Chapter 6

Synthetic Peptides as an Alternative Tool for the Diagnosis of Cryptococcosis

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Abstract

Cryptococcosis is an important systemic mycosis that threatens the lives of humans and animals. The disease is caused by two species of the genus *Cryptococcus*: *Cryptococcus neoformans* and *Cryptococcus gattii.* The diagnosis of cryptococcosis is made through microscopy, fungal culture followed by biochemical tests, and detection of the cryptococcal capsular antigen (CrAg). Despite the existence of an established diagnostic protocol, the search for new diagnostic tests is necessary due to the high incidence of the disease, with estimates of approximately 1 million cases of cryptococcal meningitis per year and more than 600,000 deaths in patients infected with human immunodeficiency virus (HIV), the potential for *C. gattii* to cause the disease in immunocompetent individuals, and the disease's rapid worldwide dissemination. With the development of biotechnology, synthetic peptides have opened up new possibilities as a source of pure epitopes and molecules for the diagnosis of various diseases, based on the detection of circulating antibodies. Synthetic peptides can also be used for the development of vaccines. Studies on Leishmaniasis, Chagas disease, paracoccidioidomycosis, tuberculosis, and, more recently, on cryptococcosis, among others, have shown that this approach shows potential for the early diagnosis of the disease, thus reducing the morbi-lethality of individuals affected by this infection and ultimately changing their prognosis.

Keywords: Cryptococcosis, diagnosis, antigens, synthetic peptides, B cell, epitopes

1. Introduction

Cryptococcosis is an important systemic mycosis that threatens the lives of humans and animals. It manifests primarily through respiratory system diseases and meningoencephalitis. Cryptococcosis is among the emergent fungal infections with significant morbi-lethality, and it is the fourth most frequent cause of opportunistic infection in human immunodeficiency

virus (HIV)-positive patients. The disease is caused by two species of the genus *Cryptococcus*: *Cryptococcus neoformans* and *Cryptococcus gattii* [1, 2].

C. neoformans has a worldwide distribution and is responsible for the high morbi-lethality in immunocompromised individuals with AIDS. In contrast, infections with *C. gattii* are prevalent in tropical and subtropical climate regions, and *C. gattii* primarily attacks immunocompetent hosts. However, *C. gattii* has also emerged in countries with temperate climates, e.g., Canada (Vancouver) and the U.S. Northwest, which demonstrates that the fungus may adapt to new environments and cause surges of infection in animals and humans [3–7].

Annually, AIDS-related cryptococcal meningitis is responsible for approximately 15% of the mortality in these individuals [8]. Sub-Saharan Africa has the largest rate of coinfection with *Cryptococcus* in these patients, and *Cryptococcus* is the most common cause of meningitis in adults [9, 10]. Recent estimates indicate an incidence of approximately 10,000 cases per year of cryptococcal meningitis in Latin America [11].

2. Etiological agents

C. neoformans and *C. gattii* are basidiomycetes in the asexual phase, appear as round cells, though they are occasionally ovoid, are isolated or budding, and encased by a mucopolysaccharide capsule. *C. neoformans* is the anamorphic phase of *Filobasidiella neoformans*, and *C. gattii* is the anamorphic phase of *Filobasidiella bacillispora* [12, 13].

C. neoformans was originally divided into two strands: var. *neoformans* (serotypes A, D, and a hybrid AD) and var. *gattii* (serotypes B and C). In 2002, *C. neoformans* var. *gattii* was recognized as a distinct species, *C. gattii.* Furthermore, previously observed phenotypic differences, newer molecular studies, and the sequencing of the fungus' genome were helpful to detect significant genetic variations between serotypes A and D and to distinguish serotype A as a new strand, *C. neoformansvar. grubii* [14, 15].

However, this classification becomes difficult, as significant divergences between serotypes are frequently observed at the molecular level [16]. Serotype limits do not entirely coincide with genetic groupings; therefore, serotyping is not regarded as a reliable technique for differentiating strands of *Cryptococcus* [17].

A series of molecular studies were conducted, including polymerase chain reaction (PCR) fingerprinting and amplified fragment length polymorphism (AFLP) analysis of the orotidine monophosphate pyrophosphorylase (*URA5*) gene and analysis of the phospholipase (*PLB1*) gene by restriction fragment length polymorphism (RFLP). As a result of these analyses, the yeasts were classified into the following nine molecular types: VNI (AFLP1) and VNII (AFLP1A and AFLP1B) (*C. neoformans* var. *grubii*, serotype A), VNIV (AFLP2) (*C. neoformans* var. *neoformans*, serotype D), VNIII (AFLP3) (Hybrid, serotype AD), VNB (only one isolated in Botswana) and VGI (AFLP4), VGII (AFLP6), VGIII (AFLP5) and VGIV (AFLP7), all corresponding to serotypes B and C, indicating that they evolved independently and in parallel [18–20].

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C. gattii genotype VGII was responsible for approximately 95% of the cryptococcosis infections that occurred in the Island of Vancouver, Canada, and in the U.S. [3, 4, 21]. Genetic studies with multilocus sequence typing (MLST), which uses the presence of virulence genes to determine subgroups, have shown that the VGIIa and VGIIb subtypes are responsible for the majority of cryptococcosis cases. Another subtype, VGIIc, which is also virulent, has emerged in Oregon, U.S. and is now, together with subtype VGIIa, contributing to the rise of the disease in that region [4, 22].

C. neoformans genotype VNI and *C. gattii* genotype VGI are regarded as the primary agents of cryptococcosis worldwide. However, in Latin America, the distribution and occurrence of *C. gattii* types differ from those in other continents [17, 19, 23, 24]. In Brazil, the *C. gattii* genotype VGII type is responsible for infections in immunocompetent hosts in the Northern (N) and Northeastern (NE) regions.

Genotype VGI is endemic in Australia and has also been described in Papua New Guinea, Asia, and southern California. The VGIII and VGIV genotypes are found less frequently, with the VGIII type isolated in the Ibero-American regions and in India and type VGIV recorded in South Africa and in the U.S. [19, 25–29].

3. Natural history of the disease

Cryptococcosis is a systemic mycosis with a pulmonary gateway, which is caused by infection with either *C. neoformans* or *C. gattii.* Infection is initiated after inhalation of the fungus' infective propagules, which are the basidiospores, or desiccated yeasts, that are dispersed in the environment [27, 30]. In the majority of cases, inhalation produces a self-limited asymptomatic pulmonary infection, which is dependent on the host's immune response, the inoculum's size, and the microorganism's virulence. Residual focuses with viable fungal elements can be established, and these can be reactivated after a number of years. At times, it may mimic tuberculosis, with nodular lesions with no calcification and eventual cavitation. Other presentations include a controlled mass similar to neoplasia, and at times, the disease manifests as pneumonia that can evolve into acute respiratory failure. The pulmonary form is the second most frequent form and attacks 35.7% of HIV-negative patients [31, 32].

Once in the lung, *C. neoformans* or *C. gattii* transit through the blood-brain barrier (BBB) to reach the central nervous system (CNS), causing meningoencephalitis and, in the most serious forms of the disease, brain cryptococcomas. The fungus shows high tropism in the CNS, which is attributed to the optimal concentration of existing nutrients in the cerebrospinal fluid that can be assimilated by the fungus (thiamine, glutamic acid, glutamine, dopamine, carbohydrates, and minerals), as well as to the lack of complement system activity in the cerebrospinal fluid (CSF) and the poor or absent inflammatory response activity of the brain tissue [33–35]. Del Poeta *et al.* [1] recently found that despite the various mechanisms proposed to be responsible for this neurotropism, it is still unknown as to how exactly this occurs during the infection in humans. The *C. gattii* incubation period associated with outbreaks is known to be short. It is believed that this strand can be more aggressive prior to its dissemination into the CNS. In the

disseminated form, one can observe the development of cutaneous infections in the form of papulae, pustules, or subcutaneous nodules. In addition, there are cases of primary cutaneous infection with no dissemination, as well as infections in other organs [34, 36, 37].

4. Virulence factors

The pathogenic species of the genus *Cryptococcus* has a number of well-elucidated determinants of virulence. Some notable determinants include the ability to produce melanin, extracellular enzymes, the capacity to survive and proliferate at 37°C (thermal tolerance), and to escape oxidative damage caused by the host and the presence of a large polysaccharide capsule [30, 38, 39].

The polysaccharide capsule is composed of 90 to 95% glucuronoxylomannan (GXM), 5% galactoxylomannan (GalXM), and approximately 1% mannoproteins (MPs) [40, 41]. It is regarded as one of the most important virulence factors for *C. neoformans* and *C. gattii*, and its cell components accumulate in body fluids, thus serving as targets for diagnosis. The mechanism proposed to explain the contribution of the capsule to virulence is its capacity to inhibit phagocytosis, inactivate components of the complement system, induce apoptosis, and regulate cytokine synthesis. Inside the macrophages, *Cryptococcus* spp. releases and accumulates fragments of the polysaccharide, which is shown to be cytotoxic to the macrophage, causing dysfunction or cell death [42–44].

5. Diagnosis of cryptococcosis

The laboratory diagnosis of cryptococcosis is based upon a number of principles: the demonstration of the yeast in the clinical material, the isolation of the yeast in the culture followed by biochemical tests for the final identification, anatomic-pathological examination, and research into circulating antigens. Several biological materials may be used for the identification of fungal infection, e.g., serum, plasma, blood, tissue, and CSF, which is the major biological material used for the diagnosis of cryptococcal infection in the CNS [45].

The direct research of the fungus can be accomplished using CSF, sputum, bronchial washing, cutaneous-mucosal lesion pus, urine, macerates of biopsy tissue, prostatic secretion, blood, and bone marrow biopsy specimens. Clinical samples analyzed with India ink indicate the presence of the capsulated yeasts (Figure 1). This method is fast and low-cost but is not very sensitive and cannot distinguish between species. Due to the high yeasts load found in samples from AIDS patients, the sensitivity of this method may reach 80% for cryptococcal meningitis, whereas in immunocompetent individuals, this sensitivity may be as low as 30–72% [46–48]. In addition, the success of this technique is dependent upon the expertise of the microbiologist, and there are reports in the literature of false negatives in 20–30% of the results from infections with *C. neoformans* or *C. gattii* due to a deficient capsule or the low fungal charge of the agent in the CSF. This is primarily an issue with the initial cases, when the diagnosis is fundamental.

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Examination of *C. neoformans* or *C. gattii* in tissues is carried out with specific dyes, e.g., mucicarmine and silver [49].

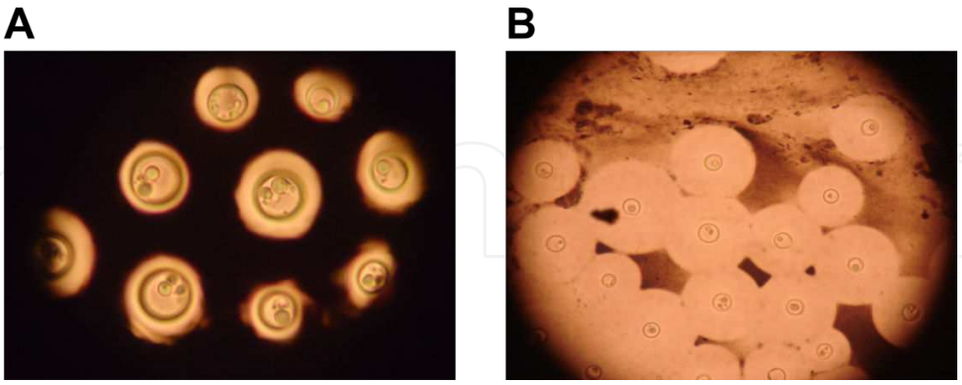

Figure 1. *Cyptococcus* spp. cells with capsule from (A) cutaneous lesion and (B) sputum identified by India Ink (40×).

The culturing of *Cryptococcus* spp. serves to corroborate the diagnosis of the disease (Figure 2). The fungus grows well in various culture media that do not contain cycloheximide (blood agar, Sabouraud agar, and brain–heart infusion agar). For cases of meningoencephalitis, the CSF culture must be repeated 7 days after the beginning of treatment and fortnightly thereafter. The cultures may remain positive for a long time, even over the course of treatment.

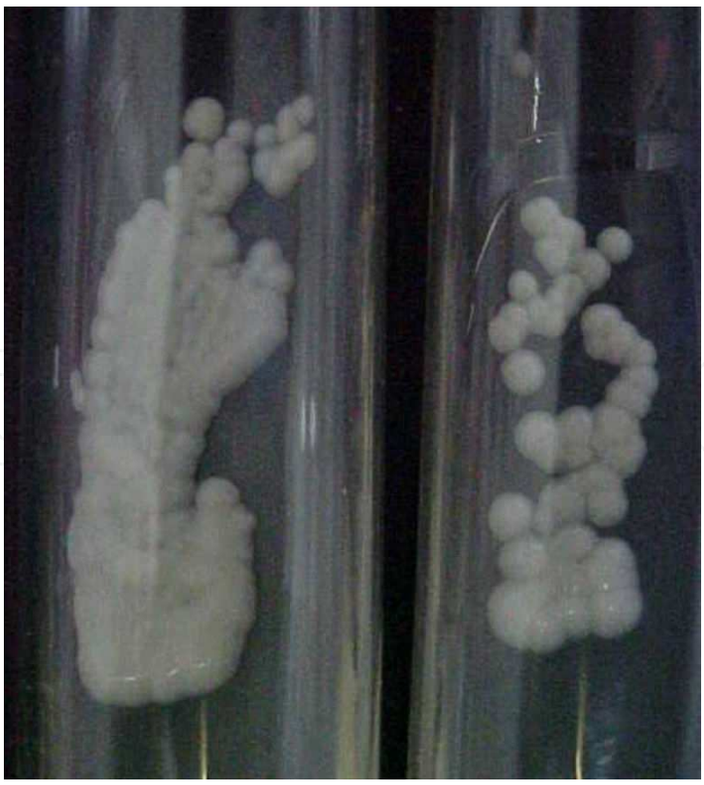

Figure 2. *Cryptococcus* culture in Sabouraud dextrose agar 2%.

After obtaining the isolate, it is necessary to differentiate the species type for clinical and epidemiological purposes [50–52]. Only *C. gattii* is resistant to canavanine and uses glycine as a carbon source; thus, canavanine-glycine-bromothymol blue agar (CGB agar) was proposed in 1982 and has been widely used in laboratories for distinguishing between *C. gattii* and *C. neoformans* species [16, 53, 54]. However, a positive reaction in CGB agar is not sufficient for definitively distinguishing the species as there have been reports of *C. neoformans* resistance to high concentrations of canavanine [55, 56].

The production of urease is a biochemical test used to identify only the genus as both *C. gattii* and *C. neoformans* are able to carry out hydrolysis of urea [57, 58]. The species *C. neoformans* and *C. gattii* are the only members of the genus that may produce melanin, thus showing a brownish color in culture media with seed extracts, e.g., *Vicia faba* or *Guizotia abyssinica*. This is due to the presence of tyrosine and chlorogenic acid in these seeds, which are oxidized by phenoloxidase produced by yeast [59, 60]. Culture media that induce the production of melanin are widely used in laboratories for the identification of yeast and differentiation of *Candida* spp.

During infection, the capsular polysaccharides of *C. neoformans* and *C. gattii* solubilize in the body fluids and can be detected and quantified with antibody-based assays. Detection of CrAg (cryptococcal antigen) has been an effective tool for diagnosing cryptococcosis [61, 62]. Detection of CrAg in the serum and CSF by the latex agglutination test (CrAg-latex) or enzyme immunoassays (EIA) has been used for more than 35 years [62]. The majority of comparative studies use cultures as the gold standard. In individuals diagnosed with AIDS and meningitis, the CrAg must always be evaluated, including cases in which the India ink assay cannot identify the yeasts. One study showed that patients living in Uganda with cryptococcal meningitis and HIV who had a negative yeast screening by the India ink assay tested positive for CrAg detection by CSF [63].

The detection of the capsular antigen by agglutination of sensitized particles of latex (LA), which until sometime ago was the immunological method with the most widespread clinical use, may be accomplished in samples from the serum, urine, bronchoalveolar lavage, and CSF. The serological reaction to latex agglutination (LA) is sensitive and specific, emphasizing titers equal to or higher than 1/8 and being able to present cross reaction with the serum of patients with rheumatoid arthritis [62, 64]. The enzyme-linked immunosorbent assay (ELISA) may detect antigens from a cryptococcal infection earlier and at lower titers; however, it is time consuming, expensive, and is laborious. Although CrAg-latex performs as well as EIA and culture, its major limitations are that latex is a manual test and that the resulting interpretation of it is subjective. CrAg-latex and EIA also require laboratory equipment and refrigeration of reagents, making them inadequate for use in environments with minimal infrastructure [61]. The need for refrigeration drastically increases the cost of the test in places with limited resources. Studies report that serological tests with CrAg-latex and EIA may show lower sensitivity when used with strands of some genotypes of *C. gattii*. The rate of false-positive examinations is lower than 1%; false positives are generally explained by technical issues, existence of other infections (e.g., *Trichosporon beigelii*, *Capnocytophaga canimorsus*, and *Stomatococcus mucilaginosus*), or contamination. False-negative results may occasionally be

observed with early infections when there is a low fungal charge, with prozone phenomena, and with poorly encapsulated organisms [62, 65].

Recently, a new sensitive, low-cost, fast, and non-laborious immunochromatographic assay known as the lateral flow immunoassay (LFA) was made available for purchase for use in serum, CSF, and urine [66]. This method has demonstrated good sensitivity for the detection of cryptococcal antigen (CrAg), primarily in HIV-positive patients [27]. The World Health Organization (WHO) has recommended the use of antigen detection using LFA for patients infected with HIV who show low CD4 cells and are asymptomatic from a neurological viewpoint [67]. This strategy enables the early identification of patients with a cryptococcal disease in the subclinical stage [68]. It has been used in various studies as a form of screening and diagnosis, thus easing its application to clinical practice. Nevertheless, reasonably good results have been accomplished in multiple types of biological specimens, e.g., blood, CSF, and urine [69].

6. Synthetic peptides

The concept of synthetic peptides and protocols for their artificial synthesis was introduced in the early 20th century [70]. Since then, peptides have become increasingly important for biochemistry, medicine, and biotechnology. In 1963, Bruce Merrifield described the development of solid-phase peptide synthesis, a technique that made the large-scale production of synthetic peptides a reality. Since then, various studies with different sizes of synthetic peptides have been reported [71].

In the early 1990s, with the development of biotechnology, recombinant antigens were widely used in clinical diagnosis to detect specific antibodies. However, their use in diagnostic tests presented some problems, such as low immunoreactivity compared with the corresponding purified human antigens, laborious and expensive production, and variation in inter-assay reactivity [72–76].

In this regard, synthetic peptides have opened up a new field and perspective as a source of pure epitopes and molecules for the diagnosis of various infectious and noninfectious diseases based on the detection of circulating antibodies and antigens and can also be used for the development of vaccines [77]. Bioinformatics tools are widely used to predict antigenic and immunogenic regions. These programs are capable of predicting B and T cells epitopes, primarily by building on the known properties of amino acids, e.g., their hydrophilicity, charge, flexibility, exposed surface area, and secondary structure [78–80].

Some factors must be taken into account when dealing with synthetic peptides. The first factor to observe is whether the epitopic area is continuous or discontinuous because the amino acids belonging to the epitope are often separated in the linear sequence and become juxtaposed only when the antigen is in its native conformation. The second factor for observation is the size of the epitope. When this field of study began, researchers worked with only small epitopes as prior to the development of solid-phase peptide synthesis, one could not synthesize very

large peptides. The very large peptides (>25–30 amino acids) are more expensive and difficult to produce and also have lower yields. For these reasons, peptides of 10–15 amino acid residues are usually recommended for the production and detection of antibodies [81–83].

The use of synthetic peptides for diagnostic tests confers several advantages, e.g., they are innocuous, easy to store and transport, have a high level of reproducibility with low levels of nonspecific reactions, and retain the possibility of changing the chemistry of the peptide by inserting cysteine residues, fatty acids, or carrier proteins or even by incorporating post-translational modifications, such as phosphorylation [84–86].

Over the past 20 years, several peptide sequences have been used to improve the sensitivity and specificity of tests that use recombinant or native protein as antigens [87–93]. However, the use of synthetic peptides as antigens has grown, with many diagnostic systems that are based on synthetic peptides in production, with some being commercially available at the present time. Some diagnostic tests that use synthetic peptides may already be part of the routine clinical diagnosis of certain diseases that involve viruses, parasites, or autoimmune diseases.

Some of the tests that are already available on the market include tests for Epstein–Barr virus, which examines various epitopes on the capsid protein; hepatitis C virus, which includes synthetic peptides that mimic its structural and nonstructural regions (NS4 and NS5); coronavirus, which is composed of synthetic peptides derived from epitopes of the nucleocapsid and spike proteins and can detect the presence of antibodies from human serum and plasma specimens; *Chlamydia trachomatis*, which has three ELISA diagnostic tests available on the market; and rheumatoid arthritis, with three generations of diagnostic tests based on the detection of antibodies by synthetic peptides [94–97]. Despite the existence of various diagnostic tests using synthetic peptides and the prevalence of studies reporting the use of synthetic peptides for the diagnosis of various pathologies, particularly those of medical importance, such as tuberculosis, Chagas disease, and leishmaniasis, little has evolved in this area with respect to systemic mycoses [98–103].

Recent advances have been made in the search for more easily available immunodiagnostic tests for fungal infections. Various methods with high specificity and sensitivity are still under development, with a particular emphasis on the search for markers that are able to detect infections at an early stage. In this regard, Caldini et al. [100] used synthetic peptides from the gp75 *Paracoccidioides brasiliensis* antigen as an alternative diagnostic method for the detection of paracoccidioidomycosis.

With regards to cryptococcosis, the search for new diagnostic tests is necessary due to the high incidence of the disease, with estimates of approximately 1 million cases of cryptococcal meningitis per year and more than 600,000 deaths in HIV-infected patients, the potential for *C. gattii* to cause the disease in immunocompetent individuals, and its rapid worldwide dissemination [8]. Therefore, controlling the disease is dependent on epidemiological control of endemic areas coupled with the mapping of new cases and early diagnosis of the disease in affected individuals.

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As previously mentioned, diagnostic methods based upon the detection of antibodies have been developed and successfully applied to various other infectious diseases. The efficacy of these methods is not impacted by the antigenic charge of the microorganism, which is particularly relevant for the diagnosis of cryptococcosis, whose major diagnostic tests, LA and LFA, are dependent on the charge of the antigen.

The early diagnosis of cryptococcosis is a challenge that science and the health system must face as in most cases, the disease is diagnosed late, which results in significant morbidity and mortality. Thus, efforts should be made toward finding a rapid, sensitive, and specific diagnosis. In this sense, the identification of multiple immunogenic targets and the possibility of synthesizing these artificial targets appear to be a promising alternative for the development of more accurate tests for the diagnosis of systemic mycosis.

In this area, Martins et al. [104] have adopted an innovative strategy that combines the technology of proteomics and bioinformatics, with the aim of identifying multiple immunogenic targets for a diagnostic test for cryptococcosis. Linear B-cell epitopes of immunoreactive proteins for *Cryptococcus* species were mapped using *in silico* analyses.

In the search for a faster and more specific test, Brandão et al. [105] tested various synthetic peptides derived from immunoreactive proteins of *Cryptococcus*spp. Of these, six showed good results, which became promising candidate antigens for future diagnostic tests. These six peptides belonged to the proteins Hsp70, Sks2, GrpE, enolase, and two hypothetical proteins. One of these, derived from Hsp70, showed 100% specificity and approximately 80% sensitivity. Table 1 shows the specificity and sensitivity of diverse diagnostic methods for cryptococcosis.

Tests	Sensitivity (%)	Specificity (%)	References
India ink	30–80	100	[46–48]
Culture	80	100	[106]
CrAg-LA	93–100	93–98	[62]
CrAg-EIA	93–100	93–98	[62]
CrAg-LFA	99–100	92–100	[106]
Synthetic peptides	55–79	90–100	[105]

CrAg, cryptococcal antigen; EIA, enzyme immunoassay; LFA, lateral flow assay; LA, latex agglutination

Table 1. Comparative performance of cryptococcosis diagnostic tests.

Hsp70 is a conserved protein that has been increasingly studied worldwide for its role in various biological processes, including the interaction of *Cryptococcus*spp with host cells. Hsp proteins have been characterized as dominant antigens in diverse models, including candidiasis, aspergillosis, and histoplasmosis [107–110]. In cryptococcosis, the Hsp proteins have been reported to be key antigens that are important for inducing the humoral response [104, 111–113]. These reports support the proposed use of epitopes from immunoreactive proteins, e.g., Hsp70, as antigens in a diagnostic test for cryptococcosis.

Higher diagnostic performance can be achieved with multi-epitope chimeric proteins. This type of antigen becomes more attractive because it has more than one antigen-binding site, thus multiplying the possibilities for increasing antigenicity. Brandão et al. demonstrated in a theoretical model (*in silico*) that the combination of peptides in a single molecule is a good strategy for improving the accuracy of a test; therefore, its use is of interest for the development of new diagnostic tests [105, 114–118].

The use of this technology for the development of a diagnostic test capable of the early identification of cryptococcosis and the possibility of building an effective vaccine for this disease are essential for significant reduction in the morbidity and mortality of individuals affected by this infection and may ultimately change their prognosis.

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