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Development of an insect model for the in vivo pathogenicity testing of yeasts

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Abstract

Conventional in vivo assays to determine the relative pathogenicity of yeast isolates rely upon the use of a range of mammalian species. The purpose of the work presented here was to investigate the possibility of using an insect (*Galleria mellonella*) as a model system for in vivo pathogenicity testing. The haemolymph of *G. mellonella* larvae was inoculated with PBS containing different concentrations of stationary phase yeasts of the genus *Candida* by injection at the last pro-leg. Larvae were incubated at 30°C and monitored over 72 hours. Results indicate that *G. mellonella* can be killed by the pathogenic yeast *Candida albicans* and by a range of other *Candida* species but not to a significant extent by the yeast *Saccharomyces cerevisiae*. The kill kinetics for larvae inoculated with clinical and laboratory isolates of *C. albicans* indicate the former class of isolates to be more pathogenic. Differences in the relative pathogenicity of a range of *Candida* species may be distinguished using *G. mellonella* as a model. This work indicates that *G. mellonella* may be employed to give results consistent with data previously obtained using mammals in conventional in vivo pathogenicity testing. Larvae of *G. mellonella* are inexpensive to culture, easy to manipulate and their use may reduce the need to employ mammals for routine in vivo pathogenicity testing with a concomitant reduction in mammalian suffering.

Keywords: Candidosis; Insect; In vivo testing; Pathogenicity; *Candida*; *Galleria*

1. Introduction

A variety of mammalian species have been employed to elucidate the role of the immune system in combating fungal infections [1–6] and have contributed to our understanding of the interactions between pathogenic fungi and host tissue in cases of human disease [7,8]. In addition, animal models have proved invaluable in establishing the pathogenicity of various fungi through in vivo tests [9,10].

The aim of the work presented here was to determine whether an insect could be used to distinguish differences in the relative pathogenicities of various *Candida* species. Such a model would have a number of advantages when compared to the use of mammals for in vivo testing including ease of culture and the absence of mammalian suffering often associated with tests of this nature. Larvae of *Galleria mellonella* (Lepidoptera: *Galleriidae*, the wax moth) have previously been used to examine properties associated with the pathogenicity of wild-type and lipopolysaccharide deficient mutants of *Pseudomonas aeruginosa* [11] and in studies incorporating *Proteus mirabilis* [12], *Escherichia coli* and *Bacillus cereus* [13]. Insects such as *Drosophila melanogaster* and *Spodoptora littoralis* have also been used to quantify the toxicity of secondary metabolites from a number of different *Penicillium* isolates [14]. The cockroach (*Blattella germanica*) has been employed in studies on the pathogenicity of *Aspergillus flavus* [15].

The immune response of insects such as *G. mellonella* is similar to that of mammals in that it consists of structural and passive barriers as well as cellular and humoral responses which are performed by haemocytes within the haemolymph [16]. Six types of haemocytes have been identified in insects and some (plasmatocytes and granulocytes) are involved in phagocytosis, encapsulation and nodule formation [17] which are important elements of the insect's cellular defences against bacteria and unicellular fungi

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[13]. Larger parasites are encapsulated and nodule formation occurs in response to a large number of invading microorganisms [18]. Other humoral factors involved in insect immunity to infection include lysozyme, lectins and the prophenoloxidase cascade [16]. The insect immune response to microorganisms has been shown to involve a change in the circulating haemocyte population and synthesis of new haemolymph proteins [12]. Active haemostatic factors which repair wounds in the cuticle and limit the development of further infection have also been identified [18].

While mammalian models have been used extensively to elucidate the immune response to fungi and the factors affecting the interaction of fungal pathogens with host tissue, they are expensive to use and undoubtedly involve some element of animal suffering. The possibility of using an insect model offers many advantages when compared to conventional mammalian systems for in vivo pathogenicity testing of yeasts. Larvae of *G. mellonella* are easy to culture and inoculate, a large number of insects may be inoculated in a relatively short period of time and results may be obtained within 48–72 h. In addition, insect larvae are inexpensive to purchase and offer the possibility of obtaining data on the relative virulence of pathogenic fungi without the need to use mammals.

2. Materials and methods

2.1. Insect larvae

Sixth instar larvae of *G. mellonella* were obtained from the Meal Worm Company, Sheffield, England. Larvae were stored in wood shavings in the dark at 15°C prior to use. All larvae were used within 3 weeks of receipt.

2.2. Yeast strains and culture conditions

Laboratory isolates of *Candida albicans* used in this study included MEN (Serotype B, originally isolated from an eye infection and a kind gift from Dr. D. Kerridge, Cambridge, UK), ATCC 10231 and ATCC 44990 (*ade*^{r}, *thr*$^{-}$) (obtained from the American Type Culture Collection, Maryland, USA). *C. albicans* 24104, 24135 and 24069 were isolated from symptomatic vaginitis patients [19]. *Candida tropicalis* NCPF 3109, *Candida pseudotropicalis* NCPF 3234, *Candida krusei* NCPF 3100, *Candida parapsilosis* NCPF 3209, *Candida glabrata* NCPF 4733 (obtained from the National Collection of Pathogenic Fungi, Bristol, UK) and *Saccharomyces cerevisiae* JJ1A (*arg*$^{-}$, *thr*$^{-}$) were also employed during the course of this work.

Yeasts were grown in YEPD broth (2% (w/v) glucose (Sigma Chemical Co., Dublin, Ireland), 2% (w/v) bactopeptone (Difco Laboratories, Detroit, USA), 1% (w/v) yeast extract (Oxoid Ltd., Basingstoke, UK) at 30°C and 200 rpm in an orbital incubator. Stocks were maintained on YEPD agar (as above but solidified by the addition of 2% (w/v) agar (Difco)) at 4°C and subcultured every 6–8 weeks.

2.3. Inoculation of insects with Candida species

Candida species were grown to the stationary phase in YEPD broth at 30°C and 200 rpm in an orbital incubator. Yeast cells were harvested by centrifugation in a Beckmann GS-6 centrifuge (5 min, 2300×*g*), washed three times and resuspended in sterile phosphate buffered saline (PBS, 0.01 M, pH 7.4). *G. mellonella* larvae, in groups of ten, were inoculated by injection into the haemocoel with 20 μl sterile PBS containing different concentrations of yeasts through the last pro-leg as described previously [11–13]. The syringe used for inoculation was an SGE 1 ml gas tight syringe (SGE Scientific Pty. Ltd., Melbourne, Australia) with a needle diameter of 0.75 mm.

Three controls were employed in all assays: the first (control) consisted of larvae which were untouched and maintained at the same temperature as the test larvae, the second (injected control) contained larvae whose pro-leg was pierced with the end of the inoculation needle but which were not inoculated with yeast or PBS while the third (PBS control) were inoculated with 20 μl sterile PBS through the last pro-leg. Larvae were placed in sterile petri dishes and incubated in the dark at 30°C in a stationary incubator. Mortality rates were determined over a 72 h period. Larval death was assessed by the lack of movement of larvae in response to stimulation together with discolouration of the cuticle. Stationary phase yeast cells to be heat killed were harvested by centrifugation, washed, resuspended in PBS and subsequently boiled for 20 min. Heat killed cells were washed three times and resuspended in sterile PBS prior to dilution to the appropriate density and inoculation into the test larvae.

Ten larvae were employed per treatment and assays were performed on three independent occasions. Results represent the mean percentage survival of larvae following the combination of the data from all experiments.

2.4. Recovery of C. albicans from G. mellonella larvae

Cells of *C. albicans* MEN were recovered from cadavers of *G. mellonella* larvae that had been incubated for 48 h following inoculation with yeast. Haemolymph samples were collected onto glass slides and immediately heat fixed by passing through a flame 3–4 times. Samples were stained with 1% (w/v) crystal violet solution for 20 min. This was washed from the slides with excess distilled water and samples were stained with Lugol's iodine for 1 min. The Lugol's iodine was washed off with excess water and 2–3 drops of acetone were placed on the samples for 5 s. The presence of *C. albicans* in the sample was determined microscopically.

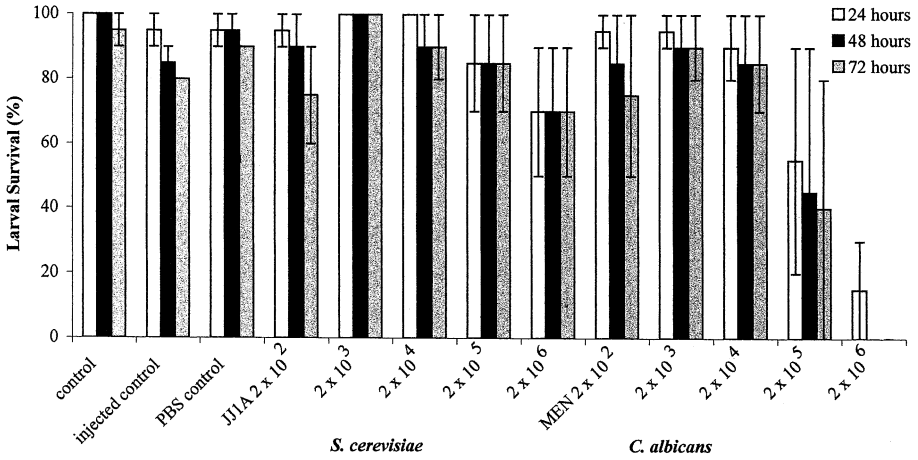

Fig. 1. Percentage survival of *G. mellonella* larvae following inoculation with *C. albicans* or *S. cerevisae*. Larvae were inoculated at the last pro-leg and incubated at 30°C for 72 h. All values represent the mean ± standard deviation of data from three independent experiments.

3. Results

3.1. Pathogenicity of C. albicans and S. cerevisiae in G. mellonella larvae

In vivo pathogenicity tests should be able to distinguish between pathogenic and non-pathogenic isolates and to detect differences in the relative pathogenicities of related microbes. In order to establish whether *G. mellonella* larvae would be capable of demonstrating differences in the pathogenicity of virulent and non-virulent yeasts initial experiments sought to examine the response of larvae to inoculation with *C. albicans* and *S. cerevisiae*. The pathogenicity of *C. albicans* is well established [1–3,7] and although the pathogenicity of some isolates of *S. cerevisiae* has been recognised in recent years it is generally regarded that this yeast is, at best, a weak pathogen with low levels of virulence [9,20].

Larvae were inoculated through the last pro-leg with 20 μl of PBS containing 2×10^2, 2×10^3, 2×10^4, 2×10^5 or 2×10^6 stationary phase cells of either species. Larvae were incubated at 30°C and the mortality rates were determined over a 72 h period. The results (Fig. 1) indicate that mortality rates were higher when larvae were inoculated with *C. albicans* MEN than with *S. cerevisiae* JJ1A at cell concentrations of 2×10^5 and 2×10^6 per 20 μl inoculum. The greatest mortality rates were observed in treatments incorporating *C. albicans* MEN with total larval kill being recorded at an inoculum of 2×10^6 following 48 h incubation. In contrast, the mortality rate for larvae inoculated with *S. cerevisiae* at the same cell concentration was 30% at 48 h incubation. Experiments incorporating isolates of *S. cerevisiae* associated with disease in humans also revealed insignificant levels of larval death (data not presented). At the lower inoculum densities (2×10^2 to 2×10^4 yeasts per 20 μl aliquot) mortality rates for the two yeast species were comparable. Low levels of larval death occurred in the three sets of controls which may have resulted from the unavoidable handling of the larvae in the initial stages of the assays.

The appearance of untreated *G. mellonella* larvae and those inoculated with *C. albicans* MEN following incubation for 48 h is presented in Fig. 2. The dark colour of larvae that have been infected with yeast is associated with encapsulation and the subsequent production of the pigment melanin, an immune response by the insect to non-self matter such as fungi and bacteria [21].

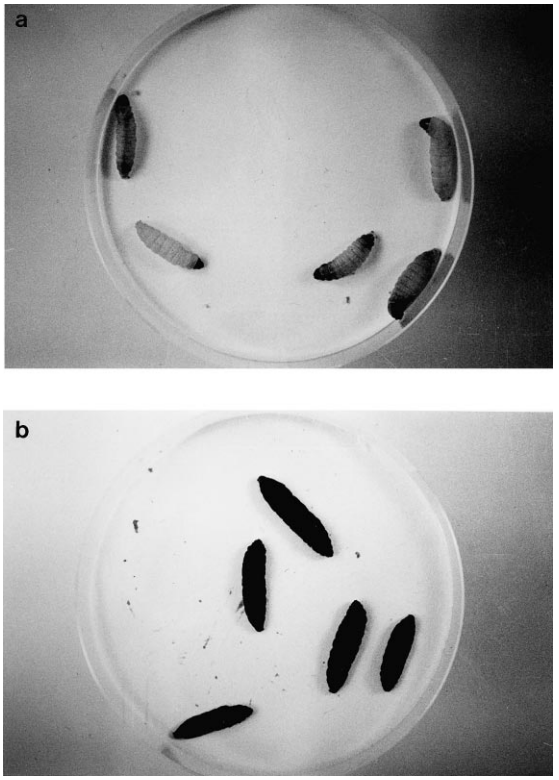

Fig. 2. *G. mellonella* larvae before (a) and after inoculation with 2×10^6 *C. albicans* for 48 h (b). The discolouration of the cadavers in b is due to melanisation. (Scale: ×0.8.)

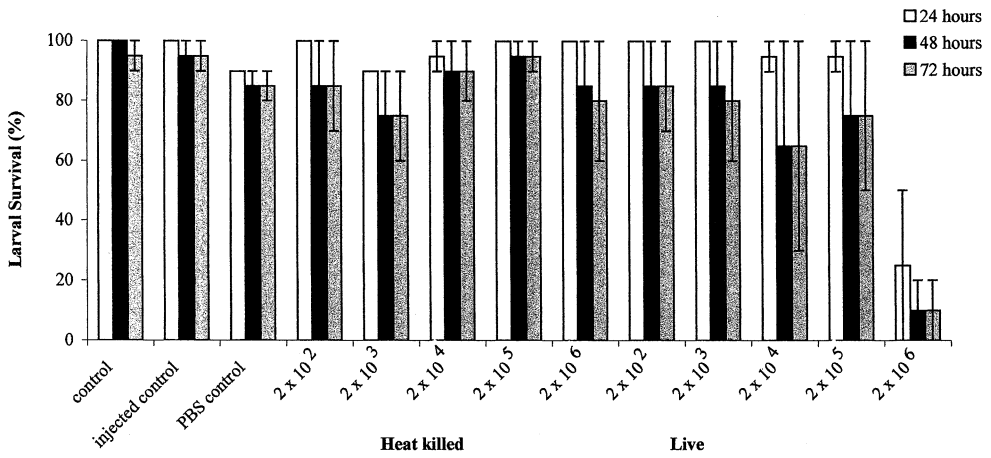

Fig. 3. Percentage survival of *G. mellonella* larvae following inoculation with live or heat killed *C. albicans* MEN. Larvae were inoculated with between 2×10^2 and 2×10^6 viable or heat killed cells and incubated at 30°C for 72 h. Values represent the mean ± standard deviation of data from three independent experiments.

3.2. Virulence of live and heat killed C. albicans MEN in G. mellonella

Larvae of *G. mellonella* may be used to distinguish between a pathogenic yeast (*C. albicans*) and a non-pathogenic yeast (*S. cerevisiae*) in in vivo tests. One possibility for the death of larvae inoculated with *C. albicans* was the development of an allergic reaction to the presence of a large number of yeast cells in the insect's haemolymph [16]. To examine this possibility, larvae were inoculated with a range of concentrations of heat killed and viable *C. albicans* MEN in 20 μl volumes.

The results indicate (Fig. 3) that mortality rates were greater at the higher cell concentrations when larvae were inoculated with viable yeast cells. Mortality rates observed for larvae inoculated with viable *C. albicans* MEN at concentrations of 2×10^6, 2×10^5, 2×10^4 and following 72 h incubation were 90%, 20% and 30% respectively. Corresponding values obtained using heat killed *C. albicans* MEN were 20%, 5% and 10% respectively. These data strongly suggest that the ability of *C. albicans* to kill larvae of *G. mellonella* requires the presence of viable yeast cells and that death is not due to the onset of an allergic reaction to the presence of a large number of yeast cells in the haemolymph.

Melanisation is an important element of the insect's immune response to the presence of *C. albicans* (see Fig. 2b), however the fate of *C. albicans* in the insect's haemolymph remains to be established. *C. albicans* is a dimorphic yeast being capable of interconverting between a budding and a hyphal or filamentous mode of growth and both forms are evident in human tissue infected with *C. albicans* [2,7,22]. Cells of *C. albicans* MEN were recovered from *G. mellonella* in order to determine whether cells remained in the budding phase or whether hyphal forms were the predominant growth form.

Following incubation of larvae that had been inoculated with 2×10^6 *C. albicans* MEN for 48 h, haemolymph was obtained from dead larvae by piercing the cuticle with a sterile needle. Samples were placed on glass slides, heat fixed and stained as described (Section 2.4). Examination of slides (Fig. 4) revealed that *C. albicans* was present in the haemolymph of *G. mellonella* indicating that not all yeasts were encapsulated by the insect's immune response. The majority of yeast were budding with very few hyphal forms evident indicating that *C. albicans* in the haemolymph retains the budding mode of growth and does not form hyphae to a significant extent.

3.3. Virulence of clinical and laboratory isolates of C. albicans in larvae of G. mellonella

Clinical isolates of *C. albicans* exhibit more pronounced virulence levels than isolates that have been cultured for long periods in the laboratory [22]. Larvae of *G. mellonella* were inoculated with cells of clinical and laboratory iso-

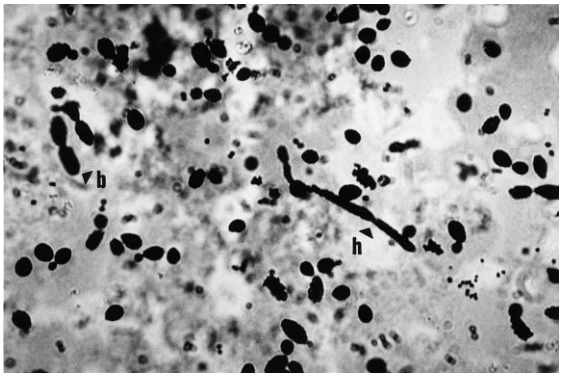

Fig. 4. Photomicrograph of cells of *C. albicans* present in insect haemolymph 48 h after inoculation. Haemolymph was extracted from insect cadavers heat fixed, and stained with crystal violet and Lugol's iodine. Budding cells (b) and a hyphal form (h) are visible. (Magnification ×1000.)

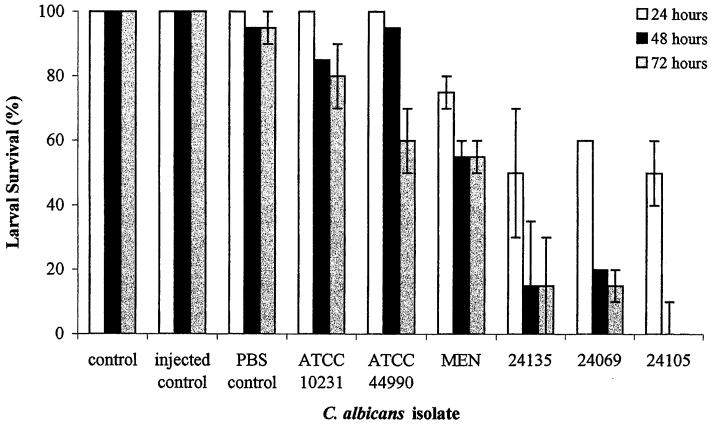

Fig. 5. Percentage survival of *G. mellonella* larvae following inoculation with laboratory or clinical isolates of *C. albicans*. Larvae were inoculated with 2×10^6 cells and incubated at 30°C for 72 h. Values represent the mean ± standard deviation of data from three independent experiments.

lates of *C. albicans* to determine whether differences in pathogenicity could be distinguished using this model. A number of clinical isolates, obtained from patients with symptoms of vulvovaginal candidosis [19], and laboratory isolates, which had been cultured in the laboratory for at least 3 years, were selected for this work.

Clinical and laboratory isolates of *C. albicans* were grown to the stationary phase in YEPD broth overnight at 30°C and 200 rpm in an orbital incubator. Cells were harvested by centrifugation, washed with PBS and introduced into *G. mellonella* larvae at the last pro-leg at a density of 2×10^6 per 20 μl aliquot. The results (Fig. 5) reveal that greater and faster larval mortality rates were observed when the three clinical isolates of *C. albicans* were employed. Following 72 h incubation, mortality rates for larvae inoculated with the clinical isolates 24135, 24069 and 24105 were 100%, 85% and 85% respectively. Mortality rates for larvae inoculated with the laboratory isolates ATCC 10231, ATCC 44990 and MEN after 72 h incubation were 20%, 40% and 50% respectively.

3.4. Comparison of pathogenicity of a number of Candida species in larvae of G. mellonella

C. albicans is the most pathogenic yeast of the genus *Candida* [22]. The non-*albicans Candida* species vary in their pathogenicity and a hierarchy has been shown to exist in terms of the ability to cause disease in mammals [23–25]. Experiments were performed incorporating various *Candida* species to determine if differences in the pathogenicity of non-*albicans Candida* species could be detected using *G. mellonella* larvae.

A range of *Candida* species frequently associated with disease in humans were selected and these varied from the highly pathogenic (e.g. *C. albicans*) [7,22,24] to those of low or negligible pathogenicity (e.g. *C. krusei*) [25]. Sta-

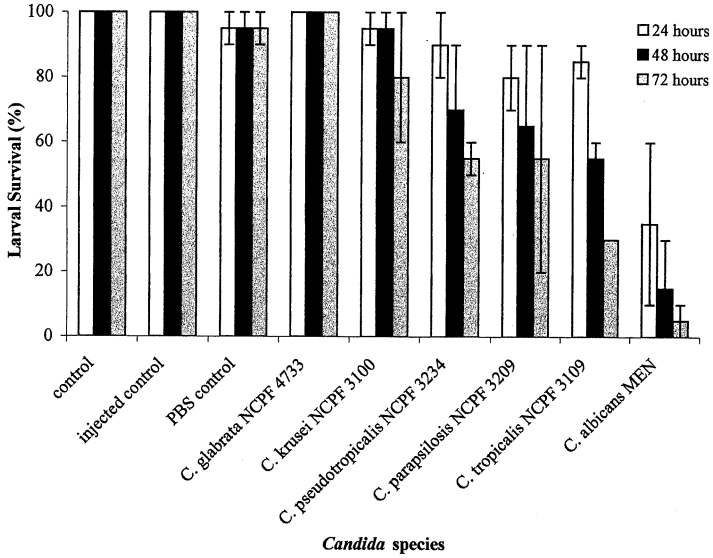

Fig. 6. Percentage survival of *G. mellonella* larvae following inoculation with a range of *Candida* species. Larvae were inoculated with 2×10^6 cells and incubated at 30°C for 72 h. Values represent the mean ± standard deviation of data from three independent experiments.

tionary phase yeast cells were prepared as described and larvae were inoculated with 2×10^6 cells of each species at the last pro-leg. The results (Fig. 6) indicate the existence of a hierarchy among the *Candida* species with *C. albicans* proving the most pathogenic of the species tested. Following an incubation period of 72 h mortality rates for larvae inoculated with *C. albicans* were 90% while those for the other species were: *C. tropicalis*: 70% mortality, *C. parapsilosis*: 45%, *C. pseudotropicalis*: 45%, *C. krusei*: 20% and *C. glabrata*: 0%. A similar trend is also evident after 48 h incubation with those larvae inoculated with *C. albicans* again showing the highest mortality rates.

4. Discussion

A number of mammalian models have been used to determine the relative pathogenicity of medically important fungi [1,2,5,26]. The data presented here indicate that *G. mellonella* larvae can be employed to differentiate between pathogenic and non-pathogenic yeast species and to demonstrate differences in the pathogenicity of yeasts of the genus *Candida*. *C. albicans* MEN kills larvae to a greater extent than the non-pathogenic *S. cerevisiae* JJ1A and the differences in relative pathogenicity may be explained by the more extensive array of virulence factors exhibited by *C. albicans* [22,27]. Heat killed cells of *C. albicans* MEN were shown to be less pathogenic than viable yeast which indicates that the expression of factors associated with viable yeast may be necessary to kill larvae. These data also indicate that larval death is not due to the development of an allergic response as a result of the presence of large numbers of yeast cells in the insect's haemolymph [16].

Assays incorporating both clinical and laboratory isolates of *C. albicans* indicated that the clinical isolates had a greater ability to kill *G. mellonella*. These observations indicate that the greater mortality rates observed for *G. mellonella* larvae when inoculated with clinical isolates of *C. albicans* may be due to the elevated expression of virulence factors [7,22]. Results obtained using *G. mellonella* as an in vivo model indicate the existence of a hierarchy among *Candida* species with respect to their ability to kill larvae of *G. mellonella*, i.e. *C. albicans* > *C. tropicalis* > *C. parapsilosis* > *C. pseudotropicalis* > *C. krusei* > *C. glabrata*, which reflects the hierarchy observed in the ability of these species to cause disease in a variety of mammals [25] and also their predominance as human pathogens [23,24,28]. The data obtained here confirm that it is possible to distinguish between different levels of pathogenicity within the genus *Candida* using *G. mellonella* larvae.

The dominant growth morphology of *C. albicans* in insect haemolymph is the budding form although a small percentage of hyphal forms have been observed. Both growth morphologies are observed in human tissue infected with *C. albicans* [22]. The exact means employed by the yeast isolates to kill insect larvae has yet to be elucidated as has the relative importance of specific virulence factors in overcoming the insect's immune response.

Larvae of *G. mellonella* may provide an attractive and reproducible model compared to other commonly used systems for investigating the relative pathogenicities of *Candida* species. Larvae are easy to culture under sterile conditions and can be manipulated with ease. Large numbers can be inoculated per assay and results are obtainable within 2 to 3 days. This model system will not provide data on the role of the mammalian immune system in combating pathogenic yeasts nor will it provide information on the interaction of pathogenic yeasts with mammalian tissue but it will allow a primary analysis of the relative pathogenicity of a variety of yeast pathogens. This system should have applications where the pathogenicity of yeast mutants needs to be established or where large numbers of yeast isolates must be screened. It could be used as an initial screen before conventional in vivo testing or to minimise the need for routine mammalian testing.

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