

AGRADECIMENTOS

Os autores agradecem à FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul) e à CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) pelo suporte financeiro.

CONFLITO DE INTERESSE

Não há conflito com o presente artigo.

REFERÊNCIAS

1. Chakrabarti A, Bonifaz A, Gutierrez-Galhardo MC, et al. Global epidemiology of sporotrichosis. Med Mycol. 2015; 53:3-14.

2. Queiroz-Telles F, Nucci M, Colombo AL, et al. Mycoses of implantation in Latin America: an overview of epidemiology, clinical manifestations, diagnosis and treatment. Med Mycol. 2011; 49(3):225-236.

3. Barros MB, Schubach TP, Coll JO, et al. Sporotrichosis: development and challenges of an epidemic. Rev Panam Salud Públ. 2010; 27:455–460.

4. Zhang Y, Hagen F, Stielow B et al. Phylogeography and evolutionary patterns in *Sporothrix* spanning more than 14,000 human and animal case reports. Persoonia 2015; 35:1 –20.

5. Rodrigues AM, Choappa RC, Fernandes GF, et al. *Sporothrix chilensis* sp. nov. (Ascomycota: Ophiostomatales), a soil-borne agent of human sporotrichosis with mild-pathogenic potential to mammals. Fungal Biol. 2016; 120: 246-264.

6. Cruz-Choappa RM, Vieille Oyarzo PI, Carvajal Silva LC. Aislamiento de *Sporothrix pallida* complex en muestras clínicas y ambientales de Chile. Rev Arg Microbiol. 2014 Oct-Dec; 46(4):311-4.

7. Dias NM, Oliveira MM, Santos C, et al. Sporotrichosis caused by *Sporothrix mexicana*, Portugal. Emerg Infect Dis. 2011 Oct; 17(10): 1975-6.

8. Lacaz CS, Porto E, Martins JEC, Heins-Vaccari EM, de Melo NT. Tratado de Micologia Médica Lacaz. 9th ed. São Paulo: Sarvier, 2002.

9. Silva-Vergara ML, de Camargo ZP, Silva PF, et al. Disseminated *Sporothrix brasiliensis* infection with endocardial and ocular involvement in an HIV-infected patient. Am J Trop Med Hyg. 2012; 86:477–80.

10. Ortega I, Soares-Felipe MS, Vasconcelos ATR, et al. Peroxide sensing and signaling in the *Sporothrix schenckii* complex: an *in silico* analysis to uncover putative mechanisms regulating the Hog1 and AP-1 like signaling pathways. Med Mycol. 2015; 53:51-59.

11. Heller J, Tudzynski P. Reactive oxygen species in phytopathogenic fungi: signaling, development and disease. Annu Rev Phytopathol. 2011; 49:369–90.

12. Ottonelli-Stopiglia CD, Magagnin CM, Castrillón MR, et al. Antifungal susceptibilities and identification of species of the *Sporothrix schenckii* complex isolated in Brazil. Med Mycol. 2014 Jan; 52(1):56-64.

13. Sun J, Zhang J, Najafzadeh MJ, et al. Melanization of a meristematic mutant of *Fonsecaea monophora* increases tolerance to stress factors while no effects on antifungal susceptibility. Mycopathologia 2011; 172(5):373-380.

14. Sutton S. Accuracy in plate counts. J Validation Technol. 2011; 17(3):42-46.

15. Wang XH, Li RY, Cao CW et al. Differential mRNA expression of *Sporothrix schenckii* catalase gene in two growth phases and effect factors. Chin Med J. 2008; 121(20): 2100-2102.

16. Brown AJ, Haynes K, Quinn J. Nitrosative and oxidative stress responses in fungal pathogenicity. Curr Opin Microbiol. 2009; 12(4):384-391.

17. Winn RM, Gil-Lamaignere C, Roilides E, et al. Selective effects of interleukin (IL) -15 on antifungal activity and IL-8 release by polymorphonuclear leukocytes in response to hyphae of *Aspergillus* species. J. Infect. Dis. 2003; 188(4): 585-590.

Tabela 1. Isolados de *Sporothrix* spp. utilizados no estudo.

Código do isolado	Espécie	Origem de isolamento
16490 MG	*S. globosa*	Humano
8600	*S. globosa*	Humano
s 110 RJ	*S. brasiliensis*	Humano
3222876	*S. brasiliensis*	Humano
237	*S. schenckii*	Humano
Santa Casa I	*S. schenckii*	Humano
Ss 181	*S. mexicana*	Ambiental
Ss 182	*S. mexicana*	Ambiental
Ss 469	*S. chilensis*	Humano
Ss 470	*S. chilensis*	Ambiental

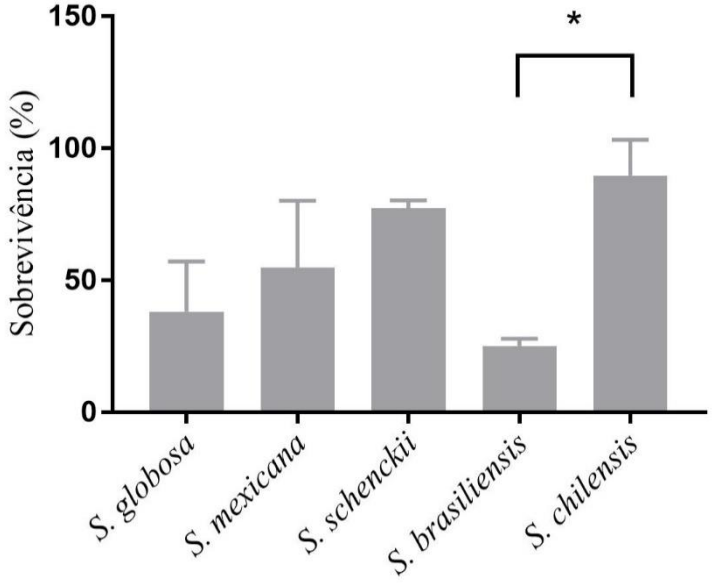

Figura 1. Percentagem de sobrevivência e desvio padrão de espécies de *Sporothrix* spp. submetidas a peróxido de hidrogênio 10 mmol l^{-1} a 37º C por 1h.

* p = 0,043 por ANOVA seguida pelo teste de Tukey. SPSS versão 18.

Anexo 1:

Normas da revista *Mycoses*

GENERAL

Mycoses is dedicated to the publication of manuscripts on topics concerning medical or veterinary mycology. Studies on plant pathology or mycological papers on fungi not related to human or veterinary medicine do not lie within the scope of Mycoses and will not be accepted. Manuscripts may be published as original communication of normal or short length (Short communication). The submission of review articles both of the mini-review and the full-length type is particularly encouraged. Only papers submitted in English will be accepted (this does not exclude the Latin text required for the description of new species or genera).

The editors reserve the right not to accept papers unless adherence to the principles given in the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW Publication NIH) is clear.

No charge is made for publication but authors will be required to pay for extensive alterations to agreed papers at proof stage.

EARLY VIEW

Mycoses is covered by Early View articles for articles that are complete and final. They have been fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated. Because they are in final form, no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot be cited in the traditional way. They are therefore given a Digital Object Identifier (DOI), which allows the article to be cited and tracked before it is allocated to an issue. After print publication, the DOI remains valid and can continue to be used to cite and access the article.

MANUSCRIPT SUBMISSION

Manuscripts are submitted to Mycoses online, i.e. electronically, from the corresponding author's Mycoses ScholarOne Manuscript (formerly known as Manuscript Central) account. You will need your files in an electronic format, an Internet connection, and a user ID and password for the site. To begin a new submission, go to Manuscript Central and log in or create an account to get your user ID and password. Full instructions are provided on the site.

If assistance is needed, the Editorial Office can be contacted and will readily provide any help users need to upload their manuscripts.

Ann O'Mahony

Mycoses Editorial Office

MYCedoffice@wiley.com

MYCedoffice@gmail.com

Authors for whom English is a second language may choose to have their manuscript professionally edited before submission. We are unable to accept any Manuscript submitted in inaccessible English or in a form unsuitable for effective peer review. (http://authorservices.wiley.com/bauthor/english_language.asp)

ELECTRONIC SUBMISSION

Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files plus separate figure files. JPEG, TIFF or EPS files are acceptable for submission, but only high-resolution JPEG, TIFF or EPS files are suitable for printing. The files will be automatically converted to a PDF document on upload and will be used for the review process. The text file must contain the entire manuscript including title page, abstract, text, references, tables, and figure legends, but no embedded figures. Figure tags should be included in the file.

Manuscripts should be formatted as described in Mycoses' Author Guidelines (below). When preparing your file, please use only standard fonts such as Times, Times New Roman or Arial for text, and Symbol font for Greek letter, to avoid inadvertent character substitutions. In particular, please do not use Japanese or other Asian fonts. Do not use automated or manual hyphenation.

For more information on preparing manuscripts for online submission, please read the detailed instructions at Manuscript Central. Additional help is available by emailing support@scholarone.com/.

Authors are encouraged to provide names of potential reviewers for their manuscript. All acceptable material submitted for publication is forwarded to the Deputy Editor in charge of the particular field who together with two further referees makes a judgement on the paper. The votes of the independent referees as well a preliminary suggestion of acceptance or rejection is forwarded by the Deputy Editor to the Editors who make the final decision on their individual specialty. Authors must inform the Editors of any possible conflict of interest capable of influenceing their judgement and if necessary, a disclaimer will be included.

Revised manuscripts must be uploaded within 2 months of authors being notified of conditional acceptance pending satisfactory revision. Authors resubmitting manuscripts should follow the same procedures as for submission of new manuscripts. If accepted, papers become the copyright of the journal**.** After acceptance please make sure that the final manuscript and the figure files are uploaded. The figures must be high-resolution scans (JPEG, TIFF or EPS files). Detailed information on our digital illustration standards is available at Author Services.

ORIGINAL ARTICLES AND REVIEWS

Text

Authors should aim for a concise readable style. Spelling should follow the Concise Oxford Dictionary, and The Oxford Dictionary for Writers and Editors. The Editors reserve the right to make corrections, both literary and technical, to the papers.

All pages must be numbered consecutively in the upper right-hand corner of each page. Starting with the title page as p.1, the pages should be numbered in the following order: title page, summary and key words, text, acknowledgements, references, tables, figure legends.

The following items should each start on a separate page.

Title Page

This should bear (1) the title, (2) the names of all authors, (3) the institutions of origin with brief addresses, (4) a short title of not more than 50 characters (including spaces) to be used as a running head, (5) a list of up to eight key words for indexing purposes, and (6) the name and full postal address (with phone and fax numbers and e-mail address) of the author who will be responsible for reading the proofs (the corresponding author). The corresponding author must keep the Mycoses office informed of any change in details until the paper is published. Authors should keep a copy of their manuscript.

Summary

Normally in less than 210 words, this should indicate clearly the scope and main conclusions of the paper. Original articles should have a structured abstract, comprising the five headings: Background, Objectives, Patients/Methods, Results and Conclusions.

Main Text

Papers should be divided into sections headed (1) introduction, (2) materials and methods (or patients and methods/subjects and methods if human patients/subjects were used), (3) results, (4) discussion (5) acknowledgements, and (6) Conflict of Interest. Avoid an excess of sub-headings - two further divisions, if necessary, should be adequate.

The introduction should explain why the work was done and briefly introduce the scope and contents of the paper. Essential details should be included in materials and methods, including experimental design and statistical analysis. Results should be recorded in the past tense. The discussion should present the author's results in the broader context of other work on the subject. Acknowledgements should be as brief as possible.

References

References should be cited using the AMA style (see examples below).

The references in the manuscript text should be numbered consecutively in the order in which they appear and indicated by Arabic numerals as superscripts. The reference list, presented in numerical order, should include the names of all authors (if there are more than six authors, only the first three should be given followed by et al.), the full title of the article, title of publication (abbreviated in accordance with Index Medicus), the year, volume number and first and last page numbers. References to books should, in addition, include the names of the editor, the edition number, where appropriate, and the town of origin and name of publisher. The accuracy of the reference is the responsibility of the author. Examples are given below.

Journals:

Bigby ME. The end of the sunscreen and melanoma controversy: a quantitative review. Ann Intern Med. 2003; 139(12):966-978.

Klein R, Hein BEK, Moss SE, et al. The relation of retinal vessel ralilxtr to the incidence and progression of diabetic retinopathy, XIX: the Wisconsin Epidemiologic Study of Diabetic Retinopathy. Arch Ophtbalmol 2004; 122(1):76-83.

Smeeth L, Iliffe S. The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. Nature. 2004; 427(6974):510-517. doi: 10.1038/nature02312.

Books:

Modlin J, Jenkins P. Decision Analysis in Planning for a Polio Outbreak in the United States. San Francisco, CA: Pediatric Academic Societies; 2004.

Adkinson N, Yunginger J, Busse W, Bochner B, Holgate S, Middleton E, eds. Middleton's Allergy: Principles and Practice. 6th ed. St. Louis, MO: Mosby; 2003.

Solensky R. Drug allergy: desensitization and treatment of reactions to antibiotics and aspirin. In: Lockey P, ed. Allergens and Allergen Immunotherapy. 3rd ed. New York, NY: Marcel Dekker; 2004:585-606.

World Health Organization. Injury: A Leading Cause of the Global Burden of Disease, 2000. Geneva, Switzerland: World Health Organization/Plenum; 2002.

Websites:

International Society for Infectious Diseases. ProMED-mail Web site. hnp://www.promedmail.org. Accessed April 29, 2004.

Software:

Epi Info [computer program]. Version 3.2. Atlanta, GA: Centers for Disease Control and Prevention; 2004

TABLES

These must be numbered consecutively with Arabic numerals and typed on separate sheets carrying an appropriate legend and presented in a way that makes the table self-explanatory.

Numerical results should be expressed as means with the relevant standard errors and/or statistically significant differences, quoting probability levels (P-values). Three significant figures are usually sufficient for mean values and standard errors should be quoted two or three more decimals than the mean.

The only lines appearing in the table should be horizontal and all decimals should be aligned in columns. The placement of all tables should be indicated in the text, being referred to as Table 1 or Tables 2 and 3.

FIGURES

Figures should be numbered in sequence in Arabic numerals as they appear in the text. Labels, lettering and symbols etc. must be professionally prepared and should be uniform. Lines should be of sufficient thickness to stand reduction (no less than 4 mm wide for a 50% reduction), and letters should be a minimum of 14 pt Times New Roman or an equivalent size. Acceptable symbols for experimental points are ¡, r, o, ˜, p, ¢. The symbols + and × will not be accepted.

Legends should be typed on a separate sheet and consist of a short title together with a brief explanatory paragraph. The legend must make the meaning of each figure understandable without further reference to the text. Photomicrographs should state the original magnification.

The position of all figures should be indicated in the text and should be referred to as Fig. 1 or Figs 1 and 3. Figure 1 should be written out in full if at the beginning of a sentence.

Mycoses is accepting coloured figures FREE of charge.

ELECTRONIC ARTWORK

Vector graphics (e.g. line artwork) should be saved in Encapsulated Postscript Format (EPS), and bitmap files (e.g. photographs) in Tagged Image File Format (TIFF). Please do not use any pixel-oriented programs. Scanned figures (only in TIFF format) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size. Colour graphics should be created using the CMYK colour palette (print colours), not RGB (monitor colours). There is a charge for alterations to figures when carried out by the publisher.

Full details of submission of figures in electronic format are available at Author Services.

If figures have more than one part, each part should be labelled (preferably) in the top left-hand corner with lower-case letters in parentheses e.g. a figure with two parts would be labelled (a) and (b). In the text this should be referred to as Fig. 1a or Figs 2a, b.

Figures should be planned to fit the printed column, i.e. 7.9 cm wide for single column and 16.5 cm wide for double column.

Photographs can be up to twice the reproduction size and must be unmounted glossy prints showing good detail and moderate contrast.

UNITS, SYMBOLS and ABBREVIATIONS

SI (Système International) units should be used and should conform with the lists printed Units, Symbols and Abbreviations - A Guide for Biological and Medical Editors and Authors 4th edn as published by the Royal Society of Medicine. Nomenclature of disease should follow the International Classification of Disease, published by the World Health Organization, as far as possible.

When first mentioned, cumbersome medical names should be abbreviated for later reference in the text. Latin bi-nominals should abbreviate the genera to the initial letter after the first mention unless it begins a sentence.

Doses of drugs should be given as unit weight per body weight, e.g. mmol kg-1. Rates should be expressed with negative indices. Concentrations should be given in terms of molarity, e.g. mmol l-1, not mM.

Numerals are to be used from 10 upwards; and the 24-hour clock, e.g. 21.00 hours, should be used.

MATERIALS

The source of all materials used should be given stating company, city and country.

Verification of the identity of living specimens used must take place through sequencing, or by consultation of taxonomists working at a reference centre. Specimens analyzed must remain accessible for later reference. Strains should be deposited in one of the major culture collections, and sequences in a public data bank. Collection and sequence numbers must be cited in the text. It is recommended that new taxa are deposited in MycoBank. Descriptions of new taxa must comply with the rules of the International Code of Botanical Nomenclature.

CASE REPORTS

Mycoses no longer accepts case reports in the traditional formats. There are two exceptions to this rule. If a report describes the first case of a previously unknown fungal disease, you may inquire with the editors whether the case should be submitted to Mycoses. Otherwise authors are encouraged to write a review article on a disease and

add a succinct description of an illustrative case to enhance understanding of the typical clinical features.

AUTHOR SERVICES

Online production tracking is now available for your article through Author Services: Author Services enables authors to track their article - once it has been accepted - through the production process to publication online and in print. Authors can check the status of their articles online and choose to receive automated e-mails at key stages of production. The author will receive an e-mail with a unique link that enables them to register and have their article automatically added to the system. Please ensure that a complete e-mail address is provided when submitting the manuscript.

Visit Author Services for more details on online production tracking and for a wealth of resources including FAQs and tips on article preparation, submission and more.

Guidelines for Cover Submission: If you would like to send suggestions for artwork related to your manuscript to be considered to appear on the cover of the journal, please follow these general guidelines.

COPYRIGHT ASSIGNMENT

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

For authors signing the copyright transfer agreement.

If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions

For authors choosing OnlineOpen

If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements (OAA):

Creative Commons Attribution Non-Commercial License OAA

Creative Commons Attribution Non-Commercial -NoDerivs License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services and Wiley Open Access.

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via

the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper.

For authors signing the copyright transfer agreement

If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions http://authorservices.wiley.com/bauthor/faqs_copyright.asp

For authors choosing OnlineOpen

If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements (OAA):

Creative Commons Attribution Non-Commercial License OAA

Creative Commons Attribution Non-Commercial -NoDerivs License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services http://authorservices.wiley.com/bauthor/faqs_copyright.asp and visit http://www.wileyopenaccess.com/details/content/12f25db4c87/Copyright--License.html.

If you select the OnlineOpen option and your research is funded by The Wellcome Trust and members of the Research Councils UK (RCUK) or the Austrian Science Fund (FWF) you will be given the opportunity to publish your article under a CC-BY license supporting you in complying with your Funder requirements. For more information on this policy and the Journal's compliant self-archiving policy please visit: http://www.wiley.com/go/funderstatement.

PROOFS

The corresponding author will receive an email alert containing a link to a web site. A working e-mail address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site. Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from Adobe.

This will enable the file to be opened, read on screen and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately.

ONLINEOPEN

OnlineOpen is available to authors of primary research articles who wish to make their article freely available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With OnlineOpen, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Library, as well as deposited in the funding agency's preferred archive. Please click here for further information about OnlineOpen. Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen if you do not wish to. All OnlineOpen articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

OFFPRINTS

A PDF offprint of the online published article will be provided free of charge to the corresponding author, and may be distributed subject to the Publisher's terms and conditions. Paper offprints of the printed published article may be purchased if ordered via the method stipulated on the instructions that will accompany the proofs. Printed offprints are posted to the correspondence address given for the paper unless a different address is specified when ordered. Note that it is not uncommon for printed offprints to take up to eight weeks to arrive after publication of the journal.

AUTHOR MATERIAL ARCHIVES POLICY

Please note that unless specifically requested, Wiley will dispose of all hardcopy or electronic material submitted two months after publication. If you require the return of any material submitted, please inform the editorial office or Production Editor as soon as possible if you have not yet done so.

Last update: February 2015.