



Original article

Interference of melanin in the susceptibility profile of *Sporothrix* species to amphotericin B



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ABSTRACT

Background: The presence of melanin in the fungal cell is a major virulence factor of the genus *Sporothrix* since it protects the fungal cells against the defense systems.

Aims: The present study aimed to investigate the interference of melanin in the susceptibility of *Sporothrix brasiliensis* and *Sporothrix schenckii* sensu stricto to amphotericin B and itraconazole, drugs recommended as therapy for disseminated and subcutaneous sporotrichosis, respectively.

Methods: Yeast cells were cultivated in minimal medium with or without L-DOPA in order to induce the production of melanin. Microdilution and killing assay methods were used to determine the antifungal activity against yeast cells with different amounts of melanin.

Results: The killing assay showed that melanization protected isolates within the *S. schenckii* complex from amphotericin B, particularly in the lower concentrations tested.

Conclusions: Studies combining amphotericin B and inhibitors of melanin are required in order to avoid this effect.

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Interferencia de la melanina en la sensibilidad de especies del género *Sporothrix* a la anfotericina B

RESUMEN

Antecedentes: La presencia de melanina en la célula fúngica es un importante factor de virulencia del género *Sporothrix* en el proceso de infección, pues protege al hongo de la acción del sistema inmunitario.

Objetivos: El objetivo del presente estudio fue investigar la interferencia de la melanina en la sensibilidad de *Sporothrix brasiliensis* y *Sporothrix schenckii* sensu stricto a la anfotericina B y el itraconazol, antifúngicos recomendados como terapia para la esporotricosis diseminada y la subcutánea, respectivamente.

Métodos: Se cultivaron células en la fase levaduriforme en medio mínimo con o sin L-DOPA con el fin de inducir de melanina. La valoración de la actividad antifúngica sobre células de *Sporothrix* con diferente contenido en melanina se realizó mediante microdilución en caldo y curvas de mortalidad.

Resultados: El ensayo mostró que la melanización protege de la acción de la anfotericina B a las especies del complejo *S. schenckii*, particularmente en las concentraciones más bajas ensayadas.

Conclusiones: Se requieren estudios de combinación entre diferentes clases de antifúngicos o de anfotericina B con inhibidores de la melanina, con el fin de disminuir o evitar este efecto.

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Palabras clave:

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Sporotrichosis is a fungal disease caused by dimorphic and thermotolerant fungi belonging to the *Sporothrix schenckii* complex that occurs in both subacute and chronic forms. This fungus primarily affects the skin and subcutaneous tissue and most frequently manifests as a lymphocutaneous infection.¹⁷ Most cases occur due to traumatic implantation of the fungus through the skin by handling plants, soil and infected animals.³⁵ Disseminated sporotrichosis has been described in immunocompromised patients, especially in those under corticosteroid treatment, patients with AIDS, diabetes and alcoholics,^{6,8–11} and, although rare, dissemination has also been reported in immunocompetent patients.^{31,38} The *S. schenckii* complex encompasses six species capable of causing sporotrichosis.²⁰ These species show different *in vitro* susceptibility profiles to antifungal agents,²¹ as well as different virulence *in vivo*.⁴

Itraconazole is the treatment of choice for lymphocutaneous sporotrichosis¹⁶; however, failure due to the *in vitro* resistance to this azole and cross-resistance with other azoles has been observed.^{21,39} In cases of disseminated sporotrichosis, amphotericin B is the advised antifungal, followed by a maintenance treatment with itraconazole.^{6,10,16}

Melanin is a requirement for fungal cells to persist when facing up to host defense systems^{3,15,26,33} and is also considered a factor of fungal drug resistance, mainly for polyenes and echinocandins. Both morphological stages of *Sporothrix* are able to synthesize melanin *via* the polyketide pathway to produce dihydroxynaphthalene (DHN) melanin, which is present in the conidia of the fungus. It has also been shown that *Sporothrix* can produce melanin through phenolic substrates such as 3,4-dihydroxy-L-phenylalanine (L-DOPA),¹ and some species can produce pyromelanin in the presence of L-tyrosine.² Almeida-Paes² demonstrated with one strain of *Sporothrix brasiliensis* that cells became more resistant to amphotericin B after melanization in medium with L-DOPA or L-tyrosine. Amphotericin B is the treatment of choice for systemic sporotrichosis.²⁴ Thus, it may be possible that therapeutic failures occur in cases of sporotrichosis as a result of drug resistance driven by melanization.

This study approaches the interference of fungal melanin in the *in vitro* susceptibility of the most common and virulent *Sporothrix* species – *S. schenckii* sensu stricto and *S. brasiliensis* – to both amphotericin B and itraconazole, the antifungals for the treatment of disseminated and lymphocutaneous forms, respectively, of sporotrichosis. Recognition of such interference may be essential in redefining doses and treatments for cases of sporotrichosis.

Material and methods

Chemicals

Amphotericin B (Sigma Chemical Co., St. Louis, MO) and itraconazole (Janssen-Cilag, São Paulo, SP) were obtained as standard powders and prepared according to the *Clinical and Laboratory Standards Institute* guidelines.⁷ RPMI 1640 medium (with L-glutamine and without sodium bicarbonate), morpholinepropanesulfonic acid (MOPS), and L-DOPA were purchased from Sigma Chemical Co. (Cleveland, OH).

Fungal strains and media

Three isolates of *S. schenckii* sensu stricto and three isolates of *S. brasiliensis* isolated from cases of lymphocutaneous sporotrichosis were studied. One strain of *Candida glabrata* (ATCC) and one strain of *Cryptococcus neoformans* (ATCC) were included as negative and positive control of melanization, respectively. The fungal yeast cells were grown at 37 °C for 7 days in a rotary shaker (150 rpm) in

brain heart infusion (BHI) broth (Himedia). Controls were obtained by growing yeast cells on minimal medium (MM) agar plates (15 mM glucose, 10 mM MgSO₄, 29.4 mM KH₂PO₄, 13 mM glycine, 3.0 μM vitamin B1, 2% agar; Difco); melanization was induced by growing yeast cells on minimal medium supplemented with L-DOPA (MML) for 10 days.³⁴ Cells were then harvested and washed with sterile phosphate-buffered saline (PBS), pH 7.2.

MIC determination

Minimal inhibitory concentrations (MIC) were determined by the standardized protocol (M27-A3) according to CLSI,⁷ adapted to the yeast phase of *Sporothrix*. Final drug concentrations ranged from 0.06 to 4 μg/ml for amphotericin B and itraconazole. After incubation at 37 °C for 48 h, MIC for amphotericin B was defined as the lowest concentration at which there was an absence of growth, and for itraconazole as the lowest drug concentration which achieved 50% growth inhibition when compared to the growth of the drug-free control.

Killing assay

For the killing assay, yeast cells were suspended in sterile normal saline at a density of 2.2×10^3 cells/ml. Cell counts were determined with a hemocytometer. Microcentrifuge tubes containing 0.1 ml aliquots of an antifungal at 10 times the final concentration were inoculated with 0.9 ml of the yeast suspensions. Final drug concentrations ranged from 2 to 8 μg/ml for amphotericin B, and from 1 to 4 μg/ml for itraconazole. After incubation at 37 °C for 2 h, aliquots were plated on brain heart infusion agar (BHI) to determine their viability, as measured by the number of CFU. Before that, a screening with a time kill curve with the samples collected at 2, 4, 8, and 24 h was performed. However, at the point of 4 h onwards there was no significant difference in mortality. Thus, it was determined the point of 2 h for the evaluation of mortality. The rate of survival was compared to that of fungal cells incubated in phosphate-buffered saline (PBS).³⁶

Spectrophotometry of yeast cell extracts

Extraction of melanin was performed in order to quantify differences in the amount of melanin in the *Sporothrix* isolates grown in different media. The method consisted of an evaluation of the crude extract of the yeast mass obtained by autoclaving it for 90 min at 120 °C in 0.02 mol/l citrate buffer pH 7 as previously described.¹⁸ Then, the suspension was centrifuged and the supernatant collected for spectrophotometric analysis. The extracts were analyzed at 260 nm.³⁴

Statistics

Statistical significance (*p* values) was determined using Wilcoxon test to compare MICs results with and without melanin interference. Student's *t*-test was used in the killing assay.

Results and discussion

All isolates produced higher amount of melanin within 10 days in medium supplemented with L-DOPA (MML), while isolates cultured in medium without L-DOPA (MM) remained with their standard melanization. The concentration of melanin was measured using spectrophotometry (Table 1). The absorbance of the melanized group was approximately twice the absorbance of the group not exposed to L-DOPA, showing the greater production of melanin when L-DOPA is present in the medium.

Table 1

Variation in melanin content, expressed as absorbance, after subculture on minimal medium and minimal medium with L-DOPA.

Isolates	Absorbance (260 nm)	
	MM	MML
Sb 01	0.701	1.495
Sb 02	0.690	1.398
Sb 03	0.691	1.450
Ss 01	0.530	1.678
Ss 02	0.561	1.691
Ss 03	0.570	1.680
Cg	0.240	0.268
Cn	0.630	1.504

MM, minimal medium; MML, minimal medium with L-DOPA; Sb, *Sporothrix brasiliensis*; Ss, *Sporothrix schenckii* sensu stricto; Cg, *Candida glabrata*; Cn, *Cryptococcus neoformans*.

The geometric mean of MICs for amphotericin B and itraconazole for the isolates subcultured in the different media were compared. There was no significant difference among the MICs of the group without increasing melanin content (MM), and the group with melanin inducing conditions (MML) ($p > 0.05$). In the killing assay, melanization protected the cells against amphotericin B. The differences were statistically significant at a concentration of 2 $\mu\text{g/ml}$ ($p < 0.001$) (Fig. 1). The results obtained with itraconazole showed no differences between the groups tested (Fig. 1).

Amphotericin B is the treatment of choice for disseminated and extracutaneous sporotrichosis.¹⁶ A previous study showed that both fungal melanin and synthetic melanin are capable of binding to amphotericin B, reducing the effectiveness of this agent. In contrast, fluconazole, itraconazole and 5-fluorocytosine activities were not modified by the presence of melanin, also observed in the study presented here. Tests with azole antifungal agents did not result in significant death of the fungus, which is consistent with the fungistatic properties of these drugs.³⁶ However, the non-interference of melanin in the itraconazole activity, presented here for the first time, makes itraconazole a potential therapeutic option for sporotrichosis.

In this study, the broth microdilution technique⁷ was unable to demonstrate the interference of melanization in the antifungal susceptibility of *Sporothrix* strains. This finding is in agreement with studies performed with *C. neoformans* and *Histoplasma capsulatum*.^{13,36,37} It was reported that this could be due to the absence, in the culture medium, of substrates for melanization. In the same study the authors attempted to minimize this problem by adding L-DOPA to RPMI, but this resulted in the formation of a black precipitate which precluded the use of this modification to the assay.³⁶

Here, the killing assay showed that melanization protected *S. schenckii* sensu stricto and *S. brasiliensis* from amphotericin B,

Table 2

Minimal inhibitory concentrations of amphotericin B and itraconazole against *S. brasiliensis* (Sb) and *S. schenckii* sensu stricto (Ss) isolates cultivated in different media.

Isolates	MIC ($\mu\text{g/ml}$)	
	AMB MM/MML	ITC MM/MML
Sb01	0.25/0.25	0.25/0.5
Sb02	0.5/0.5	0.5/0.5
Sb03	0.5/0.5	0.5/0.5
Ss01	0.25/0.5	1/2
Ss02	1/1	0.5/1
Ss03	1/1	0.25/0.25

MIC, minimal inhibitory concentrations; AMB, amphotericin B; ITC, itraconazole; MM, minimal medium; MML, minimal medium with L-DOPA.

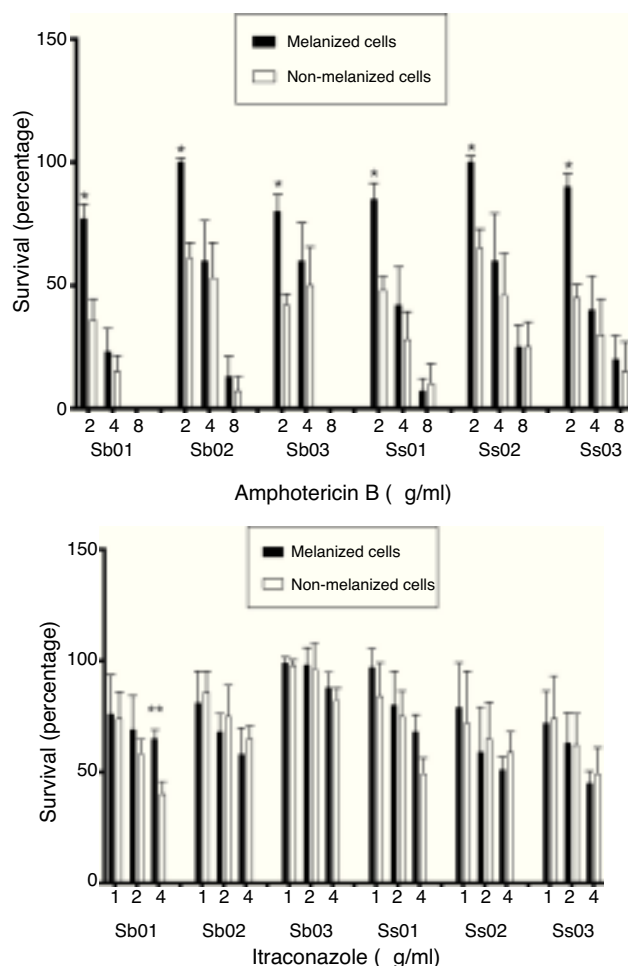


Fig. 1. Amphotericin B and itraconazole killing assay. The rates of survival of yeast cells (*S. brasiliensis* [Sb 01, Sb 02, Sb 03] and *S. schenckii* sensu stricto [Ss 01, Ss 02, Ss 03]) grown in minimal medium (MM), and minimal medium with L-DOPA (MML) after exposure to various concentrations of amphotericin B and itraconazole for 2 h were compared to those of fungal cells incubated in PBS. p -Values were calculated by comparing melanized groups with their respective non-melanized group using the Student's t -test. * $p < 0.001$, ** $p < 0.05$. Similar results were obtained in two separate experiments.

particularly in the lower concentration tested, as has already been demonstrated in one strain of *S. brasiliensis*.² At higher concentrations, although there was in general a greater survival of melanized cells, the differences were not statistically significant. Previous studies using the same technique have also demonstrated that melanization was able to protect *C. neoformans*, *H. capsulatum* and *Wangiella dermatitidis* against amphotericin B.^{13,29,36} These authors suggest that fungal melanin in its antioxidant role binds to amphotericin B and reduces its activity. In addition to its antifungal activity, amphotericin B is also a potent immunomodulator.²⁵ Thus, its binding to fungal melanin can also reduce the effect on host factors, such as activation of T cells and macrophages. Furthermore, melanin is also an immunologically active molecule that suppresses inflammation.^{5,22} *In vitro* studies have demonstrated that albino conidia are two-fold more highly phagocytized than the respective pigmented spores, what indicates that melanin contributes to the survival of the fungal cells in the host.³² The melanization of the fungal cells also affects the pathogenesis of cutaneous sporotrichosis because pigmented isolates showed greater ability to invade tissues than albino mutant strains in an experimental model of sporotrichosis.¹⁹

S. brasiliensis, the most virulent species of the complex,⁴ has frequently been reported in infections in Brazil, often affecting immunocompetent patients. Case reports of lung injury in HIV negative patients,²⁸ a familial outbreak in the southeastern of Brazil,²⁷ and even resistance to itraconazole treatment in animals have been reported.¹² *Sporothrix* species are also the most frequent pathogens in cases of osteoarticular infection in immunocompetent patients.³⁰

In a recent study, Rodrigues et al.³¹ confirmed that *S. brasiliensis* has a lower genetic diversity and less variability in the *in vitro* susceptibility than *S. schenckii* sensu stricto (both species are phylogenetically related), whose high genetic variation couples with a high fluctuation in the antifungal susceptibility and virulence profile.⁴ Despite the slight number of strains studied here, higher MICs for amphotericin B and greater variability in the itraconazole MICs for the strains of *S. schenckii* sensu stricto were observed. Regarding the melanin content assessed in this study, there was no apparent variation among strains of the same species. However, if a large number of strains of the same population were evaluated, it is likely that differences in the production of this molecule would be found.

Disseminated sporotrichosis is a problem especially for immunocompromised patients. These individuals require higher doses of amphotericin B for some months and often require secondary prophylaxis for an extended period of time. Melanization is essential for the virulence of *Sporothrix*, and it is evident that melanin can be synthesized in a human host and in mammalian tissues during infection by *Sporothrix* species.²³ The combination of different classes of antifungal agents has been shown to be promising,¹⁴ and can reduce the effect of melanin, since certain classes of drugs does not seem to be affected by it. In addition to this, our findings reinforce the importance of combination studies of amphotericin B with potent inhibitors of melanin synthesis for sporotrichosis infection.

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Conflict of interest

The authors have no conflict of interest to declare.

References

- Almeida-Paes R, Frases S, Fialho Monteiro PC, Gutierrez-Galhardo MC, Zancopé-Oliveira RM, Nosanchuk JD. Growth conditions influence melanization of Brazilian clinical *Sporothrix schenckii* isolates. *Microbes Infect.* 2009;11:554–62.
- Almeida-Paes R, Frases S, Araújo Gde S, de Oliveira MM, Gerfen GJ, Nosanchuk JD, et al. Biosynthesis and functions of a melanoid pigment produced by species of the *Sporothrix* complex in the presence of L-tyrosine. *Appl Environ Microbiol.* 2012;78:8623–30.
- Alviano DS, Franzen AJ, Travassos LR, Holandino C, Rozental S, Ejzemberg R, et al. Melanin from *Fonsecaea pedrosoi* induces production of human antifungal antibodies and enhances the antimicrobial efficacy of phagocytes. *Infect Immun.* 2004;72:229–37.
- 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;15:651–5.
- Avramidis N, Kourounakis A, Hadjipetrou L, Senchuk V. Anti-inflammatory and immunomodulating properties of grape melanin. Inhibitory effects on paw edema and adjuvant induced disease. *Arzneimittelforschung.* 1998;48:764–71.
- Bunce PE, Yang L, Chun S, Zhang SX, Trinkaus MA, Matukas LM. Disseminated sporotrichosis in a patient with hairy cell leukemia treated with amphotericin B and posaconazole. *Med Mycol.* 2012;50:197–201.
- Clinical and Laboratory Standards Institute (CLSI). Reference method for broth dilution antifungal susceptibility testing yeasts. Approved standard M27-A3, 3rd ed.; 2008.
- 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.
- Fonseca-Reye S, López Maldonado FJ, Miranda-Ackerman RC, Vélez-Gómez E, Alvarez-Iñiguez P, Velarde-Rivera FA, et al. Extracutaneous sporotrichosis in a patient with liver cirrhosis. *Rev Iberoam Micol.* 2007;24:41–3.
- Freitas DF, de Siqueira Hoagland B, do Valle AC, Fraga BB, de Barros MB, de Oliveira Schubach A, et al. Sporotrichosis in HIV-infected patients: report of 21 cases of endemic sporotrichosis in Rio de Janeiro. *Brazil Med Mycol.* 2012;50:170–8.
- Gutierrez-Galhardo MC, do Valle AC, Fraga BL, Schubach AO, Hoagland BR, Monteiro PC, et al. Disseminated sporotrichosis as a manifestation of immune reconstitution inflammatory syndrome. *Mycoses.* 2010;53:78–80.
- Guterres KA, de Matos CB, Osório LdaG, Schuch ID, Cleff MB. The use of (1–3) β -glucan along with itraconazole against canine refractory sporotrichosis. *Mycopathologia.* 2014;177:217–21.
- Ikeda R, Sugita T, Jacobson ES, Shinoda T. Effects of melanin upon susceptibility of *Cryptococcus* to antifungals. *Microbiol Immunol.* 2003;47:271–7.
- Ishida K, de Castro RA, Borba Dos Santos LP, Quintella LP, Lopes-Bezerra LM, Rozental S. Amphotericin B, alone or followed by itraconazole therapy, is effective in the control of experimental disseminated sporotrichosis by *Sporothrix brasiliensis*. *Med Mycol.* 2015;53:34–41.
- Jacobson ES. Pathogenic roles for fungal melanins. *Clin Microbiol Rev.* 2000;13:708–17.
- 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.
- Kwon-Chung KJ, Bennett JE. Subcutaneous and deep mycoses – sporotrichosis. *Medical mycology*, 26. Malvern: Lee & Febiger Cap; 1992. p. 707–29.
- Lloyd KO, Bitoon MA. Isolation and purification of a peptido-rhamnmannan from the yeast form of *Sporothrix schenckii*. Structural and immunochemical studies. *J Immunol.* 1971;107:663–71.
- Madrid IM, Xavier MO, Mattei AS, Fernandes CG, Guim TN, Santin R, et al. Role of melanin in the pathogenesis of cutaneous sporotrichosis. *Microb Infect.* 2010;12:162–5.
- 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.
- 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.
- Mohagheghpour N, Waleh N, Garger SJ, Dousman L, Grill L, Tusé D. Synthetic melanin suppresses production of proinflammatory cytokines. *Cell Immunol.* 2000;199:25–36.
- Morris-Jones R, Youngchim S, Gome BL, Aisen P, Hay RJ, Nosanchuk JD, et al. Synthesis of melanin-like pigments by *Sporothrix schenckii* in vitro and during mammalian infection. *Infect Immun.* 2003;71:4026–33.
- Neto RJP, Machado AAM, Castro G, Quaglio ASS, Martinez R. Esporotricose cutânea disseminada como manifestação inicial da síndrome da imunodeficiência adquirida – relato de caso. *Rev Soc Bras Med Trop.* 1999;32:57–61.
- Nosanchuk JD, Cleare W, Franzot SP, Casadevall A. Amphotericin B and fluconazole affect cellular charge, macrophage phagocytosis, and cellular morphology of *Cryptococcus neoformans* at subinhibitory concentrations. *Antimicrob Agents Chemother.* 1999;43:233–9.
- Nosanchuk JD, Casadevall A. The contribution of melanin to microbial pathogenesis. *Cell Microbiol.* 2003;5:203–23.
- Oliveira MM, Maifrede SB, Ribeiro MA, Zancope-Oliveira RM. Molecular identification of *Sporothrix* species involved in the first familial outbreak of sporotrichosis in the state of Espírito Santo, southeastern Brazil. *Mem Inst Oswaldo Cruz.* 2013;108:936–8.
- Orofino-Costa R, Unterstell N, Carlos Gripp A, de Macedo PM, Brota A, Dias E, et al. Pulmonary cavitation and skin lesions mimicking tuberculosis in a HIV negative patient caused by *Sporothrix brasiliensis*. *Med Mycol Case Rep.* 2013;16:65–71.
- Paolo WF Jr, Dadachova E, Mandal P, Casadevall A, Szanislo PJ, Nosanchuk JD. Effects of disrupting the polyketide synthase gene *WdPKS1* in *Wangiella [Exophiala] dermatitidis* on melanin production and resistance to killing by antifungal compounds, enzymatic degradation, and extremes in temperature. *BMC Microbiol.* 2006;6:55.
- Rammaert B, Gamaletsou MN, Zeller V, Elie C, Prinapori R, Taj-Aldeen SJ, et al. Dimorphic fungal osteoarticular infections. *Eur J Clin Microbiol Infect Dis.* 2014. <http://dx.doi.org/10.1007/s10096-014-2149-0>.
- Rodrigues AM, de Hoog GS, de Cássia Pires D, Brihante RS, Sidrim JJ, Gadelha MF, et al. Genetic diversity and antifungal susceptibility profiles in causative agents of sporotrichosis. *BMC Infect Dis.* 2014;14:219.
- Romero-Cabello R, Bonifaz A, Romero-Feregrino R, Sánchez CJ, Linares Y, Zavala JT, et al. Disseminated sporotrichosis. *BMJ Case Rep.* 2011. <http://dx.doi.org/10.1136/bcr.2010.3404>.
- Romero-Martinez R, Wheeler M, Guerrero-Plata A, Rico G, Torres-Guerrero H. Biosynthesis and functions of melanin in *Sporothrix schenckii*. *Infect Immun.* 2000;68:3696–703.
- Teixeira PA, de Castro RA, Ferreira FR, Cunha MM, Torres AP, Penha CV, et al. L-DOPA accessibility in culture medium increases melanin expression

- and virulence of *Sporothrix schenckii* yeast cells. *Med Mycol.* 2010;48: 687–95.
35. Torres-Rodríguez JM, Palácio-Hernanz A, Negroni-Briz R, Pereiro-Miguens M. Sporotrichosis. In: *Micología médica*. Barcelona: Marron S.A; 1993. p. 157–66 [chapter 16].
 36. van Duin D, Casadevall A, Nosanchuk JD. Melanization of *Cryptococcus neoformans* and *Histoplasma capsulatum* reduces their susceptibilities to amphotericin B and caspofungin. *Antimicrob Agents Chemother.* 2002;46: 3394–400.
 37. van Duin D, Cleare W, Zaragoza O, Casadevall A, Nosanchuk JD. Effects of voriconazole on *Cryptococcus neoformans*. *Antimicrob Agents Chemother.* 2004;48:2014–20.
 38. Yap FBB. Disseminated cutaneous sporotrichosis in an immunocompetent individual. *Int J Infect Dis.* 2011;15:727–9.
 39. Zhang X, Huang H, Feng P, Zhang J, Zhong Y, Xue R, et al. In vitro activity of itraconazole in combination with terbinafine against clinical strains of itraconazole-insensitive *Sporothrix schenckii*. *Eur J Dermatol.* 2011;21: 573–6.