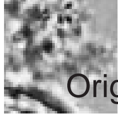

Original

Cell wall proteinaceous components in isolates of *Candida albicans* and non-*albicans* species from HIV-infected patients with oropharyngeal candidiasis

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Summary

Oropharyngeal candidiasis (OPC) remains a common opportunistic infection in HIV-infected patients. *Candida albicans* is the most frequent causative agent of OPC. However, non-*albicans* spp. are being increasingly isolated. Candidal cell wall proteins and mannoproteins play important roles in the biology and pathogenesis of candidiasis. In the present study, we have analyzed the proteinaceous components associated with cell wall extracts from *C. albicans*, *Candida tropicalis, Candida pseudotropicalis, Candida krusei, Candida glabrata, Candida parapsilosis, Candida guilliermondii* and *Candida rugosa* obtained from HIV-infected patients with recurrent OPC. Cell wall proteinaceous components were extracted with β-mercaptoethanol and analyzed using electrophoresis, immunoblotting (with antisera generated against *C. albicans* cell wall components, and with serum samples and oral saline rinses from patients with OPC), and lectin-blotting (concanavalin A) techniques. Numerous molecular species were solubilized from the various isolates. Major qualitative and quantitative differences in the polypeptidic and antigenic profiles associated with the cell wall extracts from the different *Candida* spp. were discernible. Some of the antibody preparations generated against *C. albicans* cell wall components were able to recognize homologous materials present in the extracts from non-*albicans* spp. Information on cell wall antigens of *Candida* species may be important in the therapy and prevention of HIV-related OPC.

Key words

Candida species, oropharyngeal candidiasis, cell wall proteins, antigenic composition

Componentes proteicos de la pared celular en aislamientos de especies de *Candida albicans* y no-*albicans* en pacientes infectados por el VIH con candidiasis orofaríngea

Resumen

La candidiasis orofaríngea es una infección oportunista observada comúnmente en pacientes infectados con el virus VIH. *Candida albicans* es el agente etiológico más frecuente de la candidiasis oral. Sin embargo, cada vez es más frecuente el aislamiento de otras especies del género *Candida* distintas de *C. albicans*. Las proteínas y manoproteínas de la pared celular de *Candida* desempeñan importantes funciones en la biología y patogénesis de la candidiasis. En el presente estudio, hemos analizado los componentes de naturaleza proteica presentes en extractos de pared de cepas clínicas de *C. albicans, Candida tropicalis, Candida pseudotropicalis, Candida krusei, Candida glabrata, Candida parapsilosis, Candida guilliermondii* y *Candida rugosa* obtenidos de pacientes infectados por VIH con candidiasis oral. El agente reductor β-mercaptoetanol fue utilizado para solubilizar los componentes de pared y éstos fueron analizados mediante técnicas electroforéticas, inmunoblot (con anticuerpos generados contra componentes de pared de *C. albicans* y con muestras de suero y lavados bucales de pacientes de candidiasis oral), y blot con concanavalina-A. Numerosas especies moleculares fueron extraídas de las diversas especies de levaduras. Los estu-

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dios revelaron diferencias cualitativas y cuantitativas en los patrones polipeptídicos asociados con los diferentes extractos de pared de las diferentes especies del género Candida, aunque también se detectaron ciertos materiales homólogos en varias de las diferentes especies. Información sobre antígenos de pared celular de especies del género *Candida* podría ser importante en el desarrollo de técnicas innovadoras para la terapia y prevención de la candidiasis oral en individuos inmunodeprimidos.

Oropharyngeal candidiasis (OPC) is one of the most common opportunistic infections in HIV-infected patients [1]. Fluconazole is very effective in the treatment of oral thrush, but an increasing problem is the emergence of yeast isolates, including *Candida albicans* and non-*albicans* spp., with decreased susceptibility to fluconazole [2,3]. Although isolation of *C. albicans* seems to correlate with episodes of OPC, the role and significance of *Candida* organisms other than *C. albicans* in the pathogenesis of OPC remains unclear [4-8].

In the case of *C. albicans*, it is well established that the cell wall is important for almost every aspect of the biology and pathogenicity of this fungus [9]. Cell wall proteins and mannoproteins constitute the major antigens and host recognition molecules. A number of studies from different laboratories have demonstrated a high degree of complexity associated with the protein and mannoprotein composition of this supramolecular structure [10-14]. In some instances, the identity and/or the function displayed by individual moieties have been determined. Also, it has been described that the expression, chemical characteristics, and physiological properties of these proteins and mannoproteins depend on multiple factors related to both the organism and the environment [15,16]. This variability may help the fungus evade host defense mechanisms.

Compared to the extensive information on *C. albicans*, little is known about the protein component of cell walls from other *Candida* species. Previous studies have shown both similarities and differences in the cell wall composition from non-*albicans* spp. as compared to that of *C. albicans* [17-20]. Differences in cell wall-mediated functions, such as adhesion and immunomodulation, have been reported [21-25]. However, further information is needed in order to establish how these differences correlate with the pathogenicity observed among the different *Candida* species.

The present study describes the analysis of the proteinaceous composition associated with cell wall extracts from different *Candida* spp. isolated from HIV-infected patients with OPC, with special emphasis on the study of the antigenic characteristics of such moieties. Characterization of candidal cell wall components, particularly those displaying antigenic properties, may provide the basis for the development of urgently needed alternative strategies for the global management of candidiasis, especially in the case of infections caused by fluconazole-resistant strains.

MATERIALS AND METHODS

Organisms and culture conditions. Isolates of the different *Candida* spp. were obtained by oral saline rinses (10 ml) from seven HIV-infected patients with recurrent oropharyngeal candidiasis (OPC) enrolled in a longitudinal study to assess significance of fluconazole resistance. Species identification was performed by both biochemical (API 20C) and microbiological (including germ-tube test, and color in CHROMagar plates) procedures. Fluconazole susceptibility was determined by both an agar dilution method [26] and the National Committee for Clinical Laboratory Standards (NCCLS) macrobroth procedure [27] (Table 1). Isolates were stored at room temperature as suspensions in sterile water. Working cultures were freshly prepared by subculturing the isolates onto plates containing Sabouraud dextrose agar, and kept at 4°C. For propagation as blastoconidia (yeast phase), a loopful of cells from the corresponding plate was inoculated into flasks containing the minimal medium supplemented with amino acids described by Lee and colleagues [28], and incubated for 24 h at room temperature in an orbital shaker.

Preparation of cell wall extracts. β-mercaptoethanol (β-ME) was used to solubilize protein and glycoprotein components from the walls of intact *Candida* cells as previously described [10,11,29] with minor modifications. Briefly, cells from cultures of each isolate were independently resuspended in alkaline buffer containing 1% (v/v) βME and incubated for 45 min at 37°C with gentle agitation. After treatment, the cells were sedimented, the supernatant fluid was recovered and centrifuged in a Millipore Ultrafree-15 centrifugal filter device for desalting and concentration (βME extract). The total sugar content in the different samples was determined colorimetrically with mannose as a standard [30].

Polyacrylamide gel electrophoresis and Western blot techniques. Cell wall components present in the βME extracts from the different isolates were separated by electrophoretic techniques under reducing conditions (SDS-PAGE) using precast 4-15% acrylamide gradient minigels (Bio-Rad, USA). Coomassie staining of the proteins present in the gels was performed as previously described [31]. Electrophoretic transfer to nitrocellulose membranes (Schleicher & Schuell, USA) was performed by conventional methods [32,33]. Indirect staining of glycoproteins present in the membranes with Concanavalin A was performed as previously described [34,35]. Different antibody preparations were used as probes for immunoblotting experiments: i) a pooled polyclonal antiserum generated against Zymolyase and βME cell wall extracts from *C. albicans* strain 3153A [29], ii) a polyclonal antibody preparation generated against the purified 58 kDa fibrinogen-binding mannoprotein (mp58) of *C. albicans* [36], iii) pooled serum samples, and iv) fresh pooled oral saline rinses (10 ml each) obtained from HIV-infected patients with OPC enrolled in the same study. The saline washes were centrifuged at low speed prior to their utilization in the assay. The different antibody preparations were diluted in 10 mM Tris-HCl buffer saline (pH 7.4), additioned with 0.05% Tween 20 and 1% BSA (TBSTB buffer). Anti-rabbit (IgG) or anti-human (IgG, IgM, and IgA) peroxidase-conjugated antibodies (depending on the antibody preparation used during the primary incubation) were used as indicator antibodies, with 4-chloro-1-naphtol as chromogenic reagent.

Table 1. Identity of the *Candida* isolates from patients with OPC.

Species	Patient	Fluconazole MIC (µg/ml) (NCCLS Macrobroth 48h)
C. albicans	A	4
C. tropicalis	B	16
C. pseudotropicalis	C	1
C. krusei	D	64
C. glabrata	D	8
C. parapsilosis	E	32
C. guilliermondii	F	8
C. rugosa	G	4

Miscellaneous. Gel electrophoresis reagents were from Bio-Rad Laboratories (USA). Unless otherwise stated, all other chemicals used were from Sigma Chemical Co (USA).

RESULTS

For all the *C. albicans* and non-*albicans* strains tested, treatment of intact cells with βME led to the solubilization of a complex array of cell wall proteinaceous components, exhibiting a wide range of apparent molecular masses (from >500 kDa to <20 kDa) when separated by SDS-PAGE. Coomassie blue staining (Figure 1, Panel A) allowed detection of the medium-to-low (<120 kDa) molecular mass proteins present in the different extracts and revealed major differences in the peptidic patterns associated with the βME obtained from the different *Candida* spp. In particular, the material extracted from *Candidapseudotropicalis* seemed to be enriched in low molecular mass species (<33 kDa), as compared to the extracts from the other species. The polypeptidic pattern of the extract from *Candida rugosa* seemed to be most similar to the one obtained from the *C. albicans* isolate, although this apparent similarity could not be confirmed when using more specific probes, such as antibody preparations (see below). In all cases, the high molecular material (>120 kDa) was insensitive to the dye, as has been reported previously [11,31]. Indirect Concanavalin A-peroxidase staining of the nitrocellulose blots revealed the glyco(manno)protein nature of the high molecular weight materials (HMWM) present in cell wall extracts from all species (Figure 1, Panel B). Staining with the lectin led to poorly resolved patterns due to the highly glycosylated and polydisperse nature of the HMWM [11]. However, some lower molecular weight discrete bands also reacted with the lectin. These were particularly noticeable in the cases of *Candida tropicalis* and *Candida guilliermondii*, but also for *Candida krusei* and *Candida glabrata*. A highly reactive well-defined band with an apparent electrophoretic mobility of 35 kDa was detected in the extracts from both *C. albicans* and *C. rugosa*.

Immunoblot analysis with pooled polyclonal antisera generated against components present in cell wall extracts from *C. albicans* 3153A [29], a collection strain, showed marked differences in the recognition patterns of the non-*albicans* strains (Figure 2, Panel A). As expected, high levels of reactivity were detected in the case of the homologous materials present in the cell wall extract from the *C. albicans* clinical isolate used in this study. This reactivity was detected along the whole spectrum of molecular masses. Recognition occurred with the non-*albicans* strains tested. However, a much weaker reactivity was detected for species other than *albicans*, particularly in the cases of *C. rugosa, C. glabrata* and *C. pseudotropicalis*, with the highest levels of cross-reactivity being detected in the extracts from *C. tropicalis* and *C. guilliermondii*. When a monospecific antibody generated against the purified 58 kDa fibrinogen-binding mannoprotein of *C. albicans* was used as a probe in the immunoblots (Figure 2, Panel B) strong reactivity was detected with the homologous band present in the extract from the *C. albicans* isolate. Weak reactivity with a moiety of similar electrophoretic mobility, as well as with a much higher molecular weight material (that barely entered the gel) was detected in the extract corresponding to the *C. guilliermondii* isolate. No reactivity was observed in the case of βME extracts from all other non-*albicans* isolates.

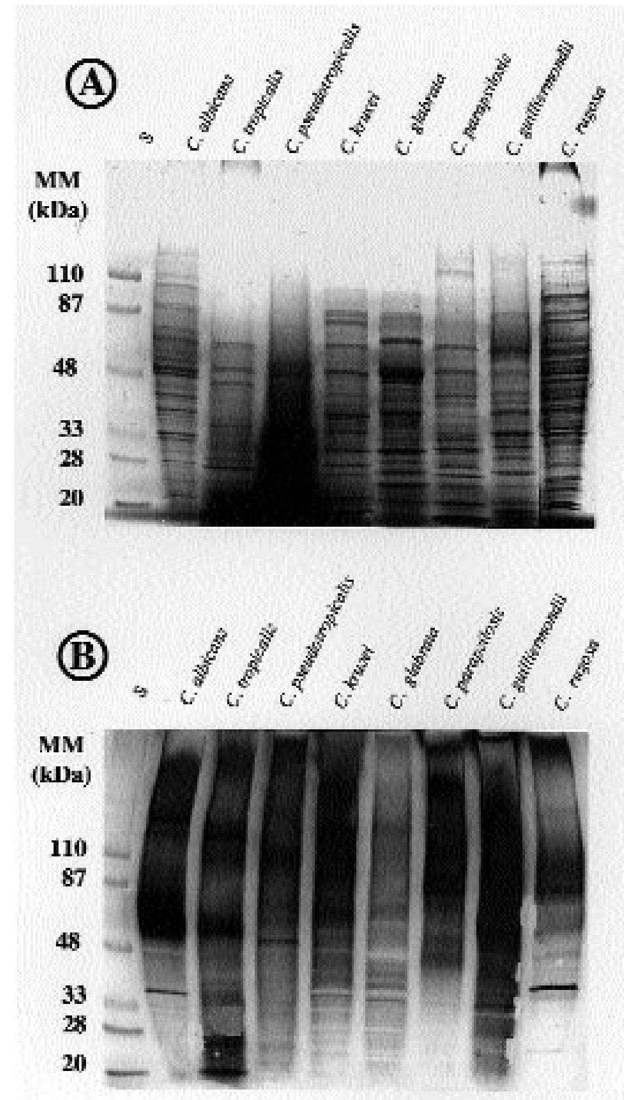

Figure 1. Coomassie blue staining (Panel A) and Concanavalin-A staining (Panel B) of the materials present in the βME cell wall extracts from the different *Candida* spp. isolated from HIV-infected patients. Cell wall components were separated by SDS-PAGE and stained with Coomassie blue directly on the gel (Panel A), or transferred to nitrocellulose paper and stained with the lectin (Panel B). The amount applied to each well, expressed as total sugar content, was 50 µg for Panel A and 25 µg for Panel B. Lanes S show the electrophoretic mobility of standard proteins of known molecular masses (MM) run in parallel.

Finally, antibody-containing serum samples and oral saline rinses from HIV-infected patients were used as probes in immunoblots of cell wall extracts from the different *Candida* spp. (Figure 3). Using pooled serum samples from patients with OPC (Figure 3, Panel A), the strongest level of reactivity was detected with the materials present

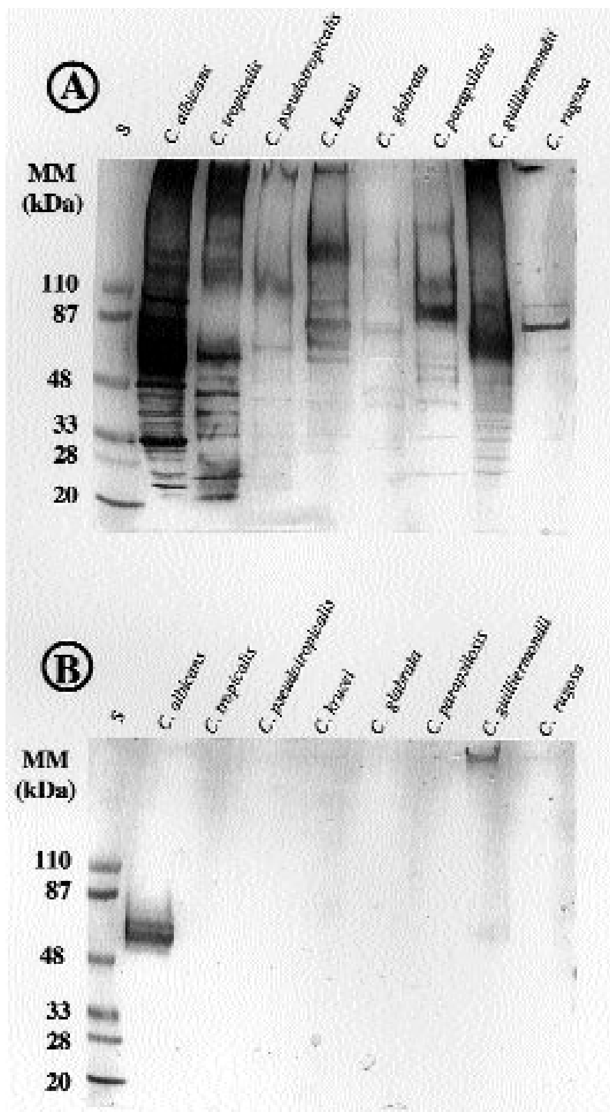

Figure 2. Western immunoblot analysis of the materials solubilized by βME from the different *Candida* spp. with pooled polyclonal antiserum (diluted 1:1,000 in TBSTB) generated against cell wall extracts from *C. albicans* strain 3153A (Panel A), or with polyclonal antiserum (diluted 1:500 in TBSTB) generated against the purified *C. albicans* 58 kDa fibrinogen-binding mannoprotein (Panel B). Materials present in the extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes. Peroxidase-conjugated anti-rabbit IgG (diluted 1:2,000 in TBSTB) was used as indicator antibody. The amount applied to each well was 50 µg of material, expressed as total sugar content. Molecular masses (MM) of prestained standard proteins run in parallel (lanes S) are indicated.

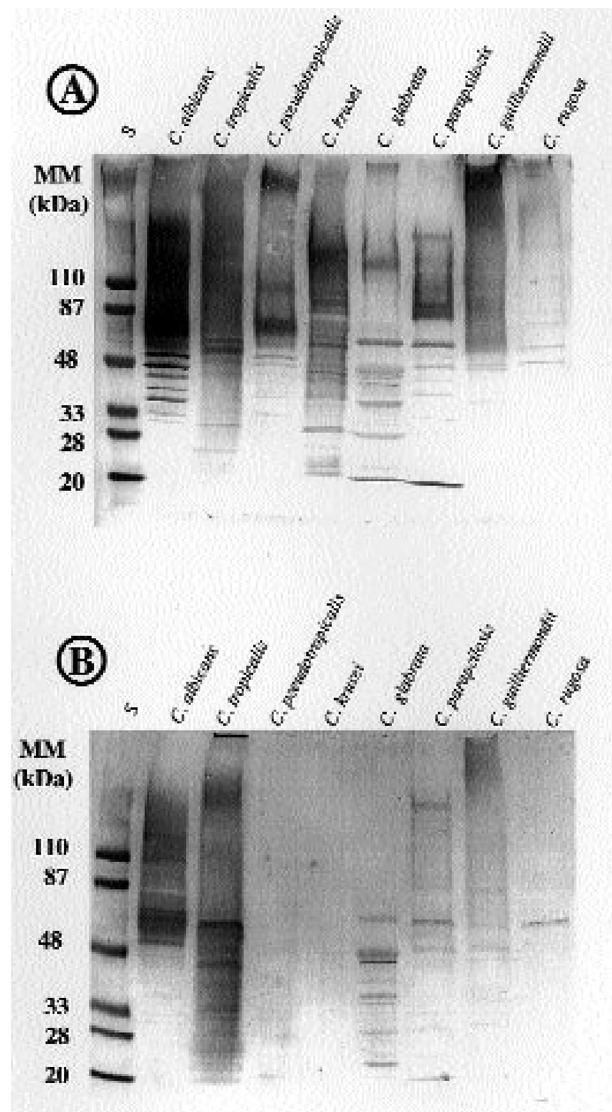

Figure 3. Immunoblot analysis of the materials present in the βME extract from the different *Candida* spp. isolated from HIV-infected patients, using as probes pooled serum samples (diluted 1:500 in TBSTB) of the same HIV-infected patients with OPC from whom the strains were isolated (Panel A) or oral saline rinses (diluted 1:10 in TBSTB) of HIV-infected patients with OPC (Panel B). SDS-PAGE and electrophoretic transfer to nitrocellulose were performed as for Figure 2. A mixture of peroxidase-conjugated anti-human IgG, IgM, and IgA (diluted 1:500 each in TBSTB) was used as secondary antiserum. Molecular masses of standard proteins run in parallel (lanes S) are indicated on the left side of each panel.

in the cell wall extract from the *C. albicans* isolate. Antibodies present in serum samples recognized the polydispersed HMWM (as expected, since anti-mannan antibodies are ubiquitous in human sera [37], as well as several well-defined medium-to-low molecular mass bands present in the *C. albicans* extract. A particularly intense labeling was observed in the region of 70 to 90 kDa, that may include reactivity towards a cluster of candidal antigens, including HSP70s, that are highly immunogenic [38-39]. Also, among other bands, a high level of reactivity was detected against a moiety with an apparent molecular mass of approximately 47 kDa, that may correspond to enolase and/or the 47 kDa fragment of *C. albicans* HSP90, since both proteins are immunodominant antigens during candidiasis [40,41]. Lower levels of reactivity were detected towards materials present in the cell wall extracts from non-*albicans* spp. Diffuse reactivity was observed against HMWM, especially in the case of *C. tropicalis* and *C. guilliermondii*. Reactivity with materials in the medium-to-low molecular mass range was particularly noticeable in the cases of *C. glabrata, C. parapsilosis* and *C. krusei*. When a preparation consisting of pooled oral saline rinses from patients with OPC was used as a probe in the immunoblots, the analysis further confirmed differences in cell wall antigens between the different species under investigation (Figure 3, Panel B). The highest levels of reactivity were observed for *C. albicans* and *C. tropicalis*. Some reactive materials were detected in extracts from *C. glabrata, C. parapsilosis, C. guilliermondii,* and *C. rugosa*, but not in extracts from *C. pseudotropicalis* and *C. krusei.*

DISCUSSION

Although much progress has been made in our understanding of the cell wall of *C. albicans* and its polypeptidic composition, comparatively little is known about the proteinaceous component of the cell surface of other species of *Candida*. In the present report, by using a combination of techniques, we have demonstrated a complex polypeptidic composition associated with cell wall (surface) extracts from different *Candida* species. Treatment with βME solubilized numerous molecular species, ranging from high (>100 kDa) to low (<20 kDa) molecular masses from the surface of all *Candida* isolates. The analysis also confirmed the existence of major differences in the polypeptidic and antigenic profiles associated with the cell wall extracts from different species of *Candida*, with distinctive patterns associated with each of the different species analyzed. These differences were already discernible by simple Coomassie staining of proteins in gels after electrophoretic separation. Lectin-blot staining with Concanavalin A revealed the presence of a conspicuous high molecular weight polydisperse material in the extracts from all isolates tested, including *C. albicans* and non-*albicans* species. However, immunoblot analysis using a polyclonal antibody against cell wall materials from *C. albicans*, together with serum samples and oral saline rinses from patients, revealed differences in antigenic cross-reactivity of this HMWM among the different species tested. This agrees with previous observations reporting differences in immunofluorescence recognition patterns for monoclonal antibodies directed to carbohydrate epitopes of *C. albicans* when used to detect homologous materials in the cell surface of non-*albicans* spp. [19,42-44], as well as with similarities and dissimilarities reported in the fine structure of carbohydrate (mannan) associated with cell wall proteins from different *Candida* species [45-47]. In the case of the non-*albicans* spp., the lack of reactivity with the monospecific antiserum generated towards the purified *C. albicans* 58 kDa fibrinogen-binding mannoprotein (except for weak reactivity in *C. guilliermondii*), seemed to indicate that the non-*albicans* species do not express this moiety, at least under the conditions that these experiments were performed.

Anti-*Candida* antibodies are present in the serum from normal and infected individuals. A local antibody response, mainly secretion of immunoglobulin A, is also induced at the mucosal level during episodes of OPC [51]. Thus, serum samples and oral saline rinses from patients with OPC provided additional antibody preparations for the study of cell wall antigens from the different *Candida* spp. Immunoblot analysis using both patients sera and oral saline rinses confirmed differences in reactivity of cell wall proteins among the different *Candida* species. In general, the βME extract obtained from the *C. albicans* isolate showed the highest degree of reactivity against these probes. This may be explained by the almost ubiquitous nature of *C. albicans* as a commensal of humans, as well as by its predominant role as an agent of infection, including in the patients from whom the serum samples and oral saline rinses used in this study were recovered. However, reactivity was also detected in the materials from non-*albicans* isolates. This reactivity could represent: i) cross-reactivity due to the existence of homology between the materials solubilized from non-*albicans* spp. with *C. albicans* antigens (as indicated also by cross-reactivity in the immunoblots using rabbit antiserum against *C. albicans* materials), ii) reactivity to antigens specific for a given species that elicit an antibody response *in vivo* when the host is either colonized or infected by such species, or iii) a combination of the previous two possibilities.

In summary, this study revealed both a high degree of complexity and the existence of major differences in the polypeptidic and antigenic profiles associated with cell wall extracts from different *Candida* spp. Since cell wall moieties may be important in mediating the interaction with the host, information leading to a better understanding of these components is important. Studying the identity, expression and function of cell wall moieties could be instrumental in the development of new approaches to the management of candidiasis, that are urgently needed, especially in the case of infections caused by *Candida* strains that are resistant to conventional antifungal agents.

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