



Evaluation of the efficacy of antifungal drugs against *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* in a *Galleria mellonella* model

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ABSTRACT

Paracoccidioides brasiliensis and *P. lutzii* belong to a group of thermomimorphic fungi and cause paracoccidioidomycosis (PCM), which is a human systemic mycosis endemic in South and Central America. Patients with this mycosis are commonly treated with amphotericin B (AmB) and azoles. The study of fungal virulence and the efficacy and toxicity of antifungal drugs has been successfully performed in a *Galleria mellonella* infection model. In this work, *G. mellonella* larvae were infected with two *Paracoccidioides* spp. and the efficacy and toxicity of AmB and itraconazole were evaluated in this model for the first time. AmB and itraconazole treatments were effective in increasing larval survival and reducing the fungal burden. The fungicidal and fungistatic effects of AmB and itraconazole, respectively, were observed in the model. Furthermore, these effects were independent of changes in haemocyte number. *G. mellonella* can serve as a rapid model for the screening of new antifungal compounds against *Paracoccidioides* and can contribute to a reduction in experimental animal numbers in the study of PCM.

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1. Introduction

Paracoccidioides brasiliensis belongs to a group of thermomimorphic fungi that is found in the form of mycelia at room temperature (25–27 °C) and in yeast form at body temperature (37 °C). Phylogenetic studies showed the existence of three different phylogenetic species of *P. brasiliensis* (S1, PS2 and PS3) [1]. More recently, genomic studies showed that *P. brasiliensis* strain 01 could not be included in these groups and was considered a new species, termed *P. lutzii* [2,3]. The individual characteristics of each species have implications in the diagnosis, clinical manifestations and treatment of the disease [4].

Paracoccidioides spp. causes paracoccidioidomycosis (PCM), a human systemic mycosis whose clinical importance has increased due to the frequency, severity of their anatomical and clinical forms, and mortality rate. PCM is endemic in South and Central America, among which Brazil, Venezuela, Colombia and Argentina are the countries with the highest number of cases. In these regions, the annual incidence rate of new cases ranges from 1 to 3 per 10⁵ inhabitants. In Brazil, PCM is the eighth most important cause of

mortality from chronic infectious diseases, with 1.65 deaths per 10⁶ inhabitants. In non-endemic regions such as the USA, Europe and Asia, some imported cases have been reported. The marked predominance of clinical disease is in adult men, rural workers and the immunocompromised [5–7].

Paracoccidioides infection occurs upon inhalation of propagules by the host. These structures then invade terminal airways, where they differentiate into yeast forms. Temperature is essential for this transformation. The lung is the first site of infection, but the yeast can spread to other organs. It is speculated that modulation of host cell apoptosis is advantageous for the fungi because the micro-organisms can evade the killing activity of the phagocytic antimicrobial machinery in tissues; this would prevent their death, and the fungi could disseminate through the host's circulatory system and stimulate the inflammatory response to invade tissues as a consequence of these injuries [8]. There is evidence that *P. brasiliensis* is able to modulate the chronic inflammatory response through nitric oxide (NO), which improves tissue degradation and/or decreases extracellular matrix synthesis by controlling inflammatory and immune mediators. In the later phase of PCM, the deleterious effects of NO may be associated with loose granulomas and high fungal dissemination [9,10]. Treatment of PCM takes place over long periods of time and depends on the severity in each patient: azoles (itraconazole, fluconazole and voriconazole) and sulfonamides are options for mild-to-moderate clinical forms; and amphotericin B (AmB) is used for severe and disseminated cases [6,7,11].

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Virulence studies of different fungi such as *P. brasiliensis*, *P. lutzii*, *Histoplasma capsulatum* [12,13], *Cryptococcus neoformans* [14], *Fusarium* spp. [15], *Candida* [16,17] and *Trichosporon* spp. [18] have been successfully performed in a *Galleria mellonella* infection model. It has important advantages as a fungal host study model: larvae can be incubated at temperatures between 25 °C and 37 °C, enabling the habitat and conditions of infection in mammals to be simulated; exact amounts of pathogens can be administered; and the viability of the larvae can easily be detected by the lack of movement and melanisation response after infection [19]. Furthermore, larvae have six important types of cells in the defence system called haemocytes, which are classified as prohaemocytes, plasmatocytes, granulocytes, coagulocytes, spherulocytes and oenocytoids. The plasmatocytes and granulocytes participate in phagocytosis, nodule formation, encapsulation and defence against microbial pathogens [20,21]. *G. mellonella* has also been used to test the efficacy and toxicity of commercial and new antifungals [22–24].

In this work, *G. mellonella* was infected with two species of *Paracoccidioides* and the efficacy and toxicity of AmB and itraconazole were evaluated in this model for the first time.

2. Materials and methods

2.1. Fungi

P. brasiliensis isolate 18 (chronic PCM; São Paulo, Brazil) and *P. lutzii* Pb01-like strain ATCC MYA-826 (acute PCM; Goiânia, Brazil) were grown in Fava Netto medium (prepared in-house) at 37 °C for 4–5 days. Fungi were transferred to brain–heart infusion (BHI) (Neogen, Lansing, MI) broth supplemented with 1% glucose (Hexapur, Solon, OH) and were grown on a shaker (New Brunswick, Edison, NJ) for 3–4 days at 150 rpm and 37 °C. For the experiment, yeast cells were washed three times with phosphate-buffered saline (PBS) (prepared in-house) containing 20 mg/L ampicillin (Sigma-Aldrich, St Louis, MO) to prevent bacterial contamination.

2.2. Insects

Eggs of *G. mellonella* (Lepidoptera: Pyralidae) were provided by Dr Carlos Eduardo Winter (Universidade de São Paulo, São Paulo, Brazil) to initiate the culture of this insect in our laboratory. Larvae were kept in plastic boxes and were reared on honeybee wax and pollen at 25 °C in darkness. Larvae of ca. 150 mg were selected for the experiments and were kept without food in Petri plates at 37 °C in the dark for 24 h prior to use.

2.3. Survival analysis

Before injection, the pro-leg area was cleaned with 70% ethanol. Each larvae group was inoculated using a Hamilton syringe through the last left pro-leg with 10 µL of 1×10^5 , 5×10^5 , 5×10^6 or 5×10^6 *P. brasiliensis* or *P. lutzii* yeast cells. The inoculum of 5×10^6 yeast cells/larva was used for the remaining studies. A group of uninfected larvae and a group of uninfected larvae inoculated with PBS were used as controls in all experiments. Larvae were incubated in Petri plates at 37 °C and were assessed at 7 days for lack of physical movement. A total of 16 larvae were used for each condition, and each experiment was replicated three times.

2.4. Efficacy and toxicity assays

At 1 h after infection with *Paracoccidioides* spp., larvae were injected with 10 µL of AmB (Sigma-Aldrich) at 0.5, 1 or 2 mg/kg or with itraconazole (Sigma-Aldrich) at 5.5, 11, 22 and 44 mg/kg through the last right pro-leg. Stock solutions of AmB and itraconazole were prepared in dimethyl sulfoxide (DMSO) (Labsynth, Diadema, SP,

Brazil) and were diluted in PBS to a DMSO concentration of 5%. Groups of uninfected larvae were treated with antifungals alone and with the vehicles to test their toxicity. Larvae were incubated at 37 °C and were assessed for 7 days for lack of physical movement.

2.5. Fungal burden

At 1, 48 and 96 h post-treatment with AmB or itraconazole, larvae from each group were surface-sterilised in 70% ethanol. A group of infected larvae without treatment was also used. Each larva was cut into small pieces with a scalpel and was suspended in 1 mL of PBS with 20 mg/L ampicillin. The tissues were transferred to conical tubes with glass beads and were homogenised using a vortex mixer (Norte Científica, Araraquara, SP, Brazil). Each sample was diluted 100× in PBS and then 100 µL of the resulting dilution was plated on BHI agar supplemented with 4% horse serum, 5% *P. brasiliensis* 339 culture filtrate and 40 mg/L gentamicin [25]. The plates were incubated at 37 °C for 10 days and then CFU of *P. brasiliensis* or *P. lutzii* were counted.

2.6. Histological evaluation

At 48 h post-treatment with AmB or itraconazole, larvae from each group were fixed by immersion in phosphate-buffered 4% formalin. A group of uninfected larvae and a group of infected larvae without treatment were also used. Samples were embedded in paraffin, were serially sectioned at a thickness of 5 µm and were stained using Periodic acid-Schiff (PAS) solution (Sigma-Aldrich). Images were analysed using an optical microscope (ZEISS AxioCam HRC; Carl Zeiss Microscopy GmbH, Jena, Germany) at 40×.

2.7. Haemocyte density

At 1 h and 48 h post-treatment with AmB or itraconazole, haemolymph samples were collected by puncturing the larval abdomen and were diluted in ice-cold PBS (1:20). Then, 10 µL aliquots of the haemocyte suspension were added to a Neubauer chamber (haemocytometer) and cells were counted in four main squares under a brightfield microscope. As the larvae without treatment were debilitated or dead at 96 h, it was not possible to acquire sufficient amount of haemolymph for analysis.

2.8. Statistical analysis

Survival curves were analysed using the log-rank (Mantel-Cox) test in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Fungal burden and haemocyte density were analysed using analysis of variance (ANOVA) with the Bonferroni post-test. A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. Killing of *G. mellonella* by *Paracoccidioides* spp. and the effect of antifungal agents

To determine whether *G. mellonella* larvae were a suitable model for studying *Paracoccidioides* pathogenesis, its infection characteristics were first defined. Larval killing was dependent on the number of *Paracoccidioides* cells injected. Very few *G. mellonella* larvae were killed with $\leq 1 \times 10^6$ yeast cells/larva within the period of infection (data not shown), whereas most of the *G. mellonella* larvae were killed with 5×10^6 yeast cells/larva, which was the concentration selected for testing the antifungal treatments.

At the end of 7 days, 11% and 4% of the larvae infected with 5×10^6 yeast cells/larva of *P. brasiliensis* or *P. lutzii* survived, respectively. All doses of AmB (0.5, 1 and 2 mg/kg) protected *G. mellonella* from

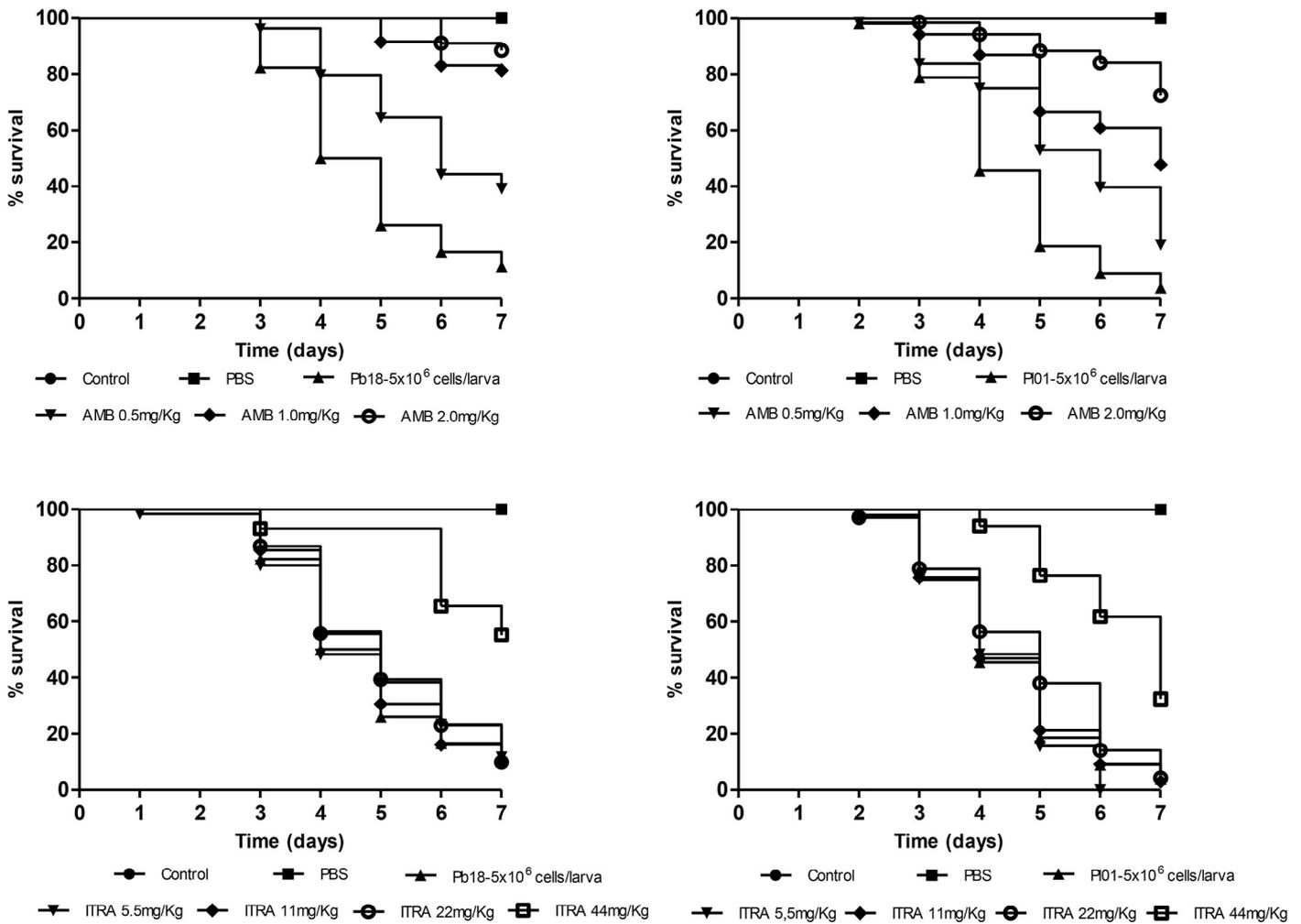


Fig. 1. Survival curves of *Galleria mellonella* infected with 5×10^6 cells of *Paracoccidioides brasiliensis* 18 (Pb18) or *P. lutzii* 01 (PI01) treated with different doses of amphotericin B (AmB) and itraconazole (ITRA). Protective effect of AmB at 0.5, 1 and 2 mg/kg and itraconazole at 44 mg/kg in *G. mellonella* infected with either *Paracoccidioides* sp. ($n = 40\text{--}50$). PBS, phosphate-buffered saline.

infection with either *Paracoccidioides* spp. in a dose-dependent manner ($P < 0.0001$). Survival rates of 39%, 81% and 88% in larvae infected with *P. brasiliensis* treated with 0.5, 1 and 2 mg/kg AmB, respectively, were observed. For larvae infected with *P. lutzii*, 19%, 48% and 72% survived after treatment with doses of 0.5, 1 and 2 mg/kg AmB, respectively. Itraconazole did not protect the larvae from infection with either *Paracoccidioides* spp. at doses of 5.5, 11 and 22 mg/kg, but increased the survival of larvae infected with *P. brasiliensis* to 55% and the survival of larvae infected with *P. lutzii* to 32% at a dose of 44 mg/kg ($P < 0.0001$) (Fig. 1). Moreover, neither 5% (v/v) DMSO as a control nor the antifungal compounds in the tested doses were toxic to the larvae.

3.2. Fungal burden in the tissues of *G. mellonella*

Subsequently, some of the larvae infected with *P. brasiliensis* and *P. lutzii* treated with 2 mg/kg AmB and 44 mg/kg itraconazole were selected for evaluation of the fungal burden over time, specifically at 1, 48 and 96 h after treatment. These doses of antifungal drugs were chosen because they demonstrated the greatest increase in larvae survival. CFUs with a cerebriform aspect, cream-coloured and not adhering to the medium were isolated. Fig. 2 shows that treatment with AmB and itraconazole significantly reduced the number of CFU in the larvae after the first hour of treatment ($P < 0.05$

for both cases). Moreover, in the AmB group, CFU were very low and could be not detected after 96 h of treatment by the plating method, whereas the itraconazole group presented non-detectable CFU after 1 h treatment followed by an increase in the fungal burden thereafter.

Some larvae that were uninfected and infected with *Paracoccidioides* spp. and treated with 2 mg/kg AmB or 44 mg/kg itraconazole were also selected for histological evaluation. PAS-stained sections showed that both *P. brasiliensis* and *P. lutzii* were present mainly under the cuticle and in the peripheral adipose bodies. It was possible to observe large aggregates of fungi with haemocyte recruitment and melanisation spots. Treatment with AmB and itraconazole reduced the number of CFU, and small aggregates could be observed in the peripheral adipose bodies of larvae (Fig. 3).

3.3. Haemocyte density in infection and after treatment

Fig. 4 shows that infection with 5×10^6 yeast cells/larva of *P. brasiliensis* or *P. lutzii* significantly reduced the haemocyte density after infection (1 h) and that the density remained the same after 48 h compared with uninfected larvae. Treatment with 2 mg/kg AmB or 44 mg/kg itraconazole did not change the number of haemocytes at the evaluated times.

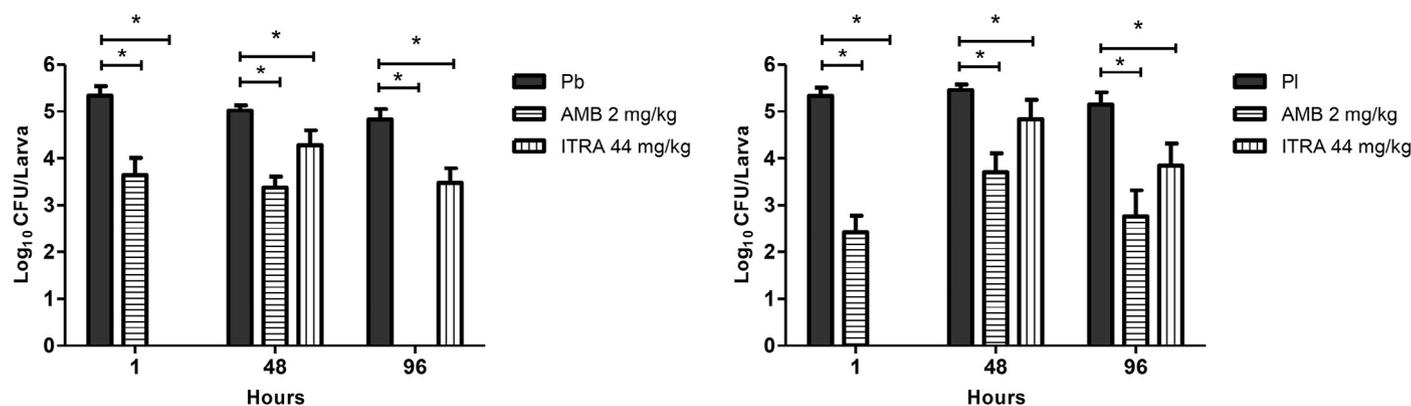


Fig. 2. Number of CFU in *Galleria mellonella* larvae infected with *Paracoccidioides brasiliensis* 18 (Pb) or *P. lutzii* 01 (Pl) assessed after 1, 48 and 96 h. Amphotericin B (AMB) 2 mg/kg and itraconazole (ITRA) 44 mg/kg reduced the number of CFU. * $P < 0.05$ vs. infected group (Pb or Pl) ($n = 6$).

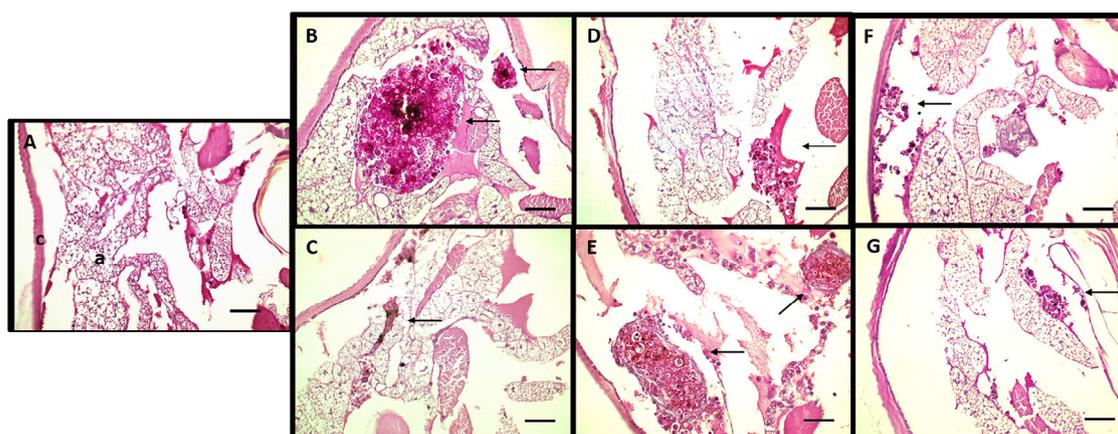


Fig. 3. Histopathological evaluation in *Galleria mellonella* assessed after 48 h (40 \times): (A) uninfected larva; (B) larva infected with *Paracoccidioides brasiliensis* and untreated; (C, D) larva infected with *P. brasiliensis* and treated with amphotericin B 2 mg/kg (C) or itraconazole 44 mg/kg (D); (E) larva infected with *P. lutzii* and untreated; and (F, G) larva infected with *P. lutzii* and treated with amphotericin B 2 mg/kg (F) or itraconazole 44 mg/kg (G). Amphotericin B and itraconazole reduced the fungal aggregates. Bars indicate 50 μ m. Black arrows indicate fungal cells. a, adipose bodies; c, cuticle. $n = 3$. A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM02703.

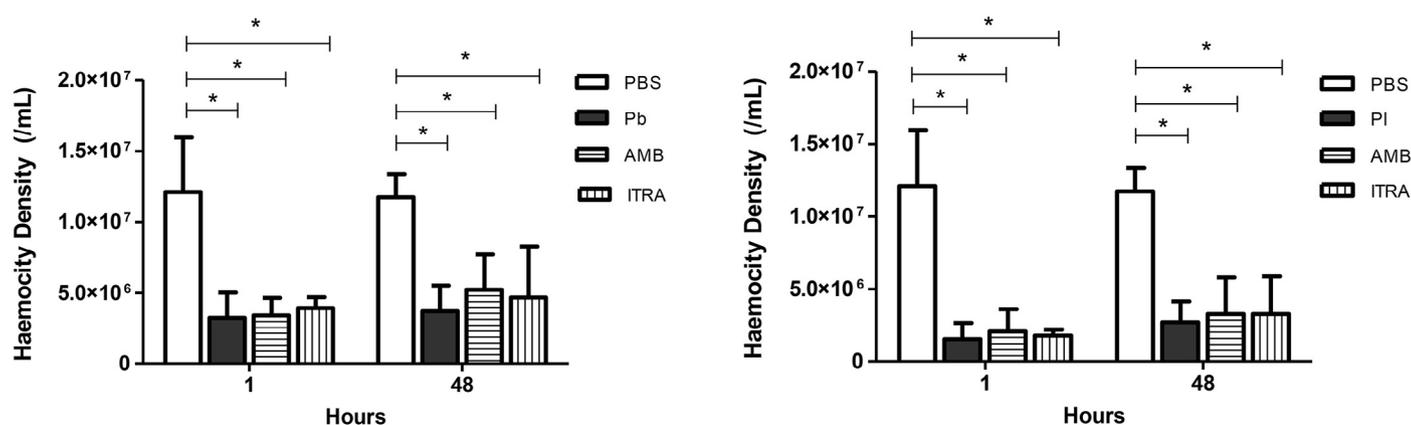


Fig. 4. Haemocytosis density in *Galleria mellonella* larvae infected with *Paracoccidioides brasiliensis* 18 (Pb) or *P. lutzii* 01 (Pl) assessed after 1 h and 48 h. *Paracoccidioides* spp. decreased the haemocytosis density, which was not altered by antifungals treatments [amphotericin B (AMB) 2 mg/kg and itraconazole (ITRA) 44 mg/kg]. * $P < 0.05$ vs. uninfected group (PBS control) ($n = 6$). PBS, phosphate-buffered saline.

4. Discussion

Because of the high cost and for ethical reasons, the principles of the three Rs (refinement, reduction, replacement) have been

applied to reduce the use of experimental mammals to the minimum necessary [26]. In this context, the use of alternative animal models has been increasingly accepted and used for the study of fungus–host interactions as well as efficacy and toxicity of antifungals.

Recently, a *G. mellonella* infection model was shown to be capable of evaluating infections caused by *P. lutzii* and *P. brasiliensis* [12,13]. An important advantage of *G. mellonella* for the study of thermally dimorphic fungi such as *Paracoccidioides* sp. over other invertebrate hosts such as *Caenorhabditis elegans* is its ability to be maintained at a temperature of 37 °C. However, there are no previous studies with antifungal agents in this *G. mellonella*–*Paracoccidioides* model. Using *G. mellonella* larvae as a model permits faster studies of the disease and its treatment. Typically, studies of virulence and antifungal efficacy against *Paracoccidioides* spp. have been performed in murine models, but one of the limitations is the long time required for the experiment (usually 30 days for the infection and 30 days for treatment).

To evaluate whether the *G. mellonella*–*Paracoccidioides* spp. model could be used to study antifungal drugs, the role of the most commonly used agents for PCM was investigated. In vitro, AmB presented minimum inhibitory concentrations (MICs) of 0.12 mg/L and 0.06 mg/L, and itraconazole presented MICs of 0.015 mg/L and 0.008 mg/L for *P. brasiliensis* isolate 18 and *P. lutzii* strain 01, respectively [27]. In the clinic, intravenous amphotericin has been used for severe cases of PCM at a dose of 1 mg/kg/day, and oral itraconazole is considered the best option for mild-to-moderate clinical forms at 200 mg/day for 6–18 months [7].

First we showed that 5×10^6 CFU/larva was a suitable inoculum both for *P. brasiliensis* and *P. lutzii* to establish a lethal infection in the *G. mellonella* model in order to evaluate antifungal agents. At this concentration, most of the larvae died at the end of the 7-day incubation period. Furthermore, the survival curve profiles for both species were similar in this model, as previously described [13]. In most other studies, antifungal drugs were administered within the first 4 h post-infection [16,18,22,28] and we chose to administer AmB and itraconazole at 1 h post-infection. Following administration of AmB at 0.5, 1 or 2 mg/kg, a dose-dependent survival curve was produced. This is in contrast to itraconazole, for which only the highest dose (44 mg/kg) increased the survival of the infected larvae.

G. mellonella has been used to evaluate the toxicity of compounds and solvents [28,29]. In parallel to the experiments for efficacy, the drugs AmB or itraconazole alone and the solvent DMSO were tested and showed no toxicity to larvae.

Second, the fungal burden of larvae infected with *Paracoccidioides* spp. was evaluated. In previous work with the *G. mellonella* model, the growth of *P. lutzii* from larvae using BHI agar plus antibiotics was not observed [12]. In this study, horse serum and *P. brasiliensis* 339 culture filtrate were added, which are important factors for the growth of *Paracoccidioides* colonies in mammalian models [25], and *Paracoccidioides* species were successfully recovered from larvae at the end of the experiment using this medium. AmB (2 mg/kg) and itraconazole (44 mg/kg) treatments were effective in the reducing the fungal burden. AmB has been shown to be effective against *Candida albicans*, *C. krusei* [16], *C. neoformans* [14] and *Fusarium* spp. [15] in *G. mellonella* models at a dose range of 1–4 mg/kg with a single administration. However, in these cases the fungal burden was evaluated at a single time point, whereas in the current study the fungal burden was evaluated at three different times (1, 48 and 96 h) and a fungicidal effect of AmB was observed in larvae. Conversely, a previous study did not observe a fungicidal effect in this model against a strain of *C. tropicalis* [17]. Azoles such as fluconazole and voriconazole have also shown activity against *C. neoformans* and *Candida* spp., respectively, in *G. mellonella* larvae [14,16]. Previously, itraconazole was tested at a dose of 100 mg/kg in combination with inhibitors of calcium signalling against *Aspergillus fumigatus* in *G. mellonella* [30]. In the current work, the efficacy of itraconazole alone at a dose of 44 mg/kg in infected larvae was investigated and a fungistatic effect was observed. However, further studies to evaluate the pharmacokinetics could be useful for understanding the bioavailability and effectiveness of these drugs in the *G. mellonella* model.

Fungi were also observed in the tissues of larvae through histopathologic analyses. Aggregates with fungal cells, haemocytes and structures such as granulomas were shown in other studies with *G. mellonella*–*Paracoccidioides* spp. [12,13] and in the lungs and oral lesions of mammals with PCM [31–33]. Treatment with AmB or itraconazole demonstrated the ability to reduce aggregates of fungi in the adipose bodies of *G. mellonella*, as also observed in mammalian models [34,35].

Another parameter evaluated in this study was the haemocyte density. The role of haemocytes in the immune response is similar to the innate immune response of mammals against pathogens. Furthermore, variation in the density of these cells is related to the pathogenicity of the fungus [21]. Infection by *P. brasiliensis* or *P. lutzii* decreased the haemocyte density in the haemolymph of larvae significantly. Such behaviour was also observed when *G. mellonella* larvae were infected with *C. albicans* and *C. krusei* and can be explained by two possibilities: the haemocytes migrate from the haemolymph to the tissues; and infection with intracellular parasites leads to phagocytosis and subsequent lysis of haemocytes [13,16]. Some studies have shown the immunomodulatory role of antifungals such as AmB, caspofungin and micafungin [36–38], but treatment with either AmB or itraconazole had no effect the number of haemocytes in infected larvae in the present study.

5. Conclusions

The results showed that *G. mellonella* larvae are a useful model for evaluating infection by different *Paracoccidioides* spp. and their response to different antifungal agents. Although *P. brasiliensis* and *P. lutzii* present different morphological and genetic characteristics, the survival curve profiles and response to AmB and itraconazole were similar in the *G. mellonella* model, which suggests important implications for the clinical treatment of PCM. Furthermore, a possible fungicidal effect of AmB and a fungistatic effect of itraconazole were observed. These effects were independent of haemocyte density changes. Finally, *G. mellonella* can serve as a rapid model to test other isolates of *Paracoccidioides* and can be used to screen new antifungal compounds against this fungi, which could subsequently help reduce the number of mammals used for PCM experimentation.

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Ethical approval: Not required.

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