Journal of Microbiological Methods 118 (2015) 182–186

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Tenebrio molitor (Coleoptera: Tenebrionidae) as an alternative host to study fungal infections

Patrícia Canteri de Souza [a], Alexandre Tadachi Morey [a], Gabriel Marcondes Castanheira [a], Karla Paiva Bocate [a], Luciano Aparecido Panagio [a], Fabio Augusto Ito [b], Márcia Cristina Furlaneto [a], Sueli Fumie Yamada-Ogatta [a], Idessânia Nazareth Costa [c], Hector Manuel Mora-Montes [d], Ricardo Sergio Almeida [a,*]

[a] Department of Microbiology, State University of Londrina, Londrina, PR, Brazil
[b] Department of Oral Medicine and Pediatric Dentistry, State University of Londrina, Londrina, PR, Brazil
[c] Department of Pathological Sciences, Center of Biological Sciences, State University of Londrina, Londrina, PR, Brazil
[d] Department of Biology, Universidad de Guanajuato, Guanajuato, Mexico

ARTICLE INFO

Article history:
Received 25 September 2015
Received in revised form 6 October 2015
Accepted 6 October 2015
Available online 8 October 2015

Keywords:
Candida albicans
Cryptococcus neoformans
Infection model
Tenebrio molitor

ABSTRACT

Models of host–pathogen interactions are crucial for the analysis of microbial pathogenesis. In this context, invertebrate hosts, including *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (nematode) and *Galleria mellonella* (moth), have been used to study the pathogenesis of fungi and bacteria. Each of these organisms offers distinct benefits in elucidating host–pathogen interactions. In this study, we present a new invertebrate infection model to study fungal infections: the *Tenebrio molitor* (beetle) larvae. Here we performed *T. molitor* larvae infection with one of two important fungal human pathogens, *Candida albicans* or *Cryptococcus neoformans*, and analyzed survival curves and larva infected tissues. We showed that increasing concentrations of inoculum of both fungi resulted in increased mortality rates, demonstrating the efficiency of the method to evaluate the virulence of pathogenic yeasts. Additionally, following 12 h post-infection, *C. albicans* forms mycelia, spreading its hyphae through the larva tissue, whilst GMS stain enabled the visualization of *C. neoformans* yeast and their melanin capsule. These larvae are easier to cultivate in the laboratory than *G. mellonella* larvae, and offer the same benefits. Therefore, this insect model could be a useful alternative tool to screen clinical pathogenic yeast strains with distinct virulence traits or different mutant strains.

1. Introduction

The wax moth larvae (*Galleria mellonella*) have been used in infectious disease research (Desalermos et al., 2012). Insects host have ethics, logistics and economic advantages over mammalian models (Li et al., 2013), and allow high efficiency testing on a large scale and at low cost (Lionakis, 2011). While USA has several commercial suppliers of *G. mellonella* larvae, Brazil has none, forcing researchers to maintain their own moth larvae supply for the experiments. This increases costs of experiments and requires a person responsible for their maintenance.

An insect host on the rise is *Tenebrio molitor* (Coleoptera), a mealworm beetle, stored-grain plague (Schroeckenstein et al., 1990), whose larvae are used for feeding pets, as well as birds (Barker et al., 1998). The advantage of this alternative model is that *T. molitor* larva, as the moth larva, can be maintained at temperatures between 25 °C and 37 °C, which makes it suitable for the study of pathogens at body temperature (Li et al., 2013). It is an advantage compared to *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (nematode), which do not tolerate this temperature range (Desalermos et al., 2012). However, differently from *G. mellonella*, the mealworm caterpillars are commercialized in Brazil and are easier to cultivate in laboratory. Another advantage concerns the form of inoculation, which is performed by injection in the *T. molitor* model. On the other hand, in the *C. elegans* model, the pathogen is added to the well where the nematode is prepared for the bioassay, resulting in a lack of accuracy regarding the number of microorganisms internalized by the host (Merkx-Jacques et al., 2013). Finally, its transcriptome was recently published (Oppert et al., 2012), offering opportunities for molecular biology advances in this field.

It is already known that *T. molitor* produces several antimicrobial peptides for defense against microbial pathogens, including fungi (Johnston et al., 2013). Different types of tenecin proteins are produced with antimicrobial activity against *Candida albicans* (Lee et al., 1999; Kim et al., 2001) and Gram-negative or Gram-positive bacteria (Chae et al., 2012; Roh et al., 2009). Furthermore, *T. molitor* larvae have been used to study *Staphylococcus aureus* and *Listeria monocytogenes*

* Corresponding author at: Departamento de Microbiologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Pr 445, Km 380, 86.057–970, Londrina, PR, Brazil.
E-mail address: rikodonto@gmail.com (R.S. Almeida).

http://dx.doi.org/10.1016/j.mimet.2015.10.004
0167-7012/

infections (Tindwa et al., 2013; Dorling et al., 2015). However, to this date, there are no reports about the use of the beetle's larvae as model host for human pathogenic fungi. Therefore, here we used *Cryptococcus neoformans* and *C. albicans* to study fungal pathogenesis in the *T. molitor* infection model system.

2. Materials and methods

2.1. Microorganisms and cultivation

Two reference strains were used in the experiments, *C. neoformans* ATCC28957 and *C. albicans* SC5314, grown in Yeast Peptone Dextrose — YPD (1% yeast extract, 2% peptone, 2% glucose) at 37 °C with agitation for 16 h. Subsequently, the cells were centrifuged, the supernatant was discarded and the inoculum was washed 3 times with Phosphate Buffered Saline — PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). Cell density was determined in a hemocytometer and suspensions with different cellular densities were produced with PBS dilutions. Controls used heat-inactivated yeast at 65 °C for 30 min. Loss of cell viability was determined by plating the suspensions on YPD agar and incubating for 48 h at 37 °C.

2.2. T. molitor larvae infection and survival curves

For the survival studies, we selected larvae weighing between 100 and 200 mg with clear, uniform color, without dark spots or grayish marks (Fig. 1A). We noticed that the uniformity of color and size ensures the reproducibility of experiments and prevents contamination of entomopathogens. The selected larvae were inoculated with the pathogen of interest, using a Hamilton syringe (701 N, 26's gauge, 10 μL capacity). Inoculation was achieved by injecting the inoculum into the hemocoel, at the second or third visible sternite above the legs, in the ventral portion (Fig. 1B). Each larva of each group of 10 animals received 5 μL of suspension containing the pathogen. Final concentrations are given in figures when necessary. We used two control groups, one was inoculated with sterile PBS and the other with 10^6 heat-inactivated cells. The larvae were then incubated at 37 °C in Petri dishes containing rearing diet and the number of dead larvae was recorded on intervals of 24 h during 10 days. To establish larvae death, we visually verified melanization and response to physical stimuli by gently touching them (Fig. 1C). The experiments were performed in triplicate with groups of ten animals, with a total of 30 larvae per group, which were used to build the survival curve. Results were analyzed with GraphPad Prism 5, arranged in a survival curve using the Kaplan–Meier method and statistical analysis was performed using log-rank test. P value <0.05 was considered significant.

2.3. Microscopic analysis of infected tissue

To evaluate fungal infection within host tissue, three larvae were infected with 10^6 yeasts/5 μL/larva and then incubated for 12 h at 37 °C. Chitin exoskeleton prevent fixative penetration in the larva and, thus tissue fixation. To circumvent this problem, animals were decapitated and the internal structures were embedded in liquid bacteriological agar 6% (Kasvi) (Fig. 2 A and B). After solidification, the agar block was cut with a scalpel and fixed overnight in formaldehyde (Merck Millipore) 3.7% diluted in PBS. Dehydration, clearing and paraffin wax embedding of the fixed agar blocks was performed in an automated carrousel-type processor following an overnight processing schedule (Spencer and Bancroft, 2008).

Slides with 5 μm tissue sections were stained with Periodic Acid-Schifft (PAS) (Guarner and Brandt, 2011) for *C. albicans* infected larvae.

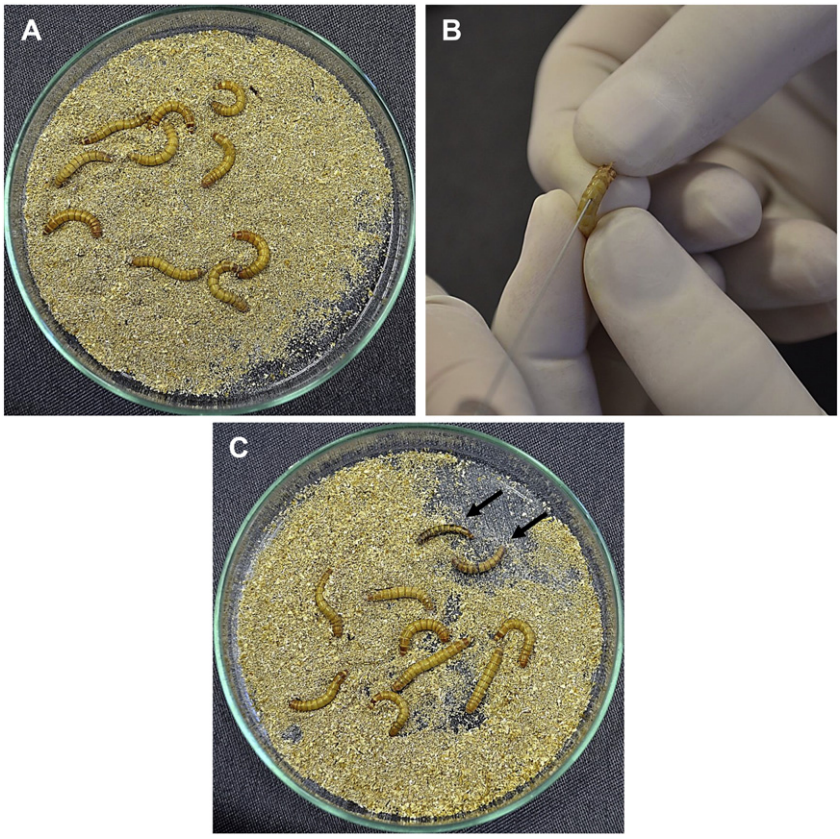

Fig. 1. *T. molitor* infection model system. A) Larvae weighing between 100 and 200 mg with clear, uniform color, without dark spots or grayish marks were selected. B) Inoculation was achieved by injecting the inoculum into the hemocoel, at the second or third visible sternite above the larva legs, in the ventral portion. C) Black arrows point dead larvae presenting their characteristic melanization.