

## Serodiagnosis of mycoses using recombinant antigens

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### Abstract

The early diagnosis of mycoses is important for the institution of an effective antifungal therapy. Detection of antibodies against crude antigenic extracts is one of the standard techniques for the diagnosis of most mycoses. However, while these crude antigenic extracts are relatively easy to obtain, they usually show low reproducibility and are not very specific, since antibodies from patients with different mycoses may show cross-reactivity. The application of molecular biology techniques to the study of fungal antigens has allowed the production of recombinant antigens that may help to solve these problems. The purpose of this review is to discuss the use of recombinant fungal antigens in the diagnosis of mycoses.

**Key words:** diagnosis, mycoses, recombinant antigens

### Introduction

Invasive fungal infections represent an important cause of morbidity and mortality in an increasingly number of patients. The early and accurate diagnosis of these infections is a key factor for an effective antifungal therapy [1]. However, the diagnosis of invasive mycoses is usually difficult since signs and symptoms are not specific and colonization and infection may be difficult to distinguish. Standard techniques for the diagnosis of fungal infection include microscopic visualization of the infecting fungus, culture, histopathology and serology. Although these methods are relatively simple to perform, they may lack specificity or sensitivity and sometimes require invasive procedures that can not be accomplished because of the clinical conditions of some of the patients.

The specific antibody response that is usually induced in patients with invasive fungal infections can help in the diagnosis of the majority of mycoses [2]. However, antibody detection may

have important limitations. On one hand, the specificity of techniques used to detect antibodies may be low when patients who are only colonized show high antibody titers or high antibody titers remain after disease when symptoms have disappeared. This situation may also exist when patients with different mycoses have antibodies that cross-react with the antigenic extract. On the other hand, antibody detection may lack sensitivity in severely immunocompromised patients who are not able to mount a detectable antibody response.

The techniques developed to detect the antibody response in patients with mycoses have traditionally used crude extracts composed by a large number of fungal components. While being relatively easy to obtain, these extracts were not free of problems. The standardization of different batches is difficult and the use of crude extracts may facilitate the cross-reactivity between antibodies from patients with different fungal and bacterial infections. However, in the last decades there has been an approach to develop more defined anti-

gens by applying the recombinant DNA technology [3]. Recombinant antigens can be prepared in a procariotic host making it easier to eliminate cross-reactivity due to post-translational modifications. In the next sections of this review, we will discuss the fungal antigens that have been produced employing this technique and focus on their possible use in the serodiagnosis of the most relevant mycoses.

### Production of recombinant antigens

The first step in the production of a recombinant antigen is the election of the antigen. Immunogenic antigens are appropriate, since they will elicit a strong immune response and therefore, high levels of antibodies against those antigens are expected in the infected patient. Localization on the fungi should be considered since a superficial antigen will probably induce a greater humoral response than an intracellular antigen not exposed to the host's defense mechanisms. On this basis the deep knowledge of the fungal cell wall is essential and the search of specific proteins unique in the fungal species to be studied is encouraged in order to avoid encountering cross-reactivity with other fungal species or with components of the host. Metabolites that are secreted by the fungi can be good candidates, as well. Expression levels should also be taken into account, since an antigen only expressed in special conditions such as, in the invasive form of the fungi can be interesting.

Basically, an exhaustive study should be performed to evaluate which antigens are unique to our fungi. The election of the antigen is the most important step and the more accurate this work is done the more promising results will be obtained at the end.

Once the antigen to be produced is chosen, primers are designed based on the sequence of the gene that encodes the antigen and of the vector that will carry it. Different vectors are available commercially offering great possibilities. Selection of the vector should be done considering the insertion fragment size, the purification method to be used after expression is achieved, the characteristics of the polylinker, the antibiotic resistance genes, etc. Amplification of genomic DNA is performed with the selected primers and the amplification product is digested with the correct

restriction enzymes in order to be inserted in the vector. Once the desired gene is cloned in an expression vector, positive recombinant colonies should be detected. The selected positive colonies should be able to overexpress the gene or gene fragment cloned so that sufficient antigen is produced and purification can take place [3]. Once the recombinant antigen is purified it can be used in serological tests such as the enzyme-linked immunosorbent assay (ELISA), western blot (WB) or the immunodiffusion (ID) assay, in order to detect the presence of specific antibodies against the selected antigen (Figure 1).

In some cases the gene sequence is unknown and therefore the approach described above should be slightly modified. In these cases two-dimensional (2D) gel electrophoresis can be performed in order to separate fungal proteins. The protein spot can be digested on-gel and the masses of the resulting fragments can be determined by MALDI-TOF mass spectrometer. Once the open reading frame is identified primers can be designed and cloning of the cDNA can be achieved by reverse transcriptase-polymerase chain reaction (RT-PCR) [4].

Once the antigen is expressed and assayed by different immunological methods obtaining good results, further studies can be accomplished to optimize the diagnostic method. Mapping of the antibody binding sites of the antigen and their expression as shorter fragments can result in greater specificity and sensitivity.

### Use of recombinant antigens in the diagnosis of mycoses

#### *Alternaria*

*Alternaria* species show a worldwide distribution and their spores are commonly found in atmospheric surveys, showing a seasonal spore-releasing pattern. *A. alternata* is a common cause of asthma and other allergic diseases. *A. alternata* can also be responsible for cutaneous and ocular lesions being diagnosed in these cases by microscopic and culture methods. The application of molecular biology techniques to the study of *Alternaria* allergens has enhanced the ability to produce allergens in quantity, to determine their biological relevance, as well as to evaluate mechanisms of *Alternaria* sensitivity.

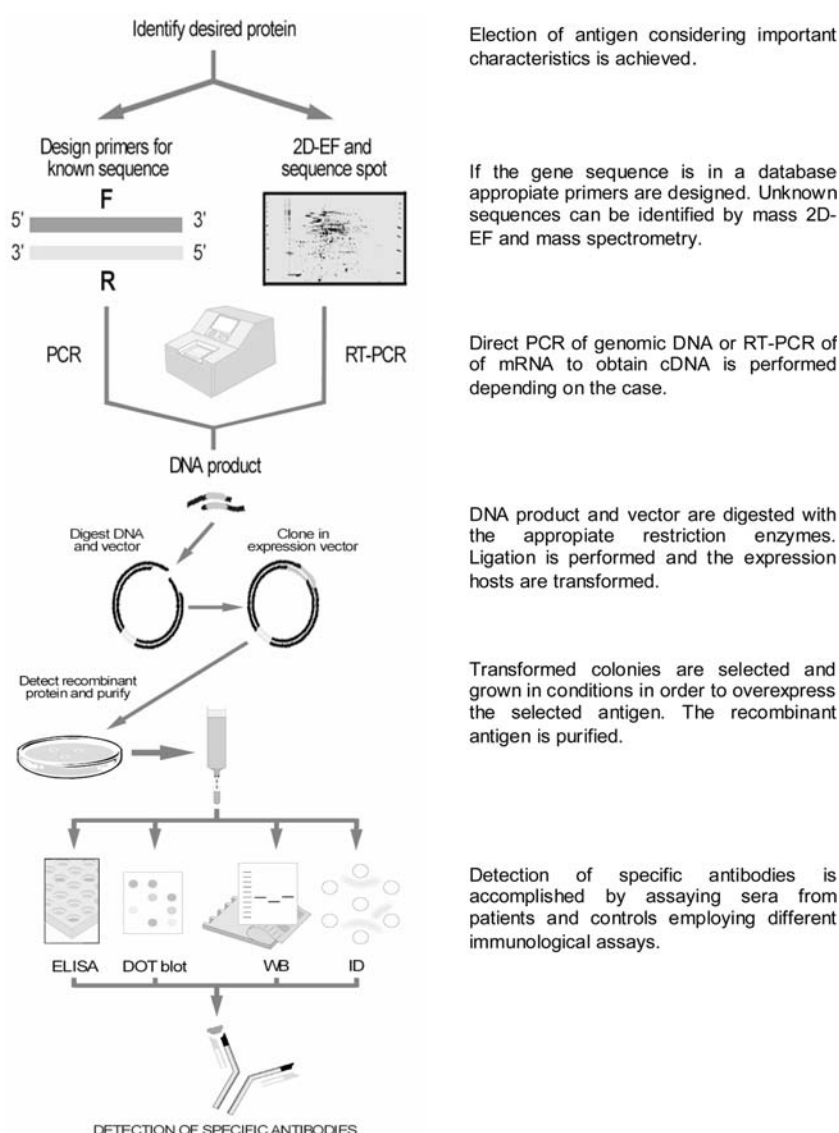


Figure 1. Production of recombinant antigens.

A number of recombinant *Alternaria* allergens have been obtained [5]. The Alt a1 protein is a two-chain dimer linked by disulfide bonds with a molecular weight of approximately 30 kDa [6]. Alt a1 is a major allergen that is recognized by IgE antibodies in 80–90% of *Alternaria* allergic individuals. Recombinant Alt a1 is commercially available (Biomay, Vienna, Austria). Skin testing of *Alternaria*-sensitive individuals demonstrated that 85.7% had positive skin tests to recombinant Alt a1 [7] and 93% of asthma/rhinitis patients had both IgE and IgG to Alt a1, emphasizing its importance as a major allergen [8]. Other *Alternaria* allergens

have also been cloned, such as Alt a6 and Alt a7, although these allergens have shown to react diversely with IgE from sera of allergic patients [9] (Table 1).

### *Aspergillus*

*Aspergillus* spp. are gaining importance as opportunistic pathogens during the last decades. In immunocompetent hosts, *Aspergillus* spp. rarely cause serious illnesses except for a number of lung disorders such as allergic bronchopulmonary aspergillosis (ABPA), allergic rhinitis, allergic

Table 1. Candidate recombinant antigens for serodiagnosis of mycoses

| Species                              | Antigen                    | Molecular mass (kDa) | References |
|--------------------------------------|----------------------------|----------------------|------------|
| <i>Alternaria alternata</i>          | Alt a 1                    | 30                   | [5, 7, 8]  |
|                                      | Alt a 6                    |                      | [9]        |
|                                      | Alt a 7                    |                      | [9]        |
| <i>Aspergillus fumigatus</i>         | Asp f1                     | 37                   | [3, 10–12] |
|                                      | Asp f 2                    |                      | [12–14]    |
|                                      | Asp f 4                    | 43                   | [15]       |
|                                      | Asp f 5                    | 57.4                 | [3]        |
|                                      | Asp f 6                    | 51.5                 | [15]       |
|                                      | Asp f 7                    | 18                   | [3]        |
|                                      | Asp f 9                    |                      | [3]        |
|                                      | Asp f 10                   |                      | [3]        |
|                                      | Asp f 11                   |                      | [16]       |
|                                      | Asp f 16                   |                      | [17]       |
|                                      | Afmp1                      |                      | [18–20]    |
|                                      | Afmp2p                     |                      | [21]       |
|                                      | HSP                        |                      | [22]       |
|                                      | Mitogillin                 |                      | [23]       |
| <i>Aspergillus flavus</i>            | Afmp1                      | 26.3                 | [21]       |
| <i>Aspergillus nidulans</i>          | Aspnd 1r                   |                      | [24]       |
| <i>Blastomyces dermatitidis</i>      | WI-1 fragment <sup>a</sup> | 30                   | [25]       |
| <i>Candida albicans</i>              | Cand a 3                   | 29                   | [26]       |
|                                      | Ecel                       | 30                   | [27]       |
| <i>Cladosporium herbarum</i>         | HCh-1                      |                      | [28]       |
| <i>Coccidioides immitis</i>          | Cla h 6                    | 46                   | [29]       |
|                                      | CF                         | 45                   | [30–33]    |
|                                      | r-PRA                      |                      | [34]       |
| <i>Histoplasma capsulatum</i>        | M antigen                  | 80.71                | [35]       |
| <i>Malassezia furfur</i>             | GH17                       | 60                   | [36]       |
|                                      | MF7                        | 21.3                 | [37]       |
|                                      | MF8                        | 14.4                 | [37]       |
|                                      | MF9                        | 9.7                  | [37]       |
|                                      | Mal f 5                    | 18.2                 | [37]       |
|                                      | Mal f 6                    | 17.2                 | [37]       |
|                                      | Mal f 7                    | 16.2                 | [38]       |
|                                      | Mal f 8                    | 19.2                 | [38]       |
|                                      | Mal f 9                    | 14                   | [38]       |
|                                      | gp43                       | 43                   | [39, 40]   |
| <i>Paracoccidioides brasiliensis</i> | p27                        | 27                   | [41, 42]   |
|                                      | hsp60                      | 60                   | [43, 44]   |
| <i>Penicillium marneffei</i>         | Mp1p                       | 75                   | [45]       |
| <i>Penicillium citrinum</i>          | Pen c 1                    | 33                   | [46]       |
|                                      | Pen c 2                    | 39                   | [47]       |
| <i>Penicillium oxalicum</i>          | Pen o 18                   | 34                   | [48]       |
| <i>Pneumocystis jiroveci</i>         | Msg                        | 95–140               | [49–51]    |

<sup>a</sup> WI-1 is now designated as Bad-1.

sinusitis, allergic asthma and aspergilloma. However, invasive aspergillosis is one of the most important infections in patients with haematological malignancies or bone marrow transplant recipients causing high mortality [52]. The major problem of invasive aspergillosis is the difficulty in

establishing an early diagnosis since blood cultures are rarely positive [53].

Elevated levels of total IgE, specific IgE, and IgG antibodies in sera are important immunodiagnostic criteria in ABPA. A variety of *A. fumigatus* allergens have been successfully cloned and expressed and their role in differential diagnosis has been explored [10, 11, 13, 16, 22]. These recombinant antigens show comparable allergenicity to their natural counterparts and are far superior to conventional extracts in serological diagnosis of ABPA since they permit discrimination between ABPA and *A. fumigatus* sensitization [3]. Asp f 1, a ribotoxin of the mitogillin family, which is identical to restrictocin from *A. restrictus*, is one of the major allergens of *A. fumigatus*. It has been shown to react with sera from 80% of patients with ABPA and with 46% of sera from *A. fumigatus*-allergic asthmatics [3]. Similar results have been observed with Asp f 3, another major allergen of *A. fumigatus*. When a number of synthetic epitope peptides of Asp f 1 have been studied, a peptide of 11 amino acids, designated P1, presented a higher diagnostic efficiency than the standardized diagnostic antigen [12]. Interestingly, the IgE response against some recombinant allergens may be disease specific. An specific IgE response against recombinant allergens Asp f 4 and Asp f 6 has been detected in patients with ABPA but not in *A. fumigatus*-allergic asthmatics [15]. Therefore, serological diagnosis of ABPA with these two allergens has a specificity of 100% and a sensitivity of 90%. Similar results have been described with recombinant allergen Asp f 16 [22]. Other recombinant *A. fumigatus* allergens used for diagnosis of ABPA include Asp f 2 [17], Asp f 5, Asp f 7, Asp f 9 and Asp f 10 [3].

*A. nidulans* is another *Aspergillus* species that has been implicated in the development of aspergilloma and ABPA in humans. An immunodominant *A. nidulans* antigen, designated Aspnd 1, that is 53.1% identical to the Asp f 2 allergen of *A. fumigatus* has been described [24]. The recombinant form of this antigen was recognized by the sera of 88% of patients with aspergilloma, while a number of control sera comprising healthy individuals and patients with different non-*Aspergillus* infections were negative.

Several antigens of *A. fumigatus* [14, 18, 23] and *A. flavus* [21] have also been cloned and expressed in order to evaluate their possible use in serodiagnosis of invasive aspergillosis. Recent studies have shown

that patients with invasive aspergillosis by *A. fumigatus* develop high levels of specific antibodies against Afmp1p [18]. An ELISA-based test to detect antibodies against the Afmp1p recombinant galactomannoprotein has been shown to be 100% sensitive for patients with aspergilloma and 33.3% for patients with invasive aspergillosis [19]. Presence of antibodies against Afmp1p in a part of the patients with invasive aspergillosis may interfere with galactomannan detection if immune complexes are formed. To solve this problem it may be necessary the combined detection of both markers. No false-positive results were found among the 80 healthy blood donors. Interestingly, no cross-reactivity was found with other fungi, since 20 patients with penicilliosis by *Penicillium marneffei*, 5 with candidiasis and 4 with cryptococcosis were found to be negative, indicating a high specificity for the test. An ELISA assay developed with polyclonal antibodies to recombinant Afmp1p detected the antigen in the sera from 53% of patients with invasive aspergillosis but in none of nine patients with aspergilloma [18]. Interestingly, when the Afmp1p antibody and antigen tests were used in combination the sensitivity in the diagnosis of *A. fumigatus* invasive aspergillosis was raised to 86.7%. Another mannoprotein from the *A. fumigatus* cell wall, designated Afmp2p, has been recently cloned and expressed in *E. coli* [14]. It has a predicted molecular mass of 51.5 kDa and is predominantly secreted by the fungus, whereas only minimal amounts can be detected in the cell wall. Since this antigen reacts specifically with sera from patients with aspergilloma and invasive aspergillosis, and it has a minimal cross-reactivity with Afmp1p, it is believed to be a good candidate for complementing Afmp1p in the serodiagnosis of *A. fumigatus* infections.

Aflmp1p is the antigenic cell wall protein in *A. flavus* homologous to Afmp1p. It has been cloned and expressed [21]. Western blot analysis showed that recombinant Aflmp1p reacted specifically with sera from four patients with aspergilloma or *A. flavus* invasive aspergillosis, suggesting that it may be also useful for serodiagnosis, although a larger number of sera should be tested.

### *Blastomyces*

*Blastomyces dermatitidis* is a dimorphic fungus that causes blastomycosis in humans and animals.

Upon inhalation of spores into the lung, at 37 °C, *B. dermatitidis* converts to yeast. It can infect immunocompetent hosts as well as establishing latency and appearing as an opportunistic pathogen in the compromised patient [54]. Antigen A is commercially available and it has been reported to be useful in serodiagnosis of blastomycosis, since the amount of IgG to this antigen is correlated with disease activity. However, the antibodies to antigen A may be detected one year after disease making it difficult to establish the stage of the disease [55]. BAD1 (formerly WI-1) is an immunodominant adhesin of 120 kDa located on the outer cell wall of *B. dermatitidis* that presents cross-reactivity with antigens of *Coccidioides immitis*, *Histoplasma capsulatum* and *Paracoccidioides brasiliensis*, among other microorganisms [56]. This cross-reactivity has been avoided by using a recombinant 25 amino acid residue tandem repeat of the BAD1 antigen that has been shown to be recognized in western blots by sera from all 35 blastomycosis patients studied, but not by sera from 10 histoplasmosis and 5 coccidioidomycosis patients [25]. These results seem promising although further work with this antigen has been directed towards vaccine production [57] and pathogenesis studies [58] rather than towards diagnostic needs.

### *Candida*

*Candida albicans* is responsible for a wide range of clinical presentations ranging from allergic disorders and superficial infections to life threatening invasive candidiasis. In some cases the detection of antibodies has been suggested to be helpful in the diagnosis of the invasive disease [59]. Other authors indicate that antibody tests are seldom used to diagnose invasive candidiasis because antibodies are often present in colonized but uninfected patients giving poor sensitivity and specificity results and severely immunocompromised patients are not able to mount a specific antibody response [1]. However, the type of antigen studied will have a strong influence on the antibody response mounted and therefore, more work should be done in this field. During the last years recombinant techniques have been used to express a number of antigens, although their potential use in serodiagnosis has not been studied in all cases.

The role of *C. albicans* in allergy is not fully understood, a situation that is partly related to the lack of knowledge on the molecular identity of allergens. A recently described allergen, named Cand a 3, is a 29 kDa protein that was found to react with IgE antibodies present in sera of 56% of asthmatic patients [26].

The glycolytic enzyme enolase is an immunodominant antigen of *Candida*, which has been studied in the diagnosis of invasive candidiasis [60]. Two *Candida* enolases have been cloned and characterized [61, 62], but most studies have involved the *ENO1* gene. In our laboratory, after cloning and expressing the *ENO1* in *Escherichia coli*, we have developed a WB assay to detect antibodies against this antigen in patients with invasive candidiasis (Figure 2). However, the sensitivity and specificity have been low. A similar approach is being followed to study the diagnostic usefulness of Ecelp, an antigen that is specifically expressed on the surface of the germ tubes of *C. albicans* [63]. WB analysis with sera from rabbits immunized with *C. albicans* germ tubes reacted with Ecelp, whereas the same sera adsorbed with germ tubes or the pre-immune sera showed no reactivity (Figure 3). Sera from rabbits infected with *C. parapsilosis* and *C. krusei* also recognized Ecelp. Cross reactivity was not observed when immunoblots were performed with sera from rabbits immunized with *A. fumigatus* or infected with *Scedosporium prolificans*. Interestingly, sera from four patients with invasive candidiasis also reacted with Ecelp, whereas sera

from four patients without invasive candidiasis did not [27]. Preliminary studies employing the recombinant Ecelp in an ELISA assay have shown high reactivity when sera from a patient with invasive candidiasis was examined, whereas control sera showed low reactivity. Although these results are promising a greater number of sera should be assayed in order to confirm the preliminary data.

### *Cladosporium*

The spores of the dematiaceous fungus *Cladosporium herbarum* are one of the most frequent fungal allergens present in the air. This fungus is known to cause IgE-mediated diseases such as rhinitis, conjunctivitis and asthma. HCh-1 is an hydrophobin from the *C. herbarum* cell wall that has been recently cloned and expressed in recombinant form. The recombinant HCh-1 was able to bind IgE from two out of 24 sera from *C. herbarum*-sensitized patients and in four out of 60 patients suffering from ABPA [28]. Interestingly, none of the sera showing reactivity with HCh-1 reacted with the *A. fumigatus* hydrophobin HYP1 that shows 28% sequence identity with HCh-1.

Enolase is an allergen (Cla h 6) in *C. herbarum*. Recombinant Cla h 6 was produced in *E. coli* and analyzed for its binding activity, showing promising results [9]. Further studies involving the cloning of IgE-reactive B-cell epitopes of *C. herbarum* enolase

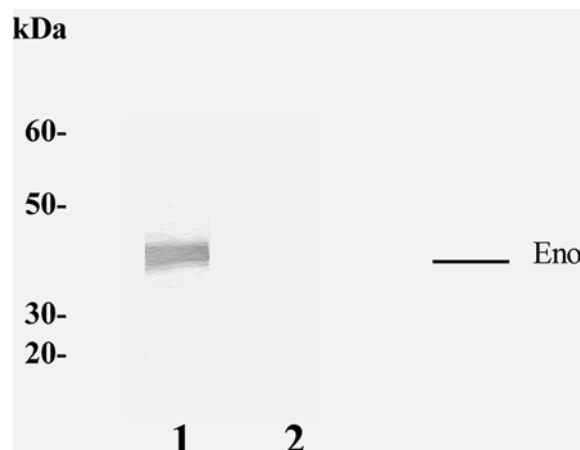


Figure 2. Western blot of partially purified extracts of Eno recombinant *E. coli* with (1) sera from a patient with invasive candidiasis and (2) a healthy donor.

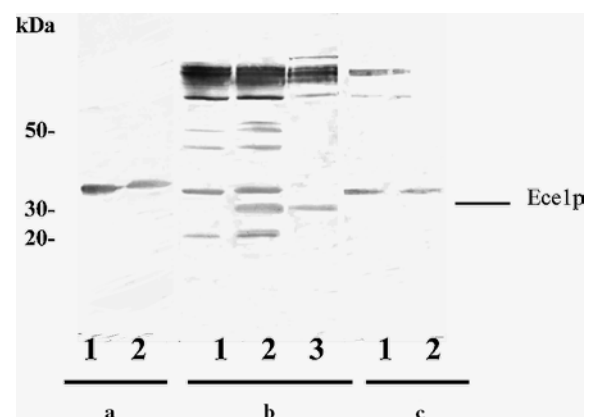


Figure 3. Western blots of *E. coli* extracts transformed with Ece1 recombinant plasmids (1) basal conditions, (2) IPTG induced, and (3) partially purified Ecelp. Reactivity of sera of rabbits immunized with germ tubes form *C. albicans*: (panel a) preimmune serum, (panel b) immune serum, and (panel c) immune serum adsorbed with germ tubes of *C. albicans*.

were performed. Mapping of Cla h 6 IgE binding epitopes identified a peptide designated peptide 9 which bound IgE from 8 of 8 patients [29].

### *Coccidioides*

*Coccidioides immitis* is the agent that causes coccidioidomycosis. This disease has been reported in immunologically compromised persons, as well as in healthy people exposed to *C. immitis* arthroconidia [30]. The definitive diagnosis of this disease is done by demonstrating the presence of *C. immitis* in tissue or by culture. Complement-fixation (CF) and immunodiffusion (ID)-CF assays to detect antibodies to coccidioidin are also useful in the diagnosis of the disease.

The coccidioidal CF and ID-CF antigen has been reported to be a chitinase that can be isolated from the culture filtrates of *C. immitis* endospore-forming spherules and from hyphae. The gene encoding this antigen has been cloned and a functional recombinant CF-chitinase antigen of 47 kDa has been produced in *E. coli* [31]. By ID assays with sera from patients with coccidioidomycosis, the recombinant CF-chitinase (rCF) generated a line of identity with the coccidioidal ID-CF antigen used as a control, demonstrating that it could be used in serodiagnosis. No detectable reaction was observed by ID with a limited number of *Blastomyces* or *Histoplasma* antisera. The reactivity of rCF was evaluated by ELISA, showing a sensitivity of 96.9% and a specificity of 100% [32].

The serodiagnostic usefulness of rCF has also been compared to commercially available coccidioidin by ELISA, showing to be more sensitive. rCF was also more effective in distinguishing the antibody levels from patients with pulmonary coccidioidomycosis than in sera from those with disseminated disease [33]. The highest titers were observed in patients with disseminated disease, suggesting that this diagnostic method could distinguish between pulmonary coccidioidomycosis and invasive coccidioidomycosis. However, the use of rCF for serodiagnostic purposes may be compromised by the finding of an epitope common to *H. capsulatum* and *B. dermatitidis*. In a study including a small number of sera, the rCF antigen reacted with sera from 6 of 12 patients with histoplasmosis and 2 of 12 patients with blastomycosis [33].

Patients with coccidioidomycosis also react with a proline-rich antigen (PRA) isolated from spherules of *C. immitis*. The gene encoding this antigen has been cloned and the recombinant antigen has been shown to react with serum IgG antibodies from 37 of 42 patients with culture-proven progressive pulmonary or extrapulmonary coccidioidal disease. In addition to the high sensitivity observed (88%), the test showed a specificity of 97% in the diagnosis of coccidioidomycosis [34]. Interestingly, the association of antibodies to rPRA with progressive infection may have prognostic value since none of 18 patients with primary self-limited coccidioidomycosis had detectable antibodies to rPRA in serum samples collected up to 141 days after illness began.

### *Histoplasma*

The temperature dependent dimorphic fungus *H. capsulatum* causes histoplasmosis, a broad spectrum of clinical conditions ranging from the self-limiting localized lung infection to the acute disseminated form, which is particularly serious in immunosuppressed patients such as those with AIDS. The identification of antibody responses in patients with histoplasmosis has been proved to be useful in the diagnosis of disease [35]. ID assays have been developed employing antigens H and M. The M gene consists of 2187-bp DNA encoding a protein of 80.7 kDa, which has significant homology to catalases from *A. fumigatus*, *A. niger*, and *A. nidulans* [64]. The M antigen has been cloned and expressed and the recombinant fusion protein demonstrated to be reactive with a serum sample from a patient with histoplasmosis. The gene encoding the H antigen has also been cloned [35] and sequence analysis revealed homology to extracellular  $\beta$ -glucosidases. Although the ability of recombinant H antigen to elicit and induce an *in vitro* cell-mediated immune response in BALB/c mice has been tested [65], its suitability in serodiagnostic assays has not been reported yet.

GH17 is a cDNA sequence, which encodes a 60 kDa protein of *H. capsulatum* that shows no significant homology with known sequences. When it was expressed as  $\beta$ -galactosidase fusion protein, it reacted with 18 of 18 sera from histoplasmosis patients but with none of the controls including sera from patients with blastomycosis, coccidioidomycosis, candidiasis and healthy

controls, suggesting it could be a useful tool in the diagnosis of histoplasmosis [36].

### *Malassezia*

*Malassezia* species are opportunistic yeasts that are involved in the pathogenesis of a number of skin diseases including atopic dermatitis (AD), pityriasis versicolor, folliculitis, and seborrheic dermatitis. Three incomplete gene products (MF7, MF8, MF9) as well as two allergens (Mal f 5 and Mal f 6) of *Malassezia furfur*, also denoted *Pityrosporum orbiculare*, were cloned and expressed using phage display technology [37]. Mal f 5 showed homology to Mal f 2, Mal f 3 and Asp f3, whereas Mal f 6 showed homology to cyclophilin. MF7, MF8 and MF9 did not reveal sequence similarity to any known proteins. All of the recombinant products obtained in the study showed IgE-binding capability to serum from AD patients.

Mal f 7, Mal f 8 and Mal f 9, also produced by phage display, did not show homology to other known proteins. Although they did show binding ability to IgE serum from AD patients by WB, a *M. furfur* extract was able to inhibit this IgE binding [38].

### *Paracoccidioides*

*Paracoccidioides brasiliensis* is a dimorphic fungus that causes paracoccidioidomycosis. Two forms of the disease are recognized, the chronic form and the acute or subacute form. The main antigen used for serodiagnosis of paracoccidioidomycosis is a glycoprotein of 43 kDa (gp43), which is analogous to the E2 culture filtrate antigen [66]. The gp43 antigen reacted with sera from 100% of patients with paracoccidioidomycosis but also with a significant amount of sera from patients with histoplasmosis or Jorge Lobo's disease [39]. The gene encoding the gp43 antigen has been sequenced, cloned and expressed in *E. coli* [40]. It encoded a polypeptide of 416 amino acids (Mr 45,947) that showed similarities of 56–58% with exo-1,3- $\beta$ -D-glucanases from *Saccharomyces cerevisiae* and *C. albicans*. The entire gp43 sequence expressed as a GST fusion protein showed reactivity by WB with sera from 6 out of 6 patients with chronic pulmonary paracoccidioidomycosis and 7 out of 7 patients with acute lymphatic forms of

paracoccidioidomycosis [67]. In this study, sera from patients with aspergillosis, candidiasis and histoplasmosis did not react with the gp43-GST fusion protein, suggesting that recombinant gp43 can be a useful tool in the diagnosis of paracoccidioidomycosis. Subfragments of the gp43 expressed as GST fusion proteins revealed that sera from patients with paracoccidioidomycosis tended to react with the C-terminal region [67].

A 27 kDa recombinant protein from *P. brasiliensis* has also been produced and evaluated with human serum samples by ELISA [41]. In this study, 47 of the 64 specimens from paracoccidioidomycosis patients were reactive (73.4%) while 35 of the 40 samples from healthy individuals were non-reactive. Cross-reactivity was observed with 11 of 15 aspergillosis patients (73.3%) and 6 of 15 histoplasmosis patients (40%). The ELISA assay had a specificity of 87.5% for paracoccidioidomycosis patients when compared to healthy subjects, while for patients with other mycoses it was 58.7%. Further studies combining the 27 kDa recombinant protein and the purified 87 kDa heat shock protein in an ELISA showed promising results since the combination of both antigens demonstrated a significant increase in sensitivity and specificity compared to results when the antigens were used separately [42].

The immunodominant heat shock protein 60 (HSP60) of *P. brasiliensis* has also been cloned, characterized and expressed [43]. When it was assayed by WB it showed reactivity with 8 of 10 patients with paracoccidioidomycosis and no reactivity with sera from normal human subjects or from patients with other mycoses such as histoplasmosis, cryptococcosis or candidiasis. Recent studies [44] done with HSP60 involving a larger amount of serum samples have shown promising results by WB (Figure 4). A high sensitivity (97.3%) and specificity (92.5%) was observed. In this case no cross reactivity was observed when individual sera from patients with aspergillosis, sporotrichosis, cryptococcosis and tuberculosis was assayed. However reactivity to HSP60 was observed in sera from 9.52% of the healthy individuals and 11.5% of patients with histoplasmosis. Although these results are promising, since a great number of serum from patients with proven mycosis has been assayed (121 serum specimens), the existence of some false positive results among healthy individuals requires the testing of a greater

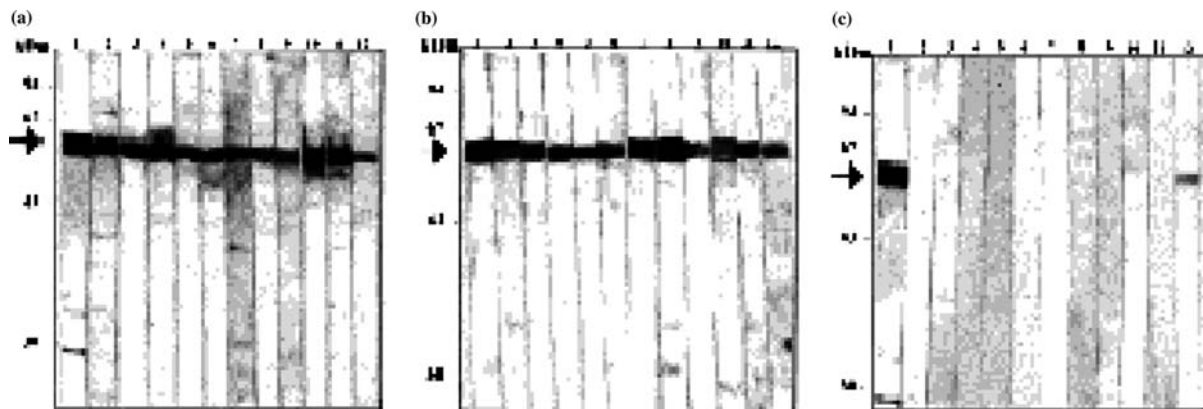


Figure 4. Reactivities of individual serum samples from *P. brasiliensis*-infected patients to the recombinant HSP60 as determined by Western Blotting. (a and b) Reactivity to serum samples (at a 1:500 dilution) from *P. brasiliensis*-infected patients with chronic and acute disease, respectively. (c) Reactivity of human control sera. Lanes 1, reaction of the purified *P. brasiliensis* HSP60 to the monoclonal antibody. Lanes 2–12, reaction to individual sera at 1:500 dilution. Reprinted with permission from ASM [44].

number of controls and further study of the reactivity to histoplasmosis samples.

#### *Penicillium*

*Penicillium marneffei* is a dimorphic pathogenic fungus that causes the systemic disease penicilliosis by *P. marneffei* in both immunocompromised and immunocompetent patients. Penicilliosis is particularly common in people infected with human immunodeficiency virus. The diagnosis of this disease is normally made employing invasive procedures. A rapid serodiagnostic assay would be helpful to identify individuals who display non-specific symptoms of *P. marneffei* infection and those who have initial asymptomatic forms of the disease [35].

Mp1 is a highly antigenic cell wall mannoprotein of *P. marneffei* that is useful to detect antibodies in patients with penicilliosis marneffei. Recombinant Mp1p has been produced and assayed as a serodiagnostic marker in an ELISA test [45]. Clinical evaluation revealed high levels of specific antibody in two immunocompetent patients and in 14 of 17 (80%) penicilliosis patients infected by human immunodeficiency virus. No false-positives were found among 90 healthy blood donors, 20 patients with typhoid fever and 55 patients with tuberculosis.

*Penicillium citrinum* and *P. oxalicum* cause IgE-mediated allergic diseases. Crude extracts have been used in skin tests giving good results. However, it would be advantageous to have pure

immunoreactive antigens or allergens that could assist the diagnosis of allergic diseases. Recombinant Pen c 1 [46] and Pen c 2 [47] from *P. citrinum* as well as Pen o 18 [48] from *P. oxalicum* have shown to bind to specific IgE antibodies from sera of patients by WB, indicating they could be used in serodiagnosis of allergic disorders.

#### *Pneumocystis*

*Pneumocystis jiroveci* is an opportunistic fungal pathogen that causes pneumonia in the immunocompromised host. Serologic studies of *Pneumocystis* pneumonia (PCP) in humans have been limited by inadequate reagents, since only small amounts of human *P. jiroveci* antigens can be obtained from clinical specimens. In addition to this problem, serologic surveys using crude *Pneumocystis* antigen preparations are generally not effective as clinical or epidemiologic tools [68]. The *Pneumocystis* antigen that has been used in most serological studies is the 95- to 140-kDa major surface glycoprotein (Msg or gpA). This antigen has been cloned and expressed in two recombinant fragments that correspond to the amino and carboxyl halves of Msg [49, 50]. The carboxyl fragment of about 100 aminoacids was reactive by WB with 49 serum samples from healthy donors as well from patients with and without PCP supporting the hypothesis that most adult humans have been infected with *P. jiroveci* at some point in their life. In more recent studies the carboxyl fragment was recognized by WB by 100% of serum specimens

while the amino fragment was recognized by approximately 44% of patients. However, the test did not distinguish between HIV-positive patients and healthy donors [50]. An ELISA using the carboxyl fragment showed that immunocompromised patients with PCP who were immunocompromised for reasons other than AIDS had higher antibody levels than did either patients with AIDS with PCP or healthy persons, but it could not distinguish among HIV-positive, PCP-positive and HIV-positive, PCP-negative patients and controls [50].

Three overlapping recombinant fragments of Msg that span the length of *P. jiroveci* Msg have been used to measure serum antibodies by WB in healthy blood donors and HIV patients [51]. Eighty (84%) of the 95 healthy persons reacted with any of 3 overlapping recombinant MSG fragments; 64% had antibodies against the middle region (approximately aa 243–760), while recombinant proteins encompassing the amino or carboxy portion were recognized by 40% (38/95) and 41% (39/95) of the serum samples, respectively. An ELISA using the three overlapping recombinant fragments of Msg demonstrated that HIV-positive patients had significantly higher antibody levels to all Msg fragments. Furthermore, HIV-positive patients who experienced a previous episode of PCP had higher levels of antibodies to MsgC than patients who never had PCP [51].

At present, Msg recombinant fragments are probably more interesting tools for the study the epidemiology of PCP infection in humans than as diagnostic tools since both healthy donors and PCP patients react with this antigen.

## Conclusions

There is an urgent need for an early diagnosis of fungal infections in order to establish an effective antifungal therapy. High sensitivity and specificity tests are desirable, whereas cross-reactivity and low reproducibility should be avoided. The evolution of molecular techniques allows the production of recombinant proteins that provide a highly reproducible source of defined antigens that permit standardization. Alone or in combination with other antigens, recombinant antigens are being employed as reagents in a variety of immunological methods, such as ID, ELISA or WB for the

diagnosis of most clinically relevant mycoses. Additionally, monoclonal and polyclonal antibodies can be raised against these recombinant antigens to be used in serodiagnostic assays. Recombinant techniques are opening new ways in the diagnosis of fungal infections.

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