

Short Communication

## A comparative serological study of the SsCBF antigenic fraction isolated from three *Sporothrix schenckii* strains

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A previous work showed that an antigenic fraction of *Sporothrix schenckii*, SsCBF, was specifically recognized by IgG antibodies present in patients' clinical specimens. The objective of this investigation was to compare the reactivity of this antigen recovered from three *S. schenckii* strains. ROC curve analysis revealed a variation in the sensitivity and specificity of the antigen derived from each of the strains, with a higher AUC for strain 1099-18. Moreover, the presence of the main O-glycosidically-linked epitopes described in the SsCBF fraction was ascertained. A significant reduction in SsCBF reactivity of all the strains was observed after  $\beta$ -elimination confirming the presence of O-glycan epitopes. The antigen isolated from strain 1099-18 proved to be a more accurate diagnostic tool for serodiagnosis of sporotrichosis.

**Keywords** Sporotrichosis, serology, *Sporothrix schenckii*, SsCBF antigen, ROC analysis

### Introduction

Sporotrichosis, a deep mycosis caused by the dimorphic fungus *Sporothrix schenckii*, is the most common subcutaneous mycosis in Latin America [1]. The infection usually occurs as a consequence of traumatic implantation of the fungus into the subcutaneous tissue and has several clinical presentations, the most frequent being the lymphocutaneous form [2]. Epidemics and microepidemics of sporotrichosis have been described throughout the world [3,4] and frequently have a natural source of infection

such as contaminated moss, wood, hay, etc. [5,6]. Until the 1980s, the feline sporotrichosis was considered rare. Recently, in Rio de Janeiro, Brazil, a zoonotic outbreak of sporotrichosis was detected involving humans and cats. The number of cases of sporotrichosis caused by cats has been increasingly reported [7].

The classic diagnosis of sporotrichosis (gold standard) relies on isolating the organism from the site of infection. However, this is an invasive method, especially when applied to the extracutaneous form of the disease [2]. Furthermore, rapid and accurate molecular and immunochemical tests used for the diagnosis of numerous infectious diseases is lacking for sporotrichosis [8].

In a previous study, our group described the SsCBF antigen expressed on the cell wall of *S. schenckii* yeast cells (strain 1099-18) [9]. This antigen was used to develop an ELISA test that showed a sensitivity and global efficiency of 90% and 86%, respectively. This serological test has proven to be a valuable method for the differential diagnosis of all clinical forms of sporotrichosis [10].

Received 12 August 2008; Final revision received 12 November 2008; Accepted 16 December 2008

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One critical parameter for determining a good diagnostic antigen is whether it is species-specific and not just expressed by a single strain. The goal of the present work was to compare the reactivity of the SsCBF antigenic fraction isolated from three strains of *S. schenckii*.

## Materials and methods

### Strains of *S. schenckii*

Three strains of *S. schenckii* (1099-18, BH-Y and BH-1) were used in this study. Strain 1099-18 was originally obtained from the Mycology Section, Department of Dermatology, Columbia University, New York, USA. The other strains were obtained from the Mycology Section of the Federal University of Minas Gerais, Brazil.

### Serum samples

A group of 132 patients with confirmed sporotrichosis (gold standard) prior to treatment participated in this study. The samples were collected between March 1998 and October 2005 at the Pedro Ernesto University Hospital of the State University of Rio de Janeiro and the Evandro Chagas Clinical Research Institute, Fiocruz, Brazil. A control group of serum samples was also used to construct the Receiver Operating Characteristic (ROC) curve. Serum samples of health individuals ( $n=23$ ), other mycoses ( $n=77$ ) (aspergillosis, cryptococcosis, histoplasmosis, chromoblastomycosis, paracoccidioidomycosis) and other pathologies unrelated to fungi ( $n=10$ ) were also examined, providing a total of 242 individual serum samples.

### Isolation of the SsCBF fraction

The SsCBF antigen was obtained by affinity chromatography of the cell wall peptidorhamnomannan (CWPR) from the yeast cell as previously described [9]. Briefly, the CWPR was applied to a Con A-sepharose 4B column equilibrated with phosphate-buffered saline (PBS) at pH 7.4. The unbound material, SsNBF, was eluted with PBS and then was desalted in a Bio-Gel P2 column. The Con A-sepharose 4B column bound material, labeled as SsCBF, was eluted with 0.1 M methyl- $\alpha$ -D-mannopyranoside in PBS and was further fractionated in a Bio-Gel P4 column. The void volume was collected and lyophilized for later analysis.

### $\beta$ -elimination of the SsCBF fraction

The SsCBF fraction from three isolates was subjected to mild alkaline hydrolysis ( $\beta$ -elimination) with 0.1 N NaOH

under reducing conditions (0.3 M NaBH<sub>4</sub>). The  $\beta$ -eliminated products were fractionated on a Bio Gel P2 column (0.5×145 cm) and eluted with 0.1 N acetic acid. The chromatographic profile was determined by carbohydrate analysis using the phenol-sulfuric assay. The  $\beta$ -eliminated SsCBF (SsCBF- $\beta$ ) was isolated and its antigenicity was evaluated by ELISA using a pool of patients' sera. This serum pool showed similar positive reactivity with all antigenic preparations.

### ELISA

The ELISA using the SsCBF antigen was carried out with serum samples from sporotrichosis patients, as previously described [10]. Briefly, the wells of 96-well polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with either SsCBF or SsCBF- $\beta$  by passive adsorption overnight at 4°C. After the blocking step, the plates were incubated with each serum sample at an optimal dilution of 1:6,400. Next, the plates were incubated with goat anti-human IgG-HRP conjugate (Gibco, BRL, St Louis, MO, USA) and reacted with *O*-phenylenediamine and H<sub>2</sub>O<sub>2</sub> in sodium citrate buffer. The reaction was stopped after 20 min with H<sub>2</sub>SO<sub>4</sub> and the optical densities were determined at 490 nm in an ELISA reader (Bio-Rad). All experiments were performed in triplicate.

### ROC analysis

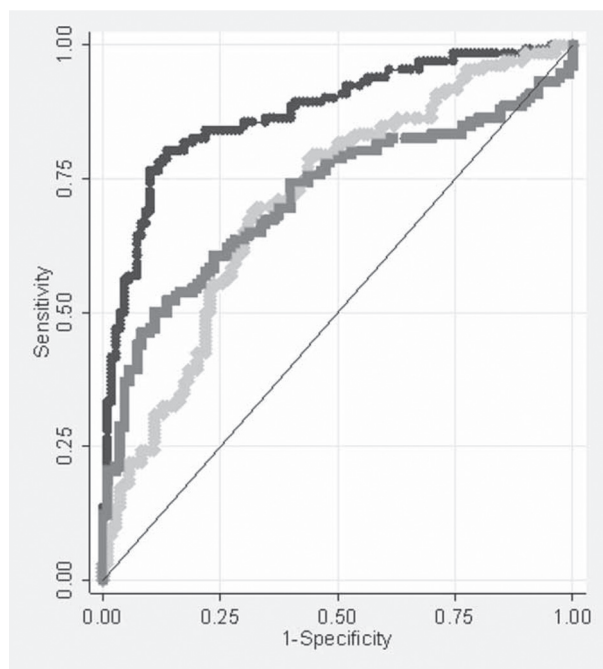
Receiver operating characteristic ROC curves (plots of sensitivity against [1-specificity]) were plotted and used to estimate the optimal cutoff for various values of sensitivity and specificity. The area under the ROC curve (AUC) was used to determine the accuracy of the test. Data analysis was performed with the Stata software, version 8.2.

### Statistical analysis

Serum level values are reported as means  $\pm$  SD. The Bonferroni *t* test was used to compare differences between groups of sera at a significance level of  $P < 0.05$ .

## Results

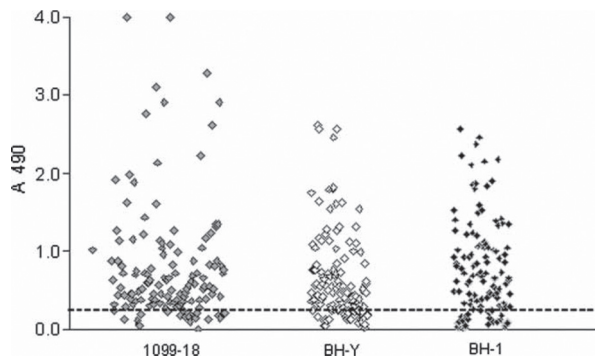
Serum samples of 242 individuals were assayed by ELISA test, as described in the methods. The ROC curve for the ELISA is shown in Fig. 1. The area under the curve (AUC) was 0.8742 (95% confidence interval (CI) 0.82978–0.91863); 0.7082 (95% CI 0.64252–0.77394); and 0.7150 (95% CI 0.64963–0.78039) for 1099-18, BH-Y and BH-1 SsCBF antigens, respectively. The ROC area was highest for the 1099-18 SsCBF antigen and no



**Fig. 1** Receiver operating characteristics (ROC) curve for the SsCBF antigen of three *Sporothrix schenckii* strains. The ROC is plotted between the true-positive rate (sensitivity) on the y-axis, and the false-positive rate (1-specificity) on the x-axis. The area under the curve (AUC) represents the accuracy of the ELISA test, which was 0.8742, 0.7150 and 0.7082 for the 1099-18 (black line), BH-1 (dark gray line) and BH-Y (light gray line) SsCBF antigens, respectively.

significant differences were observed in discriminatory power (ROC areas) between the other two SsCBF antigens. Chi-square analysis revealed statistically significant differences between the 1099-18 SsCBF antigen and the BH-Y and BH-1 SsCBF antigens ( $\chi^2=24.66$ ,  $P<0.0001$  for BH-Y and  $\chi^2=23.05$ ,  $P<0.0001$  for BH-1). Chi-square analysis between BH-Y and BH-1 SsCBF antigens showed no statistically significant difference ( $\chi^2=0.03$ ,  $P<0.8574$ ). The reactivity of the ConA nonbinding fraction, SsNBF, was also evaluated. For this fraction, the AUC was 0.3448 (95% CI 0.27580–0.41372) and 0.3810 (95% CI 0.30938–0.45259), for 1099-18 and BH-Y, respectively. The serological reactivity against the SsCBF isolated from 1099-18, BH-Y and BH-1 *S. schenckii* strains was determined by ELISA in the sera of patients with different clinical presentations of sporotrichosis (Fig. 2). The cutoff point ( $A_{490}=0.23$ ) was established by measuring and comparing the mean plus two standard deviations of the ELISA values from the sera of healthy donors.

We hypothesized that the reactivity of patient antibodies to SsCBF occurred at the main O-glycan epitopes of the antigen. To test this hypothesis, SsCBF was subjected to mild alkaline hydrolysis which removes these epitopes.



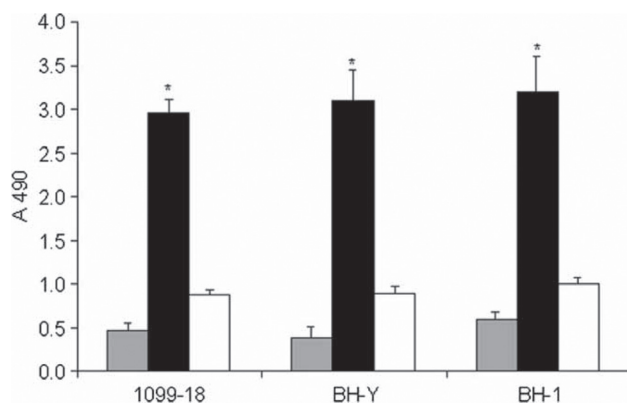
**Fig. 2** Reactivity determined by ELISA of serum specimens from 132 patients with different clinical manifestations of sporotrichosis against the SsCBF isolated from three strains of *S. schenckii*. The horizontal dashed line represents the cutoff point (0.23).

The  $\beta$ -eliminated products were analyzed by thin layer chromatography as previously described [11]. The presence of the O-linked di-, tri- tetra- and pentasaccharides in the three SsCBF fractions was ascertained by comparison of the  $R_F$  values with the known purified oligosaccharides previously described by Lopes-Alves *et al.* used as standards [12] (data not shown). The  $\beta$ -eliminated fractions, SsCBF- $\beta$ , were then assayed with either a pool of sera from patients presenting sporotrichosis or control sera from healthy individuals. The reactivity of the SsCBF- $\beta$  was compared with the untreated fraction. A significant reduction in SsCBF reactivity for all strains was observed after  $\beta$ -elimination (Fig. 3).

## Discussion

The development of new methodologies for more accurate diagnosis of infectious diseases is currently under intense investigation. Most existing diagnostic methods have significant shortcomings due to the large number of false-positive and false-negative results [13]. One of the key challenges is to identify a good pathogen-derived antigen that is not only species-specific, but also reproducible and sensitive. Another important aspect is the variation intrinsic to the strain used for antigen preparation.

Several techniques, including immunoelectrophoresis, agglutination and immunodiffusion, have been used for the serodiagnosis of cutaneous sporotrichosis. However, low specificity due to cross-reactions with other mycoses has been observed [14]. Furthermore, these techniques are less sensitive when compared to enzyme-linked immunoassays, which are promising tools in the diagnosis of numerous infectious diseases [15]. The ELISA test using the antigenic fraction SsCBF showed a sensitivity of 90% and specificity of 80% when assayed with sera from patients presenting



**Fig. 3** Reactivity of SsCBF and SsCBF- $\beta$  fractions from strains 1099-18, BH-Y and BH-1 determined by ELISA. The following groups were assayed using a pool of sera: healthy individuals assayed with SsCBF (gray bar), patients presenting sporotrichosis assayed with SsCBF (black bar) and with SsCBF- $\beta$  (white bar). \* $P < 0.05$  compared to SsCBF- $\beta$ .

several clinical forms of sporotrichosis [10]. Almeida-Paes *et al.* [16] observed a sensitivity of 97% and specificity of 89% using a mycelial phase of *S. schenckii* exoantigen in the ELISA test [16]. Although both the SsCBF and exoantigen assays gave similar reactivity, the use of exoantigen isolated from culture filtrates can be considered of limited value in serological reactions due to their heterogeneous composition, which can also impact inter-laboratory reproducibility. Previous work with a similar culture filtrate antigen of *S. schenckii* showed a significant variation in its biochemical composition, antigenic pattern and DTH reaction. The authors reported that a significant variation in this type of antigenic preparation occurs following only slight modifications in culture conditions [17]. In this case, the SsCBF antigen brings several advantages. One of them is to be a stable and reproducible antigenic preparation with well characterized epitopes [9,10].

The first aim of this study was to ascertain the inter-strain variability of the SsCBF antigen by comparing the reactivity of SsCBF derived from three strains from *S. schenckii*. Analysis of the results showed that all the strains studied expressed the SsCBF antigen, and that it reacted with similar binding characteristics to serum derived from *Sporothrix*-infected patients. This suggests that the SsCBF antigen is well conserved, a feature necessary for the development of a reliable diagnostic tool for *Sporothrix* infection.

The second aim of this study was to ascertain the role of O-glycans in determining the antigenic properties of SsCBF. Previous work has shown that reactivity against the SsCBF antigen by IgG antibodies present in sera from patients with sporotrichosis was due to the presence of the O-linked tetra- and pentasaccharides [9,12]. We therefore

subjected the SsCBF antigen to mild alkaline hydrolysis, which removes O-glycans [11]. A significant reduction in the reactivity of SsCBF- $\beta$  from all three strains showed that O-glycan residues linked to SsCBF are important determinants of host reactivity.

The data presented in this work reinforce the validity of the ELISA test using SsCBF as a new tool that should be considered during differential diagnosis of sporotrichosis.

## Acknowledgements

A.R.B.E. and L.M.L.B. were granted fellowships by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). This work was supported by Brazilian Health Ministry (E-26/170.794/2005).

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References

- da Rosa AC, Scroferneker ML, Vettorato R, *et al.* Epidemiology of sporotrichosis: a study of 304 cases in Brazil. *J Am Acad Dermatol* 2005; **52**: 451–459.
- Lopes-Bezerra LM, Schubach A, Costa RO. *Sporothrix schenckii* and sporotrichosis. *An Acad Bras Cienc* 2006; **78**: 293–308.
- Feeny KT, Arthur IH, Whittle AJ, *et al.* Outbreak of sporotrichosis, Western Australia. *Emerg Infect Dis* 2007; **13**: 1228–1231.
- Dixon DM, Salkin IF, Duncan RA, *et al.* Isolation and characterization of *Sporothrix schenckii* from clinical and environmental sources associated with the largest U.S. epidemic of sporotrichosis. *J Clin Microbiol* 1991; **29**: 1106–1113.
- Kauffman CA. Sporotrichosis. *Clin Infect Dis*. 1999; **29**: 231–236; quiz 7.
- Singer J, Muncie J. Sporotrichosis. Etiologic considerations and report of additional cases from New York. *NY State J Med*. 1952; **52**: 2147–2153.
- Barros MB, Schubach AO, Schubach TM, Wanke B, Lambert-Passos SR. An epidemic of sporotrichosis in Rio de Janeiro, Brazil: epidemiological aspects of a series of cases. *Epidemiol Infect* 2007; **21**: 1–5.
- Chan CP, Cheung YC, Renneberg R, Seydack M. New trends in immunoassays. *Adv Biochem Eng Biotechnol* 2008; **109**: 123–154.
- Penha CV, Bezerra LM. Concanavalin A-binding cell wall antigens of *Sporothrix schenckii*: a serological study. *Med Mycol* 2000; **38**: 1–7.
- Bernardes-Engemann AR, Costa RC, Miguens BR, *et al.* Development of an enzyme-linked immunosorbent assay for the serodiagnosis of several clinical forms of sporotrichosis. *Med Mycol* 2005; **43**: 487–493.
- Lopes-Alves LM, Mendonça-Previato L, Fournet B, Degand P, Previato JO. O-glycosidically linked oligosaccharides from peptidoglycanomannans of *Sporothrix schenckii*. *Glycoconj J* 1992; **9**: 75–81.
- Lopes Alves L, Travassos LR, Previato JO, Mendonça-Previato L. Novel antigenic determinants from peptidoglycanomannans of *Sporothrix schenckii*. *Glycobiology* 1994; **4**: 281–288.

- 13 Celik U, Kocabas E. New diagnostic tool of tuberculosis: interferon-gamma assays. *Tuberk Toraks* 2007; **55**: 108–117.
- 14 Casserone S, Conti-Díaz IA, Zanetta E, Peña de Pereira ME. Serology of cutaneous sporotrichosis. *Sabouraudia* 1983; **21**: 317–321.
- 15 Albuquerque CF, da Silva SH, Camargo ZP. Improvement of the specificity of an enzyme-linked immunosorbent assay for diagnosis of paracoccidioidomycosis. *J Clin Microbiol* 2005; **43**: 1944–1946.
- 16 Almeida-Paes R, Pimenta MA, Pizzini CV, *et al.* Use of mycelial-phase *Sporothrix schenckii* exoantigens in an enzyme-linked immunosorbent assay for diagnosis of sporotrichosis by antibody detection. *Clin Vaccine Immunol* 2007; **14**: 244–249.
- 17 Takata M, Ishizaki H. Correlation among culture times, sugar composition and biological activities of *Sporothrix schenckii* antigens. *Mycopathologia* 1983; **84**: 31–39.