Antifungal Efficacy during Candida krusei Infection in Non-Conventional Models Correlates with the Yeast In Vitro Susceptibility Profile

Liliana Scorzoni¹,², Maria Pilar de Lucas³, Ana Cecilia Mesa-Arango¹,⁴, Ana Marisa Fusco-Almeida², Encarnación Lozano³, Manuel Cuenca-Estrella¹, Maria Jose Mendes-Giannini²*, Oscar Zaragoza¹*

1 Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain, 2 Laboratório de Micologia Clínica, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista de São Paulo, Araçatuba, Brazil, 3 Department of Cellular Biology, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain, 4 Group of Investigative Dermatology, University of Antioquia, Medellín, Colombia

Abstract

The incidence of opportunistic fungal infections has increased in recent decades due to the growing proportion of immunocompromised patients in our society. Candida krusei has been described as a causative agent of disseminated fungal infections in susceptible patients. Although its prevalence remains low among yeast infections (2–5%), its intrinsic resistance to fluconazole makes this yeast important from epidemiologic aspects. Non mammalian organisms are feasible models to study fungal virulence and drug efficacy. In this work we have used the lepidopteran Galleria mellonella and the nematode Caenorhabditis elegans as models to assess antifungal efficacy during infection by C. krusei. This yeast killed G. mellonella at 25, 30 and 37°C and reduced haemocytic density. Infected larvae melanized in a dose-dependent manner. Fluconazole did not protect against C. krusei infection, in contrast to amphotericin B, voriconazole or caspofungin. However, the doses of these antifungals required to obtain larvae protection were always higher during C. krusei infection than during C. albicans infection. Similar results were found in the model host C. elegans. Our work demonstrates that non mammalian models are useful tools to investigate in vivo antifungal efficacy and virulence of C. krusei.

Introduction

Fungal infections have emerged worldwide due to a growing population of immunosuppressed patients, including patients with cancer, AIDS, solid-organ and hematopoietic stem cell transplant recipients, premature neonates, and patients recovering from major surgery [1–5]. These infections have significant morbidity and mortality rates and are difficult to prevent, diagnose and treat [6–8].

Candida spp are commensal yeasts responsible for different clinical manifestations, from mucocutaneous overgrowth to blood stream infections [1,9–12]. Candida albicans is still the major cause of invasive fungal disease. However, a growing number of infections produced by non-albicans Candida spp has been reported in the last years [1,13–15]. Among them, there are some species that are intrinsically resistant or have reduced susceptibility to antifungals. The massive use of antifungals in prophylaxis, such as fluconazole, has facilitated the selection of pathogenic fungi resistant to these agents [16–19].

Candida krusei is an opportunistic pathogen which presents intrinsic resistance to fluconazole. The infection is associated with the prophylactic or therapeutic use of this antifungal agent [20–23]. Two mechanisms of azole resistance in C. krusei have been described: overexpression of drug efflux pumps [24] and diminished sensitivity of the target enzyme, the cytochrome P450 sterol 14-demethylase (encoded by the CYP51 gene) [25]. Diseases caused by C. krusei have high associated mortality (30–60%) [26,27]. Despite the intrinsic resistance to fluconazole, C. krusei is usually susceptible to voriconazole in vitro, which correlates with the binding of this drug to the target enzyme [21].

Antifungal resistance in vitro does not always correlate with clinical resistance. The best correlation between in vitro and clinical efficacy is found in HIV-positive patients with oropharyngeal candidiasis [22,28,29]. In contrast, although C. parapsilosis shows reduced in vitro susceptibility to echinocandins, these antifungals have been shown to be effective in the treatment of invasive candidiasis caused by this species [30].

Citation: Scorzoni L, de Lucas MP, Mesa-Arango AC, Fusco-Almeida AM, Lozano E, et al. (2013) Antifungal Efficacy during Candida krusei Infection in Non-Conventional Models Correlates with the Yeast In Vitro Susceptibility Profile. PLOS ONE 8(3): e60047. doi:10.1371/journal.pone.0060047

Copyright: © 2013 Scorzoni et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: O.Z. is funded by grant SAF2011-25140 from the Spanish Ministry for Economics and Competitiveness. L.S. is funded by a fellowship from the Agencia Española para la Cooperación Internacional y Desarrollo. C. elegans strain AU37 was provided by the CGC (Caenorhabditis Genetics Center), which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have the following interests: In the past 5 years, M.C.E. has received grant support from Astellas Pharma, bioMerieux, Gilead Sciences, Merck Sharp and Dohme, Pfizer, Schering Plough and Soria Melguizo SA. He has been an advisor/consultant to Gilead Sciences, Merck Sharp and Dohme, Pfizer, and Schering Plough. There are no patents, products in development or marketed products to declare. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: giannini@fcfarr.unesp.br (MMG); ozaragoza@iscrii.ies (OZ)
The use of invertebrate hosts has recently emerged and facilitated the study of fungal pathogenesis. Among these non-mammalian hosts, amoebae (*Acanthamoeba castellanii*, *Dictyostelium discoideum*), nematodes (*Caenorhabditis elegans*) and insects (*Drosophila melanogaster*, *Galleria mellonella*) have been successfully used to study the virulence of some fungi [31–35]. Moreover, some aspects of the innate response are conserved between these hosts and mammals [36]. *Galleria mellonella* is a lepidopteran (*Pyralidae*) that provides important advantages as host model. The larvae can be incubated in a range of temperature between 25 to 37°C, so it is possible to simulate the natural fungal habitat and the infection conditions in mammals. In addition, as in mammalian models, it is possible to introduce by injection exact doses of pathogens to the larvae, which poses a technical improvement over other non-conventional hosts. *Galleria mellonella* has six types of phagocytic cells that play an important role in the defense system [37,38]. The density of these cells in the haemolymph is not constant, and changes during infection can be easily measured and used as a parameter of the response of the larvae after exposure to pathogens [39]. The viability of the larvae can be easily recorded by the lack of movement and the massive melanization induced by *G. mellonella* in response to infection [40–42]. Another organism that is used as model host is the soil nematode *Caenorhabditis elegans*, which feeds on microorganisms, but is susceptible to bacterial and fungal pathogens [33,43–45]. *Caenorhabditis elegans* has been used to study virulence, filamentation and antifungal efficacy of antifungal drugs [44,46].

In this study, we initially aimed to characterize the interaction between *G. mellonella* and *C. krusei* with two purposes: 1) To get insights about virulence traits of this pathogenic yeast, and 2) to investigate if antifungal efficacy in vivo correlates with the susceptibility profile shown by *C. krusei* in vitro. Furthermore, we have complemented our studies with *C. elegans*, and observed similar behaviors, indicating that non-conventional models can be used to investigate *C. krusei* virulence and antifungal efficacy.

**Materials and Methods**

**Strains and media**

*Candida albicans* SC5314 [47], *C. krusei* ATCC 6258 and two clinical isolates (CL8053 and CL80317) from the Yeast Collection of the Mycology Reference Laboratory of the Spanish National Centre for Microbiology and *Cryptococcus neoformans* variety grubii (H99 strain, ATCC 20882) were used in this study. The yeasts were grown overnight in liquid Sabouraud medium (Difco, BD, USA) at 30°C with shaking. *Escherichia coli* OP50 strain was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota) and was maintained on LB agar plates at 37°C.

**Antifungal susceptibility testing (AFST)**

Minimum inhibitory concentration (MIC) values were determined using the EUCAST protocol [48,49]. For AFST, 10 different clinical isolates of *C. albicans* and 10 clinical isolates of *C. krusei* were obtained from the yeast collection of the Mycology Reference Laboratory of the Spanish National Centre for Microbiology. Data were expressed as geometric mean, mode, range (minimum-maximum) and MIC frequency distribution.

**Insect larvae manipulation and incubation conditions**

*Galleria mellonella* larvae (0.3–0.5 g, R.J. Mous Livébaït, The Netherlands) were placed in Petri dishes and incubated at 37°C in the dark the night before the experiments. Larvae with color alterations (i.e. dark spots or with apparent melanization) were excluded. Antifungals and yeast suspensions were injected in the haemocoele through the last left pro-leg of the larvae using a 10 μL Hamilton syringe (Hamilton, USA). The pro-leg had been previously cleaned with 70% ethanol. A total of 10 μL were injected in each larva. Larvae death was monitored by visual inspection of the color (brown-dark brown) and by lack of movement after touching them with forceps. For each condition, a total of 20 larvae were used, and each experiment was repeated at least twice. After infection, larvae were incubated at 25, 30 or 37°C.

**Survival assay**

Yeast strains were grown overnight in liquid Sabouraud, washed with PBS, and suspended in the same buffer. Cell density was estimated with an Automatic Cell Counter TC10 (Bio Rad). For survival assays, larvae were inoculated with 107, 5×106 and 2.5×106 cells/larva of *C. krusei* and 106, 5×105 and 105 cells/larva of *C. albicans*. The inocula were prepared in PBS plus 20 mg/L of ampicillin to prevent bacterial contamination. The infected larvae were incubated at 25°C, 30 or 37°C, and the death was daily monitored during 7 days.

**Growth curve at different temperatures**

Yeast strains were grown overnight and diluted in fresh Sabouraud liquid medium at 103 cells/mL. Two hundred microliters of this suspension were placed in 96-well microdilution plates, and incubated at 25, 30 or 37°C in a Labsystems EMS Reader MF spectrophotometer. Optical density (OD) was determined at 530 nm every hour during 72 hrs.

**In vivo phagocytosis assay**

Yeast cells were stained with 10 μg/mL Calcofluor white (Sigma, St. Louis, MO) for 30 min at 37°C. Then, these cells were injected in *G. mellonella* larvae (105 cells/larva, 5 per group), and phagocytosis was analyzed after 3 hrs of incubation at 25 and 37°C. Haemolymph was collected in 1.5 mL tubes and diluted 1:1 in IPS buffer (Insect Physiological Saline: 150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris-HCl pH 6.9, 10 mM EDTA and 30 mM sodium citrate) to avoid coagulation and melanization of the haemolymph. Haemocytes were placed on a slide and phagocytosis was visually quantified using a Leica DMI 3000B microscope. One hundred haemocytes from each larva were counted in each case, and the percentage of haemocytes containing yeasts was calculated and plotted. *Cryptococcus neoformans* H99 strain was used as control. Phagocytosis was also analyzed in larvae infected in the same way, but treated with 64 mg/kg fluconazole or 4 mg/kg amphotericin B.

**Determination of haemocyte density**

Groups of five *G. mellonella* were infected with 107 yeast cells/larva and incubated at 37°C for 3 hrs. The haemolymph of each larva was collected in 1.5 mL tubes and diluted 1:10 in IPS buffer. The cells were counted using a haemocytometer.

**Measurement of in vivo filament formation**

*Galleria mellonella* was infected with 107 cells/larva of *C. albicans* and *C. krusei* strains. The larvae were incubated at 37°C for 24 hours. Larvae were macerated in 100 μm nylon cell striainers (Falcon, BD, USA) with 1 mL of IPS. The liquid was then collected, centrifuged and suspended in 1 mL of the same buffer. Samples were stained with Calcofluor white (Sigma, St. Louis, MO), as described above, and yeast morphology was observed with a Leica DMI 3000B fluorescence microscope.
Melanization quantification

Larvae were infected with PBS, 5 \times 10^5, 10^6 and 5 \times 10^6 cells/larva of C. krusei. Then, the haemolymph of each larva was collected after 3 and 24 hrs in 1.5 mL tubes and diluted 1:10 with IPS buffer. The samples were placed in 96 well microdilution plates. To quantify melanin levels, we took advantage of existing protocols that quantify laccase activity by detecting the final results with the visualization of the dark compound. So the OD at 405 nm was measured using a Labsystems IEMS Reader MF spectrophotometer. Melanization of larvae infected with 405 nm was measured using a Labsystems IEMS Reader MF spectrophotometer. Melanization of larvae infected with C. albicans (5 \times 10^5 cells/larva) and C. krusei (5 \times 10^6 cells/larva) and treated with 64 mg/kg fluconazole and 4 mg/kg amphotericin B was also evaluated.

Treatment with antifungal drugs

Infected larvae were treated with amphotericin B (1, 2 or 4 mg/kg, Sigma Aldrich Quimica, Madrid, Spain), fluconazole (128, 64, 32, 12, or 4 mg/kg, Pfizer SA, Madrid, Spain), voriconazole (7.5 or 10 mg/kg, Pfizer SA, Madrid, Spain) or caspofungin (1, 2 or 4 mg/kg Merck & Co, Inc, NJ, USA). In some experiments, a combination of fluconazole and amphotericin B was also used. Antifungals were applied immediately after the infection. Groups of 10 larvae were treated with the antifungals alone to test the toxicity.

Fungal burden determination

Infected larvae were selected at different times of infection, washed with 70% ethanol and cut into small pieces with a scalpel. Two mL of PBS-ampicillin were added and the mix was homogenized gently with a vortex and glass beads for 10 seconds. The mix was finally suspended in 9 mL of PBS-ampicillin. Different dilutions were made for each sample and 50 \mu L from these dilutions were placed on Sabouraud-cloramphenicol agar plates (Oxoid). The plates were incubated at 37°C for 48 h, and the number of colony forming units (CFUs) was determined.

Histology

Three larvae from different groups (uninfected, infected and/or treated with antifungals) were collected on different days of the infection. The larvae were preserved in 70% ethanol and incubated at 15°C beginning to the end of the experiment and avoids mixing with their progeny [33,44]. The sek-1 gene encodes a mitogen-activated protein kinase which is important for the defense of C. elegans against microbial infections [33,44]. Therefore worms defective for sek-1 are more susceptible to infection and die earlier than wild-type C. elegans animals. Candida strains were cultivated in liquid Sabouraud medium (Difco, BD, USA) at 35°C with shaking. One hundred mL from this culture were inoculated on solid BHI media (Difco) containing kanamycin (90 \mu g/mL) and ampicillin (200 \mu g/mL) and incubated at 30°C for 24 hours. Synchronized worms in the L4 stage were added to the center of the agar plates inoculated with the yeast strains lawns and incubated for three hours at 35°C. In parallel, L4 worms were placed on agar plates containing lawns of E. coli OP50 strain. After the three hours incubation, worms were washed with M9 and transferred to 12-well plates with 1 mL 60% M9 buffer [45], 40% BHI, 10 \mu g/mL cholesterol in ethanol, 200 \mu g/mL ampicillin and 90 \mu g/mL kanamycin. Around 20–30 worms were placed in each well. For antifungal efficacy, amphotericin B (1 and 2 \mu g/mL), fluconazole (12 \mu g/mL), voriconazole (0.25, 7.5 and 10 \mu g/mL), caspofungin (2, 4 and 6 \mu g/mL), or a combination of amphotericin B (1 \mu g/mL) plus fluconazole (12 \mu g/mL) plus caspofungin (12 \mu g/mL) were added to the media. Plates were incubated at 25°C and individual worm survival was monitored daily. Nematodes were considered dead when they did not respond to touch. A minimum of two independent experiments was carried out for each treatment. Images were captured with a video camera (JVC KY-F550) attached to a dissecting microscope (Leica MZ7.5).

Statistics

Graphs and Statistics analyzes were performed with Graph Pad Prisma 5 (La Jolla CA, USA). Survival curves were analyzed by Log-rank (Mantel-Cox) Test and phagocytosis assays, haemocyte density, melanization quantification and fungal burden were analyzed using t-Test.

Results

Candida krusei killed G. mellonella in a dose dependant manner

We first investigated if C. krusei killed G. mellonella. Our results showed that G. mellonella is susceptible to C. krusei infection (Figure 1A). The death rate of the larvae depended on the yeast dose injected. Most reproducible results were found when larvae were infected with 5 \times 10^5 C. krusei cells. However, C. krusei was less virulent than other fungi, such as C. albicans, which killed G. mellonella at lower doses (5 \times 10^4, Figure 1B). To confirm that the death was not a consequence of a shock due to big amounts of yeast injected in the larvae, we inoculated a group of larvae with yeast inactivated by incubation in 4% paraformaldehyde. As shown in Figure 1C, inactivated yeast did not kill G. mellonella, confirming that larvae death was dependent on living yeast.

To verify if C. krusei virulence in G. mellonella depended on the temperature at which the larvae are incubated, we compared virulence of C. krusei and C. albicans at different temperatures (25, 30 or 37°C). Candida albicans was more virulent at 37°C than at 30°C. In contrast, no statistical difference was observed in the survival of G. mellonella infected with C. krusei and incubated at the different temperatures, indicating that C. krusei virulence does not depend on temperature (Figure 1D). Similar findings were obtained with C. krusei clinical isolates (result not shown). We also studied the virulence of these two species at environmental temperature (25°C). In agreement with the previous data, we found that C. krusei was virulent at 25°C, while C. albicans virulence was significantly decreased at this temperature (data not shown).

To confirm these results, we investigated if C. krusei growth was affected by temperature in a similar manner as C. albicans. So we performed growth curves of both species at 30 and 37°C. As shown in Figure 1E and F, C. albicans grew better at 37°C.
compared to 30°C (two-fold reduction in generation time). In contrast, C. krusei grew similarly at both temperatures (0.85 fold decrease in generation time when the cells were grown at 37°C compared to 30°C). We found similar results at 25°C (data not shown), supporting that C. krusei growth is not affected by the incubation temperature. The final OD reached at the stationary phase was almost identical at 30 and 37°C. Yeast inoculation caused early melanization of the larvae. As shown in Figure 3A, the final OD at the stationary phase at different temperatures was different with both species. Candida albicans reached higher OD at 37°C, which differed from the situation found in C. krusei, where the final OD at the stationary phase was almost identical at 30 and 37°C. Latency period was longer at 30°C, but the same trend was observed in both species (Figures 1E and 1F).

Yeast inoculation caused early melanization of the larvae

Galleria mellonella larvae appeared melanized after a few minutes of C. krusei infection (Figure 2A). To quantify this phenomenon, we collected the haemolymph and measured its optical density at 405 nm. When larvae were infected with 5 x 10⁶ C. krusei cells, there was a significant accumulation of melanin in the haemolymph (4.3 times compared to the non-infected larvae), and this melanization increased over time (5 times at 24 hrs, Figure 2B). Clinical isolates showed a similar behavior (Figures 2C and D). We evaluated if C. krusei induced melanization of G. mellonella at lower temperatures, and we found that this phenomenon also occurred at 25°C (data not shown).

Phagocytosis and effect of C. krusei on haemocyte density

We examined if different C. krusei strains had any effect on haemocyte density. As shown in Figure 3A, C. krusei produced a decrease in haemocyte density in a similar manner to C. albicans. We then investigated if C. krusei cells were phagocytosed by G. mellonella haemocytes. We compared the phagocytosis of this pathogen to the one measured with C. albicans and C. neoformans. The phagocytosis for all Candida strains (albicans and krusei) was significantly lower to the phagocytosis observed with C. neoformans (Figure 3B). The same result was found when phagocytosis was assessed at 25°C (data not shown).

Candida krusei can filament in vitro, so we investigated if this change also took place during infection in G. mellonella. We included C. albicans in these experiments as control, since it has been reported that this yeast can form hyphae in this model host [53]. As expected, C. albicans efficiently produced filaments in the larvae. Candida krusei also produced filaments, and in G. mellonella crude extracts they were frequently found in clumps of fat body of dark color, which we believe that are composed mainly of insect melanin. This fact may explain the fast melanization of G. mellonella when infected with C. krusei.

Antifungal efficacy during C. krusei infection in G. mellonella

One of the main features for C. krusei is its in vitro susceptibility profile. As shown in Figure 4, C. krusei is less susceptible to amphotericin B, voriconazole and caspofungin than C. albicans, and intrinsically resistant to fluconazole. So we studied if this phenotype correlated with a lack of response to the antifungal during infection in G. mellonella. For this purpose, we infected G. mellonella with C. krusei or C. albicans, and treated the larvae with different antifungals (fluconazole, voriconazole, amphotericin B and caspofungin). In the case of larvae infected with C. krusei, treatment with fluconazole, even at very high doses (32 or 64 mg/ kg) did not increase the survival (Figures 5A and B). At higher

Figure 1. Comparison of the virulence of C. krusei and C. albicans in G. mellonella. (A) Survival curve of G. mellonella infected with different inocula of C. krusei ATCC 6258 (● PBS; ▲ 10⁷ cells/larva; ▲ 5 x 10⁶ cells/larva; ▼ 2.5 x 10⁵ cells/larva incubated at 37°C (B). Survival curve of G. mellonella infected with different inocula of C. albicans SC5314 (● PBS; ▲ 10⁵ cells/larva; ▲ 5 x 10⁴ cells/larva; ▼ 10³ cells/larva (C). Survival of G. mellonella infected with inactivated yeast. Control dead cells (● PBS; ▲ C. krusei ATCC 6258 5 x 10⁶ cells/larva; ▲ C. krusei ATCC 6258 5 x 10⁵ cells/larva (dead); ▲ C. albicans SC5314 10⁶ cells/larva; ▲ C. albicans SC5314 10⁵ cells/larva (dead) (D). Effect of the incubation temperature on the virulence of C. albicans and C. krusei. ● PBS; ▲ C. krusei ATCC 6258 (37°C); ▼ C. krusei ATCC 6258 (30°C); ● C. albicans SC5314 (37°C); ▲ C. albicans SC5314 (30°C); Growth curves of C. albicans (E) and C. krusei (F) at different temperatures. ○ 37°C; ▲ 30°C.

doi:10.1371/journal.pone.0060047.g001
concentrations (128 mg/kg), there was a decrease in the survival, which is explained by the toxicity of the antifungal at this high concentration, which induced 25% of death after 7 days of treatment (data not shown). When the same experiments were performed with *C. albicans*, treatment with all the fluconazole concentrations produced significant survival (Figures 5C and 5D).

Concerning other azoles, *C. krusei* is considered susceptible to voriconazole, although it presents higher MIC values to this antifungal than *C. albicans* (see Figure 4). So we studied the efficacy of voriconazole during infection in *G. mellonella*. We found that both voriconazole concentrations tested (7.5 and 10 mg/kg) protected larvae from *C. albicans* infection (Figure 6A). In contrast, larvae infected with *C. krusei* were only protected with higher concentrations.

**Figure 2. Melanization of *G. mellonella* infected with *C. krusei*.** (A) Visual appearance of *G. mellonella* larvae infected with different *C. krusei* doses. (B, C and D) Optical Density (OD) of the haemolymph of *G. mellonella* infected with *C. krusei* ATCC 6258 (B), clinical isolate CL8053 (C) and CL80317 (D) with $5 \times 10^5$, $10^6$, $5 \times 10^6$ cells/larva. The different size inoculum reveals dose-response melanization (* p<0.05). All the experiments in this figure were performed at 37°C. doi:10.1371/journal.pone.0060047.g002

**Figure 3. Interaction between *C. krusei* and haemocytes.** (A) Changes in haemocyte density during *C. krusei* infection. The haemolymph of infected larvae with *C. neoformans*, *C. albicans* SC5314, *C. krusei* ATCC 6258, CL8053 and CL80317 clinical isolates and PBS was collected and the concentration of haemocytes was estimated using a haemocytometer (B). Phagocytosis percentage of *C. neoformans*, *C. albicans* SC5314, *C. krusei* ATCC 6258, CL8053 and CL80317 clinical isolates. Asterisks denote differences statistically significant (* p<0.05). doi:10.1371/journal.pone.0060047.g003
voriconazole concentrations (Figure 6B). Lower doses did not have any effect on survival.

Amphotericin B (4 mg/kg) prolonged survival of larvae infected with *C. albicans* at all the concentrations tested (Figure 6A). In contrast, amphotericin B only protected larvae infected with *C. krusei* at the highest dose (4 mg/kg), which produced a 60% survival at the fourth day (Figure 6B). In a similar way, caspofungin was effective during *C. albicans* infection at all the doses tested (Figure 6C), while it only protected larvae inoculated with *C. krusei* at the highest dose (4 mg/kg) (Figure 6D). We also used an antifungal combination with fluconazole (12 or 4 mg/kg) and amphotericin B at a sub-therapeutic dose in *G. mellonella* (1 mg/kg), but we found no synergic effect between the antifungals (data not shown).

Fungal burden determination and histopathology

The fungal burden was determined by recovering the yeast cells from the larvae infected with *C. albicans* or *C. krusei* and treated with fluconazole (12 mg/kg) or amphotericin B (4 mg/kg). The number of CFUs increased in larvae infected with both pathogens with the time of infection (Figure 7). Treatment of larvae infected with *C. albicans* with fluconazole or amphotericin B decreased the number of CFUs by 1000-fold (Figure 7A). In larvae infected with *C. krusei*, amphotericin B reduced the fungal burden by 10-fold. Curiously, fluconazole also reduced the initial fungal burden, although it did not have an effect after longer times (5 days, Figure 7B).

To complement these studies, we performed histopathology of infected and treated larvae. *Candida albicans* (Figure 8C and 8D) and *C. krusei* (Figure 8K and 8L) were found both in yeast and filament forms. The antifungal treatment with fluconazole (12 mg/kg) in larvae infected with *C. albicans* or *C. krusei* decreased the number of yeasts. Moreover, the fungi were mainly found in defined structures surrounded by *G. mellonella* cells (Figure 8E, F, M, N). Amphotericin B (4 mg/kg) had the same effect as fluconazole, although fewer yeast cells were found with this treatment (Figure 8G, H, O, P). The antifungals did not have a different effect on larvae infected with *C. albicans* or *C. krusei.*

---

**Figure 4. Antifungal susceptibility profile of *C. krusei* and *C. albicans*.** A) Distribution of MIC values (*n* = 10) to amphotericin B, caspofungin, fluconazole and voriconazole of *C. albicans* (white bars) and *C. krusei* (black bars). B) Description of antifungal susceptibility of *C. albicans* and *C. krusei* to different antifungals. *N* = 10. The geometric mean (GM), mode, minimum (Min) and maximum (Max) are shown. doi:10.1371/journal.pone.0060047.g004
Treatment with the antifungals alone did not have any effect on the histopathology of the larvae (result not shown).

**Effects of amphotericin B and fluconazole on the physiology of *G. mellonella* during *C. albicans* and *C. krusei* infection**

Antifungals have immunomodulatory properties in mammals and in *G. mellonella* [54–56]. We studied the effect of Amphotericin B (4 mg/kg) and fluconazole (12 and 64 mg/kg) on haemocyte density, melanization and phagocytosis during *G. mellonella* infection by *C. krusei* and *C. albicans*. None of the antifungal treatments influenced the haemocyte density of *C. krusei* infected larvae. However, fluconazole (64 mg/kg) reduced the haemocyte density in larvae infected with *C. albicans* by two fold (p = 0.017, Figure 9A).

None of the antifungals had a significant effect on the melanization of larvae infected with *C. krusei*. In contrast, antifungal treatment of larvae infected with *C. albicans* reduced melanization after 24 hours of infection. Fluconazole (64 mg/kg) and amphotericin B (4 mg/kg) reduced the melanization of these larvae by 1.8 (p = 0.0139) and 1.5 fold, respectively (p = 0.003, Figure 9B). No differences were observed in melanization or phagocytosis after 3 hours of infection with *C. albicans* or *C. krusei*. Antifungal drugs alone did not cause any effect in *G. mellonella* on the parameters analyzed.

**Virulence and antifungal efficacy in *C. elegans* model**

The nematode *C. elegans* is another non mammalian model that has been used as a host to study microbial virulence in this study. We also used this model to evaluate the in vivo protection of antifungals during *C. krusei* infection such as amphotericin B, fluconazole, voriconazole, caspofungin, and a combination of amphotericin B plus fluconazole. *Candida albicans* and *C. krusei* both killed *C. elegans* worms. In both *Candida* strains, worm death was associated with filamentation of the yeast in the worms (Figure 10A). When we investigated the protection of the different antifungal treatments, we found that all the antifungals protected during *C. albicans* infection at all the concentrations tested (Figure 10B). In contrast, in nematodes infected with *C. krusei*, the behavior of the antifungals was different: amphotericin B only protected at concentrations ≥2 μg/mL and fluconazole was not protective at any of the concentrations used (Figure 10C). Caspofungin showed similar protection as the one observed when the worms were infected with *C. albicans* (Figure 10C). The antifungal combination of fluconazole (12 μg/mL) and amphotericin B (1 μg/mL) did not show any synergistic effect in this model (result not shown). We also studied how voriconazole protected the worms from infection. As shown in Figure 10D, all the concentrations used (0.25, 7.5 and 10 mg/L) protected larvae from infection by *C. albicans*. However, only the higher doses (7.5 and 10 mg/L) showed efficacy during *C. krusei* infection, while the lowest dose (0.25 mg/L) was not protective.

**Discussion**

The use of invertebrate hosts to study the virulence of microbial pathogens presents advantages over conventional mammals. Amoebae, nematodes and insect hosts are good models to study virulence and to elucidate host–pathogen interaction. Ethical issues, cost and faster results are other benefits of these models [41,42,57]. During evolution, non vertebrate animals have developed immunity against microbial pathogens [42], and for this reason, there are functional and structural similarities between the innate immune system of mammals and insects. So, these models can be used to study immune responses [57].

In this work, we have used two different hosts, *G. mellonella* and *C. elegans*, to investigate virulence of *C. krusei* and antifungal efficacy. Compared to other non-conventional models, *G. mellonella* allows the use of precise pathogen doses by injection, low cost and...
Figure 6. Efficacy of voriconazole, amphotericin B or caspofungin during C. krusei and C. albicans infection in G. mellonella. A and B) Voriconazole treatment efficacy (7 and 10 mg/kg) in G. mellonella infected with C. albicans SC5314 (A) or C. krusei ATCC 6258 (B). C and D) Amphotericin B treatment efficacy (1, 2, 4 mg/kg) in G. mellonella infected with C. albicans SC5314 (C) or C. krusei ATCC 6258 (D). E and F) Caspofungin treatment efficacy (1, 2, 4 mg/kg) in G. mellonella infected with C. albicans SC5314 (E) or Candida krusei ATCC 6258 (F). In all the cases, the larvae were infected with $5 \times 10^5$ C. albicans cells/larva and $5 \times 10^6$ C. krusei cells/larva.
doi:10.1371/journal.pone.0060047.g006

Figure 7. Effect of antifungal treatment on fungal burden in G. mellonella infected with C. albicans or C. krusei. Galleria mellonella larvae were infected with C. krusei ATCC 6258 (A, $5 \times 10^6$ cells/larva) or C. albicans SC5314 (B, $5 \times 10^6$ cells/larva) and CFUs recovered from G. mellonella. Black bars, no treatment, white bars, fluconazole (12 mg/kg), grey bars, amphotericin B (4 mg/kg).
doi:10.1371/journal.pone.0060047.g007
evaluation of survival at different temperatures. The virulence of five \textit{C. albicans} strains with mutations in genes related to filamentation was evaluated in \textit{G. mellonella} and it was demonstrated that this model is useful as a filamentation assay [53]. In the case of \textit{C. neoformans}, the virulence of different isolates, morphogenesis and antifungal treatments in \textit{G. mellonella} showed good

Figure 8. Histopathology of \textit{G. mellonella} infected with \textit{C. krusei} and \textit{C. albicans} and treated with different antifungals. \textit{Galleria mellonella} was infected with $5 \times 10^5$ cells/larva of \textit{C. albicans} SC5314 (C–H), or with $5 \times 10^6$ cells/larva of \textit{C. krusei} ATCC 6258 (K–P). After 96 hours of infection, larvae were processed for histopathology as described in Material and Methods. (A, B, I, J), uninfected controls; (C, D, K and L), untreated controls; (E, F, M and N), larvae treated with fluconazole (12 mg/kg); (G, H, O and P), larvae treated with amphotericin B (4 mg/kg). (A, C, E, G, I, K, M, O), low magnification; (B, D, F, H, J, L, N and P), high magnification. doi:10.1371/journal.pone.0060047.g008

Figure 9. Effect of antifungal treatment of haemocyte density and melanization of \textit{G. mellonella} infected with \textit{C. krusei} or \textit{C. albicans}. (A) Hemocytic density of \textit{G. mellonella} infected with \textit{C. albicans} SC5314 or \textit{C. krusei} ATCC 6258 treated with amphotericin B (4 mg/kg) or fluconazole (64 mg/kg). (B) Optical Density (OD) of the haemolymph of \textit{G. mellonella} infected with \textit{C. albicans} or \textit{C. krusei} treated with amphotericin B (4 mg/kg) or with fluconazole (64 mg/kg). Black bars, no treatment; grey bars, fluconazole; white bars, amphotericin B. * p<0.05. doi:10.1371/journal.pone.0060047.g009
correlation with mammalian system [57,58]. Previous work demonstrated that *C. elegans* is susceptible to different *Candida* species. For that reason, this host has been used to look for new compounds with antifungal activity [44,46]. Besides, the available *C. elegans* mutant animals defective in signaling pathways involved in the immune system allows the study of the molecular mechanisms of host-pathogen interaction [59]. However, there are also some cases in which there is no correlation between virulence in mammals and *G. mellonella* [60], so further studies are required to validate the use of these models.

For these reasons, *C. krusei* offers a good model to validate the use of invertebrate models. This yeast shows reduced virulence in mammalian systems and fungal burden is significantly lower in animals infected with *C. krusei* than in animals infected with other fungal pathogens, such as *C. albicans* [61], so it is possible to compare its virulence with other highly virulent yeasts. Moreover, *C. krusei* is intrinsically resistant to fluconazole, so it offers an excellent model to correlate antifungal efficacy in vitro and in vivo. Previous work showed that *G. mellonella* infected with $2 \times 10^6$ cells/larva of *C. krusei* killed 20% of the larvae after 72 hrs [62]. In our work, we have reproduced the model and observed that larvae killing can be faster by increasing the pathogen concentration. However, *C. krusei* was less virulent than *C. albicans* because the amount of yeast required to cause 100% death on the fourth day was 10 times higher. This is also in agreement with the reduced virulence of *C. krusei* in mammalian models [63,64], but also indicates that *G. mellonella* offers a simple method to study virulence traits of *C. krusei*. This finding is of particular interest, since not every microorganism (i.e., *Pneumocystis murina*) can cause disease in *G. mellonella* [65].

The possibility to incubate *G. mellonella* at different temperatures is one of the best advantages of this model because it permits to study virulence at both environment and mammalian temperatures. The virulence of some pathogenic microorganisms (such as *Cryptococcus neoformans*, *Fusarium spp* and *Acinetobacter baumannii*) in *G. mellonella* is affected by the incubation temperature of the larva.
after inoculation [66,67]. In contrast, no statistical difference in the virulence of C. krusei was observed between the two temperatures. This correlates with the growth rate of C. krusei at both temperatures. In contrast to C. albicans, C. krusei growth was less affected by the temperature. Interestingly, G. mellonella seems to better tolerate environmental temperature than physiological temperature, and it would be expected that immunity is impaired at high temperature. However, our data indicate that in the case of fungi with reduced virulence, immunity at high temperature can control infection, and virulence of the fungus is more dependent on virulence traits of the yeast. Candida krusei and C. albicans produced filaments in G. mellonella, although the morphology was different. Candida krusei tended to form cell aggregates with melanization, characteristic of encapsulation. This result indicates that G. mellonella differentially recognizes pathogenic yeasts, which can be related to the different virulence exhibited by these two Candida species.

Decrease in the amount of haemocytes has been associated with increased susceptibility to fungal infections [39]. Candida krusei induced a reduction in the proportion of haemocytes in a similar way as C. albicans. This result suggests a mechanism of phagocytosis avoidance by which Candida species induce killing of G. mellonella, but does not explain the difference in virulence shown by the different Candida spp. This reduction might be a consequence of haemocyte explosion after filamentation of these yeasts after internalization. Interestingly, Cryptococcus neoformans does not cause a reduction in the number of hemocytes in the first two hours post-infection [58,66], which might correlate with the fact that this fungus is an intracellular pathogen and can survive in phagocytic cells without affecting their viability [68,69]. In addition, haemocytes are recruited at infection sites to form clumps or nodules [38,70], so it is also possible that a proportion of the haemocytes migrate from the haemolymph to the infection sites. In agreement with our findings, it has been described that C. albicans induced a reduction in the concentration of haemocytes. In contrast, larvae infected with S. cerevisiae showed higher survival and haemocytic concentration [39]. Moreover, the compound [Ag2 (mal) (phen3)] increased the survival of larvae infected with C. albicans, and also increased the haemocyte concentration [71]. Phagocytosis of C. krusei and C. albicans was also lower compared to other fungi, such as C. neoformans. There are several mechanisms that could account for this phenomenon. Candida spp might be poorly phagocytosed due to impaired pathogen recognition by insect haemocytes. In addition to the reduction of haemocyte density and haemocyte explosion after filament formation discussed above could also explain the reduced phagocytosis observed. The future development of in vitro models to study yeast-haemocyte interaction will be of great help to fully characterize these phenomena.

Melanization is a humoral response of the insect that is catalyzed by the enzyme phenoloxidase, and the reaction occurs through the formation of capsules that surround foreign particles [72]. We observed a fast melanization process after infection with C. krusei. The degree of melanization depended on the inoculum concentration, but not on the viability of the cells, indicating that melanization is an unspecific process that depends on the presence of foreign particles.

One of the main findings of our work is the correlation between the in vivo efficacy of antifungals during C. albicans and C. krusei infection and their in vitro susceptibility profiles. Fluconazole did not have any protective effect during C. krusei infection in both G. mellonella and C. elegans models. Similar findings have been obtained with protection in immunosuppressed mice [63,73], which validates the use of non-mammalian models to study antifungal efficacy. Due to the simplicity of these models, we believe that these hosts offer suitable and reliable systems to evaluate antifungal efficacy in vivo. In this sense, C. elegans has been successfully used to perform high-throughput assays to evaluate fungal susceptibility to different types of compounds [46,74–76]. However, more information with resistant strains is required to fully validate their use. We also noticed differences in the protection between C. albicans and C. krusei in vivo after treatment with voriconazole, amphotericin B and caspofungin. During C. krusei infection, the caspofungin and amphotericin B concentrations required for protection were always higher than during C. albicans infection. Although C. krusei is considered susceptible to the three drugs, it has reduced susceptibility to caspofungin and amphotericin B compared to C. albicans [20,77,78]. So our data are again in agreement with the different susceptibility profile shown by these species in vitro. While several articles suggest molecular mechanisms for the resistance to fluconazole exhibited by C. krusei, it is not known why this species is less susceptible to amphotericin B than C. albicans. The survival experiments correlated with the fungal burden observed in the larvae. Reduction of the fungal burden was very significant during C. albicans with all the antifungals. In contrast, in larvae infected with C. krusei, fluconazole had no effect on CFUs and the effect of amphotericin B was less pronounced than in larvae inoculated with C. albicans. These data indicate that larvae response is less dynamic during C. krusei infection, which poses a limitation to perform pharmacodynamic studies in this infection model. Similar findings have been found in mammalian models. In immunosuppressed mice, fluconazole does not protect during C. krusei infection and amphotericin B had a partial effect, while anidulafungin treatment resulted in almost full survival of the animals [79]. In agreement, in another study, fluconazole had a very moderate effect in reducing fungal burden in neutropenic mice compared to other azoles, such as sauvanconazole [64]. The use of antifungal combinations has not been sufficiently explored to study the pharmacodynamics response during C. krusei infection, and we believe that non mammalian models might offer a simple and easy procedure to evaluate this important issue.

Caenorhabditis elegans is also useful to test antifungal efficacy against several pathogenic fungi, including Candida spp and Fusarium spp [44,80]. We found very similar results with C. elegans, and these results are comparable with the ones found in G. mellonella. This finding is important in the context of our work, because we have been able to reproduce very similar results using two different and independent host models. Despite the differences in the immune responses between nematodes and insects, C. krusei and C. albicans were virulent in both hosts. These results strongly support the use of these hosts as screening models. Interestingly, we could not find significant differences in the virulence of these species in C. elegans, in contrast to the results found in G. mellonella, where C. albicans was more virulent than C. krusei. We believe that this difference between the behavior of the different yeast species in G. mellonella and C. elegans is the temperature at which the virulence experiments are performed, which is significantly lower in C. elegans.

Understanding fungal pathogenesis and the antifungal discovery are key aspects in medical mycology. Non-conventional models represent an important alternative for in vivo studies, even in the case of organisms that present low virulence in mammalian systems, such as C. krusei. Our results also demonstrate the feasibility of non-mammalian models to identify new antifungal compounds against resistant species. The correlation of the virulence of pathogenic fungi in mammals and non-mammalian models is still unclear. However, there is increasing evidence that
some virulence phenotypes are reproduced in invertebrate models. For this reason, we believe that more studies to validate the full use of these hosts are required in the future.

Acknowledgments
We thank Raquel Pérez Tavárez for the histology experiments.

References
1. Pfäffer MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20: 133–165.
2. Almirante B, Rodriguez, D. Park BJ, Cuenca-Estrella M, Planas AM, et al. (2005) Epidemiology and predictors of mortality in cases of Candida bloodstream infection: results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. J Clin Microbiol 43: 1829–1835.
3. Chen S, Slavin M, Nguyen Q, Marriott D, Playford EG, et al. (2006) Active surveillance for candidemia, Australia. Emerg Infect Dis 12: 1508–1516.
4. Lavelle AJ, Miek ST, Roubinian N, Kollef MH (2008) Treatment-related risk factors for hospital mortality in Candida bloodstream infections. Crit Care Med 36: 2967–2972.
5. Pfäffer MA, Diekema DJ (2010) Epidemiology of invasive mycoses in North America. Crit Rev Microbiol 36: 1–53.
6. Colombo AL, Tobon A, Restrepo A, Queiroz-Telles F, Nucci M (2011) Epidemiology of endemic systemic fungal infections in Latin America. Med Mycol 49: 785–798.
7. Warnock DW (2006) Fungal diseases: an evolving public health challenge. Med Mycol 44: 692–705.
8. Shorr AF, Gupta V, Sun X, Johannes RS, Spalding J, et al. (2009) Burden of early-onset candidemia: analysis of culture-positive bloodstream infections from a large U.S. database. Crit Care Med 37: 2519–2526; quiz 2535.
9. Eggimann P, Garbino J, Pinet D (2003) Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. Lancet Infect Dis 3: 685–702.
10. Colombo AL, Nucci M, Park BJ, Nouer SA, Arthington-Skaggs B, et al. (2006) Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. J Clin Microbiol 44: 2816–2823.
11. Shao PL, Huang LM, Hsueh PR (2007) Recent advances and challenges in the clinical management of human infections caused by Candida krusei. Antimicrob Agents Chemother 40: 2443–2446.
12. Abbas J, Bodey GP, Hanna HA, Mardani M, Girgawy E, et al. (2000) Different classes of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect 18: E246–E248.
13. Arendrup MC (2010) Epidemiology of invasive candidiasis. Curr Opin Crit Care 16: 445–452.
14. Peman J, Canton E, Quindos G, Eraso E, Alcoba J, et al. (2012) Epidemiology, genotypic diversity, and antifungal susceptibility of Candida krusei isolated in Spain. Mycoses 55: 785–798.
15. Trick WE, Fridkin SK, Edwards JR, Hajjeh RA, Gaynes RP (2002) Secular trends in nosocomial candidemia and in vitro antifungal susceptibility of Candida albicans. J Infect Dis 186: 143–150.
16. Desalermos A, Fuchs BB, Mylonakis E (2012) Selecting an invertebrate model for infection by Candida species: review of the caupofungin database. Antimicrob Agents Chemother 56: 3940–3951.
17. Rodríguez-Tudela JL, Alcazar-Fuoli L, Cuesta I, Alastruey-Izquierdo A, Rodríguez-Creixems M, et al. (2010) Caspofungin use in patients with invasive candidiasis caused by common non-albicans Candida species: review of the caspofungin database. Antimicrob Agents Chemother 54: 1086–1087.
18. Steenbergen JW, Shuman HA, Casadevall A (2001) Cryptococcus neoformans interactions with amoeba suggest an explanation for its virulence and intracellular pathogenicity in macrophages. Proc Natl Acad Sci U S A 98: 15425–15426.
19. Rodríguez-Tudela JL, Nosanchuk JD, Casadevall A (2003) Cryptococcus neoformans virulence is enhanced after growth in the genetically maleable host Drosophila melanogaster. Infect Immun 71: 4892–4872.
20. Mylonakis E, Ausubel FM, Perfect JR, Hirtman J, Calderwood SB (2002) Killing of Candida albicans by Cryptococcus neoformans as a model of yeast pathogenesis. Proc Natl Acad Sci U S A 99: 15675–15680.
21. Glavas-Blohm J, Municchio M, Mylonakis E (2012) Of model hosts and man: using Candida albicans, Drosophila melanogaster and Galleria mellonella as model hosts for infectious disease research. Adv Exp Med Biol 710: 11–17.
22. Aralar AM, Marcal A, Chen J, Suter B, Thomas D, et al. (2004) Immune-deficient Drosophila melanogaster: a model for the innate immune response to human fungal pathogens. J Immunol 172: 5622–5629.
23. Patton AL, Sun D (2003) Immune function of mosquito immune cells using in culture. Insect Biochem Mol Biol 33: 263–273.
24. Boman HG, Hultmark D (1987) Cell-free immunity in insects. Annu Rev Microbiol 41: 103–129.
25. Alvarado-Ramirez E, Torres-Rodriguez JM, Sellart M, Vidotto V (2008) Determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2-phosphate decarboxylase by complementation of S. cerevisiae ura3 y. Infect Immun 71: 4862–4872.
26. Berg J, Fuchs BB, Aperis G, Muy TT, Ausubel FM, et al. (2007) Antifungal chemical compounds identified using a C. elegans pathogenicity assay. PLoS Pathog 3: e18.
27. Tamm K, Rivas MA, Lachance C, et al. (2005) Killing of Candida albicans by Cryptococcus neoformans as a model of yeast pathogenesis. Proc Natl Acad Sci U S A 102: 1931–1936.
28. Pfäffer MA, Diekema DJ, Persing DH, Chenoweth D, Hsu WC, et al. (2005) Determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect 11: 142–151.
29. Rodrigues-Tudela JL, Arendrup MC, Barchiesi F, Bille J, Chryssanthou E, et al. (2010) Caspofungin use in patients with invasive candidiasis caused by common non-albicans Candida species: review of the caspofungin database. Antimicrob Agents Chemother 56: 3940–3951.
51. Williamson PR (1994) Biochemical and molecular characterization of the diphenol oxidase of Cryptococcus neoformans: identification as a laccase. J Bacteriol 176: 656–664.

52. Sulston J, Hodgkin J (1988) The Nematode Caenorhabditis elegans. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

53. Fuchs BB, Eby J, Nobile CJ, El Khoury JB, Mitchell AP, et al. (2010) Role of filaments in Galleria mellonella killing by Candida albicans. Microbes Infect 12: 480–486.

54. Ben-Ami R, Lewis RE, Kontoyiannis DP (2008) Immunocompromised hosts: immunopharmacology of modern antifungals. Clin Infect Dis 47: 226–233.

55. Kelly J, Kavanagh K (2011) Caspofungin primes the immune response of the larvae of Galleria mellonella and induces a non-specific antimicrobial response. J Med Microbiol 60: 189–196.

56. Mesa-Arango AC, Scorzoni L, Zaragoza O (2012) It only takes one to do many jobs: Amphoterin, C. krusei and immunomodulatory drug. Frontiers in Immunology 3: 286.

57. Mylonakis E, Casadevall A, Ausubel FM (2007) Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. PLoS Pathog 3: e1002074.

58. Garcia-Rodas R, Casadevall A, Rodriguez-Tudela JL, Cueca-Estrella M, Zaragoza O (2011) Cryptococcus neoformans capsular enlargement and cellular gigantism during Galleria mellonella infection. PLoS One 6: e214415.

59. Pukkila-Worley R, Ausubel FM, Mylonakis E (2011) Candida albicans infection of Caenorhabditis elegans induces antifungal immune defenses. PLoS Pathog 7: e1002747.

60. Jackson JC, Higgins LA, Lin X (2009) Conidiation color mutants of Aspergillus fumigatus are highly pathogenic to the heterologous insect host Galleria mellonella. PLoS One 4: e51224.

61. Arrendrup M, Horn T, Frimodt-Moller N (2002) In vivo pathogenicity of eight medically relevant Candida species in an animal model. Infection 30: 286–291.

62. Cotter G, Doyle S, Kavanagh K (2000) Development of an insect model for the in vivo pathogenicity testing of yeasts. FEBS J Immunol Med Microbiol 27: 163–169.

63. Karyotakis NC, Anaissie EJ, Hachem R, Dignani MC, Samonis G (1993) Comparison of the efficacy of polyenes and triazoles against hematogenous infection in neutropenic mice. J Infect Dis 168: 1311–1313.

64. Majithiya J, Sharp A, Parmar A, Denning DW, Warm PA (2009) Efficacy of isavuconazole, voriconazole and fluconazole in temporarily neutropenic murine infection. J Antimicrob Chemother 63: 161–166.

65. Fuchs BB, Bishop LR, Kovacs JA, Mylonakis E (2011) Galleria mellonella are resistant to Phanerochaete fumigatus infection. Mycopathologia 171: 273–277.

66. Mylonakis E, Moreno R, El Khoury JB, Iltumur A, Heitman J, et al. (2005) Galleria mellonella as a model system to study Cryptococcus neoformans pathogenesis. Infect Immun 73: 3842–3850.

67. Coleman JJ, Muhammed M, Kasperkovic PV, Vyas JM, Mylonakis E (2011) Fusicatia pathogenesis investigated using Galleria mellonella as a heterologous host. Fungal Biol 115: 1279–1289.

68. Feldmesser M, Tucker S, Casadevall A (2001) Intracellular parasitism of macrophages by Cryptococcus neoformans. Trends Microbiol 9: 273–278.

69. Garcia-Rodas R, Zaragoza O (2012) Catch me if you can: phagocytosis and killing avoidance by Cryptococcus neoformans. FEMS Immunol Med Microbiol 64: 147–161.

70. Mesa-Arango AC, Forastiero A, Bernal-Martinez L, Cueca-Estrella M, Mellado E, et al. (2012) The non-mammalian host Galleria mellonella can be used to study the virulence of the fungal pathogen Candida tropicalis and the efficacy of antifungal drugs during infection by this pathogenic yeast. Med Mycol.

71. Rowan R, Moran C, McCann M, Kavanagh K (2009) Use of Galleria mellonella larvae to evaluate the in vivo anti-fungal activity of [Ag2(mal)phen]3. Biometals 22: 461–467.

72. Bidla G, Hauling T, Dushay MS, Theopolis U (2009) Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors. J Innate Immun 1: 301–308.

73. Anaissie EJ, Karyotakis NC, Hachem R, Dignani MC, Rex JH, et al. (1994) Correlation between in vitro and in vivo activity of antifungal agents against Candida species. J Infect Dis 170: 304–309.

74. Coleman JJ, Ghosh S, Okoi I, Mylonakis E (2011) Antifungal activity of microbial secondary metabolites. PLoS One 6: e25321.

75. Dealearmos A, Muhammed M, Glavis-Bloom J, Mylonakis E (2011) Using C. elegans for antimicrobial drug discovery. Expert Opin Drug Discov 6: 645–652.

76. Coleman JJ, Okoi I, Tegos GP, Holson EB, Wagner FF, et al. (2010) Characterization of plant-derived saponin natural products against Candida albicans. ACS Chem Biol 3: 321–332.

77. Dandro M, Scalzito MM, Morace G (2004) In vivo activity of voriconazole and other antifungal agents against clinical isolates of Candida glabrata and Candida krusei. Eur J Clin Microbiol Infect Dis 23: 619–624.

78. Munoz P, Sanchez-Somolinos M, Alcala L, Rodriguez-Creixems M, Pelaez T, et al. (2003) Candida krusei fungaemia: antifungal susceptibility and clinical presentation of an uncommon entity during 13 years in a single general hospital. J Antimicrob Chemother 55: 188–193.

79. Ostrosky-Zeichner L, Paetznick VL, Rodriguez J, Chen E, Sheehan DJ (2009) Activity of anidulafungin in a murine model of Candida krusei infection: evaluation of mortality and disease burden by quantitative tissue cultures and measurement of serum (1,3)-beta-D-glucan levels. Antimicrob Agents Chemother 53: 1639–1641.

80. Muhammed M, Fuchs BB, Woo MP, Berger J, Coleman JJ, et al. (2012) The role of mycelium production and a MAPK-mediated immune response in the C. elegans-Fusicatia model system. Med Mycol 50: 488–496.