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Immunoproteomics and immunoinformatics analysis of *Cryptococcus gattii*: novel candidate antigens for diagnosis

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Aim: To identify immunoreactive proteins of *Cryptococcus gattii* genotype VGII and their B-cell epitopes. **Materials & methods:** We combined 2D gel electrophoresis, immunoblotting and mass spectrometry to identify immunoreactive proteins from four strains of *C. gattii* genotype VGII (CG01, CG02, CG03 and R265). Next, we screened the identified proteins to map B-cell epitopes. **Results:** Sixty-eight immunoreactive proteins were identified. The strains and the number of proteins we found were: CG01 (12), CG02 (12), CG03 (18) and R265 (26). In addition, we mapped 374 peptides potentially targeted by B cells. **Conclusion:** Both immunoreactive proteins and B-cell epitopes of *C. gattii* genotype VGII that were potentially targeted by a host humoral response were identified. Considering the evolutionary relevance of the identified proteins, we may speculate that they could be used as the initial targets for recombinant protein and peptide synthesis aimed at the development of immunodiagnostic tools for cryptococcosis.

Cryptococcosis has become a significant public global health problem. The CDC estimate the occurrence of over 1 million new cases per year of cryptococcosis worldwide in patients with AIDS and, despite the recent improvements in the diagnosis and treatment of cryptococcosis, cryptococcal meningitis is responsible for over 600,000 deaths per year worldwide. This potentially fatal fungal disease is caused by one of two species of the same genus: *Cryptococcus neoformans* (genotypes VNI–VNIV) or *Cryptococcus gattii* (genotypes VGI–VGIV). The first species is best known as the cause of severe meningoencephalitis or meningitis in immunocompromised patients and has been considered to be the most medically important species for many years [1–5]. It is important for clinical microbiology laboratories to accurately differentiate one species from the other. Rapid identification of *C. neoformans* and *C. gattii* is imperative for favoring the prompt treatment of cryptococcosis and for understanding the epidemiology of the disease [6–11]. *C. gattii* merits more attention so that its environmental occurrence and role in cryptococcosis can be accurately determined, as this information will be helpful in devising strategies to manage potential outbreaks of cryptococcosis. Today, the differentiation between *C. neoformans* and *C. gattii* is a difficult task since: the available commercial differentiation methods (e.g., API® 20C AUX [bioMérieux, France], Vitek® [bioMérieux] and MicroScan® [Siemens, Germany]) do not differentiate between *C. neoformans* and *C. gattii*; and the methods capable of differentiating the two species (multiplex PCR, liquid array detection of pathogens, PCR restriction fragment length polymorphism [12,13] and matrix-assisted laser desorption/ionization mass spectrometry [MS] [14–16]) are not routinely used.

Recently, however, increased medical attention has been paid to *C. gattii*, which mainly affects immunocompetent individuals [1,2,17]. The interest in clinical studies of this species has arisen due to its rapid spread in Asia, Africa, Australia, Europe and South America, along with outbreaks in Vancouver Island, BC, Canada, and in parts

Keywords
■ B cell ■ cryptococcosis ■ *Cryptococcus gattii* ■ diagnosis ■ epitopes ■ immunoinformatics ■ immunoproteomics

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of continental North America. The potential for *C. gattii* to cause illness in immunocompetent patients and its rapid spread worldwide justify the implementation of a public health effort to increase the awareness of both the public and healthcare professionals [1,18–24]. The mechanism of *C. gattii* dispersion is not well understood. However, it is thought that the emergence of industrialization, as well as the subsequent development of high population densities, concurrent with processes such as the export of trees and wood products, transport of bacteria via air currents, water currents and biotic agents (e.g., birds, animals and insects) and global warming, may be the main catalysts for the spread of this pathogen [1,2]. As cryptococcosis does not require compulsory notification in Brazil, and there is currently no surveillance of this mycosis, its prevalence and annual incidence can only be approximately estimated based on publications of cases occurring in specialized services. Nevertheless, it is well known that the clinical cases attributed to these two species of the *Cryptococcus* genus are distributed differently throughout Brazil, with *C. neoformans* being predominant in the south and southeast and *C. gattii* (mainly genotype VGII) being predominant in the north and northeast regions. In these latter two regions, *C. gattii* affects immunocompetent adults, adolescents and children, and is associated with high morbidity and mortality rates (ranging from 37 to 49%). Information regarding the conditions that support the development of cryptococcosis in immunocompetent individuals remains elusive. However, it is possible that environmental factors play an important role in this process, considering that people living in endemic areas are at high risk for developing the disease [21,25–30]. Preventative measures that effectively combat infection by *C. gattii* are not available at present. Hence, the best approach for controlling cryptococcosis is through early diagnosis and treatment. Although recent advances directed at early diagnosis of cryptococcosis have been published [31–33], a diagnostic method with high specificity and sensitivity to guide the management and control of cryptococcosis remains to be developed.

Information from the recently published *C. gattii* genome, combined with data obtained via proteomic approaches, has created opportunities for the development of diagnostic tools and therapeutic targets in the context of cryptococcosis caused by *C. gattii* [18]. The development of such tools has been improved by the production of antigens, which increases the range of alternative tests for immunoassay-based pathogen detection. In this study, we identify both immunoreactive proteins of *C. gattii* and predicted B-cell epitopes for their potential use as antigens in new serologic tests.

Materials & methods

Study patients & sera

The study included three immunocompetent subjects with cryptococcal meningitis recruited at the Natan Portella Institute of Tropical Diseases, a reference center for infectious diseases in Teresina, the capital of the northeastern state of Piauí, Brazil. As negative controls, we used sample sera from uninfected subjects (n = 3) who did not present clinical indicators or positive serological tests for cryptococcosis. The sera obtained from these patients were stored in a freezer at -20°C until use. The demographic, epidemiological, laboratory and baseline clinical characteristics of each patient were obtained from medical records and transferred to an epidemiological sheet. Detailed information regarding the patients involved in this study was published by Martins *et al.* [26]. Sera from three immunocompetent patients with cryptococcal meningitis caused by *C. gattii* genotype VGII were selected to produce a pool of serum containing each antigen at a titer of 1:1024 as determined by the latex agglutination test for diagnosing cryptococcosis. This pooled serum was used for all western blot experiments.

C. gattii strains

C. gattii proteins were screened in four different strains. The first strain, R265 (ATCC number: MYA 4093), was previously identified by Kidd *et al.* [20] and was kindly provided by MH Vainstein from the culture collection of the Laboratory of Fungi of Medical and Biotechnological Importance from the Biotechnology Center, Federal University of Rio Grande do Sul (UFRGS), Brazil. The other three *C. gattii* strains (CG01, CG02 and CG03) molecular type/genotype VGII involved in this study were isolated from immunocompetent patients who had cryptococcal meningitis (two children and one adult, from the state of Piauí, northeastern Brazil). These isolates were submitted to strain identification [26], which was performed via PCR restriction fragment length polymorphism analysis using *URA5* as a target gene, as described by Meyer *et al.* [34].

Culture conditions

Strains were recovered from 15% skimmed milk stocks, stored at -20°C prior to use. The strains

were maintained on yeast extract–peptone–dextrose (YPD) media (1% yeast extract, 2% peptone, 2% dextrose and 2% Bacto™ Agar, Becton Dickinson, NJ, USA). Each isolate was inoculated and grown in 200 ml of YPD broth at 37°C for 24 h with shaking at 200 rpm. To obtain a better protein yield, the cultures were subsequently quantified in a Neubauer chamber, reseeded to an equivalent of 4×10^7 cells/ml in 200 ml YPD final broth and incubated under the same conditions as described above. After 24 h, the cells were collected via centrifugation at 7500 *g* for 10 min at 10°C and washed three times in cold, sterile Milli-Q® water (Millipore Corp., MA, USA). The cell pellet obtained was stored at -80°C.

Preparation of protein extracts

Prior to the extraction process, the samples were lyophilized (FreeZone® lyophilizer, Labconco, MI, USA) and macerated in liquid nitrogen until a fine powder was obtained. The samples were suspended in a lysis buffer with protease inhibitors and detergents (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 50 µM N-*p*-tosyl-L-phenylalanine chloromethyl ketone, 5 mM iodoacetamide, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and 0.25% v/v Triton™ X-100). The proteins were solubilized by vortexing the suspensions for 5 min at intervals of 1 min on ice, followed by centrifugation at 10,000 *g* for 20 min at 8°C. The supernatants were collected and preserved at -20°C. The remaining cell debris was suspended in the same buffer, followed by vortexing for 5 min with intervals on ice every 1 min. The supernatant was collected after centrifugation, pooled with the first supernatant and stored at -80°C. The protein content was determined by the Bradford method [35] using known concentrations of bovine serum albumin as the standard.

2D gel electrophoresis

Samples containing 150 µg of protein were precipitated using a 2D gel electrophoresis (2DE) clean-up kit (GE Healthcare, UK) following the manufacturer's instructions, then solubilized in 150 µl of isoelectric focusing (IEF) buffer containing 9 M urea, 4% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT) and 0.2% (v/v) ampholytes pH 4–7 (Bio-Rad, CA, USA) with 0.002% orange G. Next, 7-cm immobilized pH gradient (IPG) strips (linear gradient, pH 4–7, Bio-Rad) were passively rehydrated for 16–18 h. IEF was performed using the PROTEAN® IEF Cell System (Bio-Rad) at 20°C with the following conditions: 250 V for 15 min, 250–4000 V for 2 h and 4000 V until reaching 10,000 V/h, with a maximum current of 50 µA/strip. Focused IPG strips were equilibrated for 15 min in equilibration buffer I, containing 30% (v/v) glycerol, 6 M urea, 1% DTT, 2% (w/v) sodium dodecyl sulfate (SDS), 0.375 M Tris pH 8.8 and 0.002% Bromophenol Blue, and then alkylated for 15 min in equilibration buffer II (equilibration buffer I in which the DTT was replaced with 4% iodoacetamide). SDS-PAGE was performed using the method developed by Laemmli [36]. The IPG strips were then placed on a 12% SDS-PAGE gel, and the second dimension of separation was performed in two steps at 10°C: 50 V/gel for 30 min and 100 V/gel until the tracking dye reached the bottom of the gels in a Mini-PROTEAN Tetra Cell Chamber (Bio-Rad), according to the manufacturer's instructions. The gels were stained with Coomassie Brilliant Blue and scanned with a GS-800™ scanner (Bio-Rad). Three technical replicates of classical 2DE western blots for each of the four independent biological samples were performed for each strain. A Coomassie Brilliant Blue G250 gel and the other duplicate gel were transferred onto a polyvinylidene difluoride membrane for subsequent western blot analysis [37].

Western blot: 2DE analysis

The gels were transferred to polyvinylidene difluoride membranes (Hybond ECL™, GE Healthcare) at 400 mA for 1 h in a transfer buffer (25 mM Tris, 192 mM glycine, 2% w/v SDS and 20% v/v methanol). The membranes were blocked at 4°C overnight with 5% (w/v) nonfat dry milk in phosphate-buffered saline pH 7.4 with Tween®-20 (PBS-T; 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, and 0.1% v/v Tween-20, Sigma Aldrich, MO, USA) and washed three times for 10 min. The membranes were then incubated with pools of sera from three patients with cryptococcal meningitis, diluted 1:7500 in blocking buffer for 2 h at 24°C. This primary antibody dilution was previously determined based on performing western blotting using a 1D gel with serum dilutions ranging from 1:2000 to 1:10,000. After washing three times with PBS-T, the blots were incubated with antihuman IgG (GE Healthcare) diluted 1:2000 in blocking buffer for 1 h at 24°C. The membranes were washed three times with PBS-T buffer for 15 min and twice with phosphate-buffered saline for 10 min. Finally, the 2DE blots were processed with the ECL detection

reagent (GE Healthcare) according to the manufacturer's instructions. Images were prepared using the VersaDoc™ 4000 MP imaging system (Bio-Rad). The western blots were evaluated in technical triplicates. The immunoreactivity of each spot, representing a positive signal in the western blot analysis, was identified by matching with the position of the corresponding spot on the gel stained with Coomassie Brilliant Blue. To select spots, the images from membranes and gels containing protein extracts were analyzed using an ImageMaster 2D Platinum 6.0® (GE Healthcare). In order to identify any nonspecific reactions, we discarded all spots that were also reactive with the pool of sera from negative controls. Only the infected reactive spots sera were manually excised, destained, digested with trypsin and subjected to MS to identify the immunoreactive proteins.

Protein identification by liquid chromatography-electrospray ionization-quadrupole-time of flight tandem MS

Gel plugs were treated through three washing steps with 100 µl of 50% acetonitrile (ACN) and 50 mM ammonium bicarbonate (NH_4HCO_3) for 15 min, followed by one washing step with 100 µl of ACN. After washing, the gel plugs were dried via vacuum centrifugation (CentriVap® Benchtop Centrifugal Vacuum concentrator, Labconco) and subjected to trypsin digestion for 18–24 h at 37°C using 20 µl of 10 µg/ml trypsin (Trypsin Gold, MS grade, Promega, WI, USA) diluted in 25 mM NH_4HCO_3. Peptide extraction was performed twice for 15 min with 100 µl of a 100% ACN and 5% formic acid solution. Trypsin digests were then concentrated in a SpeedVac® concentrator to approximately 10 µl and resuspended in 10 µl of 0.1% trifluoroacetic acid.

The resulting peptides were analyzed by liquid chromatography-electrospray ionization-quadrupole-time of flight tandem mass spectrometry (MS/MS) using a Waters nanoACQUITY UPLC™ system coupled to a Q-TOF Ultima™ API mass spectrometer (Waters MS Technologies, UK) at the Unit of Protein Chemistry and Mass Spectrometry (Uniprote-MS, Biotechnology Center, UFRGS, Brazil). The peptides were eluted from the reverse-phase column into the mass spectrometer at a flow rate of 600 nl/min with a 10–50% water/ACN 0.1% formic acid linear gradient over 30 min. The MS survey scan was set to 1 s (0.1 s interscan delay) and recorded at 200–2000 Da. MS/MS scans were performed from 50 to 2000 m/z, and the scan and interscan rates were set as for MS. For each survey scan, the three most intense multiple-charged ions over a threshold of eight counts were selected for the MS/MS analysis. The collision energies for peptide fragmentation were set using the charge state recognition files for +2 and +3 peptide ions provided by MassLynx™ (Waters). The raw MS/MS data were processed using Mascot Distiller 2.2.1 software (Matrix Science, MA, USA) to form peak lists that were exported in the Mascot generic format.

Database searches

The peak lists for each protein spot were analyzed with the aid of the 'MS/MS Ion Search' engine of Mascot (version 2.1) software [101]. The Mascot search parameters were as follows: oxidation of methionine, modification of cysteine by carbamidomethylation, partial cleavage leaving one internal cleavage site, a peptide tolerance of 0.2 Da and a MS/MS tolerance of 0.1 Da. The significance threshold was set at $p < 0.05$, and identification required that each protein contained at least one peptide with an expected p-value < 0.05. Thus, we compared the calculated molecular mass and isoelectric point values from the identified proteins with the observed values on the 2DE gel. Additionally, if a protein with a nonsignificant score was part of a horizontal series of spots with the adjacent identical proteins, we included this identification. All amino acid sequences were downloaded as FASTA-format files from the Broad Institute protein database [102]. The Blast2GO tool [103] was used to obtain functional categories of proteins. This tool assigns gene ontology (GO) terms based on the Basic Local Alignment Search Tool (BLAST) definitions. This assignment was accomplished by submitting the FASTA sequences of the identified proteins to the Blast2GO platform and comparing them against the National Center for Biotechnology Information (NCBI) databases [104]. Briefly, Blast2GO used BlastP with the default parameters to identify similar proteins with GO annotations.

Mapping B-cell epitopes

To map linear B-cell epitopes in the immunoreactive proteins selected by western blotting, we used two different programs: ABCPred [105], which is based on machine-learning methods that apply a recurrent neural network [38], and BCPreds [106], which is also based on machine-learning methods, but involves methods that apply a support vector machine [39]. Only those

peptides that were simultaneously identified by the two programs were considered to be putative antigens for the development of immunoassays for cryptococcosis. This approach was based on the work of Faria *et al.*, which showed that the use of the default scores of prediction software programs associated with the overlap predictions of more than one software program can be preferable to the use of a single type of prediction [40].

Results

2DE proteome profiling of *C. gattii* strains

Proteins from *C. gattii* strain R265 and our isolates (CG01, CG02 and CG03) were separated using 2DE and analyzed for reactivity to the serum by immunoblotting. In fact, the capsule was a major obstacle to obtaining protein. The key points to improve protein recovery were background culture in liquid medium and lyophilization with maceration in liquid nitrogen. After these steps, proteins were obtained by solubilization in a lysis buffer containing 4% detergent (CHAPS). Protein samples obtained in this manner are mainly cytoplasmatic proteins and a trace quantity of membrane proteins associated to carbohydrates.

A representative image of the protein patterns is shown in Figure 1. Image analysis demonstrated approximately 350 spots for each strain of *C. gattii* (Figure 1), all of which optimally resolved at pH 4–7, and their molecular weights ranged from 12 to 225 kDa.

Only 68 of these spots showed reactivity based on immunoblotting against a pool of sera from cryptococcosis patients. One hypothesis to explain the low number of proteins identified by immunoblotting is that most of the proteins were cytoplasmic, together with the fact that many surface antigens of this fungus are located in capsules and should be represented mainly by carbohydrates.

The distribution of the spots for each strain is as follows: 12 spots from CG01; 12 from CG02; 18 from CG03; and 26 from R265. All of the immunoreactive spots selected for identification with MS were classified according to their biological activity (Table 1 & Supplementary Table 1) (see online at: www.futuremedicine.com/doi/suppl/10.2217/FMB.13.22). The data generated by the Blast2GO analysis are presented at GO multilevels to illustrate the general functional categories according to biological processes and molecular functions (Supplementary Figures 1 & 2) [103]. Of the 68 spots corresponding to 48 proteins, 34 (70.8%) were selected from only one strain, and 14 (29.2%) were simultaneously derived from at least two strains; among the latter 14 proteins, six were simultaneously selected from three strains (Figure 2).

Prediction of B-cell linear epitopes

We sought to determine whether the 48 reactive proteins identified in this study shared epitopes that were potentially recognized by patient antibodies. Therefore, we screened these proteins for predictive B-cell epitopes using two different bioinformatics tools. The ABCPred program yielded 4747 peptides, whereas BCPreds yielded 475. By comparing the B-cell epitopes generated by these two different approaches, we were able to identify 374 concordant peptides (the peptide sizes ranged from nine to 14 amino acids) (Supplementary Table 2). We believe that the B-cell prediction was efficient because the majority of the proteins (41/48; 85.4%) presented in a high percentage (greater than 25%) of the epitopes (Table 2). This percentage of epitopes takes into account the total number of predicted peptides and the total number of amino acids in the mapped protein. Two hypothetical proteins (CNBG_1302 and CNBG_1079) contained the highest number of predicted peptides. Proteins and peptides with a greater potential of being B-cell epitopes deserve further investigation as candidate antigens for use in diagnostic testing.

Discussion

In this immunoproteomic study, we identified immunoreactive proteins in *C. gattii*, as well as the putative B-cell epitopes of these predicted proteins using immunoinformatics tools. The proteins recognized *in vitro* by antibodies in this work probably have the same antigenic determinants that induce antibodies *in vivo*. This approach has frequently been used to identify immunogenic proteins and, consequently, new targets to diagnose several diseases [41–54]. Although similar results have been achieved in cryptococcosis using murine [55] and koala models [56], this is the first report in human beings. In addition, the use of sample isolates obtained directly from patients would be very difficult, because they are hard to obtain directly from the brain or lung during infection, and the sample collected would probably not have a sufficient amount of protein for a proteomic approach.

We identified 68 immunoreactive proteins and highlight that only six of them were reactive in three isolates simultaneously without a reaction for negative sera. Due to their

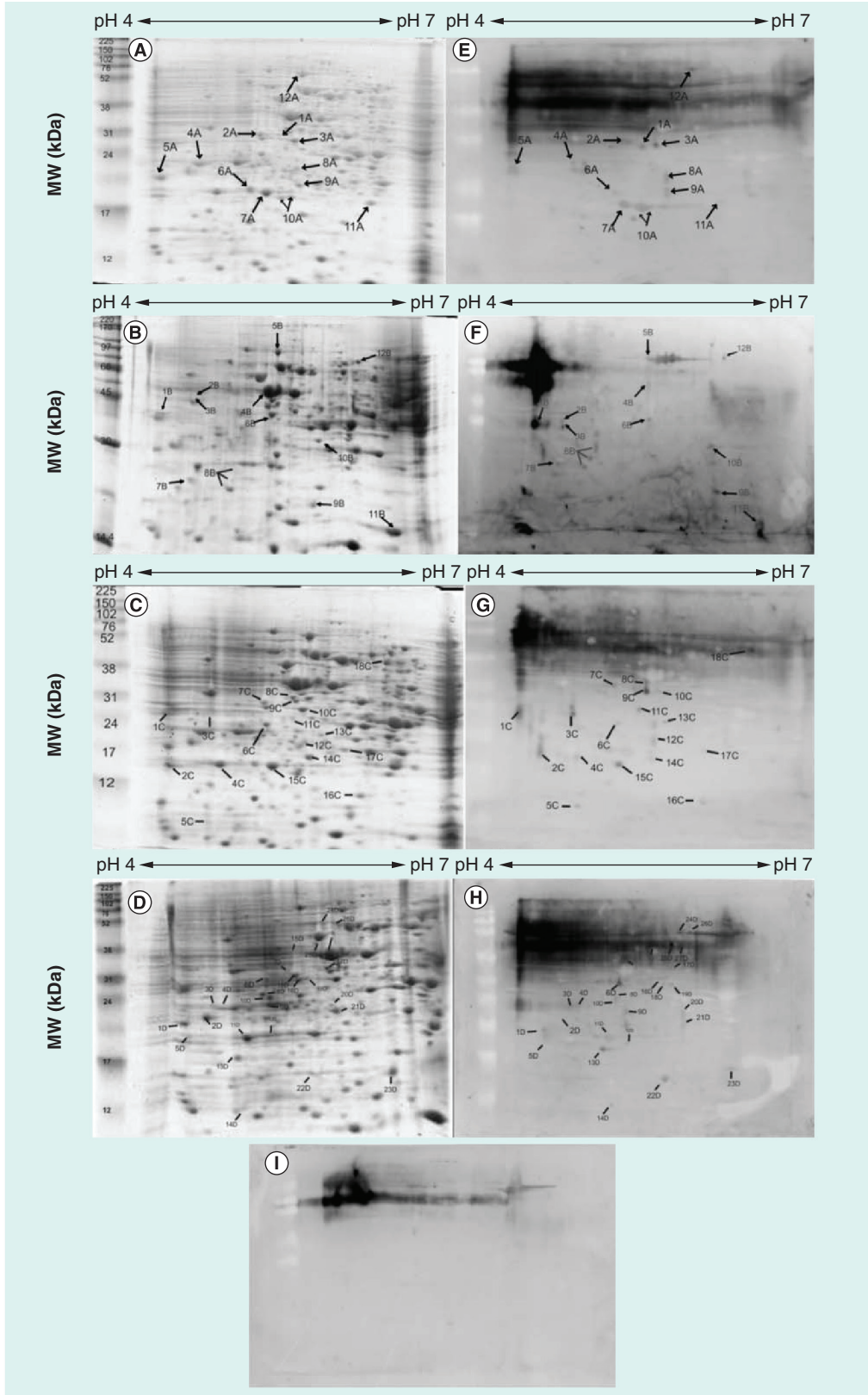

Figure 1. Western immunoblot analysis of representative serum samples. *Cryptococcus gatti* proteins in 2D gel electrophoresis: **(A)** CGOI, **(B)** CGO2, **(C)** CGO3 and **(D)** R265. Representative results of immunoblot from the pool of patient sera with cryptococcosis: **(E)** CGO1, **(F)** CGO2, **(G)** CGO3 and **(H)** R265 strains. **(I)** Result of immunoblot from the sera of uninfected person with cryptococcosis. The numbers refer to the spot identification used in Table 1.
MW: Molecular weight.

Table 1. Immunodominant proteins identified by serologic proteome analysis.

Spot/strain	Protein	Accession code	Mascot ID	Biological process	Molecular function
11A/CG01	Microtubule motor	E6R0Y5	CNBG_0115	Microtubule-based movement	ATP binding; microtubule motor activity
25D/R265	Heat shock protein sks2	E6R0K8	CNBG_0239	Response to stress	ATP binding
21D/R265	HHE domain-containing protein	E6R0I8	CNBG_0257	Unknown	Unknown
11C/CG03	Cytoplasm protein	E6R0G3	CNBG_0282	Unknown	Binding
5A/CG01	Conserved hypothetical protein	E6R0C0	CNBG_0290	Unknown	Unknown
7C/CG03	Conserved hypothetical protein	E6R068	CNBG_0372	Unknown	RNA binding
3D/R265	Ubiquitin carboxyl-terminal hydrolase	E6R058	CNBG_0379	Ubiquitin-dependent protein catabolic process	Ubiquitin thiolesterase activity
17D/R265	Disulfide-isomerase	E6QZV4	CNBG_0482	Cell redox homeostasis	Electron carrier activity; isomerase activity; protein disulfide oxidoreductase activity
9B/CG02	Conserved hypothetical protein	E6R3U6	CNBG_0624	Unknown	Unknown
12B/CG02	Aconitase	E6R432	CNBG_0705	Tricarboxylic acid cycle	Four-iron, four-sulfur cluster binding; aconitate hydratase activity
22D/R265	Cytoplasm protein	E6R4T7	CNBG_0959	Unknown	Unknown
8D/R265	Conserved hypothetical protein	E6R568	CNBG_1079	Unknown	Unknown
6B/CG02	Succinyl-CoA ligase β-chain	E6QYK0	CNBG_1185	Tricarboxylic acid cycle	ATP binding; succinate-CoA ligase (ADP-forming) activity
9C/CG03	Succinyl-CoA ligase β-chain	E6QYK0	CNBG_1185	Reductive tricarboxylic acid cycle	ATP binding
18D/R265	Succinyl-CoA ligase β-chain	E6QYK0	CNBG_1185	Tricarboxylic acid cycle	ATP binding
12C/CG03	Conserved hypothetical protein	E6QY79	CNBG_1302	Unknown	Unknown
3B/CG02	Endopeptidase	E6QY25	CNBG_1355	Proteolysis	Aspartic-type endopeptidase activity
8C/CG03	3-isopropylmalate dehydrogenase	E6QXQ4	CNBG_1460	Oxidation reduction	NAD or NADH binding
16D/R265	3-isopropylmalate dehydrogenase	E6QXQ4	CNBG_1460	Oxidation reduction	3-isopropylmalate dehydrogenase activity; NAD binding
4B/CG02	ATP synthase β-subunit	E6R8N5	CNBG_1632	ATP hydrolysis-coupled proton transport	ATP binding; hydrogen ion-transporting ATP synthase activity
15D/R265	ATP synthase β-subunit	E6R8N5	CNBG_1632	ATP catabolic process	Proton-transporting ATPase activity, rotational mechanism
6D/R265	Ketol-acid reductoisomerase	E6R847	CNBG_1816	Oxidation reduction	Coenzyme binding; isomerase activity; ketol-acid reductoisomerase activity
1C/CG03	Glyceraldehyde-3-phosphate dehydrogenase	E6R7Z5	CNBG_1866	Glycolysis	NAD binding; glyceraldehyde-3-phosphate dehydrogenase (NAD^+) phosphorylating activity
1B/CG02	Mannitol-1-phosphate dehydrogenase	E6RA07	CNBG_2079	Oxidation reduction	Oxidoreductase activity; zinc ion binding

The data are sorted by Mascot ID in crescent order.

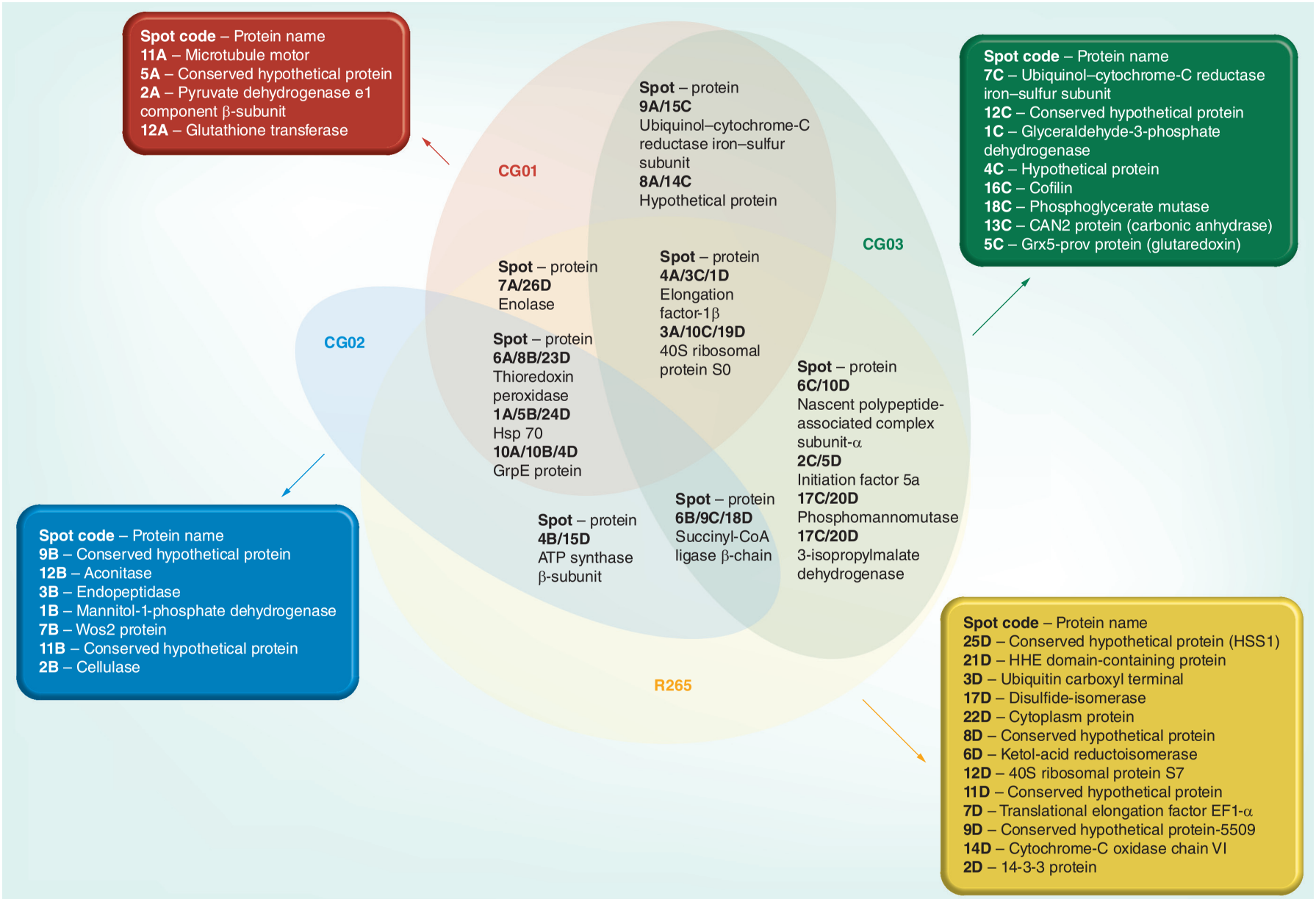

Figure 2. Venn diagram showing immunodominant proteins identified by serologic proteome from four *Cryptococcus gattii* strains. Strains, spots and protein names are according to Table 1.

Table 2. Proteins and B-cell epitopes mapped by ABCPred and BCPreds algorithms identified by immunoblot sera from patients with cryptococcosis.

Protein ID (aa†)	Accession code	Name of protein	WB strain‡	Epitopes (n)	Epitopes (%)§
CNBG_1302§ (222)	E6QY79	Conserved hypothetical protein	CG03	9	47
CNBG_1079§ (302)	E6R568	Conserved hypothetical protein	R265	11	42
CNBG_3163 (225)	E6RDR5	Wos2 protein	CG02	8	41
CNBG_0115 (595)	E6R0Y5	Microtubule motor	CG01	21	40
CNBG_2923 (292)	E6RBG9	40S ribosomal protein S0	CG01, CG03, R265	9	35
CNBG_0705 (780)	E6R432	Aconitase	CG02	23	34
CNBG_0282 (334)	E6R0G3	Cytoplasm protein	CG03	10	34
CNBG_4548 (239)	E6R5R6	CAN2 protein	CG03	7	34
CNBG_4834 (459)	E6RF86	Translation elongation factor EF1-α	R265	13	33
CNBG_4027 (281)	E6R6Y7	Ubiquinol–cytochrome-C reductase iron–sulfur subunit	CG01, CG03	8	33
CNBG_3631 (138)	E6R286	Cofilin	CG03	4	33
CNBG_5365 (431)	E6R762	Cellulase	CG02	12	32
CNBG_5509 (213)	E6QZ83	Conserved hypothetical protein	R265	6	32
CNBG_3378 (216)	E6R3K5	Elongation factor 1β	CG01, CG03, R265	5	32
CNBG_1866 (336)	E6R7Z5	Glyceraldehyde-3-phosphate dehydrogenase	CG03	9	31
CNBG_4560 (225)	E6R5Q4	Conserved hypothetical protein	R265	6	31
CNBG_6164 (187)	E6RED0	Conserved hypothetical protein	CG01, CG03	5	31
CNBG_3060 (228)	E6RDF4	GrpE protein	CG01, CG02, R265	6	30
CNBG_4692 (191)	E6R5B7	Nascent polypeptide-associated complex subunit-α	CG03, R265	5	30
CNBG_0959 (152)	E6R4T7	Cytoplasm protein	R265	4	30
CNBG_1632 (547)	E6R8N5	ATP synthase β-subunit	CG02, R265	14	29
CNBG_3753 (531)	E6RDZ5	Phosphoglycerate mutase	CG03	14	29

†*Position of the first amino acid of the epitope identified.*
‡*According to* **Table 1**.
§*Highest number of predicted peptides.*
WB: Western blot.

Table 2. Proteins and B-cell epitopes mapped by ABCPred and BCPreds algorithms identified by immunoblot sera from patients with cryptococcosis (cont.).

Protein ID (aa[†])	Accession code	Name of protein	WB strain[‡]	Epitopes (n)	Epitopes (%)[§]
CNBG_1355 (432)	E6QY25	Endopeptidase	CG02	11	29
CNBG_2617 (199)	E6RAJ9	40S ribosomal protein S7	R265	5	29
CNBG_5941 (158)	E6RBX1	Initiation factor 5a	CG03, R265	4	29
CNBG_0239 (614)	E6R0K8	Heat shock protein sks2	R265	15	28
CNBG_4851 (167)	E6RFA3	Cytoplasm protein	R265	4	28
CNBG_1185 (418)	E6QYK0	Succinyl-CoA ligase β-chain	CG02, CG03, R265	10	27
CNBG_2079 (420)	E6RA07	Mannitol-1-phosphate dehydrogenase	CG02	10	27
CNBG_0290 (251)	E6R0C0	Conserved hypothetical protein	CG01	6	27
CNBG_5765 (256)	E6RCQ9	14-3-3 protein	R265	6	27
CNBG_0379 (168)	E6R058	Ubiquitin carboxyl-terminal hydrolase	R265	4	27
CNBG_4912 (756)	E6RFH1	Heat shock protein (Hsp70)	CG01, CG02, R265	17	26
CNBG_2318 (175)	E6R9A9	Hypothetical protein	CG03	4	26
CNBG_4789 (135)	E6RF38	Conserved hypothetical protein	CG02	3	26
CNBG_0482 (408)	E6QZV4	Disulfide-isomerase	R265	9	25
CNBG_4625 (407)	E6R5I5	Pyruvate dehydrogenase e1 component β-subunit	CG01	9	25
CNBG_1460 (373)	E6QXQ4	3-isopropylmalate dehydrogenase	CG03, R265	8	25
CNBG_2499 (270)	E6RA79	Phosphomannomutase	CG03, R265	6	25
CNBG_6043 (233)	E6RCV4	Glutathione transferase	CG01	5	25
CNBG_0372 (291)	E6R068	Conserved hypothetical protein	CG03	6	24
CNBG_1816 (401)	E6R847	Ketol-acid reductoisomerase	R265	8	23
CNBG_5485 (152)	E6QZ59	Grx5-prov protein	CG03	3	23
CNBG_0257 (221)	E6R0I8	HHE domain-containing protein	R265	4	21
CNBG_0624 (125)	E6R3U6	Conserved hypothetical protein	CG02	2	18

[†]Position of the first amino acid of the epitope identified.
[‡]According to Table 1.
[§]Highest number of predicted peptides.
WB: Western blot.

Table 2. Proteins and B-cell epitopes mapped by ABCPred and BCPreds algorithms identified by immunoblot sera from patients with cryptococcosis (cont.).

Protein ID (aa†)	Accession code	Name of protein	WB strain‡	Epitopes (n)	Epitopes (%)§
CNBG_2132 (197)	E6R9V1	Thioredoxin peroxidase tpx1	CG01, CG02, R265	3	17
CNBG_3703 (433)	Q5KLA7	Phosphopyruvate hydratase (enolase)	CG01, R265	6	16

†*Position of the first amino acid of the epitope identified.*
‡*According to* Table 1.
§*Highest number of predicted peptides.*
WB: Western blot.

antigenicity in different strains, we believe that these proteins would be most promising for testing as antigens. However, after the analysis of the overlapping protein sequences in *C. gattii* (data not shown), we observed a high similarity (above 94%) between proteins from *C.gattii* genotypes VGI and VGII, except for the enolase protein (51%). Taken together, assessing the specificity of these antigens will be the next step to validate them for the diagnosis of cryptococcosis.

In addition, the fact that only six immunoreactive proteins were common to the three strains of *C. gattii* genotype VGII tested in our study is not surprising. For example, we recently demonstrated the significant genetic diversity of the *C. gattii* genotype VGII in Brazil using the multilocus sequence typing technique [57]. Of the six immunoreactive proteins identified here (Table 1), three are constitutive and involved in the cell cycle, cell division or the tricarboxylic acid cycle and were therefore excluded from this discussion.

Two of the three antigenic targets identified here, Hsp70 and GrpE, are members of the 70-kDa heat shock protein family. Hsp70 was identified as an antigenic target in sera from cryptococcosis patients infected with the strains CG01, CG02 and R265. This finding corroborates previous studies that identified proteins of the Hsp family as potential biomarkers for cryptococcosis [58–60]. Hsp70 is a phylogenetically conserved chaperone protein whose expression increases in response to temperature variation and environmental stress [61]. The role of this protein as an antigenic target in the context of cryptococcosis has been reported by various studies in both humans (by Rodrigues *et al.* [62]) and animal models in the clinical and subclinical phases (by Jobbins *et al.* [56] and Young *et al.* [55]), thus supporting our findings.

To our knowledge, this is the first report of the involvement of the second type of protein in the chaperone family with a size of 70 kDa (GrpE) as an antigenic target in the context of cryptococcosis. These new data have the potential to be used in the development of immunoassays because the same serum pool of neurocryptococcosis patients showed reactivity to GrpE in the three distinct strains of *C. gattii* genotype VGII.

The third protein identified in our study as an interesting antigenic target for the development of an immunoassay is the thiol peroxidase tpx1. We identified this protein in the aforementioned CG01, CG02 and R265 *C. gattii* genotype VGII strains. It is interesting to note that thiol peroxidase, a protein that acts to remove peroxides, also acts in response to oxidative stress, similar to Hsp70. Such proteins have previously been identified as being essential for intracellular survival, virulence and resistance to oxidative and nitrosative stress in *C. neoformans*, as reported by Missall *et al.* [63–65] and Wang *et al.* [66]. The finding presented here that thiol peroxidase is an antigenic target in *C. gattii* genotype VGII corroborates the findings of Jobbins *et al.* [56] and supports the hypothesis that this protein is involved in the pathogenesis of *C. gattii* genotype VGII infection in humans [62,67–69].

Other important proteins identified in the current study include the following: enolase and ATP synthase (found in strains CG01 and R265); phosphomannomutase (strains CG03 and R265); aconitase and Wos2 (strain CG02); and cofilin and CAN2 (strain CG03).

Enolase and ATP synthase appear to be important in the process of host invasion by the fungus. During the invasion of the brain and the CNS, penetration of the blood–brain barrier is a prerequisite for the establishment of meningoencephalitis by the opportunistic fungal pathogen *C. neoformans*. The fungal cells require a high level of energy to traverse this barrier, and enolase, which is an enzyme in the glycolysis pathway, could be essential for this purpose during the infection process [58]. In addition, the relative importance of plasminogen in infectious diseases is indicated by the surface-associated plasminogen-binding properties manifested by diverse species of human pathogens. Several

proteins, including enolase, have been found to play a major role in the microbial recruitment of plasminogen [68].

Phosphomannomutase can be considered to be an indirect virulence factor for *Cryptococcus* spp. This statement is justified by the fact that this enzyme is responsible for the synthesis of mannose, which is a carbohydrate comprising up to two-thirds of the main cryptococcal virulence factor, the polysaccharide capsule. The capsular polysaccharide of *C. neoformans* helps to protect yeast against the host immune system due to its function as either an antiphagocytic factor and/or an antigenic polysaccharide that is extruded into tissue and fluids and produces an immunosuppressive effect. The capsular polysaccharide, GXM, comprises three major sugars: mannose, xylose and glucuronic acid. The identification of this protein as an antigenic target is not surprising because it is involved in the production of mannoprotein–mannose complexes that elicit a cytokine-mediated inflammatory immune response. Important factors for proliferation in host tissue include enzymes involved in central carbon metabolism (gluconeogenesis and acetylCoA synthesis), regulators of capsule and melanin synthesis and proteins involved in resistance to phagocytosis [17,70–72].

The identification of the proteins aconitase (an enzyme involved in the metabolism of carbohydrates) and Wos2 (a cochaperone of Hsp90) corroborates the results of a study by Crestani *et al.* [73], which showed that aconitase and Wos2 expression levels increase in *C. gattii* (strain R265) in response to a lack of iron in the culture medium. It is important to note that the common factors between our study and that of Crestani *et al.* [73] are the stress conditions to which *C. gattii* was exposed and that Wos2 has been suggested to be an early marker of *C. gattii* infection in koalas [56]. Similar to enolase, the protein cofilin identified herein may play a relevant role in the process of the fungus spreading to the CNS. The rationale for this hypothesis is that cofilin interferes with the polymerization of F-actin and G-actin strands, thereby modulating the cytoskeleton of the target cell. This idea is in accordance with the results reported by Wang *et al.* [66], who demonstrated differential expression of cofilin in human umbilical vascular endothelial cells infected by *C. neoformans*.

The identification of CAN2 as an antigenic target in the context of cryptococcosis may be explained, at least in part, by this protein experiencing an increase in pressure in response to the change in the environmental CO_2 tension (0.033%) relative to that found in the host (5%). This protein works as a sensor for this CO_2 tension change and alerts the cell to the need for capsule thickening. This thickening is, in turn, an important virulence mechanism in cryptococcosis [74,75].

Importantly, the applied B-cell epitope prediction was efficient because the majority of the proteins (85.4%) presented a high percentage of epitopes. The high percentage of epitopes in nearly all of the identified proteins can be explained by the selection based on western blot analysis. It is important to highlight the fact that two proteins that were previously defined as hypothetical proteins showed a higher percentage (>42%) of epitopes. These hypothetical proteins exhibit a conserved homology in other species of *Cryptococcus*. The identified epitopes can be produced as synthetic peptides and could be tested for use in the diagnosis of cryptococcosis.

Conclusion

In conclusion, the applied combination of immunoproteomics and immunoinformatics methods was demonstrated to be a specific and powerful tool for identifying novel antigens from mycological pathogens. The major finding of this work was the identification of *C. gattii* proteins recognized as molecular targets by antibodies produced by patients with cryptococcal meningitis. In addition, we identified potential antigens that may be used for the development of more accurate serological diagnoses in human cryptococcal meningitis.

Future perspective

Cryptococcosis caused by *C. gattii* genotype VGII has resulted in significant repercussions over the years owing to the occurrence of outbreaks in Canada, mainly affecting immunocompetent individuals, causing high morbidity and mortality. Therefore, significant efforts have been devoted to the search for diagnostic tests that are sensitive, specific and can detect infection as early as possible. The immunogenic proteins and B-cell epitopes identified herein need to be investigated as antigens in serological tests to diagnose cryptococcal infection. These proteins could present cross-reaction with cryptococcal infection caused by other species. Although these proteins can react with multiple fungal species, some of these fungi may not be present in the patient. However, this is the first important step to selecting new target antigens to facilitate the immunodiagnosis of cryptococcosis.

Executive summary

Objectives of the study

- The objective was to identify immunoreactive proteins using sera from patients with cryptococcosis.
- The study aimed to map B-cell epitopes from immunoreactive proteins from *Cryptococcus gattii* genotype VGII.

Methods

- 2D gel electrophoresis integrated with immunoblotting and mass spectrometry was used to identify immunoreactive proteins.
- ABCPred and BCPreds programs were utilized to predict epitopes for B cells.

Conclusion

- A total of 68 immunoreactive proteins were identified.
- The ABCPred and BCPreds programs generated 374 concordant peptides.
- The Hsp70, thioredoxin peroxidase and GrpE proteins were immunoreactive in at least three strains of *C. gattii* genotype VGII.

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Ethical conduct of research

The study protocol was approved by the State University of Piauí-Brazil Institutional Review Board (CEP 079/2008) and by the Brazilian National Ethics Committee (CONEP). All patients enrolled in the study signed forms providing free and informed consent.

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