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Current Status of the Diagnostic and Genomics of *Cryptococcus neoformans/C. gattii* Species Complex

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Cryptococcosis is a systemic human and animal infection that has emerged as a life-threatening opportunistic mycoses since the advent of the acquired immune deficiency syndrome (AIDS), caused by the *Cryptococcus neoformans*/*C. gattii* species complex that comprises the pathogenic cluster, while other species of the yeast can sporadically be pathogenic to humans [1]. Moreover, biochemical differences subdivide *C. neoformans* into two varieties, *C. neoformans* var. grubii and *C. neoformans* var. *neoformans*, whereas differences in capsule polysacharides further subdivide the species into three serotypes, A, D and AD. Recently, a comprehensive molecular-based study proposed a rearrangement of the complex C. *neoformans*/*C. gattii* into seven species: *C. neoformans* (as var. grubii), C. de*neoformans* (as var. *neoformans*), as well as *C. gattii*, C. bacillisporus, C. deuterogattii, C. tetragattii, C. decagattii (as a subdivision of *C. gattii*) [2], still under evaluation by the scientific community. The former variety, *C. neoformans* var. gattii, is now recognized as a separate species, *C. gattii*, with serotypes B and C [3-7], based mostly on molecular methods and on the absence of genetic recombination between var. *neoformans* and var. gattii [2].

C. neoformans lives in the environment throughout the world, being associated to soil and avian excreta, especially pigeon droppings [8]. Additionally, *C. gattii* can be found on decaying wood and on multiple living tree species as an environmental habitat for the yeast [9,10], occurring mostly in immunocompetent individuals [11]. Both species infect primarily the alveolar space via inhalation of spores causing a pneumonia-like symptom that can spread to the brain and evolve to cryptococcal meningitis [4,7,12], whereas in immunocompetent hosts the lesions are focal, forming cryptococcomas, due to localized and effective host response to infection. The predilection for the central nervous system (CNS) could be determined by the production of laccase which catalyzes the formation of melanin in the fungal cell wall from catecholamine precursors [13]. In the specific case of C. *neoformans*, it can lead to a meningoencephalite, the most common presentation of fungal meningitis in individuals with AIDS, and can be fatal without treatment. There is an estimative of one million annual cases of cryptococcal meningitis caused by *C. neoformans* globally, mainly in immunosuppressed patients. Worldwide, the fungal disease contributes to nearly 630,000 deaths [14], the majority HIV-infected individuals in India, Africa, and Southeast Asia (one-third of all deaths in HIV/AIDS patients). In Brazil, cryptococcosis by *C. neoformans* occurs in all regions, but *C. gattii* is the responsible for infections in young people and children in the Northern and Northeastern regions [15].

It is now well established that *Cryptococcus* is a model system for study and development of pathogenesis, diagnostic methods and therapeutics. The diversity at the genetic level (~100 genetic loci linked to its virulence composite) [1] allows for studies on virulence, genomics, transcriptomics and proteomics of this fungus.

Bases of identification of *Cryptococcus neoformans*/*C. gattii* species complex

C. neoformans and *C. gattii* can be traditionally identified by phenotypic tests, as the production of a polysaccharide capsule, which is basically composed by glucuronoxylomannan (90%) and galactoxylomannan structures [15], being accepted as the major virulence factor and diagnostic proof for the confirmation of the disease, once it can be produced in vitro and in vivo, and detected after staining with India ink. Other tests focus on the melanine deposition on the cell wall (phenoloxidase test) and growth at 37°C. *C. gattii* can be differentiated by the ability to use glycine as carbone and nitrogen sources, and resisting growth inhibition by cycloheximide and canavanine [11].

Serotypes A or D of *C. neoformans* can be separated from the serotypes B or C of *C. gattii* after growing on L-canavanine-bromothymol blue-glycine (CGB) agar medium, which induces colony color changes, blue for *C. gattii* and greenish yellow for *C. neoformans* [16]. Such phenotypic method (chemotyping) is still used for species distinction [1], as an alternative when molecular methods are unavailable. Furthermore, inaccuracy of some traditional tests can be complemented by molecular approaches.

The cryptococcal antigen lateral flow assay (CrAg® LFA) (Immy, Inc., Noeman, OK, USA) is a rapid and accurate lipstick assay that has been recently developed for the detection of cryptococcal polysaccharide antigen (CrAg) in serum and cerebrospinal fluid (CSF) [17]. It is an immunochromatographic based test, which has been proposed for qualitative and semiquanitative detection of cryptococcal antigen from serotypes of clinical *C. neoformans* and *C. gattii*. The assay is simple and can be performed in 10 minutes, especially from samples of patients with meningitis [17], and presents higher sensitivity and specificity, 99.3% and 99.1%, respectively, when compared to India ink microscopy, culture and CrAg latex agglutination [18]. A systematic review found high accuracy of the CrAg LFA in serum and CSF, further suggesting its use from urine samples in early stages of the disease [19]. The CrAg LFA showed an excellent performance when tested by our group and was able to detect the cryptococcal antigen from CSF (-70°C) stored for more than one year (data not published yet). CrAg LFA has the potential to be integrated as a screening test into HIV care, specifically targeting people with severe immunosuppression and to be used in prospective epidemiological studies, to define treatment strategies. The assay was apparently used for the first time in the diagnosis of cryptococcosis and prevention of cryptococcal meningitis in 2013 [20].

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Despite the overall similarity of responses at the phenotypic and genetic levels used by the *C. neoformans*/*C. gattii* species complex, there are important differences between these species, once they diverged genetically ~40 million years ago, thus, allowing the sequence data to be used for epidemiological purposes [21].

Molecular identification and genomic analysis

The taxonomy, genetic diversity and epidemiology of *Cryptococcus* have been evaluated using several molecular approaches, such as PCR fingerprinting [22,23], restriction fragment length polymorphism (RFLP) [22], amplified fragment length polymorphism (AFLP) [24-26], fingerprinting multilocus sequence typing (MLST) [1,27,28], multilocus microsatellite typing (MLMT) [29], and whole genome sequence [1,30].

Currently, the widely accepted classification of *Cryptococcus* describes eight molecular patterns (genotypes): VNI/AFLP1 and VNII/AFLP1A (*C. neoformans* var. grubii), VNIII/AFLP3 (AD hybrid), VNIV/AFLP2 (*C. neoformans* var. *neoformans*), besides VGI/AFLP4A/AFLP4B, VGII/AFLP6, VGIII/AFLP5A/ AFLP5B/ AFLP5C, and VGIV/AFLP7 (*C. gattii*) [24, 31]. Using M13 PCR-fingerprinting and PCR-RFLP analyses of the URA5 gene (orotidine monophosphate pyrophosphorylase) with double digested (HhaI/ Sau96I), the IberoAmerican Cryptococcal Study Group typed clinical, veterinary, and environmental isolates of *C. neoformans* and *C. gattii* from Argentina, Brazil, Chile, Colombia, Guatemala, Mexico, Peru, Venezuela and Spain into the eight previously established molecular types, with the most prevalent type being VNI of the var. *grubii*, of serotype A [22]. Isolates of *C. neoformans* AFLP1/VNI were predominant in patients with AIDS in the State of Mato Grosso, Brazil, while HIV-negative patients were most frequently affected by *C. gattii* AFLP6/VGII [32]. Likewise, a higher prevalence of molecular type VNI was detected from clinical and environmental isolates from Rio Grande do Sul, Brazil [33].

Multi locus sequence typing (MLST) is a molecular typing approach proposed by Maiden et al. [34], which exploits the distinct characteristics and electronic flexibility of nucleotide sequence data, for the characterization of microorganisms, whose data can be interconnected between laboratories. The bank indexes sequence variations in ~400-500pb of five to ten genes, composed primarily by housekeeping genes [22]. *C. neoformans* and *C. gattii* are widely represented in one of the most complete MLST fungal databases, that will allow fast expansion of the data analyses [1], now available into the international fungal multi locus database (http://mlst.mycologylab.org/DefaultInfo.aspx?Page=Home). The site provides searching tools for sequences or allele types, pairwise ID using the polyphasic ID algorithm of BioloMICS, pairwise identification based on single locus using Blast, deposit data for new sequences and primers and PCR amplification conditions.

In order to increase automation and make the MLST more versatile, Chen et al. [35] proposed a multiplexing approach (based on seven MLST loci), the new generation multi-locus sequence typing (NGMLST), connected to an automated software program for data analysis, the MLSTEZ. The authors focused on nine MLST loci (CAP59, GPD1, IGS1, LAC1, PLB1, SOD1, URA5, TEF1 and MPD1 of *Cryptococcus* [28], to generate profiles with high quality and specificity when compared to the ones of the reference strains.

Genetic studies have allowed the evaluation of pathogenicity and virulence aspects on *Cryptococcus* strains. The genome of related *C. neoformans* var. *neoformans* strains (JEC21 and B-30501A) covers approximately 20Mb organized in 14 chromosomes, with ~6,500 introns, ~5% constituted by regions of rRNA repeats and 5% of transposons (40-100Kb), contributing to karyotype instability and phenotypic changes [36]. *C. neoformans* var. grubii H99 has been used for genetic, molecular and virulence studies in recent years and also presents 14 chromosomes. Therefore, Janbon et al. conducted a whole genome comparison between different serotypes and concluded that there are fewer chromosome rearrangements (translocations and inversions) between C. var. *neoformans* (JEC21) and C. var. grubii (H99) than when the genome is compared with *C. gattii*.

Interestingly, changes in the genome structure of the yeast can occur even with a strain laboratory passage or in a situation of environmental stress that usually occurs at the site of infection, as at the human subarachnoid space [30, 37].

Using the whole genome sequencing (WGS) approach, Ormerod et al. [38] compared isolates obtained from cerebrospinal fluid during infectious process of *C. neoformans* var. *grubii* H99 and suggested rapid genomic changes and evolution (microevolution) subsequent to penetration of the CNS and the administration of antifungal therapy. In addition, specific genotypes have been associated with an unfavorable clinical outcome [39].

The JGI-MycoCosm web portal provides data access, visualization, and analysis tools for comparative genomics of fungi, with information on JEC21 and H99 strains and all data were incorporated into JGI MycoCosm to functionally annotate the predicted genes (http://genome.jgi.doe.gov/Cryne_JEC21_1/Cryne_JEC21_1.home.html). Using an expressed sequence tags (ESTs) to compare the genomes of *C. neoformans* var. grubii and *C. neoformans* var. *neoformans* after gene annotations, Loftus et al. [36] found that introns, alternative splicing and antisense transcription are very frequent in C. *neoformans*. In a transcriptional approach (using RNA-seq), Janbon et al. [30] compared the whole genome of H99 strain with others: *C. neoformans* (JEC21) and *C. gattii* genomes, CVI (WM276) and VGII (R265). The comparison between *C. neoformans* genome showed overall similarities under the same environmental conditions and found few chromosomal rearrangements, despite single nucleotide variations and novel genes. However intron splicing, strand-specific transcription, and non-coding RNAs were identified [30].

A new approach, sequence based and abundance-weighted was implemented to improve the performance of fungal detection and quantification, named the new-generation sequencing (NGS). The approach was applied for several fungi with 454 pyrosequencing of rDNA ITS regions, including *Cryptococcus*, and the results showed higher proportion of this yeast in samples obtained from bronchoalveolar lavage of HIV-positive patients than in the samples obtained from healthy individuals [40].

A simple, fast and accurate technique for identification of different microorganisms based on mass spectrometry has been applied over the last decade, named MALDI-TOF (matrix-assisted laser desorption ionization-time of flight). It is an alternative to phenotypic and genotypic approaches, and allows the construction of a proteomic profile from mass spectral evaluation of yeasts medically important, allowing their rapid and accurate identification [41,42], and distinction of *Cryptococcus neoformans* from *C. gattii* [43].

The methodology is based on the fact that each microorganism forms a unique mass spectral pattern of peptides and proteins and an unknown microorganism can be identified after comparing its spectrum

with the ones in a reference spectral library [44]. Using this approach, Firacative et al. [43] could identify 100% of the 164 isolates tested, and distinguish *C. neoformans* from *C. gattii*. Moreover they could be further separated into the eight major molecular patterns. Danesi et al. [45] created an in-house library for a set of nine species uncommonly reported in human and animal cryptococcosis, including, *C. albidus* and *C. laurentii*, to make timely and correct identifications using MALDI-TOF MS in a routine of laboratory diagnostics. Currently one limiting factor for the identification using this technology is that commercial reference libraries contain a limited number of spectra for *Cryptococcus* strains.

In conclusion, it is important to direct investigations about mechanisms of the cryptococcal disease and to search for answers on the clinical aspects of affected patients, mostly immunosuppressed. The "Rede Brasileira de Criptococose" (RBC), in which the group of Alagoas takes part, integrates several laboratories around Brazil to research the diversity and ecology of *Cryptococcus* species through molecular typing and about clinical-epidemiology of the disease. Novel methods for accurate and rapid identification of pathogenic *Cryptococcus* spp. in the laboratory routine should also be explored. In addition, the maintenance of global epidemiology surveillance is imperative to monitor disease outcomes.

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