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Original article

Cell-free antigens of *Sporothrix brasiliensis*: antigenic diversity and application in an immunoblot assay

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Summary

Sporotrichosis is a subcutaneous mycosis diagnosed by isolation of the fungus in culture. Serological tests for help in diagnosis in general do not use purified or recombinant antigens, because there is a paucity of described immunoreactive proteins, especially for the new described *Sporothrix* species, such as *Sporothrix brasiliensis*. This study aims to characterise antigens from *S. brasiliensis* and verify their application in serodiagnosis of sporotrichosis. An immunoblot assay allied with computer-based analysis was used to identify putative antigenic molecules in a cell-free extracts of both morphological phases of this fungus, and to delineate antigenic polymorphism among seven *S. brasiliensis* isolates and one *S. schenckii* Brazilian strain. The mycelial and yeast phase of the fungus originated 14 and 23 reactive bands, respectively, which were variable in intensity. An 85 kDa antigen, verified in the yeast phase of the fungus, was observed in all strains used and the immunodominant protein was identified. This protein, however, cross-react with serum samples from patients infected with other pathogens. The results show that the *S. brasiliensis* cell-free antigen extract is a single and inexpensive source of antigens, and can be applied on the sporotrichosis serodiagnosis.

Key words: Antigen, immunoblot, *Sporothrix brasiliensis*, sporotrichosis.

Introduction

Sporothrix schenckii is a dimorphic fungal pathogen that exists in soil, plant debris or rotting wood in the mycelial form which transforms into cigar-shaped yeast cells in a host. The disease caused by this fungus is called sporotrichosis, which results in most of cases from the traumatic inoculation of the fungus in the subcutaneous tissue of humans or other animals.[1] Nowadays, the aetiological agents of this mycotic infection belong to five well-defined species of dimorphic fungi called *S. schenckii* complex. These species comprise *S. schenckii*, *S. globosa*, *S. mexicana*, *S. brasiliensis* and *S. luriei.*[2]

Several sporotrichosis outbreaks have been reported in the literature.[3–6] Nowadays, in Rio de Janeiro State, Brazil, an outbreak, with zoonotic involvement, is occurring[7,8] and *S. brasiliensis* is the major species recovered from human patients.[9] The definitive diagnosis of this infection is performed by isolation of *S. schenckii* in culture from clinical specimens, such as tissue fragments and pus from lesions.[1] However, culture methods are often difficult to apply in unusual forms of sporotrichosis. For this reason, the detection of patients' antibody responses would offer a more rapid and alternative methodology to microbiological means of diagnosis.[10]

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Few studies have been reported with regard to characterisation of antigenic composition from different evolutive forms of members from the genus *Sporothrix*. Although some *S. schenckii* proteins have been reported as having diagnostic value,[11,12] there is still a paucity of purified and well-characterised antigens. A peptide-rhamnomannan was identified as an antigen of the yeast cell surface[11] and apparently is the component responsible for cross reactivity on serological tests. Other yeast and mycelia antigens have also been described, but their role in the infection remains unknown. Therefore, a correct assessment of the biological nature of some antigens and the individual capacities of these antigens to evoke active immunity needs to be demonstrated.

Cell-free antigens were described by Camargo *et al.* [13] for the serodiagnosis of paracoccidioidomycosis. They are comprised of non-covalent binding proteins in the surface of the fungal cell wall and make up an arsenal of proteins which is readily available for the immune system's action. This study was performed to evaluate seven *S. brasiliensis* and one *S. schenckii* cell-free antigens extracts from both morphological phases of the fungus for their antigenic differences and to investigate their applicability on the serodiagnosis of sporotrichosis.

Material and methods

Ethics

This study was approved by the Research Ethics Committee of IPEC/Fiocruz (CAAE: 0019.0.009.000-09).

Strains

Eight *Sporothrix* strains were included in this study (Table 1). The seven *S. brasiliensis* strains were from the zoonotic epidemic of sporotrichosis in Rio de Janeiro State, Brazil. The *S. schenckii* strain was isolated in Espírito Santo State, Brazil, from a patient with the limphocutaneous form of sporotrichosis which became infected while working with soil. Species identification of these strains was performed by phenotypic and molecular approaches as described on previous studies.[9,14] To maintain the strains in the mycelial phase, fungi were cultured in Sabouraud Agar (Becton Dickinson and Company, Sparks, MD 21152, USA) at 25 °C whilst the yeast form was obtained by culturing strains in Brain Heart Infusion Agar (Becton, Dickinson and Company) supplemented with 0.5% yeast extract (Fluka Analytical, Buchs, Germany) at 36 °C. Strains are maintained at the Colecao de Fungos Patogenicos (WDCM 951), Fundacao Oswaldo Cruz.

Table 1 *Sporothrix* strains analysed in this study.

Strain	Source	Clinical form	Species
19536	Human	Fixed cutaneous	*S. brasiliensis*
17331	Human	Disseminated cutaneous	*S. brasiliensis*
22582	Human	Extracutaneous	*S. brasiliensis*
22932	Human	Limphocutaneous	*S. brasiliensis*
23252	Human	Limphocutaneous	*S. schenckii*
23508	Environmental	Not applied	*S. brasiliensis*
1468-1	Animal	Cat with several lesions	*S. brasiliensis*
2834	Animal	Cat with a single lesion	*S. brasiliensis*

Antigens

The cell-free antigens from both morphological phases of *S. schenckii* and *S. brasiliensis* were prepared with the same protocol used by Camargo *et al.* [13] to obtain antigen extract from *Paracoccidioides brasiliensis*. In brief, fungi with 5 days of growth for the yeast phase and 7 days for the mycelial phase were suspended in a proportion of 300 mg of fungal cells to 1 ml of sterile distilled water in a microcentrifuge tube, mixed for 30 s in a Vortex mixer and immediately centrifuged at $12\,000 \times g$ in a Eppendorf 5415C centrifuge for 1 min. The supernatant fluid containing the cell-free antigens had their protein content determined by the method of Bradford.[15]

Polyclonal antiserum

Rabbit hyperimmune antiserum was prepared by priming injection with an antigen obtained by mechanical disruption using glass beads of *Sporothrix* yeast cells in two male white rabbits. The first immunisation was made with complete Freud's adjuvant (Sigma Chemical CO., St. Louis, MO 63178, USA); the second immunisation was made with incomplete Freud's adjuvant (Sigma Chemical CO.) 15 days after the first immunisation. The last boost was given 30 days after the priming boost and was performed without adjuvant. Four days after the third immunisation, rabbits were bled and serum was collected after clot formation.

SDS-PAGE and Immunoblot

The cell-free antigens (1.0 μg protein of mycelial antigens and 2.5 μg protein of yeast antigens) were first dissociated at 100 °C for 5 min in 0.125 mol l^{-1} Tris–

HCl buffer (pH 6.8) containing 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol and 0.025% bromophenol blue. SDS-polyacrylamide gel electrophoresis was then conducted with 7.5% polyacrylamide separating and 4% polyacrylamide stacking gels in an electrophoresis cell (Mini-Protean II; Bio-Rad Laboratories, Richmond, CA, USA). The different antigenic concentrations loaded into gels were optimised as the concentration resulting in higher number of bands with lower background after gel staining. Electrophoresis was conducted at 10 mA constant current for stacking and at 20 mA for protein separation. Gel contents were electrotransferred to nitrocellulose membranes in a Mini Trans-Blot cell (Bio-Rad) containing transfer buffer with 25 mmol l^{-1} Tris–HCl, 192 mmol l^{-1} glycine and methanol [20% (vol/vol); pH 8.3] and operated at 397 mA for 1 h. Free binding sites in the membranes were blocked by incubation for 30 min in 5% (wt/vol) non-fat dry milk in 20 mmol l^{-1} Tris–HCl-500 mmol l^{-1} NaCl-0.2% Tween 20 (pH 7.5) (TTBS). Membranes were incubated for 60 min at room temperature with the rabbit polyclonal antiserum diluted 1/100 in TTBS containing 5% non-fat milk. This dilution was determined after checking different antisera dilutions (from 1/50 to 1/1000), and it was the dilution giving best band resolutions and low background. Membranes were washed in TTBS four times for 5 min each; then goat anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories Inc. West Grove, PA 19390, USA) diluted in TTBS were added and incubated as described above. Blot membranes then were washed and incubated with substrate solution consisting of 5-bromo-4-chloro-3-indolylphosphate [BCIP; 15 mg ml^{-1} in dimethylformamide (DMF)] and nitroblue tetrazolium (NBT; 30 mg ml^{-1} in 70% aqueous DMF). Substrate stock solutions were diluted 1:100 before use in Tris/NaCl buffer [100 mmol l^{-1} Tris–HCl (pH 9.5), 100 mmol l^{-1} NaCl, 50 mmol l^{-1} $MgCl_2$]. After colour development membranes were rinsed exhaustively in deionised water.

Two-dimmensional electrophoresis analysis

Isoeletric focusing was performed according to OFarrel.[16] Gels were loaded with 300 μg protein of the yeast cell-free antigens in sample buffer containing 9.5 mol l^{-1} urea, 1.6% (vol/vol) ampholines 5.0–8.0, 0.4% (vol/vol) ampholines 3.5–10.0, 2% (vol/vol) NP-40, 5.0% (vol/vol) β-mercaptoethanol. The 11 cm strips (pH 3–10) were treated for 30 min with equilibration buffer [0.08 mol l^{-1} Tris–HCl pH 6.0, 5% (vol/vol) β-mercaptoethanol, 2.3% (wt/vol) SDS, 1% (vol/vol) glycerol, 0.01% (wt/vol) bromophenol blue]. The second dimension was performed on a 5–15% gradient 10 cm polyacrylamide gel, according to Laemmli[17] after the treatment of the strips on denaturation buffer (62.5 mmol l^{-1} Tris–HCl pH 6.8, 5% β-mercaptoethanol, 0.5% SDS, 5% glycerol, 0.05% bromophenol blue). Proteins from three different gels were silver stained and other three gels were transferred to nitrocellulose membranes for immunoblot analysis, using a pool of sera from patients with sporotrichosis, as well as histoplasmosis, paracoccidioidomycosis and American Tegumentary Leishmaniasis.

Human sera and Immunoblot assay for sporotrichosis

Twenty-eight serum specimens obtained from different patients with sporotrichosis were also incorporated in this study. All these patients have their diagnosis based on the isolation of *Sporothrix* spp. in culture and presented the fixed cutaneous (n = 9, 32.1%), lymphocutaneous (n = 15, 53.6%), disseminated cutaneous (n = 3, 10.7%) and extracutaneous (n = 1, 3.6%) forms of sporotrichosis. A total of 40 heterologous serum samples from patients with culture-proven diseases, such as histoplasmosis (n = 10, 25%), paracoccidioidomycosis (n = 10, 25%), American tegumentary leishmaniasis (n = 10, 25%), aspergillosis (n = 5, 12.5%), cryptococosis (n = 2, 5%), coccidioidomycosis (n = 2, 5%) and chromoblastomycosis (n = 1, 2.5%) were also included in this study. Sera from homologous and heterologous individuals were collected before the beginning of specific therapy and none of patients were submitted to sporotrichin skin test. All these clinical samples were chosen randomly and were obtained from the Immunodiagnostic Section Serum Bank, Mycology Branch, IPEC, Fiocruz, where they were stored at −20 °C until use. In addition, 10 serum samples from healthy blood donors, showing no detectable antibody levels to HIV, HBV, *Trypanossoma cruzi* or *Treponema pallidum* were included in this study as negative controls. All serum specimens were collected from individuals living in Rio de Janeiro City, Brazil, an endemic region of sporotrichosis.[1,8] Immunoblot assay was developed using vertically sliced membranes containing yeast-phase *S. brasiliensis* cell-free antigens. Strips were incubated for 60 min at room temperature with serum specimens diluted 1/100 in TTBS containing 5% non-fat milk. Strips were washed in TTBS four times for 20 min each; then goat anti-human IgG-alkaline phosphatase conjugates (Sigma Chemical Co.) diluted in TTBS were added and incubated as described above. Blot strips were washed and incubated with

substrate solution as described before. After colour development, strips were rinsed exhaustively in deionised water.

Computer analysis

The revealed immunoblots were digitalised using an HP ScanJet 5500C scanner. The images were analysed using the ImageQuant TL software, version 2003.03 (GE Healthcare, Piscataway, NJ, USA) for molecular weight and intensity of each presented band.

Results

Mycelial cell-free antigens

The polyacrylamide gel with the eight mycelial *Sporothrix* cell-free antigens presented protein bands with molecular weights ranging from 220 to 40 kDa (Fig. 1a). Computacional analysis with the ImageQuant TL software showed that the polyclonal rabbit antiserum revealed by the immunoblot assay 14 antigenic protein bands, with molecular mass of 240, 230, 220, 203, 150, 143, 135, 90, 85, 81, 76, 70, 60 and 40 kDa (Fig. 1b). The number of individually recognised bands in each strain ranged from 9 (23508 strain) to 12 (22582 strain). The 230, 220, 135 and 85 kDa bands were observed in all strains included in this study. The 40 kDa band was observed in six strains and, after computacional analysis, differences in intensity of bands were observed among them. This experiment was repeated twice, with similar results.

Yeast cell-free antigens

The protein profile of the antigenic extract obtained from the yeast phase of the eight *Sporothrix* was quite similar (Fig. 2a), with protein bands of 220–20 kDa. However, the immunoblots showed variable patterns of immunoreactivity among the strains included in this study. The rabbit polyclonal antiserum revealed 23 IgG-reactive glycoprotein bands with apparent molecular weight of 240, 203, 186, 173, 152, 140, 130, 121, 112, 103, 97, 91, 85, 81, 72, 64, 60, 58, 55, 50, 47, 44 and 38 kDa (Fig. 2b). The cell-free antigenic extract from *S. schenckii* 23252 showed the highest number of reactive bands (22 bands). However, the intensity of these bands was smaller than those originated from other strains as shown by the analysis generated with the ImageQuant TL software (data not shown). The

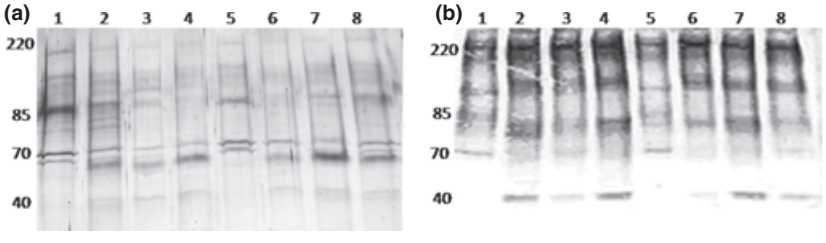

Figure 1 Protein pattern (a) and immunological reactivity (b) of *S. schenckii* and *S. brasiliensis* mycelial form cell-free antigens obtained from the following *Sporothrix* strains: lane (1) 19536; lane (2) 17331; lane (3) 22582; lane (4) 22932; lane (5) 23252; lane (6) 23508; lane (7) 1468-1; lane (8) 2834. Molecular weights (in kDa) are indicated at left.

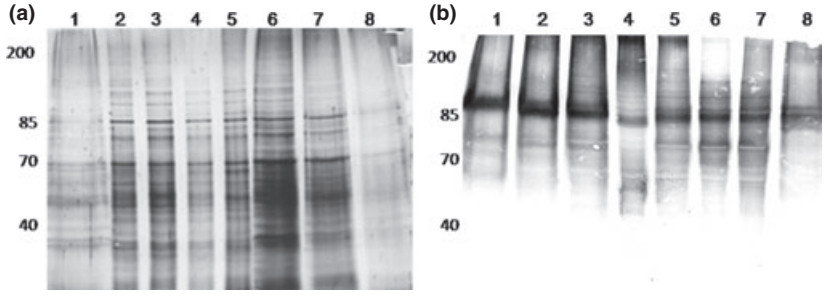

Figure 2 Protein pattern (a) and immunological reactivity (b) of *S. schenckii* and *S. brasiliensis* yeast form cell-free antigens obtained from the following *Sporothrix* strains: lane (1) 19536; lane (2) 17331; lane (3) 22582; lane (4) 22932; lane (5) 23252; lane (6) 23508; lane (7) 1468-1; lane (8) 2834. Molecular weights (in kDa) are indicated at left.

2834 and the 19536 strains presented only seven immune-reactive bands. The 85-kDa antigenic band was observed in all strains and corresponded to the most reactive antigenic band in all strains used in this study as observed visually and by computer analysis. This experiment was repeated twice and the same bands were observed in all SDS-PAGE runs.

Two-dimensional analysis of yeast phase cell-free antigens

As the yeast-phase *S. brasiliensis* free-cell antigens presented an elevated number of protein bands on the SDS-PAGE analysis, we performed a two-dimensional analysis of these proteins. When submitted to this assay, around 154 spots were observed on three different runs of the yeast cell-free antigens from *S. brasiliensis* strain 22582 (Fig. 3a). The most prominent spots were those of 85 kDa (pI 6.1), 70 kDa (pI 5.0), 60 kDa (pI 4.8), 50 kDa (pI 5.8), 39 kDa (isoforms of 5.3 and 5.4), 35 kDa (pI 4.6), 30 kDa (pI 4.8), 25 kDa (pI 5.0) and 13 kDa (pI 7.1). The other strains presented similar two-dimensional electrophoresis patterns. When the proteins were transferred to a nitrocellulose membrane, 93 reactive protein spots to antibodies present in a pool of sera from patients with sporotrichosis could be observed (Fig. 3b). We were able to find spots in the same range of molecular weight found in the previous unidimensional immunoblot assays with variable intensity. It was observed that proteins with high molecular weight showed lower intensity than the other proteins. Seven of the reactive protein spots were also reactive against normal human sera (Fig. 3c), and other proteins presented cross-reactions with sera from other infections: 35 reacted slightly with histoplasmosis sera (Fig. 3d), fourteen spots reacted with paracoccidioidomycosis sera (Fig. 3e), and 26 reacted with leishmaniasis sera (Fig. 3f). We could clearly identify seven proteins from the 93 *S. brasiliensis* reactive spots that did not present cross-reactions (Fig. 3b). Their molecular weight and isoelectric point are presented on Table 2.

Immunoblot for diagnosis of sporotrichosis

The cell-free antigens from yeast phase 22582 *S. brasiliensis* strain were probed with individual serum from patients with sporotrichosis and other infections. This strain was chosen for this purpose because it yielded an antigenic extract with several immunologically reactive proteins, with good recognition by the rabbit polyclonal antiserum. This experiment was performed three times and we observed 13 IgG-reactive bands on the analysed strips (Fig. 4). The 85-kDa was the immunodominant antigen, presenting 100% reactivity with the homologous sera, but also reacted with 9% heterologous sera. Most antigens cross-reacted with antibodies presented in sera from patients with other mycotic infections, however, the 131- and 120-kDa molecules appear to be specifically recognised by antibodies presented in sera from patients with sporotrichosis. The 120-, 112- and

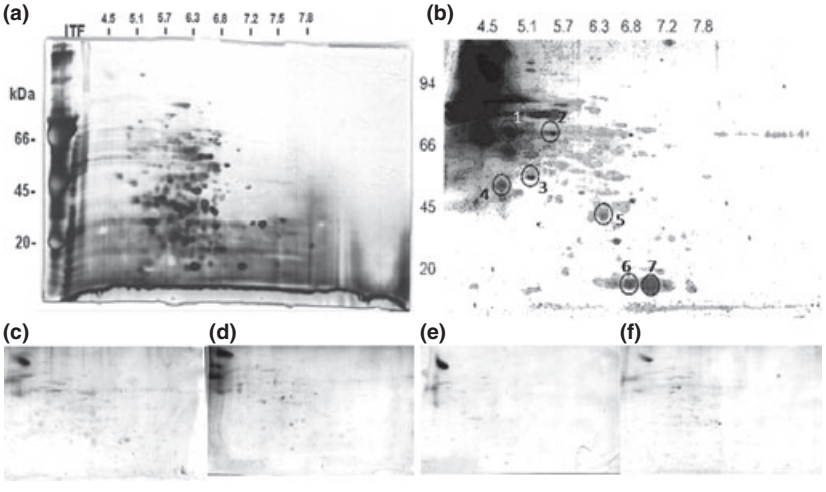

Figure 3 Two-dimensional electrophoresis of yeast *S. brasiliensis* cell-free antigens: (a) Silver-stain of cell-free antigens from yeast phase of 22582 strain, where 154 spots can be observed. (b) Western-blot of the same antigenic extract probed with sera from patients with sporotrichosis, 93 reactive spots can be observed. Outlined numbered spots do not present cross-reactions and have good response against homologous sera (Table 2). Cross-reactions of yeast-phase *S. brasiliensis* antigens detected by western-blots probed, from left to right, with normal human (c), histoplasmosis (d), paracoccidioidomycosis (e) and leishmaniasis (f) serum samples.

Table 2 *Sporothix brasiliensis* specific proteins identified on the two-dimensional electrophoresis.

Spot number	Molecular weight (kDa)	Isoelectric point
1	70	5.0
2	70	5.5
3	53	5.1
4	50	4.7
5	42	6.4
6	13	6.8
7	13	7.1

103-kDa antigens also reacted with several homologous sera and their reactivities with heterologous sera were lower than the observed with the 85-kDa antigen. Proteins with molecular weight lower than 85-kDa reacted with a few homologous sera (Table 3). It is interesting to note that using this methodology, antigens of 72-, 55-, 50- and 40-kDa were recognised by some, but not all, individual human sera included in this study. These antigens are about the same molecular weight of spots 1–5 observed on 2D Western Blots (Fig. 3B). This methodology presented 100% sensitivity, but only 50% specificity when a single band was visible on the strips of a positive serum. On the other hand, if we considered as positive a serum that reacted with at least two different antigenic proteins, we obtained 92.9% sensitivity; 80% specificity and 84.6% efficiency.

Discussion

Sporotrichosis is a cosmopolitan disease, and is considered the most prevalent subcutaneous mycosis in Latin America.[18] The gold standard method for the diagnosis of this infection is culture, but the time necessary for fungal growth and identification can be up to 3 weeks.[1] In addition, in cases of extracutaneous disease, collection of clinical specimens may be difficult, requiring invasive procedures. Obtaining clinical specimens is also not possible in cases of spontaneous regression of lesions. Consequently, the establishment of serological methods for serodiagnosis and follow-up of therapy in sporotrichosis is highly necessary.

In the 1970s and 1980s, many serologic tests for the diagnosis of sporotrichosis were described, such as immunodiffusion, immunoelectrophoresis, complement fixation, latex and tube agglutination.[19–21] However, these tests used crude antigenic preparations, without adequate standardisation, which can contribute to a lack of reproducibility. As we observed in this study, the use of different strains to prepare antigens results in extracts with distinct antigenic profiles, and maybe this can cause discordant results in different laboratories.

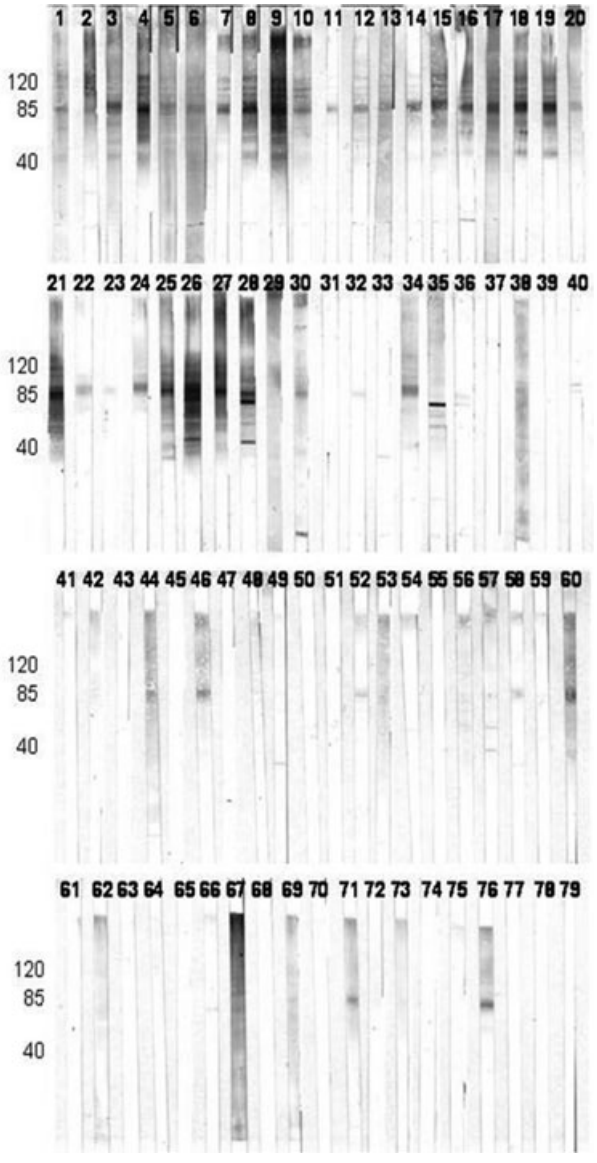

Figure 4 Immunological reactivity of IgG antibodies present in 28 serum samples from patients with sporotrichosis (lanes 1–28) and in heterologous sera (lanes 29–78) to the yeast *S. brasiliensis* cell-free antigens. Heterologous sera correspond to patients with coccidioidomycosis (lanes 29 and 30), paracoccidioidomycosis (lanes 31–40), histoplasmosis (lanes 41–50), leishmaniasis (lanes 51–60), aspergillosis (lanes 61–65), chromoblastomycosis (lane 66), cryptococcosis (lanes 67 and 68) and healthy individuals (lanes 69–78). Lane 79 corresponds to the negative control, where no serum was applied after strip blocking.

In a recent study, it was demonstrated that the *S. schenckii* strains from the Rio de Janeiro outbreak of sporotrichosis present more than 85% similarity in DNA analysis with the RAPD technique.[22] However, the protein and antigenic profiles encountered in this study

Table 3 Number of positive IgG antibodies reactions to proteins present on the yeast-phase *S. brasiliensis* cell-free antigens.

Protein[1]	Sporotrichosis *n* = 28	PCM[2] *n* = 10	HC[3] *n* = 10	Leish[4] *n* = 10	Other Mycoses[5] *n* = 10	Healthy individuals *n* = 10
186	0	0	0	0	1	0
131	4	0	0	0	0	0
120	16	0	0	0	0	0
112	23	3	0	0	1	1
103	25	3	0	0	2	0
91	9	2	0	0	0	0
85	28	3	3	6	1	4
81	3	2	0	0	2	0
72	11	1	1	1	0	0
60	14	1	1	3	0	0
55	6	1	0	0	0	0
50	15	1	0	1	0	0
40	1	1	1	0	1	0

[1]Molecular weight (kDa).

[2]Paracoccidioidomycosis.

[3]Histoplasmosis.

[4]American tegumentary leishmaniasis.

[5]Aspergillosis, coccidioidomycosis, cryptococcosis and chromoblastomycosis.

among the seven strains from this geographical area were somewhat different. This observation may be explained by the fact that DNA duplication in eukaryotic organisms is a very controlled mechanism whereas protein synthesis is a highly dynamic process, which could be affected by diverse factors. This difference in DNA and protein polymorphism was previously verified in other pathogenic fungi, such as *Histoplasma capsulatum*[23] and *P. brasiliensis.*[24,25] Therefore, the results of this study, connected with previous results from our group,[22] are in accordance with other fungal models.

The *S. schenckii* 23252 strain, from Espírito Santo State, where any zoonotic outbreak of sporotrichosis has occurred up to now, demonstrated similar antigenic pattern to the antigenic extracts obtained from *S. brasiliensis* strains, suggesting that the polymorphic variations on the antigenic protein profiles are not associated to the geographical distribution of *Sporothrix* nor to the differences observed on the transmission pattern of sporotrichosis on these Brazilian States. Then, according to our results, different *Sporothrix* species may present similar antigenic profile.

In this study, we did not verify similar profiles when the mycelial and yeast cell-free antigens of the same strain were analysed. Comparable results were observed in a similar study describing protein patterns of *P. brasiliensis* in both morphological forms.[24] The *S. schenckii* yeast form yielded an antigenic extract with more reactive bands than the mycelial form of the studied strains. These results might be associated with the life cycle of this fungus. Yeast is the parasitic form of the members of the *S. schenckii* species-complex and therefore may expose more antigenic determinants than the mycelial form into the host. In addition, we cannot exclude the utilisation of the rabbit antiserum against *S. schenckii* yeast phase on our analyses.

At least three mycelial antigens (230-, 220- and 76-kDa molecules) were recognised by the rabbit antiserum produced against the yeast *S. schenckii* form. However, they were not recognised on the immunoblot probed against yeast antigens. In a work with monoclonal antibodies (mAbs) against the M antigen of *H. capsulatum*, Hamilton and co-workers found that the mAbs against a 70–75 kDa protein were also able to recognise high molecular mass bands at 230-kDa, that were probably an intact catalase molecule and also molecules of 190- and 130-kDa, that probably represent molecules comprising either two or three subunits, or proportions thereof.[26] We believe that a similar event occurred in our analyses. Antibodies raised against a subunit of a molecule present in the yeast phase of the fungus were able to recognise a larger molecule expressed only in the mycelial phase of *S. brasiliensis*. It is interesting to note that one of our molecules also have 230-kDa, as the intact catalase recognised by the mAb against the 70-kDa M antigen present in histoplasmin.[26] As there are no studies about *Sporothrix* catalases, we cannot confirm that the 230-kDa molecule recognised on the mycelial antigen is a catalase. Indeed, our results suggest different protein expression patterns on both morphological phases of *S. brasiliensis*.

The 85-kDa protein was the major constituent among the proteins identified on the cell-free extract obtained from the *S. brasiliensis* yeast phase, and could be considered an immunodominant antigen of this species. Our two-dimmensional electrophoresis gel also demonstred a spot around this molecular weight with an isoeletric point of 6.1 that was strongly reactive with antibodies present in sera form patients with sporotrichosis. Moreover, an 85-kDa protein that reacted with polyclonal *S. schenckii* antiserum was observed in all mycelial strains, although we cannot conclude if the 85-kDa presented in the mycelial cell-free extract is the same 85-kDa antigen found in the yeast extract. It is interesting to note that this antigen could be observed also on the 23252 *S. schenckii* strain, suggesting that this is a very conserved protein on the *S. schenckii* complex. An important rhamnomannan with an 84-kDa molecular weight present in *S. schenckii* cell wall was described previously,[11] which was the main anti-

genic component. The biological nature of this antigen could be the same as 85 kDa identified in our immunoblots. In addition, the 60 kDa protein could be the same 58 kDa antigen previously observed.[11] Recently a glycoprotein of 70-kDa involved in fungal adherence to dermal extracellular matrix was isolated[27] and we could observe on our analysis a *Sporothrix* cell-free reactive spot with a similar isoelectric point of the described protein (pI 4.1) and also two other specific spots of pI 5.0 and 5.5 that could be isoforms of the described *S. schenckii* adhesin. An antigenic spot of 70-kDa and a pI of 5.0 was also detected on cell wall analysis of *S. schenckii*[28] Therefore, the purification and characterisation of biological nature of these antigens, especially the 85-kDa molecule, is essential to confirm these hypotheses.

The antigenic composition of *S. schenckii* is poorly understood, as there is a paucity of described antigens. The two-dimensional electrophoresis of proteins has been demonstrated as a very good approach to study the antigenic composition of this fungus.[28] With this methodology we could identify seven proteins of high-*S. brasiliensis* specificity from the yeast cell-free antigens, that present several bands after SDS-PAGE. Antigens about the same molecular weight of proteins 1–5 (Fig. 3B) were also detected on immunoblots performed with individual human sera (Fig. 4), indicating that they can be assessed by both SDS-PAGE or 2D electrophoresis. The 13-kDa molecules (spots 7 and 8 of Fig. 3B) were not observed on SDS-PAGE derived immunoblots, probably because differences in gel concentrations between these two methodologies. Therefore, the use of gradient gels, even for SDS-PAGE analysis is strongly recommended for analysis of *S. brasiliensis* antigens. These antigens could be used in the future, after their appropriate purification and characterisation, in the serodiagnosis of sporotrichosis.

The cell-free antigens were first described by Camargo *et al.* [13] for use in immunodiffusion test for serodiagnosis of paracoccidioidomycosis, and latter their application in a immunoblot assay was demonstrated, where sera from patients with the chronic form of paracoccidioidomycosis recognised up to 23 bands and with acute paracoccidioidomycosis around 30 antigens.[29] Some *S. brasiliensis* antigens, especially the 131 and 120 kDa proteins presented as very specific antigens when probed with individual serum samples from patients with sporotrichosis and other mycosis. Therefore, they can also be used, isolated or conjugated, in immunoassays for sporotrichosis. Preparation of this antigen is easy, fast and requires less reagents and materials than other preparations, such as mechanically disrupted citoplasmic antigens. We also did not observe in the immunoassay described in this work differences in pattern recognition among patients with different clinical forms of sporotrichosis, in accordance to the results observed in the ELISA assay using mycelial form *S. schenckii* exoantigens.[10] Sensitivity and specificity values of the immunoblot test described in this work were calculated when a sample is considered positive if at least two proteins are reactive in the assay and are in accordance with other new described immunoassays for the serodiagnosis of sporotrichosis.[10,30,31]

Antigenic variations in *S. schenckii* protein composition were already described when different culture times were used to prepare antigenic extracts from a single strain.[32] The present study showed that the use of different strains or even the morphological form of *Sporothrix* isolates could have an effect on the antigenic reactivity of a *Sporothrix* extract. Therefore, an adequate standardisation of sporotrichin antigens must be made before their use in the serodiagnosis of sporotrichosis. We also find that the use of yeast cell-free antigens of *S. brasiliensis* are an easy source of antigenic molecules that can be applied on the serodiagnosis of sporotrichosis, presenting good specificity and sensitivity. In the future, purified molecules can be used as a pool of antigens for better results on the diagnosis of this infection.

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Conflicts of interest

None.

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