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Galleria mellonella are resistant to *Pneumocystis murina* infection

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Abstract

Studying *Pneumocystis* has proven to be a challenge from the perspective of propagating a significant amount of the pathogen in a facile manner. The study of several fungal pathogens has been aided by the use of invertebrate model hosts. Our efforts to infect the invertebrate larvae *Galleria mellonella* with *Pneumocystis* proved futile since *P. murina* neither caused disease nor was able to proliferate within *G. mellonella*. It did, however, show that the pathogen could be rapidly cleared from the host.

Keywords

Galleria mellonella; infection model; *Pneumocystis murina*

Introduction

Pneumocystis is a fungal pathogen that causes morbidity and mortality in patients that are immunodeficient or immunosuppressed. Studying *Pneumocystis* has proven difficult due to the particular challenges of growing *Pneumocystis* for research purposes. The lack of an effective means to grow the microbe in cell culture prohibits sufficient and facile propagation for research on the biology of the microbe. Attempts at *in vitro* propagation through cell culture have shown only a modest rate of replication [1]. Mammalian models have been identified but non-mammalian models have yet to be established to propagate or study *Pneumocystis*.

The study of many pathogens has been greatly aided by the use of invertebrate models such as *Caenorhabditis elegans*, *Drosophila melanogaster*, *Galleria mellonella*, *Dictyostelium discoideum* and *Acanthamoeba castellanii* [2]. The study of *Pneumocystis* has thus far relied on more complicated mammalian models. Models that have been used to isolate *Pneumocystis* include rats, mice and ferrets [3, 4]. The mouse and rat models require immunosuppression in order to study *Pneumocystis*. Thus development of an invertebrate model to study *Pneumocystis* would be an asset to the study of this fungal pathogen.

In this study, we explore *G. mellonella* as a potential model host for *P. murina*. *G. mellonella* has been used as a model host to study fungal pathogens such as *Aspergillus* spp. [5–7], *Candida albicans* [8–10] and *Cryptococcus neoformans* [11]. As an infection model it offers the attractive attributes of being able to be maintained at 37°C post infection in order

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to study pathogenicity at mammalian temperature conditions and it can be injected with a specific amount of pathogen [2].

Our investigation found that *G. mellonella*, although susceptible to a number of medically relevant microbes, is not susceptible by *P. murina. P. murina* do not replicate within *G. mellonella* and are cleared from the *G. mellonella* larvae. We hope that the information contained in this report will allow others to benefit from our experience in attempting to establish a non-vertebrate model for *P. murina* studies utilizing *G. mellonella*.

Materials and Methods

Strains and media

P. murina were partially purified from mouse lung homogenate with Ficoll-Hypaque gradient centrifugation, frozen using Recovery Cell Culture Freezing Medium (Gibco) and stored at −80°C. The stored cells were thawed in a water bath at 35°C and then an equal volume of RPMI was added containing 20% heat-inactivated fetal bovine serum (FBS). *P. murina* were collected with centrifugation at 1000 × g for five min then suspended in 1 ml of phpsphate buffered saline (PBS). *Pneumocystis* organisms were quantified prior to infection using a real-time quantitative PCR (qPCR) assay based on the *P. murina dhfr* gene as previously described [12].

As a positive control in monitoring disease development, *C. neoformans* were included in our *G. mellonella* infection assays. *C. neoformans* (wild type strain KN99α) inocula were prepared by growing a culture overnight at 30°C in YPD (1% yeast extract, 2% peptone and 2% dextrose). The cells were collected with centrifugation and washed twice with PBS. *C. neoformans* were then counted with a hemocytometer and used immediately.

G. mellonella survival assay

G. mellonella larvae (Vanderhorst Wholesale, St. Marys, Ohio) were injected at the last left pro-leg using a Hamilton syringe as previously described [11]. *G. mellonella* infected with *C. neoformans* received 1.5×10^5 cells per larvae. *P. murina* was delivered at an inoculum of 4.85×10^5 or 4.85×10^6 cells per larvae. All *G. mellonella* received a 10 μl inoculum volume of fungal cells suspended in PBS. Each infection group contained sixteen larvae of the appropriate weight (330 ± 25 mg). Larvae were kept at 37°C in the dark and scored daily for survival. Killing curves were plotted and statistical analysis (log rank test) was performed by the Kaplan-Meier method using STATA 6 statistical software (Stata). Killing curves were performed in three independent replicates and a representative graph was reported.

Hemocyte density

Hemolymph was collected from larvae 72 h post-infection into pre-chilled collection tubes. The hemolymph from three larvae were pooled into a single sample and three samples were examined per infection group. Hemolymph was diluted in PBS then hemocytes were counted with a hemocytometer. We did not differentiate between the various types of hemocytes within the hemolymph.

Tissue staining

G. mellonella were infected as described above. The internal structures of three larvae per infection group were collected at 96 h post infection and placed in formalin. The tissue was fixed overnight at 4°C, serially dehydrated with ethanol then transferred to xylene for three h and embedded in paraffin. Sections were cut and stained with a 4D7 monoclonal antibody specific for *P. murina* and an Alexa Fluor 488 conjugated anti-mouse IgG secondary

antibody [13, 14]. DAPI was included when mounting the slides. The tissue sections were examined with light and fluorescent microscopy. A Nikon eclipse TE2000-U microscope was used with 100x magnification.

P. murina quantification from *G. mellonella*

Hemolymph and internal body structures were collected from *G. mellonella* at the indicated time points, weighed and stored at −80°C for DNA isolation. To the frozen material, 100 μl of glass beads (425–600 μm) were added along with 1 ml DNA isolation buffer (100mM Tris HCl pH 7.5, 700mM sodium chloride, 10 mM ethyldiaminetetraacentic acid, and 0.05% sodium dodecyl sulfate). The mixture was agitated vigorously for 5 min to disrupt the tissue then β-mercaptoethanol was added to 1% followed by 300 μg/ml proteinase K. The slurry was then incubated at 65°C for 30 min then allowed to cool to room temperature. DNA was isolated with phenol:chloroform then isopropanol precipitated. The isolated DNA was used as a template in a qPCR reaction to quantify the Pneumocystis organisms within *G. mellonella*.

Results and Discussion

Our goal for this study was to determine if *G. mellonella* was a useful infection model to study *P. murina*. 81–93% of *G. mellonella* inoculated with *P. murina* survived for 216 h without exhibiting signs of a lethal infection (Fig. 1). We included a *C. neoformans* infected group of *G. mellonella* larvae as a control. None of the larvae infected with *C. neoformans* were alive at 216 h post infection. Survival curves were plotted using the Kaplan-Meier analysis, and the differences in survival were evaluated for significance using the log rank test. There was no significant difference between *P. murina* infected larvae and larvae injected with PBS. Symptoms that can accompany fungal infections include lethargy and melanization of the larvae and eventually death. In the case of *P. murina* infected larvae, they remained active and did not melanize (Fig. 1A).

Another infection symptom that can be followed with *G. mellonella* larvae is a change in hemocytes density. Fungi that are pathogenic to larvae lead to a reduction in *G. mellonella* hemocytes density while inoculations with fungi that are non-pathogenic result in *G. mellonella* maintaining a hemocytes density similar to uninfected larvae [9]. At 72 h post-infection with *C. neoformans* we observed a reduction in *G. mellonella* hemocyte density (Fig. 1B). This was congruent with observations associated with other fungi that cause lethal infections [9]. Larvae inoculated with *P. murina*, however, retained a hemocytes density comparable to larvae injected with PBS (Fig. 1B).

Although *P. murina* did not cause a lethal infection, we were still interested in determining of the fungi were retained with the larvae. Further, we examined the internal structures of *G. mellonella* to see if *P. murina* could be identified within the larvae or replicate within *G. mellonella*. Tissue collected from *G. mellonella* 96 h post infection were stained and then observed for the presence of *P. murina*. We did not find any *P. murina* in sections stained with 4D7 monoclonal antibody observation with fluorescent microscopy (Fig. 2).

The fungal burden within *G. mellonella* was assessed through qPCR of the dihydrofolate reductase gene (*dhfr*), which is a single copy gene that is amenable for *P. murina* quantification in mouse models [12]. The hemolymph and tissue was collected from *G. mellonella* at 1, 48 and 96 h post infection with *P. murina*. As a control, hemolymph and tissue was also collected from *G. mellonella* at 1 h post injection with PBS. DNA was isolated from the collected material and qPCR was used to measure the number of copies of *DHFR*, thus indicating the number of *Pneumocystis* nuclei within the tissue. The decrease in *G. mellonella* fungal burden indicates that *P. murina* is rapidly cleared from the host (Fig.

2C). It is not known at this time how the *G. mellonella* facilitate the clearing of *P. murina* from the system but one possibility is through an immune response either in which the *G. mellonella* presents adverse conditions to which *P. murina* cannot survive or they may be engulfed and subsequently destroyed by hemocytes which are *G. mellonella* phagocytic cells.

Thus it does not appear that *G. mellonella* sustains an infection by *P. murina* and is not a suitable model for the proliferation of the fungus for the study of pathogenesis. It has been a difficult challenge to identify alternative hosts to study *Pneumocystis*. One particular aspect to the challenge is that *Pneumocystis* appears, by present available evidence, to be species specific or to need species-specific requirements to establish an infection [15, 16]. What we did find from our endeavor of infecting *G. mellonella* with *Pneumocystis* was that *Pneumocystis* could be eliminated from the host system rapidly and without symptoms of disease. There is the potential that species-specific infection requirements were not fulfilled within *G. mellonella* and perhaps the fungal cells were not able to remain viable in that environment. Another possibility is that insects share a soil environment with a number of various fungal pathogens and this close proximity and constant bombardment by fungi have aided in the evolution of a means to effectively combat *Pneumocystis*.

The innate immune system of insects including *G. mellonella* uses multiple means to defend against fungal pathogenesis including phagocytosis of organisms by hemocytes. The hemocoel environment is further made hostile by host production of enzymes and reactive oxygen species. Also, within the *G. mellonella* defense arsenal is a gallerimycin peptide [17] and several moricin-like peptides that exhibit antifungal activity [18].

Further research is needed to identify what host biological facets are essential for the propagation of *Pneumocystis* within a viable host or conversely, from the host perspective, identifying which host responses prevent propagation of *Pneumocystis* and the development of PCP.

Abbreviations

FBS	fetal bovine serum
PBS	phosphate buffered saline

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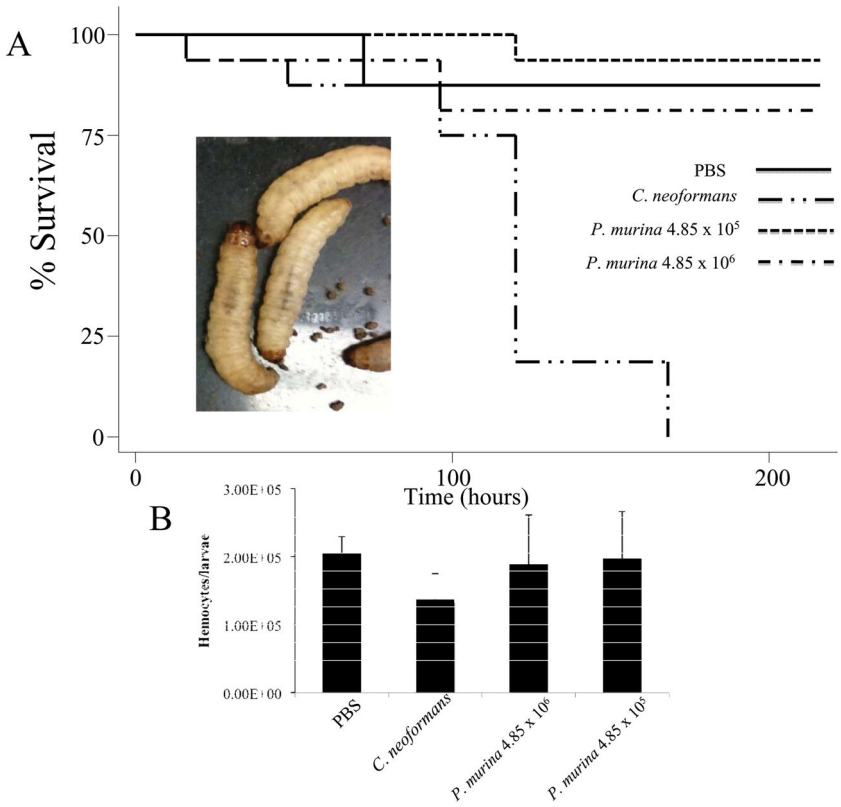

Figure 1.

A. A Kaplan-Meier plot of *G. mellonella* survival after injection with 1.5×10^5 cfu/larvae of *C. neoformans* showed that *C. neoformans* caused a lethal infection whereby 50% mortality was reached by 120 h post-infection. *G. mellonella* infected with 4.85×10^5 or 4.85×10^6 *P. murina* did not shows signs of infection. At 216 h post infection, 15 out of 16 *P. murina* infected *G. mellonella* remained alive from the group infected with 4.85×10^5 cells per larvae and 13 out of 16 were alive in the group infected with 4.85×10^6 cells per larvae. The image to the left shows that *G. mellonella* did not melanize after injection with *P. murina*. **B.** Hemocyte density was reduced when larvae were infected with *C. neoformans*. However, larvae infected with *P. murina* had a hemocytes density similar to that of uninfected larvae.

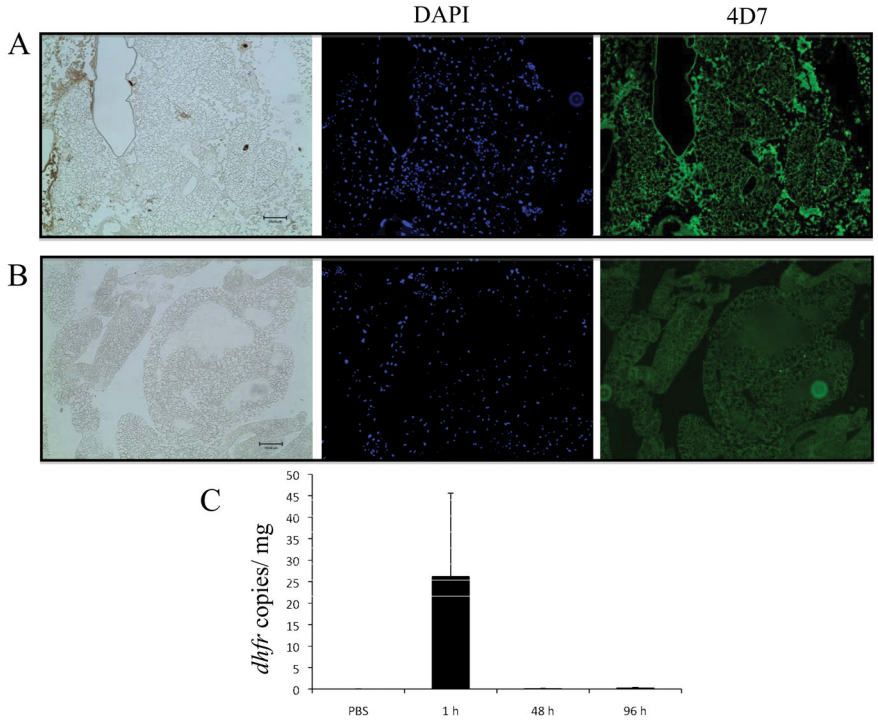

Figure 2. *G. mellonella* tissue was fixed 96 h post infection and then embedded in paraffin. Paraffin sections were stained with 4D7 monoclonal antibody and a secondary IgG anti-mouse antibody conjugated to Alexa Fluor 488 was used to identify the presence of *P. murina* cells in the tissue. The tissue was also stained with DAPI

A. No fungal cells were observed in *C. neoformans* infected tissue as expected since the antibody is specific to *Pneumocystis*. **B.** *Pneumocystis* was also not identified in tissue from larvae previously inoculated with *P. murina*. Scale bar, 100 μm (bottom right corner). **C.** The amount of *P. murina* within *G. mellonella* tissue was quantified at 1, 48 and 96 h post infection using qPCR to amplify *dhfr*.