Caenorhabditis elegans as a model system to assess *Candida glabrata*, *Candida nivariensis* and *Candida bracarensis* virulence and antifungal efficacy

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Abstract

Although *Candida albicans* remains the major etiological agent of invasive candidiasis, *Candida glabrata* and other emerging species of *Candida* are increasingly isolated. This species is the second most prevalent cause of candidiasis in many regions of the world. However, clinical isolates of *Candida nivariensis* and *Candida bracarensis* can be misidentified and are underdiagnosed due to shared phenotypic traits with *C. glabrata*. Little is known about both cryptic species. Pathogenesis studies are therefore needed to understand their virulence traits and their susceptibility to antifungal drugs. The susceptibility of *Caenorhabditis elegans* to different *Candida* species makes this nematode an excellent model for assessing host–fungal interactions. We evaluated the usefulness of *C. elegans* as a nonconventional host model to analyze the virulence of *C. glabrata*, *C. nivariensis* and *C. bracarensis*. The three species caused candidiasis and the highest virulence of *C. glabrata* was confirmed. Furthermore, we determined the efficacy of current antifungal drugs against the infection caused by these species in the *C. elegans* model. Amphotericin B and azoles showed the highest activity against *C. glabrata* and *C. bracarensis* infections, while echinocandins were more active for treating those caused by *C. nivariensis*. *C. elegans* proved to be a useful model system for assessing the pathogenicity of these closely related species.

1. Introduction

Invasive candidiasis is the most frequent mycosis, mainly in patients suffering from immunodeficiency. Although *Candida albicans* remains the predominant etiological agent, there is an increase in infections caused by other *Candida* species, such as *Candida parapsilosis*, *Candida glabrata*, *Candida krusei* and *Candida auris*, which has been associated with reduced antifungal susceptibility or even increased rates of resistance (1, 2, 3). Among these species, *C. glabrata* has been considered the second or third most isolated species of *Candida* from blood cultures according to geographical distribution. This species is a frequent cause of candidemia in the USA, Australia and North and Central Europe, and there is a trend toward an etiological rise in Spain and Portugal (2, 4, 5, 6). *C. glabrata* invasive infection treatment is often a clinical challenge due to the increasing prevalence of azole resistance. Although echinocandins are considered the treatment of choice (7), *C. glabrata* is also the species most likely to be resistant to echinocandins (8, 9).

C. glabrata sensu stricto shares high phenotypic similarities and genetic closeness with *Candida bracarensis* and *Candida nivariensis*. As of yet, the reported incidence of *C. bracarensis* and *C. nivariensis* is low, and data about their virulence and antifungal susceptibility are unclear (5, 10, 11). Among several virulence factors, production of hydrolytic enzymes such as hemolysins or secreted phospholipases and aspartyl proteinases are considered important virulence factors contributing to the pathogenesis of candidiasis (12, 13, 14).

Invertebrate models are promising alternatives to mammals in the study of invasive candidiasis because they provide great advantages considering ethical issues, costs and physiological simplicity. The nematode *Caenorhabditis elegans* is one of these models successfully applied to advance the knowledge of *Candida* infection pathogenesis. This worm is approximately 1 mm in length, and transparent, and has a short reproductive cycle of 2-4 days and life span of 2-3 weeks. Moreover, its genome has been sequenced, and a wide variety of mutant strains are available (15, 16, 17). However, few studies have analyzed the utility of this model host to

63 assess the virulence of *Candida* species and antifungal efficacy for candidiasis (18, 19, 20, 21).
64 In particular, to the best of our knowledge, this nonconventional model has never been applied
65 to study the pathogenesis of candidiasis caused by *C. glabrata* and other phylogenetically
66 closely-related species.

67 Within this framework, we were interested in assessing the utility of the *C. elegans* host model
68 to study, for the first time, the pathogenesis of *C. glabrata*, *C. nivariensis* and *C. bracarensis*.
69 For this purpose, we determined that this nonconventional infection model can be applied to
70 determine the virulence behavior of these three phylogenetically related species in vivo.
71 Furthermore, we evaluated the in vivo antifungal efficacies of amphotericin B, echinocandins
72 and several azoles using the *C. elegans* model and tried to correlate them with their in vitro
73 susceptibility profiles.

74

75 **2. Materials and methods**

76 **2.1. Microorganisms and growth conditions**

77 Reference strains of *Candida* used to carry out the experiments are detailed in Table 1. They
78 include two reference strains of each species of the *C. glabrata* complex: *C. glabrata* ATCC
79 90030 and NCPF 3203, *C. nivariensis* CBS 9984 and CECT 11998, and *C. bracarensis* NCYC
80 3397 and NCYC 3133. These strains were cultured in yeast extract peptone dextrose (YEPD;
81 1% yeast extract, 2% bacteriological peptone, 2% D-glucose) liquid medium (Panreac, Spain) at
82 30 °C for 18 h under shaking conditions. The double mutant *C. elegans* AU37 strain (*glp-*
83 *4*(*bn2*); *sek-1*(*km4*)) used in this study was obtained from the Caenorhabditis Genetics Center
84 (University of Minnesota, USA). This double mutation increases the susceptibility to microbial
85 infections (*sek-1*) and maintains a constant number of sterile worms at 25 °C (*glp-4*). The *C.*
86 *elegans* strain was propagated at 15 °C on nematode growth medium (NGM) agar plates
87 previously seeded with the nonpathogenic strain OP50 of *Escherichia coli*, which was used as a

food source for the nematodes. The experiments were performed with a synchronous population of worms in the L4 larval stage obtained as previously described by Ortega-Riveros et al. (20).

2.2. Production of phospholipase, proteinase and hemolytic activity

The production of phospholipases and proteinases and the hemolytic activity of these phylogenetically related species were analyzed. Phospholipase activity was tested following the method described by Polak (22) but using malt agar plates containing 1 M NaCl, 5 mM $CaCl_2$ and 8% sterile egg-yolk emulsion (23). To evaluate the production of aspartyl proteinase, solid medium containing bovine serum albumin (Sigma-Aldrich Inc., USA) was used as described by Cassone et al. (12). The phospholipase activity was defined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone. The proteinase activity was established as the estimated diameter of the lytic area around the growth of the strain. Finally, the hemolytic activity was studied using the methodology described by Luo et al. (13) but using the plate assay described by Manns et al. (24).

2.3. In vitro antifungal susceptibility

The antifungal efficacy of seven antifungal drugs against the six strains of *C. glabrata*, *C. nivariensis* and *C. bracarensis* was tested. The drug concentration ranged from 0.03 to 16 µg/ml for amphotericin B (AmB) (Sigma-Aldrich Inc., USA), anidulafungin (AND) (Pfizer SA, Madrid, Spain), caspofungin (CAS) (Merck and Com Inc., NJ, USA), micafungin (MCF) (Astellas Pharma Inc., Japan), posaconazole (PCZ) (Merck & Com Inc., NJ, USA) and voriconazole (VCZ) (Pfizer SA, Madrid, Spain). The concentration ranged from 0.12 to 64 µg/ml for fluconazole (FCZ) (Pfizer SA, Madrid, Spain). The minimum inhibitory concentration (MIC) of the antifungal drugs against each strain was determined according to the methodology described for yeasts in documents M27-A3 and M27-A3/S4 from the Clinical Laboratory Standards Institute (25, 26). Type strains obtained from the American Type Culture Collection (ATCC), *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality controls for in vitro antifungal susceptibility testing.

2.4. *Caenorhabditis elegans* infection

The assays were performed as previously described by Breger et al. (27). *C. elegans* populations were placed for 2 h at 25 °C on brain heart infusion (BHI) agar plates (Panreac, Spain) seeded with lawns of the different *Candida* strains, allowing the worms to ingest them. Afterward, the nematodes were washed with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 ml of 1 M $MgSO_4$ and H_2O to 1 l) supplemented with kanamycin (90 µg/ml) and placed on NGM agar plates to remove the yeast cells from their cuticles. Then, the nematodes were transferred in groups of 20 worms to each well of microtiter plates that contained M9 buffer supplemented with kanamycin and 10 µg/ml cholesterol in ethanol. Sixty nematodes were used to study the mortality caused by each strain of *Candida*, and groups of uninfected nematodes were included as controls in each experiment. Microtiter plates were incubated at 25 °C and visually scored as live or dead nematodes every 24 h using a stereomicroscope (Nikon SMZ-745, Japan) for the subsequent 120 h. All experiments were conducted at least in triplicate on different days.

2.5. Antifungal treatments

To evaluate the effect of antifungal drugs against *Candida* infection, previously infected L4 nematodes were treated with concentrations of 8 µg/ml AND, 4 and 8 µg/ml CAS and MCF, 32, 64 and 128 µg/ml FCZ, or 1 and 2 µg/ml VCZ, PCZ and AmB.

The stock solutions of CAS, FCZ and MCF were prepared in water, while AmB, AND, PCZ and VCZ were prepared in 1% dimethyl sulfoxide (DMSO) following the instructions of the manufacturer. Different concentrations of antifungal drugs were prepared and added to the microtiter plates, and in each condition, 60 nematodes were included. In each experiment, 14 different treatments were assessed for each strain, and groups of infected but untreated nematodes were also analyzed in the presence and absence of 1% DMSO as controls to test the effect of DMSO. At least 960 nematodes were assayed for each strain and experiment. Microtiter plates with nematodes under different conditions were incubated at 25°C, and

survival was visually monitored every 24 h for the subsequent 120 h. All experiments were conducted at least in triplicate on different days.

2.6. Statistics

Survival analysis curves were prepared by the Kaplan-Meier method with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The long-rank test with the statistical program SPSS v24.0 (IBM, Chicago, IL, USA) was applied to estimate the differences in the survival of *C. elegans* infected with the different *Candida* strains and conditions ($p<0.05$ was considered statistically significant).

3. Results

3.1. Characterization of *Candida* strains: enzymatic activity and in vitro antifungal susceptibility

Phospholipase and proteinase production and hemolytic activity were studied to analyze the virulence traits of these species. No phospholipase or proteinase activity was detected in any of the strains tested. However, alpha (partial) hemolysis was observed in all strains, except the *C. glabrata* ATCC 90030 strain, which showed gamma hemolysis (no hemolysis).

The in vitro antifungal activities against *C. glabrata*, *C. nivariensis* and *C. bracarensis* are summarized in Table 2. All six strains of these three closely related species were susceptible to all antifungal drugs tested. MICs of the quality controls were within the published ranges.

3.2. Survival of *Caenorhabditis elegans* infected with *Candida*

The ability of the three closely related species to develop infection in *C. elegans* was assessed (Figure 1). All six strains of *Candida* were able to kill *C. elegans* and showed statistically significant differences with the survival of uninfected nematodes, which remained nearly constant throughout the experiment (99.6% survival at 120 h). It took at least two days to detect

nematodes killed by any of the three *Candida* species. Although the *C. nivariensis* CECT 11998 strain caused higher initial mortality, the *C. glabrata* ATCC 90030 strain was the most lethal at 120 h. Only the *C. glabrata* ATCC 90030 strain achieved a mortality rate of more than 50% at 120 h. Moreover, the *C. glabrata* ATCC 90030 strain was significantly more virulent than the NCPF 3203 strain (p=0.001). There were also differences between the two strains of *C. bracarensis* (p=0), which is the species that killed the lowest percentage of nematodes, but not between the two strains of *C. nivariensis* (Table 1).

We also evaluated the ability of these six *Candida* strains to cause infection in the presence of DMSO in the medium. A significant 4.4% reduction in the viability of uninfected nematodes in the presence of DMSO was detected compared to that in the absence of DMSO (95.2% and 99.6% survival at 120 h, respectively, p=0). The survival percentages of *C. elegans* infected with *Candida* were also lower in the presence of DMSO. However, the presence of DMSO in the medium, with respect to its absence, resulted in significantly lower survival of the nematodes at 120 h infected with either strain of *C. glabrata* (p<0.001) or with the *C. bracarensis* NCYC 3397 strain (p=0), but not with the remaining strains (Table 1). Moreover, when DMSO was in the medium, significant survival differences were detected between the uninfected nematodes and those infected with any of the *Candida* strains except for the *C. bracarensis* NCYC 3133 strain (p=0.98).

Overall, our findings indicate the following virulence categorization of these three *Candida* species in the *C. elegans* model: *C. glabrata* > *C. nivariensis* > *C. bracarensis*.

3.3. Antifungal therapy efficacy for candidiasis in *Caenorhabditis elegans*

Nematodes infected with each of the six *Candida* strains were treated with three antifungal drugs prepared in water (CAS, FCZ and MCF) and with four prepared in DMSO (AmB, AND, PCZ and VCZ) at different concentrations.

We detected that, with respect to that of infected and untreated *C. elegans*, all antifungal drugs significantly reduced the mortality of *C. elegans* during *C. glabrata* infection. However, the

189 reduction in nematode mortality during infections caused by *C. nivariensis* and *C. bracarensis*
190 was drug- and strain-dependent (Figure 2).

191 The antifungal drugs prepared in water achieved a nematode survival of up to 96.8% (value
192 obtained with 8 µg/ml MCF) in the treatment of *C. glabrata* infection, and those prepared in
193 DMSO reached a nematode survival of no more than 85.2% (with 2 µg/ml AmB) (Table 1).
194 Nevertheless, these antifungal drugs in DMSO managed to reduce the mortality for a higher
195 percentage of nematodes. When the nematodes following infection with the *C. glabrata* ATCC
196 90030 strain were treated with 8 µg/ml MCF or 1 µg/ml AmB, a higher worm mortality
197 reduction was obtained (51.2% for both). For nematodes infected with the NCPF 3203 strain
198 and treated with 8 µg/ml MCF or 2 µg/ml AmB, the mortality was reduced by 31.4% and
199 40.1%, respectively (Figure 2).

200 Among the antifungal drugs prepared in water, 8 µg/ml MCF was the most effective for treating
201 infections caused by either *C. glabrata* strain (Table 1). During ATCC 90030 strain infection,
202 significant differences in survival were detected when the worms were treated with 8 µg/ml
203 MCF or FCZ at all concentrations tested (p=0.006) but not when comparing to other drugs (4
204 µg/ml MCF and both concentrations of CAS) that also resulted in effective treatments.
205 However, against NCPF 3203 strain infection, MCF (8 µg/ml) was significantly more effective
206 than all the other antifungal drugs except FCZ (128 µg/ml), which was similarly effective
207 (96.8% and 94.6% survival at 120 h, respectively). AmB (1 and 2 µg/ml) resulted in the highest
208 percentage of *C. elegans* survival. Nevertheless, no significant differences were observed
209 between these and the other antifungal drugs prepared in DMSO against the infection of either
210 *C. glabrata* strain, except for the treatment with VCZ (1 µg/ml) (p=0.015) or AND (8 µg/ml)
211 (p=0) against the infection of the ATCC 90030 strain and AND (8 µg/ml) (p=0.001) against
212 NCPF 3203 strain infection. These latter drugs allowed the least number of worms to survive
213 (Figures 2).

MCF and CAS (8 µg/ml) were effective in protecting against *C. nivariensis* and *C. bracarensis* infections (Table 1). Although the treatment using any of the antifungal drugs prepared in water significantly protected against *C. nivariensis* CBS 9984 strain infection, echinocandins produced the highest *C. elegans* survival results. The same was observed for treating *C. nivariensis* CECT 11998 strain infection, except that with the lowest doses of FCZ (32 and 64 µg/ml), no differences were detected in worm survival compared to that of infected and untreated *C. elegans* (Figure 2). AmB (2 µg/ml), among those prepared in DMSO, was the most effective against *C. nivariensis* CBS 9984 strain infection, and together with VCZ (1 µg/ml), these drugs significantly increased worm survival compared to the survival of infected and untreated worms. The other drugs did not reduce the mortality of *C. elegans* (Figure 2). No treatment prepared in DMSO significantly increased the survival rate of *C. elegans* infected with the *C. nivariensis* CECT 11998 strain compared to that of infected and untreated worms.

C. bracarensis was the least virulent of the three *Candida* species, and the survival of nematodes infected with either strain was so high in the absence of DMSO that it was difficult to evaluate the efficacy of some treatments. Treatment with the antifungal drugs prepared in water increased the survival of nematodes infected with either strain but did not achieve a significant improvement in worm survival, likely due to the low effect of the infection. On the other hand, the antifungal drugs prepared in DMSO resulted in increased survival of *C. elegans* infected with the NCYC 3397 strain, with 1 µg/ml VCZ achieving the highest worm survival (96.3%) (p=0). However, no drugs achieved protection against *C. elegans* infection with the *C. bracarensis* NCYC 3133 strain.

4. Discussion

C. elegans has been explored as an alternative model for characterizing host–fungal interactions. Most studies of invasive candidiasis using this host model focus on the infection caused by *C. albicans* (15, 28), and few studies involve other *Candida* species (18, 19, 20, 21). The

emergence of *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. auris*, among others responsible for invasive candidiasis, makes both the study of the pathogenesis and worldwide surveillance of these species necessary (2). The actual epidemiology of emergent species that cause candidiasis, such as *C. glabrata* and the phylogenetically closely related species *C. nivariensis* and *C. bracarensis* is still unknown. Multiple studies have reported misidentified isolates of these cryptic species (29, 30, 31, 32, 33). Molecular approaches based on PCR, sequencing or MALDI-TOF MS are increasingly being applied because of their success in identifying rare *Candida* species. Therefore, enhanced knowledge of *C. glabrata* and closely related species improves the diagnosis and choice of the most appropriate antifungal treatment.

The six strains of *C. glabrata*, *C. nivariensis* and *C. bracarensis* used in this study were able to infect and kill *C. elegans*. Despite current knowledge of the pathogenic potential of *Candida* using in vivo models, no studies have yet been published examining the pathogenesis of *C. nivariensis* and *C. bracarensis*. Nevertheless, there are sound data on the capacity of these cryptic species to cause infection in humans (10, 11, 29, 34, 35, 36, 37, 38, 39). Fairly little is known about virulence factors of *C. glabrata*. We did not detect proteinase, phospholipase or hemolytic activities in any of the six *Candida* strains tested. Several studies compared different protocols for analyzing the production of enzymes, such as phospholipase, highlighting the limitations in their detection (14, 40). Therefore, the absence of these virulence factors would need to be confirmed by further analysis. However, it has been demonstrated that adhesins, including proteins of the Epa family, are involved in virulence and are highly present at the cell surface of *C. glabrata* (41). The pathogenic potential of *C. nivariensis* and *C. bracarensis* could be explained, among others reasons, by the high numbers of *EPA* genes detected in these two species but not in other nonpathogenic species of the *Nakaseomyces* clade to which these species belong (42, 43).

In our study, *C. glabrata* was the most virulent species, and *C. bracarensis* was the least virulent species in *C. elegans*, which is coincident with the incidence of cases of these species in the literature (30, 31, 44, 45). Virulence studies of *C. glabrata* developed in in vivo models

reported that approximately 30% of mice infected with 1×10^5 CFU per mouse survived up to 19 days post infection (46). However, no more than three days were necessary to kill *Galleria mellonella* larvae with an infective dose of 2.5×10^6 cells per larva (47). This difference highlighted the effect of the infective dose, although the specific characteristics of each host model also have to be considered (48). One of the limitations in the *C. elegans* model is not being able to control the precise infective dose administered, so the time employed in the infection of nematodes is one of the factors to consider (20). In the present work, the survival percentages of *C. elegans* at 120 h were lower in the presence of 1% DMSO. This effect of DMSO was observed, to a greater or lesser extent, in the survival of nematodes infected with the six *Candida* strains and even in uninfected nematodes used as controls. The addition of DMSO when the eggs hatch has been shown to have a beneficial effect on the longevity of *C. elegans*. However, nematodes should be in this first stage of life and not in the adult stage after egg laying because DMSO could alter the membrane fluidity of worms, affecting the exchange of metabolites and external molecules (49). This phenomenon could be a potential explanation for the decrease in nematode survival observed.

Notably, the antifungal drugs prepared in DMSO managed to reduce the mortality of a high percentage of nematodes infected with either strain of *C. glabrata* or with the *C. bracarensis* NCYC 3397 strain, indicating their effectiveness despite the toxicity of the drug. AmB was very effective against these strains of *C. glabrata* and *C. bracarensis*, as the highest percentages of reduction in nematodes mortality were achieved. This polyene also showed good results in other studies in vitro against *C. glabrata* and *C. bracarensis* (5, 50) and in treating *C. glabrata* infection in *G. mellonella* (47) and murine models (51). Moreover, AmB was also effective in combination with VCZ for the treatment of persistent *C. glabrata* candidemia (9). In our study, the activities of VCZ and PCZ against these three *Candida* strains were similar; both drugs achieved a similar increase in worm survival percentage in each *Candida* strain infection. In vivo studies conducted in murine models also exhibited the same improvement for both antifungal drugs against *C. glabrata* infection (52, 53). In vitro susceptibility of *C. glabrata* and

294 *C. bracarensis* to these second-generation triazoles has been reported, although reduced
295 susceptibility of *C. glabrata* (5, 54) and *C. bracarensis* (30, 38) isolates has also been described.

296 Regarding *C. nivariensis*, none of the drugs in DMSO increased the survival of nematodes
297 infected with either of the two strains studied by more than 10%; despite the susceptibility
298 detected in vitro. Several in vitro studies showed the susceptibility of this species to AmB,
299 AND, PCZ and VCZ (30, 32, 39, 55, 56); however, *C. nivariensis* isolates with reduced
300 susceptibility or resistance to FCZ, VCZ or PCZ have also been reported (36, 37, 57).

301 Echinocandins are the treatment of choice for *C. glabrata* invasive infections, but there are
302 reports of *C. glabrata* isolates with resistance to these drugs (3, 58, 59). Resistance has been
303 associated with echinocandin exposure and increased use in clinical practice (2, 9, 56, 60). In
304 time-kill studies, all echinocandins were less active against *C. nivariensis* (61). However, our
305 findings showed that in the *C. elegans* model, CSF and MCF were the most effective treatments
306 against *C. nivariensis* infections and were also very effective against *C. glabrata* infections. The
307 low virulence of *C. bracarensis* strains in this host model made it difficult to assess the effect of
308 these two drugs. Nevertheless, AND showed good results against infection with both *C.*
309 *bracarensis* NCYC 3397 and *C. glabrata* NCPF 3203 strains. AND was reported as the least
310 effective echinocandin against *C. glabrata* infection in mice (62). Conversely, in a rabbit model
311 of candidiasis, AND was more effective than liposomal AmB for treating *C. glabrata* infection
312 associated with catheter colonization (63). The effectiveness of CSF was reported as an
313 adequate treatment for *C. nivariensis* catheter-related fungemia (10), and it was also the most
314 effective echinocandin in a murine model of *C. glabrata* infection with a dose of 1 mg/kg or 20
315 mg/kg (62, 64, 65, 66, 67). The treatment with CSF at this last dose in a murine model showed
316 the appearance of *C. glabrata* strains harboring *FKS* mutations after five to nine days of
317 treatment (8). Moreover, in a *G. mellonella* model of *C. glabrata* infection, 4 µg/g CSF
318 increased larval survival by 60% (47). MCF presents a low interlaboratory MIC variability
319 compared to that of CSF, and its clinical use is widespread compared to that of AND (68).
320 However, *C. glabrata* isolates resistant to MCF have been reported (3, 9). Likewise, susceptible

isolates of *C. glabrata*, *C. nivariensis* and *C. bracarensis* were also detected in several in vitro studies (30, 38, 39, 56).

Finally, FCZ is an antifungal drug frequently used in the treatment of invasive candidiasis, but increasing acquired resistance of *C. glabrata* has been reported (2). No protective effect against *C. glabrata* infection was observed in *G. mellonella* treated with 3, 6 or 12 µg/mg FCZ (47). However, in a murine model, treatment with high doses of FCZ (from 30 to 100 mg/kg FCZ) was required to achieve a significant decrease in the fungal burden (51, 52, 69). Although all strains were susceptible to FCZ in vitro, the highest doses of FCZ (64 or 128 µg/ml) were needed to detect a significant increase in the survival of *C. elegans* infected with *C. glabrata* or *C. nivariensis*. FCZ-resistant *C. nivariensis* isolates have been reported (36, 37), as well as susceptible isolates (39).

In conclusion, *C. elegans* was an appropriate and simple infection model to study the virulence of *C. glabrata* and the closely related species *C. nivariensis* and *C. bracarensis*. *C. glabrata* was the most virulent species. Moreover, this model system was successfully used for in vivo screening of antifungal drugs against infections caused by these three *Candida* species. However, the effect of antifungal treatments against *C. bracarensis* strains was sometimes compromised due to the low virulence of this species, and therefore, other models are needed where the infective dose can be more accurate.

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614 **Table 1.** Survival of *Caenorhabditis elegans* infected with each of the six *Candida* strains used in this study and evaluation of antifungal treatment.

Strain	Origin	Collection reference	Survival of *C. elegans* at 120 h in absence / presence of DMSO	The three most effective antifungal drugs (survival of *C. elegans* at 120 h)	
				Dissolved in water	Dissolved in DMSO
Candida glabrata					
ATCC 90030	Blood	American Type Culture Collection	40.3% / 26.5%	Micafungin, 8 µg/ml (91.5%) Micafungin, 4 µg/ml (90.6%) Caspofungin, 4 µg/ml (89.6%)	Amphotericin B, 1 µg/ml (82.4%) Voriconazole, 2 µg/ml (82.1%) Posaconazole, 2 µg/ml (81.5%)
NCPF 3203	Blood	National Collection of Pathogenic Fungi	65.4% / 45.1%	Micafungin, 8 µg/ml (96.8%) Fluconazole, 128 µg/ml (94.6%) Caspofungin, 8 µg/ml (91.8%)	Amphotericin B, 2 µg/ml (85.2%) Voriconazole, 2 µg/ml (83.8%) Voriconazole, 1 µg/ml (83.7%)
Candida nivariensis					
CECT 11998	Blood	Colección Española de Cultivos Tipo	72.9% / 65.3%	Micafungin, 8 µg/ml (88.3%) Caspofungin, 8 µg/ml (86.4%) Caspofungin, 4 µg/ml (86.2%)	Voriconazole, 1 µg/ml (64.8%) Voriconazole, 2 µg/ml (63.5%) Amphotericin B, 1 µg/ml (57.2%)
CBS 9984	Bronchoalveolar lavage	Westerdijk Fungal Biodiversity Institute	75% / 73%	Caspofungin, 8 µg/ml (94.9%) Micafungin, 8 µg/ml (94%) Micafungin, 4 µg/ml (92.7%)	Amphotericin B, 2 µg/ml (83.2%) Voriconazole, 1 µg/ml (81.2%) Amphotericin B, 2 µg/ml (74.7%)
Candida bracarensis					
NCYC 3133	Catheter	National Collection of Yeast Cultures	89.4% / 89.4%	Caspofungin, 8 µg/ml (94.5%) Caspofungin, 4 µg/ml (94.4%) Micafungin, 8 µg/ml (92.6%)	Amphotericin B, 1 µg/ml (80.1%) Voriconazole, 2 µg/ml (76%) Amphotericin B, 2 µg/ml (75.8%)
NCYC 3397	Blood	National Collection of Yeast Cultures	97.6% / 70.8%	Caspofungin, 8 µg/ml (100%) Micafungin, 4 µg/ml (99.6%) Micafungin, 8 µg/ml (99.5%)	Voriconazole, 1 µg/ml (96.3%) Posaconazole, 2 µg/ml (96.1%) Amphotericin B, 2 µg/ml (95.4%)

615 DMSO: 1% dimethyl sulfoxide

616 **Table 2.** In vitro antifungal activity of caspofungin (CAS), micafungin (MCF), anidulafungin
617 (AND), amphotericin B (AmB), posaconazole (PCZ), voriconazole (VCZ) and fluconazole
618 (FCZ) against *Candida glabrata*, *Candida nivariensis* and *Candida bracarensis* strains.

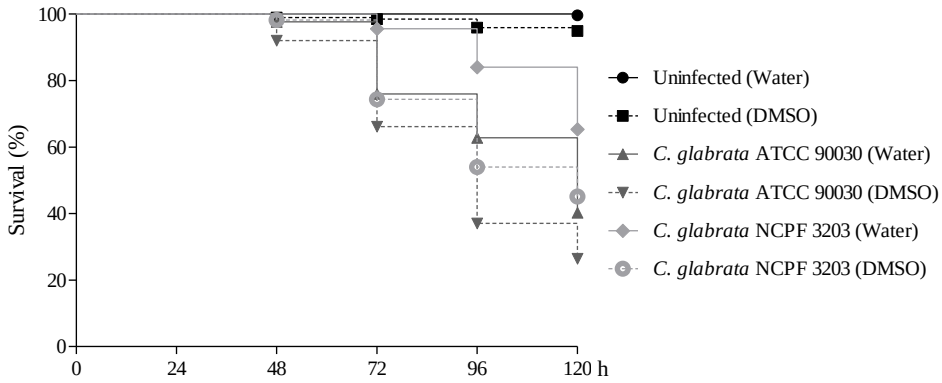
Strain	MIC (µg/ml)						
	CAS	**MCF**	**AND**	**AmB**	**PCZ**	**VCZ**	**FCZ**
C. glabrata ATCC 90030	0.5	0.03	0.06	1	1	0.5	8
C. glabrata NCPF 3203	0.25	0.03	0.06	1	0.5	0.25	4
C. nivariensis CBS 9984	0.25	0.03	0.06	2	0.5	0.06	8
C. nivariensis CECT 11998	0.25	0.03	0.06	2	0.5	0.12	4
C. bracarensis NCYC 3397	0.25	0.03	0.06	1	1	0.12	4
C. bracarensis NCYC 3133	0.25	0.03	0.06	2	1	0.12	4

619 MIC: minimum inhibitory concentration

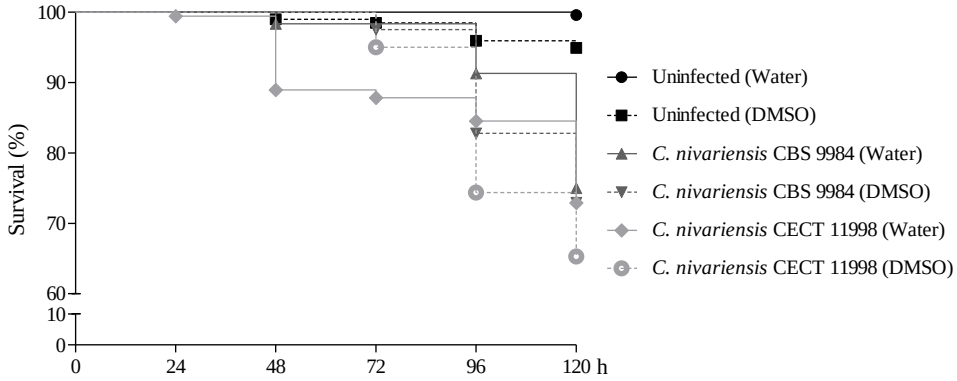
620

Figure 1. Survival curves of *Caenorhabditis elegans* infected with strains of *Candida glabrata* (a), *Candida nivariensis* (b) or *Candida bracarensis* (c) in the absence (water) or presence of 1% dimethyl sulfoxide (DMSO).

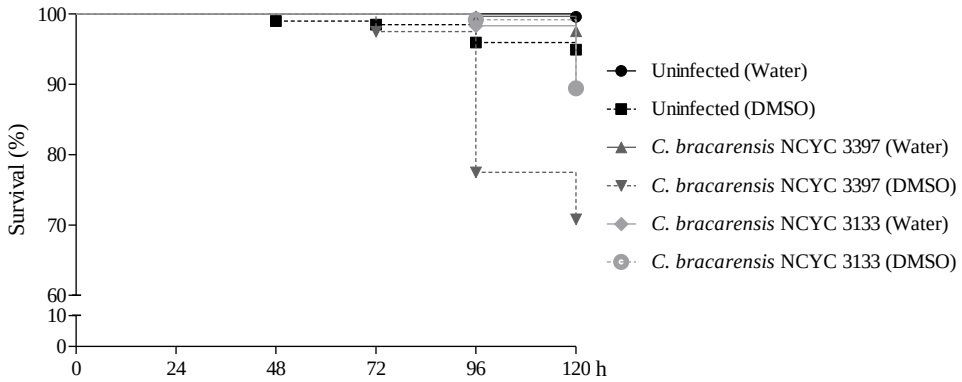
a)



b)



634 **c)**



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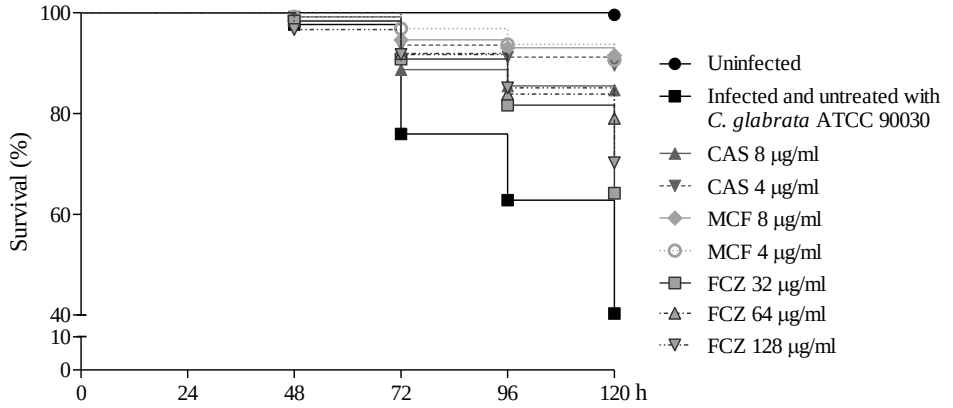
638 **Figure 2**. Efficacy of the antifungal drugs at different concentrations during *Caenorhabditis*
639 *elegans* infection with *Candida glabrata* ATCC 90030 (a), *Candida glabrata* NCPF 3203 (b),
640 *Candida nivariensis* CBS 9984 (c), *Candida nivariensis* CECT 11998 (d) or *Candida*
641 *bracarensis* NCYC 3397 (e). The antifungal drugs fluconazole (FCZ), caspofungin (CAS) and
642 micafungin (MCF) were prepared in water, while amphotericin B (AmB), anidulafungin
643 (AND), posaconazole (PCZ) and voriconazole (VCZ) were prepared in 1% dimethyl sulfoxide
644 (DMSO).

645 **a) *Caenorhabditis elegans* infection with *C. glabrata* strain ATCC 90030**



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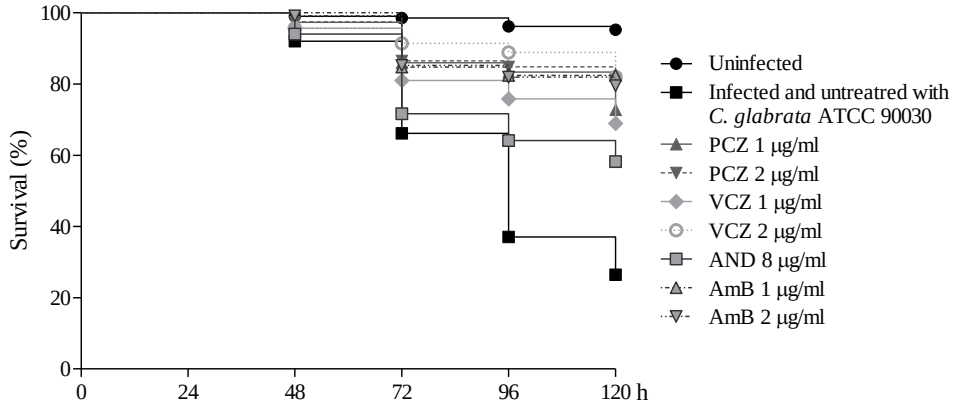

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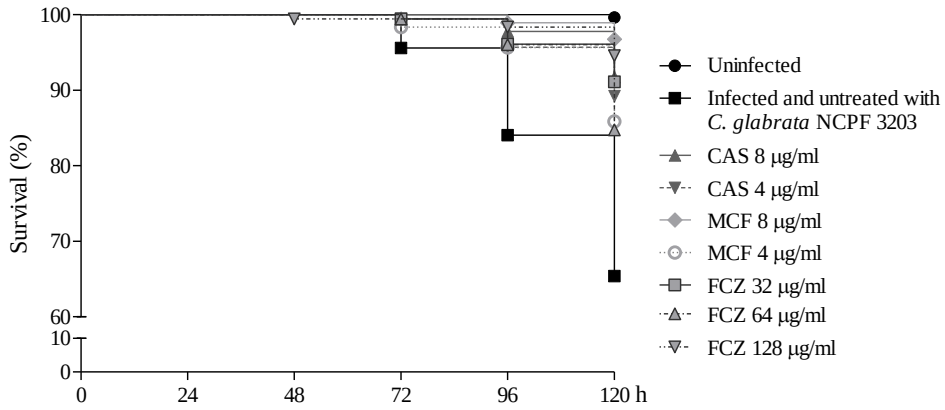
651 **b) *Caenorhabditis elegans* infection with *C. glabrata* strain NCPF 3203**



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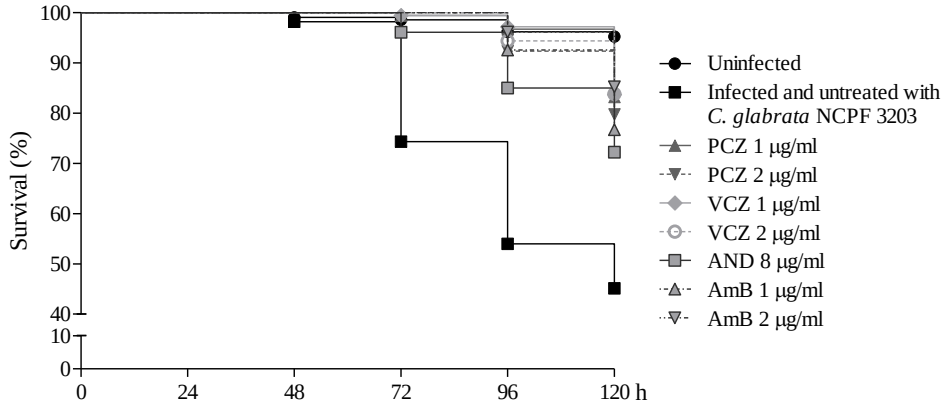

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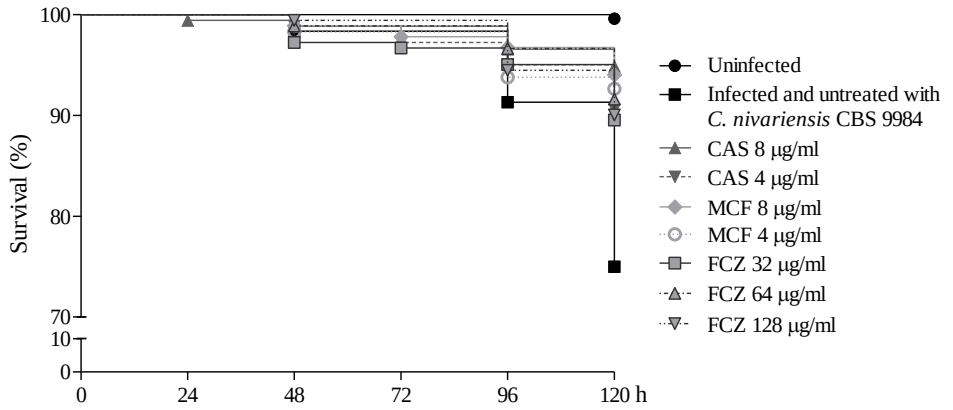
656 **c) *Caenorhabditis elegans* infection with *C. nivariensis* strain CBS 9984**



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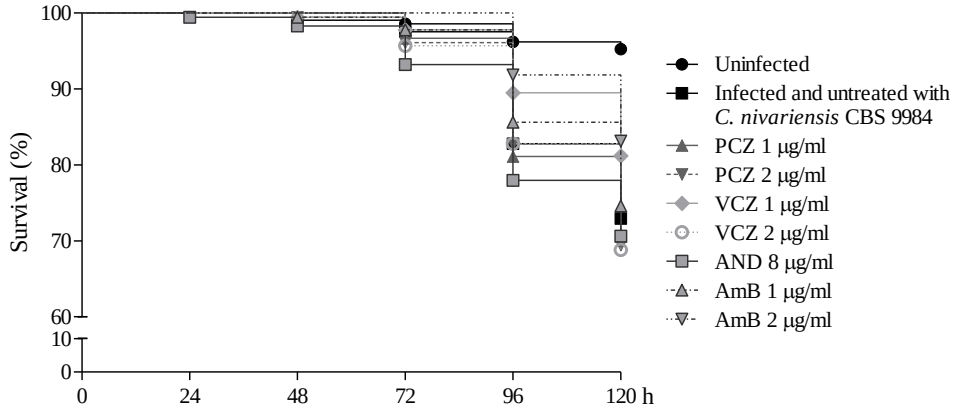

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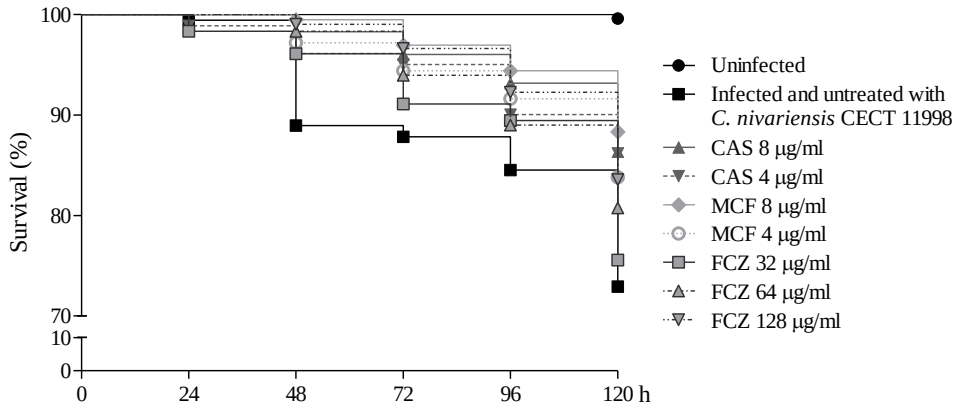
662 **d) *Caenorhabditis elegans* infection with *C. nivariensis* strain CECT 11998**



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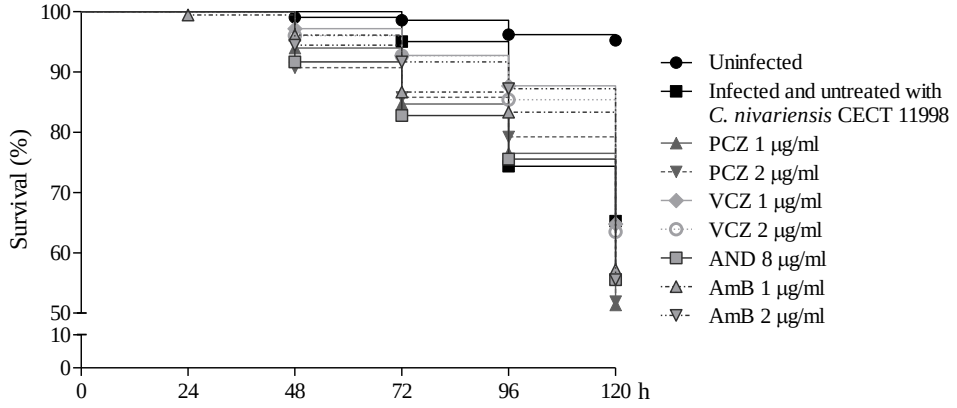

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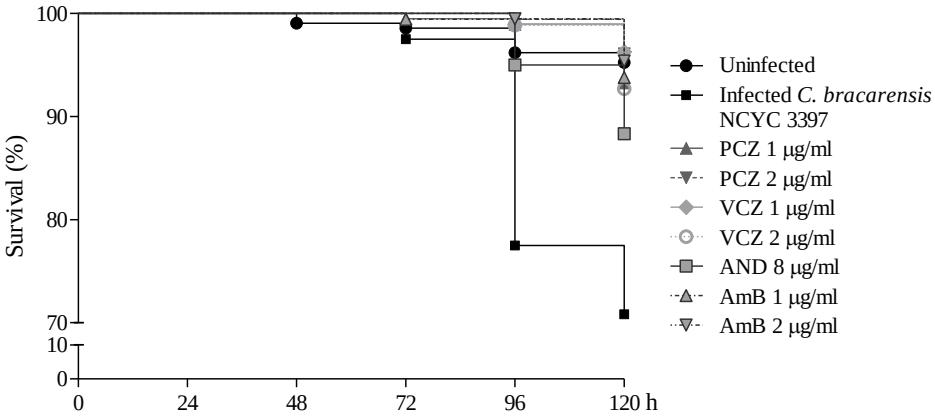
669 **e) *Caenorhabditis elegans* infection with *C. bracarensis* strain NCYC 3397**



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