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Brief Report

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Can passage in *Galleria mellonella* activate virulence factors of *Paracoccidioides brasiliensis* as in the murine model?

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Abstract

Paracoccidioidomycosis (PCM) is a fungal disease restricted to Latin countries, and its etiologic agents derive from the *Paracoccidioides* genus. Attenuation or loss of virulence in *Paracoccidioides* spp. following successive subculturing has been described. However, virulence can be recovered by passage in mammalian host. In this study, the recovery of adhesion of *P. brasiliensis* through passage in mice was compared to that in the insect *Galleria mellonella.* Analysis of *in vitro* fungal-host cell interaction, gene expression of adhesins, and analysis of the survival curves revealed that *Galleria mellonella* is useful for the reactivation of *P. brasiliensis* adhesion.

Key words: adhesins, *Paracoccidioides brasiliensis*, *Galleria mellonella*, mice, fungal-host cell interaction, virulence factors.

Paracoccidioidomycosis (PCM) is caused by the dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii*. PCM infection occurs by inhalation of fungus propagules and has important medical, social and economic impacts.[1]

Numerous events related to the host and the microorganism are important for the development of a fungal infection.[2] Endocytosis, active penetration, and the Trojan horse mechanism have been described as fungal invasion mechanisms. However, the adhesion process is the first step to invasion and is fundamental for successful fungal infection.[3]

Adhesins are microbial cell surface molecules that mediate host-microorganism interaction and are important for the virulence of *Paracoccidioides* spp.[4] Following adhesion, fungus internalization may occur; although *Paracoccidioides* spp. are not obligatory intracellular pathogens, this event in their infection is probably associated with disease dissemination.[5]

Several studies describe problems during *in vitro* storage and successive subculturing of dimorphic fungi. Mineral oil preservation is one of the proposed alternatives for storage. However, this can strongly affect fungi characteristics and virulence mechanisms.[6] The recovery of these characteristics can be observed following passage in mammals.[7] Because of ethical issues, other animal models have been developed as an alternative to the mammalian models. Invertebrates represent a good option for *in vivo* experiments because of their rapid life cycle, small size, low cost, and the evolutionary conservation of the innate immune system between invertebrates and mammals.[8] *Galleria mellonella* was successfully used to study *Paracoccidioides* spp. virulence[9] and antifungal treatment.[10] Moreover, the involvement of the adhesin 14-3-3 in the virulence of *P. brasiliensis* was also evaluated in this model using a 14-3-3 knockdown strain.[11]

In this study, we analyzed the reactivation of virulence factors in a subcultured *P. brasiliensis* strain following passage in two animal models: mice—the gold standard for animal experimentation—and the insect *Galleria mellonella*—an alternative animal model that has recently gained increased importance. The fungal-cell interaction profile, gene expression of adhesins of *P. brasiliensis,* and survival curves were evaluated before and after animal passage.

Hence, *P. brasiliensis* (São Paulo, Brazil) was subcultivated 10 times in Fava Netto's medium and denominated Pb18-S. The same strain was recovered from the lungs of male BALB/C mice after 30 days of infection (Pb18-M), as well as from *G. mellonella* after 4 days of infection (Pb18-G), as described by de Lacorte Singulani et al.[10] The fungal-cell interaction was evaluated by flow cytometry. Briefly, 10^6 fungal cells/ml of each isolate previously stained with 100 μM CFDA-SE (Sigma Aldrich, USA) were incubated for 1, 3, and 6 h in a monolayer formed with 10^6 cells/ml of A549 pneumocyte linage. Following incubation, pneumocytes were stained with 0.165 μM Alexa Fluor® 647 phalloidin conjugates (Invitrogen). The fungal-cell interaction and its data were analyzed using the FACSCanto flow cytometer (Becton Dickinson) and the BD FACS Diva software (Becton Dickinson), respectively. The interaction assay data were analyzed by ANOVA followed by Tukey's *post hoc* test.

The fungal-cell interaction of both recovered isolates did not reveal a statistical difference after 1 and 3 h of infection compared to Pb18-S (Fig. 1). The isolates Pb18-G and Pb18-M showed a significant difference in interaction compared to Pb18-S after 6 h of infection, with a 1.8- and 2.2-fold increase, respectively.

Brummer et al. [7] were the first to observe that *P. brasiliensis* isolates were less virulent after subculture compared to freshly isolated strains. Donofrio et al.[12] demonstrated that contact of this fungus with mammalian cells strongly increased its ability to adhere to pneumocytes. Mendes-Giannini et al.[13] revealed that reisolated fungus has a higher ability to interact with extracellular matrix (ECM) proteins. In alignment with these studies, our results demonstrated that attenuated *P. brasiliensis,* isolated by intense subcultivation, shows an increased ability to interact with pneumocytes when recovered from *G. mellonella*, as is also observed in strains recovered from the mouse model.

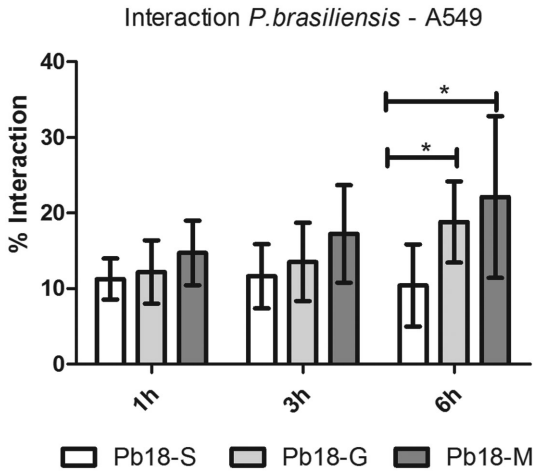

Figure 1. Fungal-cell interaction. Interaction percentage of *P. brasiliensis* sub-cultivated (Pb18-S), recovered from mice (Pb18-M) and *G. mellonella* (Pb18-G), analysed after 1, 3, and 6 h of incubation by flow cytometry. $^*P < .05$ in relation to Pb18-S.

In order to support the fungal-cell interaction data, the gene expressions of *P. brasiliensis* adhesins—enolase,[14] glycoprotein Gp43,[15] triosephosphate isomerase (TPI),[16] and 14-3-3[17]—were evaluated using real-time polymerase chain reaction (PCR). RNA extraction was performed using the RNeasy Mini Kit (Qiagen), and first-strand cDNA synthesis was achieved using the reverse transcriptase RevertAidTM H Minus Reverse Transcriptase (Fermentas, Canada). Real-time PCR assay was carried out using Maxima® SYBR Green/ROX qPCR Master Mix (2 ×) (Fermentas, Canada) in the Applied Biosystems 7500 (Applied Biosystems) cycler equipment. The results of relative expression were calculated using the $2^{-\Delta\Delta CT}$ method.[18] The 60S ribosomal L34 gene was used as the housekeeping gene.[4] Data collected for the Pb18-S strain were used for data normalization. Statistical analysis was performed using Student's *t*-test. Animal passage increased the expression of all the evaluated adhesins. However, statistically significant differences between passage in *G. mellonella* and passage in mice were observed only for Gp43 expression (Fig. 2). A previous study analysed adhesin gene expression of *P. brasiliensis* and *P. lutzii* after 1 hour of infection in *G. mellonella* and revealed an overall increase in expression of these genes,

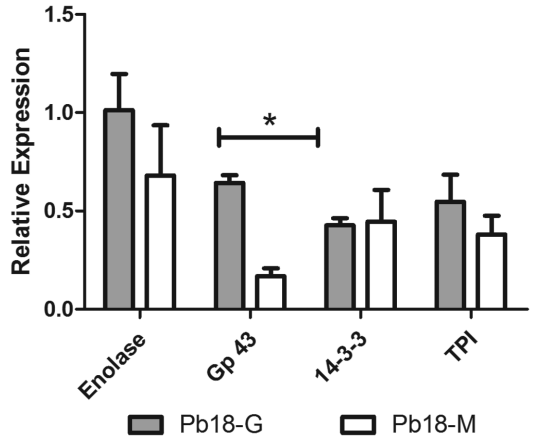

Figure 2. Relative expression of the *P. brasiliensis* adhesins enolase, Gp43, 14-3-3 and TPI after the isolation from *G. mellonella* (Pb18-G) and mice (Pb18-M) analyzed by real-time PCR. $^{*}P < .05$.

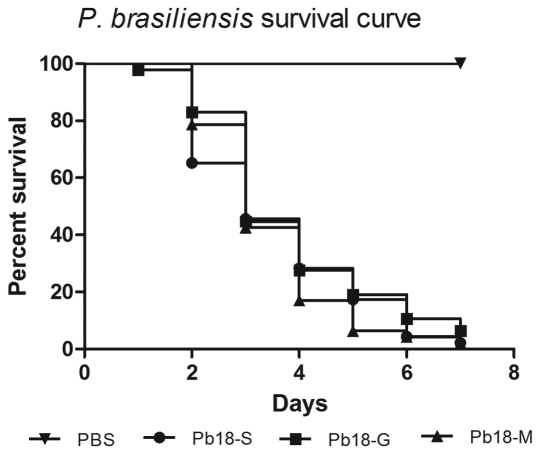

Figure 3. Survival curve performed in *G. mellonella* with *P. brasiliensis* sub-cultivated (Pb18-S), recovered from mice (Pb18-M) and recovered from *G. mellonella* (Pb18-G).

including Gp43. However, the profile of adhesin expression was observed to be different for *P. lutzii* and *P. brasiliensis*.[9]

Furthermore, survival curves were performed aiming to evaluate if the recovery of the adhesion could influence in the pathogenicity of *P. brasiliensis*. For this, *G. mellonella* were infected with 5×10^6 cells/larvae of each *P. brasiliensis* isolate and as a control were used larvae treated with phosphate-buffered saline (PBS). The larvae were incubated at 37°C and death was monitored daily for 7 days by visual inspection based on lack of movement after touching them with forceps.[9] Survival curves assay were analysed by Log-rank (Mantel-Cox) test. Despite the increase of fungal cell interaction and adhesion expression, the analysis of the survival curves reveled a similar pathogenicity for Pb18-S and both mammalian and insect isolates (Fig. 3). The possible explanation is that despite virulence mechanisms are considered determining factors for the pathogenesis, the pathogenicity depends on numerous and complex events correlated to the host and to the microorganism. In other words, pathogenesis is a multifaceted phenomenon and involves a combination of properties.[2, 19]

The activation of virulence factors has been described for other fungi using nonmammalian host models such as *Acanthamoeba castellani* and *Dictyostelium discoideum*.[20] Some studies have proposed that fungal pathogens may undergo microevolution during their interaction with host cells.[21] Indeed, our study suggests that *Paracoccidioides* spp. demonstrate an improved capacity of expressing adhesins during mouse or insect infection along with an improvement in their general virulence.

The use of mammalian animals in research has been the focus of acts and laws controlling the ethical issues of animal experimentation. The use of invertebrate animals is well accepted by the scientific community, and their application in the study of fungal virulence and treatment is increasing.[8,22] Moreover, the correlation between invertebrates and mammalian hosts has been demonstrated in recent studies.[23] Our results indicate that *G. mellonella* is a suitable model to reactivate adhesion—one of the most studied virulence mechanisms of *P. brasiliensis*. *G. mellonella* has the advantage of faster isolation of fungi (4 days) compared to mice (30 days). Moreover, this model reduces the use of mammals in laboratory experimentation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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