Using *Galleria mellonella–Candida albicans* Infection Model to Evaluate Antifungal Agents

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*Candida albicans* is the most common fungal pathogen. *Galleria mellonella* is widely used as an infection model host. Nevertheless, the *G. mellonella–C. albicans* infection model had not been optimized for drug evaluation before this study. In this work, we revealed that 5×10⁵ colony forming unit (CFU)/larva was a suitable inoculum to optimize the *G. mellonella–C. albicans* infection model in order to evaluate antifungal agents. Using our optimized model, the antifungal effect of fluconazole, amphotericin B and flucytosine, and the synergy between amphotericin B and flucytosine were successfully verified. Thus, this study provides a rapid, inexpensive and reliable way to evaluate antifungals *in vivo*.

**Key words** *Galleria mellonella; Candida albicans; antifungal agent*

_Candida albicans_ is the most common opportunistic fungal pathogen of humans, causing diseases varying from superficial mucosal infections to lethal systemic disorders.¹⁻³ Available antifungal drugs are limited in clinic, and drug resistance to the antifungals is a significant challenge.⁴ There is an urgent need for the development of new antifungal drugs.⁵

Animal models are important for new drug discovery. Notably, insect infection models have significant ethical, logistical and economical advantages over mammalian models, and provide a rapid evaluation of the efficacy and toxicity of agents *in vivo*.⁶⁻⁹ Among the insects available, *Galleria mellonella* emerged at the forefront.⁶⁻⁹ The greater wax moth *G. mellonella* (Lepidoptera: Pyralidae) is found in most of the world.¹⁰ The *G. mellonella* larvae are at a convenient size (≥2–3 cm in length) and easy to work with.¹¹ Similar with vertebrates, it has both cellular and humoral immune response to infection.¹² Of note, the *G. mellonella* larvae can be maintained at temperatures between 15°C and 37°C,¹³ which makes the larvae well suited to study pathogens at human body temperature. This is significant because it impacts the pathogenicity of organisms as virulence factors are known to be regulated by temperature.¹⁰ These advantages make *G. mellonella* an attractive host for studying pathogens and antimicrobial agents.¹¹⁻¹⁵

Fuchs et al.¹¹ established the *G. mellonella–C. albicans* infection model by injecting 1×10⁶ Cells per larva to study fungal pathogenesis. However, the established model was not suitable to evaluate antifungal agents, because the *C. albicans* burden was heavy and the antifungals cannot rescue the caterpillars. In this study, we optimized the *G. mellonella–C. albicans* infection model and used this model for antifungal drug evaluation successfully.

**MATERIALS AND METHODS**

**Strains and Media** Six *C. albicans* strains including SC5314, two fluconazole (FLC)-sensitive clinical strains (045 and 01010) and three FLC-resistant clinical strains (891, 100 and 11286) were used in this study. SC5314 was kindly provided by Dr. Joachim Morschhäuser (Institut für Molekulare Infektionsbiologie, Universität Würzburg, Würzburg, Germany). Clinical strains were kindly provided by Dr. Jun Gu (Changhai Hospital, Shanghai, China). The strains were routinely grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) liquid medium at 30°C in a shaking incubator.

*G. mellonella* Killing Assay *G. mellonella* caterpillars in the final instar larval stage were selected to be similar in size (approximately 0.33 g) and absent of any grey markings. Overnight grown *C. albicans* cells were collected, washed, and used to prepare suspensions with a range of *C. albicans* concentrations. The inoculum was injected directly to the last left pro-leg using a Hamilton syringe.¹⁴ Before injection, the area was cleaned using an alcohol swab. *C. albicans* suspensions were injected in a 5 µL volume. A control group received 5 µL phosphate buffered saline (PBS) instead of the *C. albicans* inoculum. Each group contained 16 randomly chosen larvae. After injection, larvae were incubated at 37°C in plastic containers and the number of dead larvae was scored daily.

Antifungal drugs were injected using the same technique. All antifungal drugs were diluted in sterile water. A series of dosages were used for each antifungal agent. More specifically, FLC was administered 1, 4 and 16 mg/kg, respectively; amphotericin B (AMB) was administered 0.4, 1.6, and 6.4 mg/kg, respectively; and flucytosine (5-FC) was administered 1.25, 5, and 20 mg/kg, respectively. Each antifungal agent was delivered once by an injection to the last right pro-leg. A mock inoculation with PBS was performed in each experiment to monitor killing due to physical injury or infection by pathogenic contaminants.

Killing curves were plotted and examined by using the Kaplan–Meier method and differences were determined by using the log-rank test (STATA 6; STATA, College Station, TX, U.S.A.).

**Tissue-Burden Culture Studies** For the evaluation of the tissue burden of *C. albicans* in caterpillars, three caterpillars...
per group were weighed after 24 h incubation. The larvae were homogenized in 3 mL sterile PBS. Serial dilutions were plated on YPD agar with antibiotics (100 µg/mL ampicillin, 100 µg/mL streptomycin and 45 µg/mL kanamycin). Plates were incubated at 30°C for 24 h and colonies were counted to determine the colony forming unit (CFU)/larva.16)

RESULTS

The Optimization of G. mellonella–C. albicans Infection Model

To determine a suitable fungal inoculum, we used the most widely used C. albicans strain SC531417–19) and tested a range of inocula including 1.25 × 10^5, 2.5 × 10^5, 5 × 10^5 and 1 × 10^6 CFU/larva, respectively. G. mellonella larvae melanized within 30 min after being infected with C. albicans (Fig. 1A). Subsequently, C. albicans inoculation resulted in the death of the larvae, and the mortality depended on the fungal burden. More specifically, 1 × 10^6 CFU/larva resulted in 100% mortality within 2 d; 5 × 10^5 CFU/larva caused 50% mortality 2 d after inoculation and resulted in 100% mortality within 4 d; with the inoculation of 2.5 × 10^5 CFU/larva, more than 60% larvae survived until the end of 12 d of our observation period; and 1.25 × 10^5 CFU/larva could not cause death of G. mellonella. In order to evaluate antifungal agents, we chose the C. albicans inoculation of 5 × 10^5 CFU/larva to optimize the G. mellonella–C. albicans infection model.

The G. mellonella–C. albicans Infection Model Is Reliable to Evaluate the Monotherapy Efficacy of Antifungal Agents

Using the above G. mellonella–C. albicans infection model, we first tested a series of drug administration time, 30 min before inoculation, 30 min after inoculation, 1 h after inoculation and 2 h after inoculation. One milligram per kilogram FLC was used for antifungal treatment based on our preliminary experiments. The G. mellonella survival data indicated that similar therapeutic effects were obtained with the four administration time tested. As shown in Fig. 2, all the FLC administration groups protected the G. mellonella from C. albicans infection significantly (p < 0.001) and there was no significant difference in the FLC administration groups with different drug delivery time (p > 0.05). We chose 30 min after inoculation as the drug delivery time for further studies.

We further evaluated the dose–effect of the commonly used antifungals, including FLC, AMB and 5-FC. The results indicated that all these antifungals could protect the G. mellonella from C. albicans infection (Fig. 3). More specifically, with the monotherapy of 16 mg/kg FLC, 6.4 mg/kg AMB, or 20 mg/kg 5-FC after SC5314 infection, more than 75% of the larvae survived until the end of 12 d of our observation period (p < 0.0001, Figs. 3A, C, E). Of note, 6.4 mg/kg AMB protected all the larvae and resulted in 100% survival of G. mellonella at the end of our observation period (Fig. 3C). In the range of drug concentrations used (FLC: 1, 4 and 16 mg/kg; AMB: 0.4, 1.6 and 6.4 mg/kg; 5-FC: 1.25, 5 and 20 mg/kg), the antifungals protected the larvae in a dose-dependent manner. Tissue fungal burden results are in accordance with the survival data, and FLC, AMB or 5-FC decreased Candida burden in a dose-dependent manner (Figs. 3B, D, F).

Fig. 1. (A) Physical Changes in G. mellonella Larvae after Infection with C. albicans

Larvae injected with 5 µL PBS (left) remain light in color. In contrast, larvae infected with 5 × 10^5 C. albicans (right) melanized after infection. The images were taken 1 h after infection. (B) Survival of G. mellonella after infection with different inocula of C. albicans. A p value of <0.05 was considered statistically significant.

Fig. 2. The Influence of Drug Delivery Time on the Antifungal Effect of FLC in the G. mellonella–C. albicans Infection Model

FLC was delivered to the larvae at different time points (30 min before inoculation, 30 min after inoculation, 1 h after inoculation and 2 h after inoculation). Sixteen randomly chosen larvae in each group. A p value of <0.05 was considered statistically significant. Significant difference was found between SC5314 infection group and all the FLC administration groups (p < 0.001).
We utilized two other FLC-sensitive clinical isolates 045 and 01010 to test the *G. mellonella–C. albicans* infection model and the effects of the antifungals. Similar results were obtained with the two strains (Fig. 4). More specifically, with the monotherapy of 16 mg/kg FLC, 6.4 mg/kg AMB, or 20 mg/kg 5-FC after 045 or 01010 infection, more than 65% of the larvae survived until the end of 12 d of our observation period \((p < 0.0001, \text{Fig. 4})\). In the range of drug concentrations used (FLC: 1, 4 and 16 mg/kg; AMB: 0.4, 1.6 and 6.4 mg/kg; 5-FC: 1.25, 5 and 20 mg/kg), the antifungals protected the larvae in a dose-dependent manner (Fig. 4).

We further used three FLC-resistant clinical isolates 891, 100 and 11286 to infect the *Galleria* separately for drug evaluation, SC5314 as the FLC-sensitive control strain. Interestingly, although 4 mg/kg FLC exhibited antifungal effect against SC5314 significantly \((p < 0.0001, \text{Fig. 5})\), FLC at the same dosage could not protect the *G. mellonella* from the FLC-resistant strains’ infection \((p > 0.05, \text{Fig. 5})\), which was in accordance with the FLC-resistance character of the isolates.

The *G. mellonella–C. albicans* Infection Model Is Reliable to Evaluate the Synergism of Antifungal Agents

Using the *G. mellonella–C. albicans* infection model, we further investigated the synergism between AMB and 5-FC. Our result indicated that AMB could synergize with 5-FC. More specifically, the monotherapy of 0.8 mg/kg AMB or 1.25 mg/kg 5-FC could not protect the larvae from *C. albicans* killing \((p > 0.05, \text{Fig. 6})\). In contrast, the combination of 0.8 mg/kg AMB and 1.25 mg/kg 5-FC improve the survival of the *C. albicans*-infected *G. mellonella* \((p = 0.0016, \text{Fig. 6})\), which was in accordance with the clinical synergism of these two drugs.\(^{20,21}\)

The *G. mellonella–C. albicans* Infection Model Cannot Be Used to Evaluate the Toxicity of Antifungal Agents Using DMSO as the Solvent

Besides to evaluate the efficacy of antifungals, we also tried to evaluate the toxicity of antifungal agents using the *G. mellonella–C. albicans* infection model.
model. In order to deliver high-dose antifungals, high concentrations of the agents are necessary. Under conditions that we cannot obtain the high concentrations of the agents using water as the solvent, we used dimethyl sulfoxide (DMSO) instead of water. Unexpectedly, we found that G. mellonella was sensitive to DMSO, 5 µL of which resulted in rapid swelling of the G. mellonella body and subsequent death. Two microliter DMSO did not result in the death of G. mellonella, but still cause swelling of the G. mellonella body. Half microliter DMSO was safe and can be used for drug delivery in the G. mellonella model.

DISCUSSION

G. mellonella infection model emerged at the forefront of insect models in infectious disease studies. In this work, we optimized the G. mellonella–C. albicans infection model for drug evaluation studies. The antifungal activity of FLC, AMB and 5-FC, and the synergy between amphotericin B and flucytosine were verified using our G. mellonella–C. albicans infection model, indicating that the model is reliable to evaluate antifungals in vivo and promising in drug screening work.

To evaluate antifungals against C. albicans, we firstly modified the G. mellonella–C. albicans infection method. We found that the previously published G. mellonella–C. albicans infection protocols were not suitable to evaluate antifungal agents. More specifically, when we injected $10^7$ or $10^6$ C. albicans cells to the G. mellonella larvae as published previously, the larvae melanized within 30 min (Fig. 1A), died within 24 h, and even 14 mg/kg FLC could not prolong the survival of the larvae. We inferred that the failure of the FLC treatment might be caused by the heavy Candida burden in the larvae. Thus, we tested a range of inocula and chose $5 \times 10^5$ CFU/larva as the suitable inoculum for the infection model to evaluate antifungals.

G. mellonella–C. albicans infection model was demonstrated reliable to evaluate antifungals in this work. Several previous studies had used G. mellonella–C. albicans infection model to evaluate antifungals. However, C. albicans was inoculated excessively in the previous studies. In this study, we infected the caterpillars with $5 \times 10^3$ CFU/larva C. albicans and the antifungals were administered 30 min after inoculation. We found that AMB was the most effective antifungal agent compared to FLC and 5-FC, and the phenomenon was consistent with the clinical efficacy of these three antifungal agents. Besides, AMB and 5-FC showed synergistic effect in the G. mellonella–C. albicans infection model, which was in accordance with the synergism of these two drugs in clinic.

Compare with other animal models, G. mellonella infection...
model has several advantages for antifungal evaluation. First, G. mellonella–C. albicans infection model provides a rapid evaluation of the efficacy and toxicity of agents in vivo, and has significant ethical, logistical and economical advantages over mammalian models.6,7 Second, the G. mellonella larvae can be maintained at temperatures between 15°C and 37°C.8 This makes the larvae well suited for studies at human body temperature. Other invertebrate hosts, such as Caenorhabditis elegans, Drosophila melanogaster and Bombyx mori usually cannot be maintained at temperatures over 30°C.8,25 Third, G. mellonella has both cellular and humoral immune response to infection, while C. elegans just has a very simple immune system compared to the mammalian.26 Fourth, G. mellonella model is easy to work with, while D. melanogaster model requires special lab equipment and technical expertise in handling the flies.9 These advantages make G. mellonella infection model attractive for antimicrobial evaluation.11,14,15

In this study, we found that 5µL DMSO could result in rapid swelling of the G. mellonella body and subsequent death. With the decrease of DMSO volume, the toxic effect decrease. The toxicity of DMSO could be caused by the protein denaturation effect of DMSO.27 It was reported that DMSO could bind to hydrophobic and aromatic side chains of proteins, resulting in protein denaturation.27 Based on our data, we suggest that caution should be paid to the usage of DMSO as the solvent for drug delivery in the G. mellonella experiments, and if DMSO is necessary, the smaller volume the better.

Collectively, we provided a protocol of using G. mellonella–C. albicans infection model to evaluate antifungals. It can be inferred that the facile inoculum and drug delivery of the G. mellonella will make it a desirable model to screen and evaluate antifungals in vivo.

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