Importance of adhesins in virulence of *Paracoccidioides* spp.

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Members of the *Paracoccidioides* genus are the etiologic agents of paracoccidioidomycosis (PCM). This genus is composed of two species: *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. The correct molecular taxonomic classification of these fungi has created new opportunities for studying and understanding their relationships with their hosts. *Paracoccidioides* spp. have features that permit their growth under adverse conditions, enable them to adhere to and invade host tissues and may contribute to disease development. Cell wall proteins called adhesins facilitate adhesion and are capable of mediating fungi-host interactions during infection. This study aimed to evaluate the adhesion profile of two species of the genus *Paracoccidioides*, to analyze the expression of adhesin-host interactions by real-time PCR and to relate these results to the virulence of the species, as assessed using a survival curve in mice and in *Galleria mellonella* after blocking the adhesins. A high level of heterogeneity was observed in adhesion and adhesin expression, showing that the 14-3-3 and enolase molecules are the most highly expressed adhesins during pathogen-host interaction. Additionally, a survival curve revealed a correlation between the adhesion rate and survival, with *P. brasiliensis* showing higher adhesion and adhesin expression levels and greater virulence when compared with *P. lutzii*. After blocking 14-3-3 and enolase adhesins, we observed modifications in the virulence of these two species, revealing the importance of these molecules during the pathogenesis of members of the *Paracoccidioides* genus. These results revealed new insights into the host-pathogen interaction of this genus and may enhance our understanding of different isolates that could be useful for the treatment of this mycosis.

Keywords: *Paracoccidioides* spp., virulence, adhesion, adhesins

Introduction

Members of *Paracoccidioides* spp. are dimorphic fungi and the etiological agents of paracoccidioidomycosis (PCM), a systemic mycosis affecting people in Latin America. Brazil has the largest number of endemic PCM areas in the world (Franco et al., 1993).

Matute et al. (2006) classified the *Paracoccidioides* genus into three phylogenetic species: S1, PS2, and PS3. These species have different geographic distributions: S1 is a paraphyletic group found in Brazil, Argentina, Paraguay, Peru, and Venezuela; PS2 is a monophyletic group found in Brazil and Venezuela; and PS3 is a monophyletic group found only in Colombia. Carrero et al. (2008)
classified these isolates into two phylogenetic species, S1 and PS3, which were previously described by Matute et al. (2006), and also classified isolate Pb01 as a new phylogenetic species. Teixeira et al. (2009a) proposed that the Pb01 isolate was a new species, P. lutzii. The newly revised molecular taxonomy for these fungi has created new opportunities for studying and understanding their eco-epidemiological relationships with their hosts (Bagagli et al., 2006; Teixeira et al., 2009a). P. lutzii and P. brasiliensis are found in the west-central and in the southern/southeastern regions of Brazil, respectively (Gegembauer et al., 2014).

The agents of systemic mycoses have some properties that permit their growth in the adverse conditions of the host and may contribute to the development of disease (Casadevall and Pirofski, 1999). Paracoccidioides spp. have mechanisms that enable them to adhere to and invade barriers imposed by the host tissues (Mendes-Giannini et al., 1994, 2006). The fungi synthesize several substances that participate directly or indirectly in the parasite-host relationship (Mendes-Giannini et al., 2000). Therefore, the successful colonization of the host tissues by the fungus is a complex event, usually involving a pathogen ligand and a host cell receptor. The microorganism has three host components with which it can interact: products secreted by the cell, surfaces of the host cell, and proteins of the extracellular matrix (ECM), such as type I and IV collagens, fibronectin and laminin (Mendes-Giannini et al., 2006). The understanding and identification of molecules involved in the adhesion of microorganisms to different substrates in the host can aid in the discovery of efficient treatments for systemic mycoses.

Paracoccidioides virulence is a multifaceted event involving the expression of multiple genes at different stages of infection (da Silva et al., 2013). Several adhesins have been described in Paracoccidioides spp. and these characterizations have most often been performed using the Pb18 and Pb01 strains. The importance of the following adhesins in fungus-host interactions has been examined: GP43 (Hanna et al., 2000; Mendes-Giannini et al., 2006), 14-3-3 protein (30 kDa) (Andreotti et al., 2005), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Barbosa et al., 2006), triosephosphate isomerase (TPI) (Pereira et al., 2007), enolase (Donofrio et al., 2009; Nogueira et al., 2010; Marcos et al., 2012), and malate synthase (da Silva Neto et al., 2009) using different methodologies like ligand affinity binding, inhibition using antibodies, binding, binding competition, Far-Western blot, ELISA, and Western blot assays.

Besides its direct participation in adhesion, most of the described adhesins for Paracoccidioides spp. are enzymes that participate in the glycolytic pathway, tricarboxylic acid cycle and glyoxylate cycle in Paracoccidioides spp. acting as “moonlighting proteins” (Marcos et al., 2014). Recent studies have shown the presence of these enzymes in extracellular vesicles produced by the fungi as well as being present in the cell wall, wherein blocking them leads to a decrease in the ability of the fungi to adhere to cellular host components (Puccia et al., 2011; Vallejo et al., 2011, 2012a,b).

The presence of these enzymes at the yeast cell surface in the absence of a conventional N-terminal signal sequence responsible for targeting the protein into the classical secretory pathway is an intriguing question in the study of the paracoccidioimycosis. Some of these enzymes could behave as anchorless adhesins, which bind to the cell wall and allows direct interaction with the host (da Silva Neto et al., 2009). However, its known that in the N-terminal half of the C. albicans GAPDH polypeptide encoded by the TDH3 gene, for example, is able to direct its incorporation into the yeast cell wall (Delgado et al., 2003) but, in the case of the fungi from the Paracoccidioides genus, more studies are needed to identify putative signals related to its cell wall targeting.

How they interact with the host cells is another intriguing question. Marcos et al. (2012) demonstrated that the P. brasiliensis enolase has a plasminogen-binding motif (RGD) (254FYKADEKKK262), that is involved in the direct interaction with the host cell surface. It was shown, that when the RGD motif was added as a competitor in the binding assay of enolase to pneumocytes, the binding of the enzyme to the host cells decreased 10%, demonstrating the role of this motif in the enzyme binding to cellular components of the host, however more studies should be done to better understand how these adhesins interacts with the host.

The virulence of fungi may be evaluated using murine models. More recently, the larvae of G. mellonella are increasingly being used as an infection model for studying the virulence factors and pathogenesis of many fungal and bacterial pathogens (Cook and McArthur, 2013). Murine models are still considered the gold standard for the study of pathogenesis; however, economic, logistical and ethical considerations limit the use of mammalian host models of infection, especially when the experiment requires the analysis of a large number of strains (Jacobsen, 2014). Thomaz et al. (2013) used G. mellonella to evaluate the efficacy of this model for the study of the dimorphic fungi P. lutzii and Histoplasma capsulatum and concluded that this is a potentially useful model for studying the virulence of dimorphic fungi.

Thus, this study aims to investigate adhesins in the two species of the Paracoccidioides genus and to understand how the capacity of these species to express adhesins affects their virulence.

Materials and Methods

Ethics Statements
Animal experiments were performed according to Brazilian Federal Law 11.794, which has established procedures for the use of animals in scientific research, and to state law, which has established the code of animal protection of the State of São Paulo. For this study, male BALB/C and C57BL/6 mice (3–4 weeks old) were used, and all efforts were made to minimize the suffering of the animals. The experiments were approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences of Araraquara—UNESP FCFAr (Case 10/2011/CEUA/FCF).

Microorganisms
Experiments were performed with two isolates belonging to two species of the genus Paracoccidioides: P. brasiliensis (Pb18) and P. lutzii (Pb01). Pb18 is representative of the major phylogenetic group S1 and has been extensively used in the literature due to its demonstrated virulence in mice when inoculated by the intraperitoneal, intratracheal and intravenous routes.
Adhesins role in Paracoccidioides spp. virulence

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Pb01 is a clinical isolate from an acute form of PCM in an adult male and is the most thoroughly studied isolate at the molecular level, as shown by an extensive analysis of its transcriptome that yielded ESTs covering approximately 80% of the estimated genome of P. lutzii (Felipe et al., 2005b). These isolates were maintained in the mycelial phase (M) and were transferred to Fava-Netto medium supplemented with fetal bovine serum to revert to the yeast phase (L) before the experiments. After reversion, isolates were maintained in Fava-Netto medium without any supplements. Consecutive subcultures were generated each week. The isolates were used for experiments after the 4th subculture. Before the experiments, the fungi were grown for 4 days in brain heart infusion medium (BHI) supplemented with glucose (2%) at 37°C with shaking at 250 rpm.

Adhesion to A549 Pneumocytes of P. brasiliensis and P. lutzii

Cells from the A549 epithelial lineage (type II pneumocytes from the Rio de Janeiro Cell Bank) were grown in 6-well cell plates in HAM F-12 medium supplemented with 10% fetal bovine serum. After monolayer formation, the cells were washed three times with sterile phosphate-buffered saline (PBS) and inoculated with a Paracoccidioides spp. inoculum of 5 × 10^6 cells/mL. The standard inoculum of Paracoccidioides spp. in PBS had previously been incubated for 15 min at 37°C with 10 µM CFSE [5(6)-carboxyfluorescein diacetate N-succinimidyl ester], which stains the fungal cell wall, washed and resuspended in PBS. The infected cells were then incubated for 2–5 h at 36.5°C and 5% CO2. After incubation, the cells were removed from the plates using 0.2% ATP-trypsin solution and 0.02% Versene (Adolfo Lutz). The trypsinized cells were washed in medium containing fetal bovine serum, centrifuged and fixed with 4% paraformaldehyde. The fixed cells were then evaluated using flow cytometry (FACSCanto, BD) to determine the amount of adherent fungi and to thereby determine the infective capacity of the different species of the Paracoccidioides genus. Experiments were performed in triplicate with three independent experiments for each species. Statistical analyses were performed using One-Way ANOVA with Tukey’s coefficient. The results of the statistical analyses were considered significant when the p-value was <0.05. Analyses and the construction of graphs were performed using Prism 5 software (GraphPad Software Inc.).

Expression Analysis of the Genes Encoding Adhesins by Real-Time PCR

The expression of genes encoding seven adhesins, which were previously described in Paracoccidioides spp., was evaluated in the different species. Specific primers for each gene were synthesized (Table 1). The L34 gene was used as the housekeeping gene in the analysis.

For this experiment, groups of 10 male BALB/c mice were infected by tail vein injection with the following Paracoccidioides isolates: Pb18 (P. brasiliensis) and Pb01 (P. lutzii). The blood of the animals was collected for RNA extraction (Bailão et al., 2006) after 10 min and 1 h of infection, and gene expression was investigated. In this study, we analyzed the expression of adhesins at earlier time points (10 min and 1 h) than those evaluated for adhesion studies (2 and 5 h) to determine the expression of adhesins in response to the first contact of the fungus with the host (infected BALB/C mice) because adhesins are molecules of great importance during the initial contact of the fungus with the host.

RNA from the fungi recovered from mouse blood was extracted using the TRIzol method (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNA synthesis was performed using reverse transcriptase (RevertAid™ H Minus Reverse Transcriptase, Fermentas, Canada) and 1 µg of total RNA.

The reaction mixtures contained 1 µL of cDNA, 12.5 µL of Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, Canada), and 0.5 µM of each primer, and the volume was brought to 25 µL with nuclease-free water. The reaction program was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by annealing and synthesis at 60°C for 1 min. Following the PCR, a melting curve analysis was performed, which confirmed that the signal corresponded to a single PCR product. Reactions were performed in an Applied Biosystems 7500 cycler. The data were analyzed using the 2^-∆∆CT method. The cycle threshold values for the triplicate PCRs of each RNA sample were averaged, and then the 2^-∆∆CT values were calculated using the control gene 60S ribosomal L34, which is a constitutive housekeeping gene that is widely used as a control in different studies of paracoccidioidomycosis (Felipe et al., 2005a; Bailão et al., 2006, 2007; Brito et al., 2011; Parente et al., 2011;
Analysis of the Relationship between Adhesion and Virulence in Different Species of the Genus Paracoccidioides

For this analysis, aliquots (5 × 10⁶ cells/ml) of each isolate of the genus were intratracheally inoculated into C57BL/6 male mice. The mice were anesthetized by administration of a combination of 250 µL of 10 mg/kg xylazine and 80 mg/kg ketamine hydrochloride. A small incision was made in the trachea region, and 0.05 mL of fungal suspension inoculum was injected using a 1.0 mL syringe. The incision was sutured, and the animals were kept warm to avoid hypothermia due to the action of the anesthetic. After inoculation, the mice were observed for mortality until 200 days post-infection. Each group contained eight mice, and a control group was inoculated with only PBS. For the survival curves, the statistical analyses were performed using the Mantel-Cox test in GraphPad Prism software, and the p-value was set at p < 0.05.

Evaluation of the Role of the Two Major Expressed Adhesins, 14-3-3 and Enolase, in the Adhesion of the Paracoccidioides Genus to A549 Pneumocytes

This experiment was made in the same way as described in the section Adhesion to A549 pneumocytes of P. brasiliensis and P. lutzii with a modification. After the treatment of the Paracoccidioides spp. cells with 10 µM CFSE, the cells were washed, resuspended in PBS and treated with 14-3-3 and enolase rabbit antisera (1:100) for 1 h at 37°C. After this new treatment, the cells were washed, resuspended in PBS and added to A549 pneumocytes culture for 2 and 5 h. These experiments were also performed in triplicate, with three independent experiments for each species. Statistical analyses were performed using One-Way ANOVA with Tukey’s coefficient. As controls, we used Paracoccidioides spp. without any treatment and treated with rabbit antiserum (1:100) for 1 h at 37°C. The results of the statistical analyses were considered significant when the p-value was <0.05. Analyses and the construction of graphs were performed using Prism 5 software (GraphPad Software Inc.).

Evaluation of the Role of the Two Major Expressed Adhesins, 14-3-3 and Enolase, in the Virulence of the Paracoccidioides Genus Using G. mellonella

For this analysis, P. brasiliensis (Pb18) and P. lutzii (Pb01) were treated with 14-3-3 and enolase rabbit antisera (1:100) for 1 h at 37°C. The 14-3-3 and enolase rabbit antisera used in this study were obtained and used in three previous studies performed by our group (Donofrio et al., 2009; Marcos et al., 2012; da Silva et al., 2013). After treatment, the inocula were washed 3 times with PBS and inoculated into the larvae of G. mellonella. The rearing of G. mellonella was performed according to Ramarao et al. (2012). The larvae were fed wax and pollen and maintained at 25°C until they reached 100–200 mg in weight. Larvae without color alterations and with the appropriate weight were selected in groups of 16 larvae, placed in Petri dishes and incubated at 37°C in the dark on the night before the experiments. The larvae were treated with 14-3-3 and enolase rabbit antisera (1:100) for 1 h at 37°C.

TABLE 1 | Primers used for the real-time PCR assays.

| Gene               | Primers                        | Product length (bp) |
|--------------------|--------------------------------|---------------------|
| Enolase            | Sense 5′-TAGGCACCCCTCAGATCC-3′ | 130                 |
|                    | Antisense 5′-GCTCTCAATCCGCAAGAT-3′ |                    |
| GADPH              | Sense 5′-AAATCTGCTTTGAGGAATG-3′ | 129                 |
|                    | Antisense 5′-CTGTCGCTGGGATACGCTT-3′ |                  |
| GP43               | Sense 5′-CTGGCTGCGGCGAAAAACT-3′ | 135                 |
|                    | Antisense 5′-CGGACTGGAAGTGATAT-3′ |                   |
| Malate synthase    | Sense 5′-GTCCTCCTCATGAGGCAA-3′ | 101                 |
|                    | Antisense 5′-TCCTTGATGGGGATTGG-3′ |                   |
| Triosephosphate isomerase | Sense 5′-CCTAAGCTCCATGAGGCG-3′ | 118                 |
|                    | Antisense 5′-GCCATTTCCATGCGGG-3′ |                    |
| 14-3-3             | Sense 5′-GTGCCTCCTTGAGACAGAC-3′ | 76                  |
|                    | Antisense 5′-AGCAACCTCAGTGGGTT-3′ |                    |
| L34 (endogenous gene) | Sense 5′-TGTCCTAAGTCGCCAGGAG-3′ | 149                |
|                    | Antisense 5′-ATGTTCTGCTGGGAGGGA-3′ |                  |

Peres da Silva et al., 2011; Lima et al., 2014). A sample that contained all reagents except P. brasiliensis cDNA was used as a negative control. After 40 rounds of amplification, no PCR products were detected in this reaction. Two biological samples were obtained for each studied situation and for each sample, and three independent experiments were performed. As a control experiment, an RNA sample was obtained from the blood of mice not infected with P. brasiliensis. Before beginning the experiments, all of the primers were evaluated to assess the efficiency of the amplifications by analyzing the standard curves using serial dilutions of cDNA as a template and determining the slope value. Subsequently, we used this value to evaluate the efficiency using the formula Efficiency = 10¹⁻¹/slope. After the experiments, the amplification products (in 1.5% agarose gels) and the dissociation curves were analyzed to ensure the amplification of only one PCR product. Statistical analyses were performed using One-Way ANOVA with Tukey’s coefficient. As controls, we used Paracoccidioides spp. without any treatment and treated with rabbit antiserum (1:100) for 1 h at 37°C. The results of the statistical analyses were considered significant when the p-value was <0.05. Analyses and the construction of graphs were performed using Prism 5 software (GraphPad Software Inc.).
were inoculated through the last left pro-leg using a 10 µL Hamilton syringe (Hamilton, USA) after cleaning the pro-leg with 70% ethanol.

A total of 5 × 10⁶ cells were injected into each larva. Larval death was recorded over the next 7 days and was based on lack of movement after manipulation with forceps. For this experiment, 6 different controls were used: (1) PBS, (2) larvae treated with 14-3-3 rabbit antiserum alone (not infected with fungi), (3) larvae treated with enolase rabbit antiserum alone (not infected with fungi), (4) larvae treated with rabbit antiserum alone (no infection), (5) larvae infected with *P. brasiliensis* and treated with rabbit antiserum for 1 h at 37°C, (6) larvae infected with *P. lutzii* and treated with rabbit antiserum for 1 h at 37°C, (7) larvae treated with *P. brasiliensis* alone and (8) larvae treated with *P. lutzii*.

For the survival curves, three independent experiments were performed. The statistical analyses were performed using the Mantel-Cox test in GraphPad Prism software, and the *p*-value was set at *p* < 0.05.

The influence of antisera on the survival and morphology of the fungi was evaluated to analyze how these factors interfere with the results found in the adhesion inhibition assays and survival curves. For this, *Paracoccidioides* spp. Pb18 and Pb01 were grown for 7 days in BHI with 2% glucose and with or without 14-3-3, enolase or rabbit antiserum at a titer of 1:100. Every 24 h, aliquots of the fungi were obtained, and the morphology and viability were analyzed. To analyze morphology, the fungi were fixed with 1% paraformaldehyde, stained with calcofluor white stain (40 µL/mL) (Sigma-Aldrich) and observed using fluorescence microscopy. The viability of the cells was assessed by measuring metabolic activity using a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[((phenylamino) carbonyl]-2H-tetrazolium-hydroxide (XTT) (Sigma) reduction assay as described by Meshulam et al. (1995).

**Results**

**Adhesion to A549 Cells of *Paracoccidioides* spp.**

The results of the adhesion assay with the different species are presented in **Figure 1**. The adhesion rates were significantly different (*p* < 0.05) when comparing *P. brasiliensis* and *P. lutzii*. After 2 h of interaction, the percentage of adhesion of *P. brasiliensis* was approximately 42.3%, while the adhesion of the *P. lutzii* isolate was approximately 36.8%. After 5 h of infection, the difference in the adhesion rates was still significantly different, with rates of approximately 45.8% in the *P. brasiliensis* isolate and approximately 39.7% in the *P. lutzii* isolate. The adhesion rate may reflect the virulence of each isolate because adhesion is an essential step for successful infection by fungi of the *Paracoccidioides* genus.

**Adhesion of *Paracoccidioides* spp. to the ECM Components Laminin, Fibronectin, and Type I and Type IV Collagen**

These assays were performed with Pb18 (*P. brasiliensis*) and Pb01 (*P. lutzii*) isolates that were placed into contact with ECM components (laminin, fibronectin, and collagens type I and type IV), after which adherent cells were removed and counted by flow cytometry. Three independent experiments were performed in triplicate and statistically evaluated (**Figure 2**).

The two species appeared to have different affinities for certain extracellular matrix components. Pb18 (*P. brasiliensis*) showed increased adhesion to fibronectin, while Pb01 (*P. lutzii*) was more adherent to type I and IV collagen.

**Expression Analysis of Genes Encoding Adhesins by Real-Time PCR**

Before we analyzed the relative expression, the efficiency of the amplifications was evaluated. For this analysis, we had to determine the value of the slope for each tested primer, and we found that the values were close to −3.32 (L34: −3.4; GP43: −3.24; ENO: −3.26; TPI: −3.24; GAPDH: −3.22; 14-3-3: −3.24; MLT: −3.25); thus, the efficiency was greater than 95% (L34: 96%; GP43: 100%; ENO: 100%; TPI: 100%; GAPDH: 100%; 14-3-3: 100%; MLT: 100%). In addition, after the amplification, the amplicons were checked by running them on agarose gels to confirm the size of the expected products. The melting curves presented only one peak, indicating that only one product was amplified (**Supplementary Figure 1**).

The relative expression levels of different adhesins in the different *Paracoccidioides* species are presented in **Figure 3**. Adhesin expression varies, with some adhesins appearing to be more important than others for infection by the different species. Generally, *P. brasiliensis* adhesins are more highly expressed than *P. lutzii* adhesins. Specifically, enolase and 14-3-3 seem to be the most important adhesins for the *Paracoccidioides* genus during its interaction with the host.

During the initial step of the interaction (10 min), only the TPI adhesin was down-regulated in *P. brasiliensis*, while all of the others analyzed were up-regulated during this step. After 1 h of this interaction, the level of expression was still significantly increased in *P. brasiliensis*, showing the importance of these molecules during the interaction of this species with the host. For *P. lutzii*, we observed the opposite, with most of the adhesins down-regulated during the initial step (10 min) of interaction with the host. However, we note that the TPI adhesin was up-regulated and therefore
seems to be one of the most important adhesins during the initial process of infection. After 1 h, we were able to observe the same pattern observed in *P. brasiliensis*, namely, a significantly increase in the expression of adhesins.

**Survival Curve of C57BL/6 Mice for the Evaluation of the Adhesion/Virulence Relationship in the Genus Paracoccidioides**

The C57BL/6 mouse strain was chosen because this lineage is more sensitive to *Paracoccidioides* infection and is frequently used for studies examining paracoccidiomycosis (de Pádua Queiroz et al., 2010; Bernardino et al., 2013; Costa et al., 2013; da Silva et al., 2013).

After 200 days of infection, we observed that 4 individuals infected with *P. brasiliensis* died, while only 1 individual infected with *P. lutzii* died, resulting in mouse survival rates of 50 and 90%, respectively (Figure 4).

**Evaluation of the Role of the Two Major Expressed Adhesins, 14-3-3 and Enolase, in the Adhesion of the Paracoccidioides Genus to A549 Pneumocytes**

It is important to know how these adhesins in fact influence in the adhesion. For this we proposed an experiment where we blocked the two major expressed adhesins 14-3-3 and enolase and, with an adhesion assay, we could measure how much these adhesins influences in the infection process of *Paracoccidioides* spp. After blocking 14-3-3 and enolase we could observe, in 2 and 5 h, that the capacity to adhere to pneumocytes was affected in *Paracoccidioides* spp. (Figure 5). After 2 and 5 h, we could observe that the blocking of 14-3-3 adhesin, lead to a decrease of 19.3 and 24.8% of *P. brasiliensis* adhesion, respectively. In the case of enolase, adhesion was reduced 8.9 and 10.4% after 2 and 5 h, respectively. For *P. lutzii* we could observe that blocking of 14-3-3 led to 9.8 and 6.2% of the reduction of adhesion after 2 and 5 h. In the case of enolase, adhesion was reduced 9.9 and 5.9% after 2 and 5 h, respectively. These results show that both adhesins are important for full virulence in *Paracoccidioides* spp.

**Evaluation of the Role of the Two Major Expressed Adhesions, 14-3-3 and Enolase, in the Virulence of the Genus Paracoccidioides Using G. mellonella**

If adhesin expression actually interferes with the virulence of the fungus, blockage of adhesin should affect virulence. To evaluate this hypothesis, we decided to block two of the most highly expressed adhesins in *Paracoccidioides* spp. (based on real-time PCR experiments), 14-3-3 and enolase, using polyclonal
antibodies. For this experiment, *P. brasiliensis* and *P. lutzii* were separately treated with 14-3-3 and enolase polyclonal antibodies (1:100) for 1 h at 37°C. Then, these fungi (5 × 10⁶ cells/larva) were used to infect *G. mellonella* larvae. The virulence of these treated fungi was evaluated for 7 days after fungal challenge. This fungal concentration was previously determined by measuring survival curves with different inoculum sizes for both *P. brasiliensis* and *P. lutzii* (data not shown). Treatment of *P. brasiliensis* and *P. lutzii* with 14-3-3 and enolase polyclonal antibodies led to significant reductions in virulence, as measured by mortality (*p < 0.05*). We observed survival rates of 68.7 and 31.2% for *P. brasiliensis* and 20 and 22% for *P. lutzii* after treatment with 14-3-3 and enolase polyclonal antibodies, respectively (Figure 6). Control larvae showed 100% survival at the end of the 7-day experiment.

These results reflect the influence of these adhesins on the virulence of *Paracoccidioides* spp. because our experimental controls showed us that treating the fungi with the different antisera did not influence survival, with greater than 90% of cells remaining viable for all 7 days of the experiment. Additionally, the morphology of the fungi was not altered during the experiment. All of the results of these control analyses are presented in Supplementary Figure 2. Table 2 shows the standard deviations for each mean value in the graphs.

**Discussion**

Studies have demonstrated the ability of *Paracoccidioides* spp. to adhere and invade (Mendes-Giannini et al., 1994), and these characteristics vary depending on the isolate (Hanna et al., 2000).
Previous studies have demonstrated that Pb18, now classified as belonging to the S1 phylogenetic strain, is the most pathogenic strain in animals (Singer-Vermes et al., 1989) and is more adherent to Vero cells (Hanna et al., 2000). Additionally, after several subcultures, Pb18 loses its adhesion ability, indicating the important relationship between virulence and adhesion. However, upon re-isolation from animals or cell culture, this fungus recovers its ability to adhere to and invade epithelial cells (Andreotti et al., 2005).

In our study, we observed that adhesion was significantly higher for the Pb18 isolate of Paracoccidioides brasiliensis than for the Pb01 isolate of P. lutzii. The adhesion process reflects the virulence of the isolates because efficient adhesion will culminate in rapid invasion of the host cells, allowing the fungus to escape the host immune system, establish infection, and, in the case of Paracoccidioides spp., cause systemic mycosis. After 5 h of infection, the adhesion rate continues to increase, indicating a high capacity to adhere to host cells, as previously observed by our group (Andreotti et al., 2005).

These differences in adhesion in different species may reflect differences in the molecules used during interactions with the host. Molecules with greater affinity for different types of ECM components may be associated with the virulence of each isolate and the establishment of the disease in different tissues. Different cell types produce components of the matrix in different quantities, which facilitates the interaction of the fungus with the host depending on the site of the infection. The ability of a microorganism to adhere and invade is recognized as an important factor in pathogenicity. Adhesion implies that the fungus recognizes ligands on the surface of the host cell or a component of the ECM. The adhesion mechanism has been extensively studied in bacteria and pathogenic fungi (de Groot et al., 2013), such as Candida albicans (Nobile et al., 2008; Murciano et al., 2012; Puri et al., 2014; Formosa et al., 2015), Aspergillus fumigatus (Upadhyay et al., 2009; Al Abdallah et al., 2012; Xu et al., 2012; Zhao et al., 2012), Sporothrix schenckii (Ruiz-Baca et al., 2009; Teixeira et al., 2009b; Sandoval-Bernal et al., 2011), Coccidioides immitis (Hung et al., 2002), Histoplasma capsulatum (Taylor et al., 2004; Suárez-Alvarez et al., 2010; Pitangui et al., 2012) and Penicillium marneffei (Srinoulprasert et al., 2006, 2009; Lau et al., 2013). In a previous study, our group demonstrated that P. brasiliensis interacts with human
fibronectin (Mendes-Giannini et al., 2006), which confirmed the results of the present study, although there was apparently greater adhesion to laminin. However, these previous assays were performed with extracts, whereas we used whole fungal cells in the present study.

Adhesion is related to the expression of adhesins, and several adhesins have been described in Paracoccidioides spp. (Mendes-Giannini et al., 2000, 2008) however, these molecules have not been previously studied to determine their role in the dynamics of infection. In the present study, we analyzed the expression of adhesins at earlier times (10 min and 1 h) than those used for adhesion studies (2 and 5 h) because we aimed to determine whether the fungi respond to host contact and when adhesin expression occurs before the fungi begin the invasion process.

The expression of adhesins in the different species was not constant, and a wide range of expression was observed among the isolates, including a lack of expression. We observed greater expression of certain adhesins, depending on the species and the duration of fungal contact.

The increased expression of GP43 was observed only for P. brasiliensis (approximately 1.5-fold higher expression at 10 min and 2.3-fold higher expression at 1 h), while its expression was reduced in P. lutzii (approximately 2-fold less expression at 10 min and 1-fold at 1 h). These data are extremely important for the study of paracoccidioidomycosis because the GP43 protein, aside from its role as an adhesin, is the main serological marker for this disease (Puccia and Travassos, 1991). All phylogenetic species cause paracoccidioidomycosis, but a lack of expression of this protein may lead to inaccuracy in serological diagnosis, as demonstrated in some studies (Rocha et al., 2009; Batista et al., 2010; Machado, 2011; Puccia et al., 2011). Our study confirms the data that have already demonstrated the differential role of GP43 in P. lutzii specifically that it is not involved in the adhesion of the fungus in this species, and shows that new serological markers for the disease should be screened to avoid making diagnostic mistakes when patients are infected with P. lutzii isolates. Additionally, these data confirm that the expression of GP43 is of extreme importance for P. brasiliensis virulence, reflecting the importance of this molecule for the diagnosis of patients affected with paracoccidioidomycosis caused by this species.

Another important finding in our study is related to the increase in enolase adhesin expression for the two species, which was approximately 8-fold at 10 min and approximately 35-fold after 1 h of infection with P. brasiliensis and was approximately 1-fold after 10 min and approximately 10-fold after 1 h of infection with P. lutzii. Recent studies have shown the importance of this protein in Paracoccidioides adhesion and virulence. Donofrio et al. (2009) first demonstrated that enolase is a fibronectin adhesin for P. brasiliensis, and Nogueira et al. (2010) subsequently described its binding to laminin and collagen type I in addition to fibronectin. Moreover, enolase expressed on the surface of P. lutzii acts as a plasminogen receptor, and through this receptor, the fungus can acquire proteolytic activity because of the plasmin generated by this binding event. Plasmin is
A high level of expression of 14-3-3 adhesin was observed in *P. brasiliensis* after 10 min of interaction, and a significant increase in the expression of 14-3-3 was observed after 1 h for the two species (approximately 12-fold for *P. brasiliensis* and 6-fold for *P. lutzii*). This protein has recently been identified as an important factor in the interaction between *Paracoccidioides* and its host. In a recent study, da Silva et al. (2013) demonstrated that a significant increase in this protein occurs on the pathogen cell wall during its interaction with A549 epithelial cells. This increase suggests an important role for this protein in the fungus-host interaction, leading to a cellular immune response that is important for the success of the fungus in the microenvironment of the host cells. In addition, Vallejo et al. (2011, 2012b) verified that this protein is transported by *P. brasiliensis* vesicles and is secreted into the culture supernatant during *ex vivo* interactions, as described by da Silva et al. (2013).

We observed an increase of triosephosphate isomerase expression in *P. lutzii* of approximately 1-fold after 10 min and 1 h. This protein has only been characterized as an adhesin in isolate Pb01 from *P. lutzii* (Pereira et al., 2007), and it has not been studied in other isolates of the genus *Paracoccidioides*.

Glyceraldehyde 3-phosphate dehydrogenase has also been characterized as an adhesin of *P. lutzii* (Barbosa et al., 2006). In our study, we observed the expression of this adhesin during early interactions between *P. brasiliensis* and the host (an increase of approximately 3.5-fold). For both species, the expression decreased after an hour of interaction (approximately 0.5-fold less expression for *P. brasiliensis* and 2.5-fold less expression for *P. lutzii*), revealing the importance of this protein in early infections with *P. brasiliensis*. Although this finding is controversial, the conditions of our experiment may have influenced these results because this adhesin was described in an *in vitro* experiment, while our study was carried out *in vivo*.

Malate synthase has been described as an adhesin for Pb01 (*P. lutzii*), binding to fibronectin and type I and type IV collagen. Moreover, (de Oliveira et al., 2013) demonstrated that *Paracoccidioides* malate synthase interacts with proteins of different molecular categories, such as those involved in cellular transport, protein biosynthesis, protein degradation and modification and signal transduction, suggesting that this protein plays pleiotropic roles in fungal cells. In the present study, it was expressed less during the early interaction of *P. lutzii* (approximately 2-fold less expression) and of *P. brasiliensis* (expressed approximately 1.5-fold more), but its expression was increased in both species after 1 h of interaction (approximately 4-fold for *P. brasiliensis* and 3-fold for *P. lutzii*).

Apparently, the enolase and 14-3-3 were the most highly expressed adhesins. We observed that the expression of adhesins depends on the duration of the interaction between the host and the fungus. The highest levels of expression for most adhesins were observed in *P. brasiliensis*. This study presents a new perspective on the complex interaction between the *Paracoccidioides* genus and the host and demonstrates the importance of studying different species during the course of infection to understand...
the molecular arsenal used by the fungus to ensure pathogenic success.

A survival curve was constructed, and *P. brasiliensis* was shown to be more virulent than *P. lutzii*. *P. brasiliensis* was able to kill 50% of the group, while *P. lutzii* killed only 10%. The *Paracoccidioides*-host interaction may cause the induction of apoptosis in the host cells (Mendes-Giannini et al., 2004; Del Vecchio et al., 2009), and this induction can facilitate the establishment and dissemination of the infection in the host. In this way, *P. brasiliensis* adhere in a more efficient way to pneumocytes than *P. lutzii*, this interaction in an *in vivo* model could lead to the efficient establishment of infection by *P. brasiliensis*, confirming the survival curve observed. These results show that there is an apparent relationship between adherence and virulence in *Paracoccidioides*.

The adhesion of *Paracoccidioides* spp. after blocking 14-3-3 and enolase is significantly affected showing us the importance of these molecules to the infection process of *Paracoccidioides* spp. The 14-3-3 seems to have more influence in this process of these molecules to the infection process of *P. brasiliensis*, and enolase is significantly affected showing us the importance of *P. brasiliensis* efficient establishment of infection by shown to be more virulent than *P. lutzii*. Furthermore, *P. brasiliensis* exhibited higher levels of adhesion expression when compared to *P. lutzii*, resulting in greater adherence of these species. These data, when analyzed in relation to the survival curves, make it clear that the greater ability of *P. brasiliensis* to express adhesins leads to greater adhesion and results in increased virulence *in vivo*. Thus, there is a clear relationship between adhesion ability, adhesin expression and fungal virulence in the genus *Paracoccidioides*.

**Acknowledgments**

This work was supported by the Brazilian organizations Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP) 2011/18038-9 and Rede Nacional de Métodos Alternativos - Conselho Nacional de Desenvolvimento Científico e Tecnológico (RENAME-CNPq) 403586/2012-7. We thank Daniella Sayuri Yamazaki for support with the animal experiment and Dr. Isabel Cristiane da Silva for all the support with A549 pneumocytes culture.

**Supplementary Material**

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00303/abstract

**Supplementary Figure S 1** | Specific amplification of the studied adhesin genes by real-time PCR. (A) Melting curve analysis of all of the primers used showing single peaks. (B) Agarose gel electrophoresis of the PCR products showing single bands.

**Supplementary Figure S 2** | Influence of antisera treatment on the survival and morphology of the fungi. Analysis of the influence of fungi treatment with 14-3-3, enolase and NRS antisera. (A) and (C) Viability of *P. lutzii* and *P. brasiliensis*, respectively, after treatment with 14-3-3, enolase and NRS antisera. No significant differences were found between the different treatments and the untreated controls. (B) and (D) Analysis of the morphology of *P. lutzii* and *P. brasiliensis*, respectively, after treatment with 14-3-3, enolase and NRS antisera (image magnification: 1000x).
References

Al Abdallah, Q., Choe, S. I., Campoli, P., Baptista, S., Gravel, F. N., Lee, M. J., et al. (2012). A conserved C-terminal domain of the Aspergillus fumigatus developmental regulator MedA is required for nuclear localization, adhesion and virulence. PLoS ONE 7:e49959. doi: 10.1371/journal.pone.0049959

Andreotti, P. F., Monteiro da Silva, J. L., Ballão, A. M., Soares, C. M., Benard, G., Soares, C. P., et al. (2005). Isolation and partial characterization of a 30kDa adhesin from Paracoccidioides brasiliensis. Microbes Infect. 7, 875–881. doi: 10.1016/j.micinf.2005.02.005

Bagagi, E., Bosco, S. M., Theodoro, R. C., and Franco, M. (2006). Phylogenetic and evolutionary aspects of Paracoccidioides brasiliensis reveal a long coexistence with animal hosts that explain several biological features of the pathogen. Infect. Genet. Evol. 6, 344–351. doi: 10.1016/j.meegid.2005.07.027

Barbosa, M. S., Bão, S. N., Andreotti, P. F., de Faria, F. P., Felipe, M. S., dos Santos Feitosa, L., et al. (2006). Glyceroldehyde-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. doi: 10.1128/IAI.74.1.382-389.2006

Batista, J., de Camargo, P. Z., Fernandes, G. F., Vicentini, A. P., Fontes, C. J., and Hahn, R. C. (2010). Is the geographical origin of a Paracoccidioides brasiliensis isolate important for antigen production for regional diagnosis of paracoccidiomycosis? Mycoses 53, 176–180. doi: 10.1111/j.1349-0507.2008.01687.x

Bergin, D., Reeves, E. P., Renwick, J., Wientjes, F. B., and Kavanagh, K. (2005). Superoxide production in Galleria mellonella hemocytes: identification of proteins homologous to the NAPDH oxidase complex of human neutrophils. Infect. Immun. 73, 4161–4170. doi: 10.1128/IAI.73.7.4161-4170.2005

Bernardino, S., Pina, A., Felonato, M., Costa, T. A., Frank de Araújo, E., Feriotti, C., et al. (2013). TNF-α and CD8+ T cells mediate the beneficial effects of nitric oxide synthase-2 deficiency in pulmonary paracoccidiomycosis. PLoS Negl. Trop. Dis. 7:e2325. doi: 10.1371/journal.pntd.0002325

Brito, W. A., Rezende, T. C., Parente, A. F., Ricart, C. A., Sousa, M. V., Bão, S. N., et al. (2011). Identification, characterization and regulation studies of the aconitase of Paracoccidioides brasiliensis. Fungal Biol. 115, 697–707. doi: 10.1016/j.funbio.2011.02.011

Caliche, V. L., Vo, C. A., and Burger, E. (1998). Immunity to Paracoccidioides brasiliensis infection. Res. Immunol. 149, 407–17. discussion: 499–500. doi: 10.1016/S0923-2494(98)80764-5

Carrero, I. L., Niño-Vega, G., Teixeira, M. M., Carvalho, M. J., Soares, C. M., Pereira, M., et al. (2008). New Paracoccidioides brasiliensis isolate reveals unexpected genomic variability in this human pathogen. Fungal Genet. Biol. 45, 603–612. doi: 10.1016/j.fgb.2008.02.002

Casadevall, A., and Pirofski, L. A. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. Infect. Immun. 67, 3703–3713.

Champion, O. L., Cooper, I. A., James, S. L., Ford, D., Karlyshev, A., Wren, B. W., et al. (2009). Galleria mellonella as an alternative infection model for Yersinia pseudotuberculosis. Microbiology 155, 1516–1522. doi: 10.1099/mic.0.026823-0

Cook, S. M., and McArthur, J. D. (2013). Developing Galleria mellonella as a model host for human pathogens. Virulence 4, 350–353. doi: 10.4161/viru.25240

Costa, T. A., Bazan, S. B., Feriotti, C., Araújo, E. F., Bassi, É., Loures, F. V., et al. (2013). In pulmonary paracoccidioidomycosis IL-10 deficiency leads to increased immunity and regressive infection without enhancing tissue pathology. PLoS Negl. Trop. Dis. 7:e2532. doi: 10.1371/journal.pntd.0002532

da Silva, J. F., de Oliveira, H. C., Marcos, C. M., da Silva, R. A., da Costa, T. A., Calich, V. L., et al. (2013). Paracoccidioides brasiliensis 30kDa Adhesin: identification as a 14-3-3 Protein, Cloning and Subcellular Localization in Infection Models. PLoS ONE 8:e62533. doi: 10.1371/journal.pone.0062533
Lima, P. S., Casaletti, L., Bailão, A. M., de Vasconcelos, A. T., Fernandes, G. R., and Soares, C. M. (2014). Transcriptional and proteomic responses to carbon starvation in Paracoccidioides. PLoS Negl. Trop. Dis. 8:e2855. doi: 10.1371/journal.pntd.0002855

Machado, G. C. (2011). “Espécies críticas em Paracoccidioides brasiliensis: Diferenciacao antígénica e de resposta a fatores estressantes,” in Departamento de Microbiologia e Immunologia (Botucatu, São Paulo, Brazil: Univ Estadual Paulista), 70.

Marcos, C. M., de Fátima da Silva, J., de Oliveira, H. C., Moraes da Silva, R. A., Mendes-Giannini, M. J., and Fusco-Almeida, A. M. (2012). Surface-expressed enolase contributes to the adhesion of Paracoccidioides brasiliensis to host cells. FEMS Yeast Res. 12, 557–570. doi: 10.1111/j.1567-1364.2012.00806.x

Matute, D. R., McEwen, J. G., Puccia, R., Monteis, B. A., Sanblas, G., Bagagli, E., Marcos, C. M., de Oliveira, H. C., Jde da Silva, F., Assato, P. A., Fusco-Almeida, A. M., and Mendes-Giannini, M. J. (2014). The multifaceted roles of metabolic enzymes in the Paracoccidioides brasiliensis species complex. Front. Microbiol. 5:719. doi: 10.3389/fmicb.2014.00719

Matute, D. R., McEwen, J. G., Puccia, R., Monteis, B. A., Sanblas, G., Bagagli, E., et al. (2006). Cryptic speciation and recombination in the fungus Paracoccidioides brasiliensis as revealed by gene genealogies. Mol. Biol. Evol. 23, 65–73. doi: 10.1093/molbev/msj008

Mendes-Giannini, M. J., Andreotti, P. F., Vincenzi, L. R., da Silva, J. L., Lenzi, H. L., Benard, G., et al. (2006). Binding of extracellular matrix proteins to Paracoccidioides brasiliensis. Microbes Infect. 8, 1550–1559. doi: 10.1016/j.micinf.2006.01.012

Mendes-Giannini, M. J., Hanna, S. A., da Silva, J. L., Andreotti, P. F., Vincenzi, L. R., Benard, G., et al. (2004). Invasion of epithelial mammalian cells by Paracoccidioides brasiliensis leads to cytoskeletal rearrangement and apoptosis of the host cell. Microbes Infect. 6, 882–891. doi: 10.1016/j.micinf.2004.05.005

Mendes-Giannini, M. J., Monteiro da Silva, J. L., de Fátima da Silva, J., Donofrio, F. C., Miranda, E. T., Andreotti, P. F., et al. (2008). Interactions of Paracoccidioides brasiliensis with host cells: recent advances. Mycopathologia 165, 237–248. doi: 10.1007/s11046-007-9074-z

Meschulam, T., Levitz, S. M., Christin, L., and Diamond, R. D. (1995). A simplified new assay for assessment of fungal cell damage with the tetrazolium dye, (2,3-bis-(2-methoxy-4-nitro-5-sulphonyl)-2H-tetrazolium-5-carboxanilide (XTT)). J. Infect. Dis. 172, 1153–1160. doi: 10.1086/317692

Nogueira, S. V., Fonseca, F. L., Rodrigues, M. L., Mundodi, V., Abi-Chacra, E. A., Nobile, C. J., Schneider, H. A., Sheppard, D. C., Filler, S. G., and Andes, D. R. (2009). Engagement of Pin1 by Pin1-interacting motifs (PIMs) from C. albicans agglutinin-like sequence (ALS) proteins in human oral epithelial cell interactions. PLoS ONE 4:73362. doi: 10.1371/journal.pone.0033362

Peres da Silva, R., Matsumoto, M. T., Brait, J. D., Valtan, A. R., de Oliveira, H. C., Soares, C. P., and Mendes Giannini, M. J. (2011). Differential gene expression analysis of Paracoccidioides brasiliensis during keratinocyte infection. J. Med. Microbiol. 60, 269–280. doi: 10.1099/jmm.0.022467-0

Pitanguy, N. S., Sardi, J. C., Silva, J. F., Benaducci, T., Moraes da Silva, R. A., Rodrigues-Arellanes, G., et al. (2012). Adhesion of Histoplasma capsulatum to pneumocytes and biofilm formation on an abiotic surface. Biofouling 28, 711–718. doi: 10.1080/08949258.2012.703639

Puccia, R., and Travassos, L. R. (1991). 43-kilodalton glycoprotein from Paracoccidioides brasiliensis: immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease. J. Clin. Microbiol. 29, 1610–1615.

Puccia, R., Vallejo, M. C., Matsuo, A. L., and Longo, L. V. (2011). The paracoccidioides cell wall: past and present layers toward understanding interaction with the host. Front. Microbiol. 2:257. doi: 10.3389/fmicb.2011.00257

Qiao, A. A., Morais, F. V., and Puccia, R. (2009). Polymorphism in the flanking regions of the Pbg4P43 gene from the human pathogen Paracoccidioides brasiliensis: search for protein binding sequences and poly(A) cleavage sites. BMC Microbiol. 9:277. doi: 10.1186/1471-2180-9-277

Ruiz-Baca, E., Toriello, C., Perez-Torres, A., Sabanero-Lopez, M., Villagomez-Castro, I. C., and Lopez-Romero, E. (2009). Isolation and some properties of a glycoprotein of 70 kDa (GP70) from the cell wall of Sporothrix schenckii involved in fungal adherence to dermal extracellular matrix. Med. Mycol. 47, 185–196. doi: 10.1111/j.1369-3788.2007.01657.x

Sandoval-Bernal, G., Barbosa-Sabanero, G., Shihayama, M., Perez-Torres, A., Tsutsumi, V., and Sabanero, M. (2011). Cell wall glycoproteins participate in the adhesion of Sporothrix schenckii to epithelial cells. Mycopathologia 171, 251–259. doi: 10.1007/s11046-010-9372-8

Silva, R. C., Padovan, A. C., Pimenta, D. C., Ferreira, R. C., da Silva, C. V., and Briones, M. R. (2014). Extracellular enolase of Candida albicans is involved in colonization of mammalian intestinal epithelium. Front. Cell. Infect. Microbiol. 4:669. doi: 10.3389/fcimb.2014.00666

Singer-Vermes, L. M., Burger, E., Franco, M. F., Di-Bacchi, M. M., Mendes-Giannini, M. J., and Calich, V. L. (1989). Evaluation of the pathogenicity and immunogenicity of seven Paracoccidioides brasiliensis isolates in susceptible inbred mice. J. Med. Vet. Med. 27, 71–82. doi: 10.1026/02681898000111

Taborda, C. P., Zarragoza, O., Murcia, H. L., Escalante, E. D., et al. (2000). Pathogenesis II: fungal responses to host cells. FEMS Yeast Res. 6, 113–123. doi: 10.1016/S1356-3258(00)00113-2

Taylor, M. L., Duarte-Escalante, E., Pérez, A., Zenteno, E., and Toriello, C. (2004). Histoplasma capsulatum yeast cells attach and agglutinate human erythrocytes. Med. Mycol. 42, 287–292. doi: 10.1080/136937803100012011

Teixeira, M. M., Theodoro, R. C., de Carvalho, M. J., Fernandes, L., Paes, H. C., Hahn, R. C., et al. (2009a). Phylogenetic analysis reveals a high level of speciation in the Paracoccidioides genus. Mol. Phylogenet. Evol. 52, 273–283. doi: 10.1016/j.ympev.2009.04.005

Teixeira, P. A., de Castro, R. A., Nascimento, R. C., Tronchin, G., Torres, A. P., Lazéra, M., et al. (2009b). Cell surface expression of adhesins for fibronectin correlates with virulence in Sporothrix schenckii. Microbiology 155, 3730–3738. doi: 10.1099/mic.0.029439-0

Tomaz, L., Garcia-Rodas, R., Guinaraaé, A. J., Taborda, C. P., Zaragoza, O., and Nosanchuk, J. D. (2013). Galleria mellonella as a model host to study Paracoccidioides brasiliensis virulence and Histoplasma capsulatum. Virulence 4, 139–146. doi: 10.4161/viru.23047
Xu, X. Y., Shi, Y., Zhang, P. P., Zhang, F., Shen, Y. Y., Su, X., et al. (2012). E-cadherin mediates adhesion and endocytosis of Aspergillus fumigatus blastospores in human epithelial cells. Chin. Med. J. (Engl.) 125, 617–621. doi: 10.3760/cma.j.issn.0366-6999.2012.04.011

Zhao, G., Li, S., Zhao, W., He, K., Xi, H., Li, W., et al. (2012). Phage display against corneal epithelial cells produced bioactive peptides that inhibit Aspergillus adhesion to the corneas. PLoS ONE 7:e33578. doi: 10.1371/journal.pone.0033578

Conflict of Interest Statement: The Associate Editor, Helio Takahashi, declares that despite having published with author, Maria Mendes-Giannini, the review process was handled objectively and no conflict of interest exists. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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