**Working Group on *Paracoccidioides brasiliensis*,** under the umbrella of ISHAM

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The aim of this network is to bring together the expertise of several groups that work in Brazil and Venezuela on this fungus, with emphasis on (a) Molecular aspects of dimorphism and virulence; (b) Genes related to virulence of *P. brasiliensis*; (c) Target genes of *P. brasiliensis* to search for inhibitors; and (d) Experimental antifungals.

## Introduction

*Paracoccidioides brasiliensis* is the causative agent of paracoccidioidomycosis (PCM), a human systemic, chronic and progressive mycosis, geographically confined to rural areas of Latin America, mainly Brazil, Colombia, and Venezuela, where it constitutes one of the most prevalent deep mycoses (1). After inhalation of air-borne propagules (2), most infected individuals develop only asymptomatic or subclinical PCM, which may progress into a disease, depending on host, strain and environmental factors [see (1)]. High positive reactivity (60-75%) in the adult population of endemic areas suggests that around 10 million people may have been infected (3). After penetrating the host, the fungus must convert to its yeast form, for the successful establishment of the infection (1). The fungus may remain confined to the lung or disseminate widely giving rise to variable clinical manifestations of PCM. Acute and subacute forms predominate in young people of both sexes, mainly affecting the lymphatic system. A chronic form, instead, predominates in adult males, characterized by a rapid fungal dissemination to multiple organs and tissues (2). The exact causes of clinical pleomorphism are not understood, but it is reasonable to presume the involvement of factors associated with both the fungus and the human host. Regarding *P. brasiliensis*, although wide biochemical diversity has been reported, no correlation with pathogenesis has been demonstrated (3).

## (a) Molecular aspects of dimorphism

The genetics of *P. brasiliensis* is poorly understood. Hyphal segments and fungal propagules or conidia are uninucleate, while yeasts are multinucleate (4). Pulsed-field gel electrophoresis (PFGE) and confocal fluorescence microscopy have allowed the genomic characterization and chromosomal mapping of *P. brasiliensis* (5-7). The nuclear genome size was estimated by PFGE at around 30 Mb; confocal fluorescence microscopy provided strong evidence to the haploid/diploid or even aneuploid nature of *P. brasiliensis* (6). DNA sequencing of ~50 Kb showed a density of one gene per 3.0-3.7 Kb, suggesting that 7500-9000 genes were present in its genome (8).

Recently, molecular advances in the field have produced significant reports on transcript characterization of this pathogen, with insights on differential gene expression in two *P. brasiliensis* isolates, namely, strains Pb18 and Pb01 (9-11). These studies add evidence to numerous previous reports confirming the genetic and metabolic diversity shown by *P. brasiliensis* in its multiple isolates.

The genetic diversity of any organism requires special attention when the development of new drugs, vaccines and diagnostic tests, are under consideration. Although several groups have examined the genetic diversity of *P. brasiliensis* at protein, chromosomal and DNA levels (9-11), we still lack any reference to the fungal genetic variability as deduced from genomic analysis in clinical isolates. Randomly amplified polymorphic DNA (RAPD) analyses have delineated a strong variability among fungal isolates, with genotypic differences that correlated with virulence, geographic distribution, susceptibility or resistance to drugs (12-15). Polymorphic microsatellites (SSR markers) have been recently described as potential molecular markers for clinical and epidemiological studies in *P. brasiliensis*, results that point, once more, to genetic variability among isolates (16). Based on phylogenetic analysis of 65 isolates, Matute *et al*. (17) recently proposed three distinct species within *P. brasiliensis*, designed S1, PS2 and PS3. Additional results by the genealogical concordance method (Carrero et al., manuscript in preparation, 18) indicate that isolate Pb01 formed a strong supported sister group to the three species reported by Matute et al. (17).

Though an important medical problem, there is still a paucity of studies on development of new diagnostic tests for PCM. So far, only two molecular tests have been designed to detect *P. brasiliensis* in clinical samples, both using PCR as the experimental

technique (19, 20). Sputum samples from 11 patients with chronic paracoccidioidomycosis were subjected to PCR with primers derived from the *gp43* sequence (19). In all cases, a single band of 0.6 kb specific for *P. brasiliensis* was produced. The second test (20) used primers derived from a 0.72 bp band common to all *P. brasiliensis* DNA samples subjected to PCR with Operon OPG18 as arbitrary primer (13). Such primers, with a 10 pg sensitivity limit, were specific for *P. brasiliensis* and produced positive identification bands in patients with a confirmed diagnosis of chronic PCM (13). Interestingly, in two cases of suspected relapses our molecular test produced results that preceded clinical, serological or mycological information by one or more weeks. One of them turned out to be a PCM relapse, while the other one was not, as correctly predicted by the molecular test. As for another patient, he suffered from chronic multifocal PCM, and developed neurological symptoms of impairment, suggestive of an involvement of the central nervous system (CNS). Our molecular test was able to detect *P. brasiliensis* in his cerebrospinal fluid, although antibody detection and microscopic observation were negative for the presence of the fungus in this sample, as usually reported in CNS-PCM (20).

**(b) Genes related to virulence of *P. brasiliensis***

The identification of virulence genes of *P. brasiliensis* may prove important to decipher clues on the development of the disease. We have established that the iron uptake system, the synthesis of melanin, as well as the synthesis of glyceraldehyde-3-phosphate dehydrogenase can be determinant in *P. brasiliensis* virulence in an animal model (21, 22). Our aim is to determine the interactome of *P. brasiliensis* as a means to identify the molecular interactions involved in these processes. For that purpose we will use two-hybrid screens to determine the interactome during the fungus infection in an animal model.

**(c) Target genes of *P. brasiliensis* to search for inhibitors**

Treatment of PCM is lengthy, the drugs may have undesirable side effects, and some are costly. Occasional resistant strains have been reported (23). Therefore, the search for more selective and efficient antifungals to treat this and others mycoses

continues. Plants, with its diversity of biochemical pathways, have provided an array of possibilities for drug development. The Cerrado (savannah), the second biggest source of Brazilian biodiversity, has sparse and scrubby vegetation features presenting more than 4,000 species that grow only in this biome (24). The rich flora of the Cerrado biome in Brazil has been poorly studied regarding to the efficacy and therapeutics of crude extracts or isolated compounds obtained from plant families (25-27).

We are working in *P. brasiliensis* specific inhibitory targets (11, 28-30) of Cerrado plants. It is expected that plant compounds interact with target sites other than those currently recognized by antifungals. Since those targets do not exist in mammalian cells, they may have potential antifungal effect, without toxicity for humans.

We have examined the *in vitro* effects of *Eugenia uniflora* fractions and purified oenothein B on the growth and viability of *P. brasiliensis*. Scanning and transmission electron studies of *P. brasiliensis* yeast cells revealed the changes characteristic of glucan synthesis inhibitors, such as squashing, depression, cracking, flattened appearance, rough surface and disruption. The oenothein B action on *P. brasiliensis* gene expression was evaluated. Oenothein B inhibits the accumulation of the 1,3-β-glucan synthase transcripts. The data suggest that oenothein B is a good natural-product candidate as an antifungal agent (31).

In this project, our aim is obtain the recombinant proteins *Pbfks1*, chitinase (*Pbcts1*) and glucanase (*Pbglc1*), enzymes involved in cell wall metabolism, and C-24 sterol methyl-transferase (*Pberg6*), malate synthase (*Pbmls1*) and isocitrate lyase (*Pbicl1*), enzymes which act on specific-fungal metabolic pathways, and determinate the kinetics parameters to search for inhibitors in Cerrado plants.

**(d) Experimental antifungals**

The sterol biosynthetic pathway has been largely studied for the search of antifungals. Azoles and allilamines act on differents steps of this pathway. However, they may interfere with similar steps in the host. Hence, the search for drugs that may act on more specific steps in on its way. One such step refers to the sterol *C*-methylations catalyzed by the enzyme (S)-adenosyl-L-methionine: $\Delta^{24}$ – sterol methyl transferase

(SMT). SMT inhibitors such as azasterols and derivatives (AZA1, AZA2, AZA3) (32, 33) have proven highly effective as antiproliferative agents against protozoa and some fungi, among them, *P. brasiliensis*. *P. brasiliensis* (Y phase) was sensitive to the action of azasterols in the following sequence: AZA-3>AZA-1>AZA-2. However, they did not act on the same steps in the pathway of sterol biosynthesis. AZA-1 and AZA-2 significantly inhibited the $\Delta^{24(28)}$ sterol methyl reductase (SMR), while AZA-3, produced a major antiproliferative effect on *P. brasiliensis*, that was due to $\Delta^{24(28)}$ sterol methyl transferase (SMT) inhibition, as deduced form the important accumulation of lanosterol recorded in this case. This contrasts with the reported effect of ketoconazole (4) on the accumulation of 24-methylene-dihydrolanosterol (B) through the inhibition of the cytochrome P-450-dependent C14α-demethylase (4). Currently, the analysis of novel sterol-hydrazones has been started in the search for more active substances against this pathogenic agent.

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**Scopes:**

**(a) Molecular aspects of dimorphism and virulence**

- Molecular analysis of the different domains of alpha-1,3-glucan synthase and their specific role during the synthesis of virulence-related alpha-1,3-glucan in *P. brasiliensis*.
- Transcriptome analyses of *P. brasiliensis* under nutritional and oxidative stress, *in vitro* or while infecting the host. Promoters of highly expressed genes under such conditions.

**(b) Genes related to virulence of *P. brasiliensis***

- Interactome of *P. brasiliensis* as a means to identify the molecules interactions that may have effect on the fungal virulence. For that purpose we will use two-hybrid screens to determine the interactome during the fungus infection in an animal model.

**(c) *Paracoccidioides brasiliensis* postgenome**

RNA interference (RNAi) for validation of antifungals and search of antifungal peptides.

**(d) Gene expression and host-pathogen interactions by cDAN microarrays**:

macrophages versus *Paracoccidioides brasiliensis* / *Histoplasma capsulatum*.

**(e) Target genes of *P. brasiliensis* to search for inhibitors**

Obtain the recombinant proteins *Pbfks1*, chitinase (*Pbcts1*) and glucanase (*Pbglc1*), enzymes involved in cell wall metabolism, and C-24 sterol methyl-transferase (*Pberg6*), malate synthase (*Pbmls1*) and isocitrate lyase (*Pbicl1*).

**(f) Experimental antifungals**

- Effect of sterol-hydrazones and similar derivatives on growth of *P. brasiliensis*.
- Effect of caspofungin on growth and morphology of *P. brasiliensis*, and importance of genes related to the synthesis of cell wall beta-1,3-glucan in the pathogenic yeast phase.

## REFERENCES


1. San-Blas, G.; Niño-Vega, G.; and Iturriaga, T. 2002. *Paracoccidioides brasiliensis* and paracoccidioidomycosis: Molecular approaches to morphogenesis, diagnosis, epidemiology, taxonomy and genetics. Medical Mycology *40*, 225-242.
2. Montenegro, M. R.; Franco, M. Pathology. In *Paracoccidioidomycosis* Franco, M., Lacaz, C. S., Restrepo-Moreno, A., Del Negro, G. Eds.; CRC Press, Inc.: Boca Raton, Fla., 1994; 131-150.
3. Restrepo, A. 1985. The ecology of *Paracoccidioides brasiliensis*: a puzzle still unsolved. Journal of Medical and Veterinary Mycology *23*, 323-329.
4. Queiroz-Telles, F. *Paracoccidioides brasiliensis* ultrastructural findings. In *Paracoccidioidomycosis* Franco, M., Lacaz, C. S., Restrepo-Moreno, A., Del Negro, G. Eds.; CRC Press, Inc.: Boca Raton, Fla., 1994; 27-47.

5. Cano, M.I.N., Cisalpino, P.S., Galindo, I., Ramírez, J.L., Mortara, R.A. and da Silveira J.F. 1998. Electrophoretic karyotypes and genome sizing of the pathogenic fungus *Paracoccidioides brasiliensis*. J. Clin. Microbiol. *36*, 742-747.
6. Feitosa, L.S., Cisalpino, P.S., dos Santos, M.R., Mortara, R.A., Barros, T.F., Morais, F.V., Puccia, R., da Silveira, J.F. and de Camargo, Z.P. 2003. Chromosomal polymorphism, syntenic relationships and ploidy in the pathogenic fungus *Paracoccidioides brasiliensis.* Fungal Genet. Biol. *39*, 60-69.
7. Montoya, A.E., Moreno, M.N., Restrepo, A. and McEwen, J.G. 1997. Electrophoretic karyotype of clinical isolates of *Paracoccidioides brasiliensis*. Fungal Genet. Biol. *21*, 223-227.
8. Reinoso, C., Niño-Vega, G., San-Blas, G., Domínguez, A. 2005. Random sequencing of *Paracoccidioides brasiliensis* genes. Med. Mycol. *43*, 681-689.
9. Goldman, G.H., dos Reis Marques, E., et al. 2003. Expressed sequence tag analysis of the human pathogen *Paracoccidioides brasiliensis* yeast phase: identification of putative homologues of *Candida albicans* virulence and pathogenicity genes. Eukaryot. Cell *2*, 34-48.
10. Felipe, M.S.S., Andrade, R.V., et al. 2003. Transcriptome characterization of the dimorphic and pathogenic fungus *Paracoccidioides brasiliensis* by EST analysis. Yeast *20*, 263-71.
11. Felipe, M.S.S., Andrade R. V. et al. 2005. Transcriptional profiles of the human pathogenic fungus *Paracoccidioides brasiliensis* in mycelium and yeast cells. J. Biol. Chem. *280*, 24706-24714.
12. Soares, C.M.A., Mollinari-Madlum, E.E.W.I., Silva, S.P., Pereira, M. and Felipe, M.S.S. 1995. Characterization of *Paracoccidioides brasiliensis* isolates by random amplified polymorphic DNA analysis. J. Clin. Microbiol. *33*, 505-507.
13. Calcagno, A.M., Niño-Vega, G., San Blas, F. and San Blas, G. 1998. Geographic discrimination of *Paracoccidioides brasiliensis* strains by randomly amplified polymorphic DNA analysis. J. Clin. Microbiol. *36*, 1733-1736.
14. Molinari-Madlum, E.E.W.I., Felipe, M.S.S. and Soares, C.M.A. 1999. Virulence of *Paracoccidioides brasiliensis* can be correlated to groups defined by random amplified polymorphic DNA analysis. Med. Mycol. *37*, 269-276.

15. Hahn, R.C., Macedo, A.M., Fontes, C.J.F., Batista, R.D., Santos, N.L. and Hamdan, J.S. 2003. Randomly amplified polymorphic DNA as a valuable tool for epidemiological studies of *Paracoccidioides brasiliensis.* J. Clin. Microbiol. *41*, 2849-2854.
16. Nascimento E., Martinez R. et al. 2004. Detection and selection of microsatellites in the genome of *Paracoccidioides brasiliensis* as molecular markers for clinical and epidemiological studies. J.Clin. Microbiol. *42*, 5007-5014.
17. Matute, D.R., McEwen, J.G., Puccia,R., Montes, B.A., San-Blas,G., Bagagli, E., Rauscher, J.T., Restrepo, A., Morais, F., Niño-Vega, G. and Taylor, J.W. 2006. Cryptic speciation and recombination in the fungus *Paracoccidioides brasiliensis* as revealed by genealogies. Mol. Biol. Evol. *23*, 65-73.
18. Carrero, L., Niño-Vega, G., San-Blas, G., Teixeira, M., Felipe, M.S. , Soares, C. M., Pereira, M., McEwen, J., Mendoza, L., and Taylor, J. W. 2006. Molecular phylogeny and variability of *Paracoccidioides brasiliensis* strains by the genealogical concordance method. Manuscript in preparation.
19. Gomes, G. M., Cisalpino, P. S.; Taborda, C. P.; et al. 2000. Polymerase chain reaction for diagnosis of paracoccidioidomycosis. J. Clin. Microbiol. *38*, 3478-3480.
20. San-Blas, G.; Niño-Vega, G.; Barreto, L.; et al. 2005. Primers for the clinical detection of *Paracoccidioides brasiliensis*. J. Clin. Microbiol. *49*, 4255-4257.
21. Barbosa, M.S., Bao, N.S., Andreotti, P.F., Felipe, M.S.S., Feitosa, L.S., Mendes Giannini M.J.S. and Soares, C.M.A. 2006. Glyceraldehyde 3-phosphate dehydrogenase of *Paracoccidioides brasiliensis* is a cell surface protein, involved in fungal adhesion to extracellular matrix proteins and interaction with cells. Infect. Immun. *74*, 382-389.
22. Bailão, A.M., Schrank, A., Borges, C.L., Dutra, V., Molinari-Madlum, E. E. W. I., Felipe, M.S.S., Mendes-Giannini, M.J.S., Martins, W.S., Pereira, M. and Soares, C. M. A. Differential gene expression by *Paracoccidioides brasiliensis* in host interaction conditions: Representational difference analysis identifies genes associated to fungal pathogenesis. Microbes Infect., submitted.
23. Hahn, R.C., Morato Conceição, Y.T., Santos, N.L., Ferreira, J.F., Hamdan, J.S. 2003. Disseminated paracoccidioidomycosis: correlation between clinical and *in vitro*

resistance to ketoconazole and trimethoprim sulphamethoxazole. Mycoses *46*, 342-347.

24. Marris E. 2005. Conservation in Brazil: the forgotten ecosystem. Nature *437*, 944-945.
25. Souza, L.K.H., Oliveira, C.M.A., Ferri, P.H. et al. 2002. Antifungal properties of Brazilian Cerrado plants. Braz J Microbiol *33*, 247-249.
26. Holetz, F.B., Pessini, G.L., Sanches, N.R., Cortez, D.A.G., Nakamura, C.V., Filho, B.P.D. 2002. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. Mem Inst Oswaldo Cruz *97*, 1027-1031.
27. Alves, T.M.A., Silva, A.F., Brandão, M. et al. 2000. Biological screening of Brazilian medicinal plants. Mem Inst Oswaldo Cruz *95*, 367-373.
28. Tomazett, P.K., Cruz, A.H.S., Bonfim, S.M.R.C., Soares, C.M.A., Pereira, M. 2005. The cell wall of *Paracoccidioides brasiliensis*: Insights from its transcriptome data. Genet Mol Res *4*, 309-325.
29. Amaral, A.C., Fernandes, L., Galdino, A.S., Soares, C.M.A., Pereira, M. 2005. Therapeutic targets in *Paracoccidioides brasiliensis*: perspectives post-transcriptome. Genet Mol Res *4*, 430-449.
30. Bonfim, S.M.R.C., Cruz, A.H.S., Jesuino, R.S.S., Ulhoa, C.J., Molinari-Madlum, E.E.W.I., Soares, C.M.A., Pereira, M. 2006. Chitinase from *Paracoccidioides brasiliensis*: Molecular cloning, structural, phylogenetic, expression and activity analysis. FEMS Immunol. Med. Microbiol. *46*, 269-283.
31. Dias Santos, G., Ferri, P.H., Santos, S.C., Bao, S.N., Soares, C.M.A., Pereira, M. Oenothein B from the Brazilian Cerrado plant *Eugenia uniflora* inhibits 1,3-β-glucan synthase transcript accumulation and induces hallmark changes in the morphology of the fungus *Paracoccidioides brasiliensis*. Submitted.
32. Visbal, G., Alvarez, A., Moreno, B., San-Blas, G. 2003. Inhibitors of (S)-Adenosyl-L-Methionine: $\Delta^{24-}$sterol methyl transferase and $\Delta^{24(28)}$-sterol methyl reductase as possible antifungal agents against *Paracoccidioides brasiliensis*. Antimicrob. Agents Chemother. *47*, 2966-2970.

33. Visbal, G., San-Blas, G., Murgich, J., Franco, H. 2005. *Paracoccidioides brasiliensis*, paracoccidioidomycosis, and antifungal antibiotics. Invited review in: Current Drug Targets: Infectious Disorders *5*, 211-226.