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TITLE:

Two infection assays to study non-lethal virulence phenotypes in *C. albicans* using *C. elegans*

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Summary

Fungal opportunist pathogens can cause life-threatening as well as minor infections, but non-lethal phenotypes are frequently ignored when studying virulence. Therefore, we developed a nematode model that monitors both the survival and reproduction aspects of host to investigate fungal virulence.

Abstract

While pathogens can be deadly to humans, many of them cause a range of infection types with non-lethal phenotypes. *Candida albicans*, an opportunistic fungal pathogen of humans, is the fourth most common cause of nosocomial infections which results in ~40% mortality. However, other *C. albicans* infections are less severe and rarely lethal and include vulvovaginal candidiasis, impacting ~75% of women, as well as oropharyngeal candidiasis, predominantly impacting infants, AIDS patients and cancer patients. While murine models are most frequently used to study *C. albicans* pathogenesis, these models predominantly assess host survival and are costly, time consuming, and limited in replication. Therefore, several mini-model systems, including *Drosophila melanogaster*, *Danio rerio*, *Galleria mellonella*, and *Caenorhabditis elegans,* have been developed to study *C. albicans*. These mini-models are well-suited for screening mutant libraries or diverse genetic backgrounds of *C. albicans*. Here we describe two approaches to study *C. albicans* infection using *C. elegans*. The first is a fecundity assay which measures host reproduction and monitors survival of individual hosts. The second is a lineage expansion assay which measures how *C. albicans* infection affects host population growth over multiple generations. Together, these assays provide a simple, cost-effective way to quickly assess *C. albicans* virulence.

Introduction

Candida albicans is an opportunistic fungal pathogen of humans residing in different niches, including the oral cavity, gastrointestinal, and urogenital tracts[1]. While typically commensal, *C. albicans* causes both mucosal and bloodstream infections, the latter of which can be deadly. The severity of *C. albicans* infection is dependent on host immune function, with immunocompromised individuals more susceptible to infection than healthy individuals[1]. In addition to host-related factors, *C. albicans* has several virulence traits which include, hyphae, biofilm formation, and production of secretory aspartyl proteinases (SAPs), which function to promote adhesion and invasion of *C. albicans* into host epithelial cells[2], and candidalysin, a cytolytic peptide toxin[3,4]. Together, this suggests that *C. albicans* virulence is a complex phenotype resulting from an interaction between the pathogen and its host environment. Therefore, investigating virulence is best studied using model organisms that serve as host environments, in contrast to in vitro approaches.

Several host models, including both vertebrate and invertebrate organisms, have been developed to study *C. albicans* infection. The murine model, considered the gold standard, is often used for its adaptive and innate immune system, and ability to monitor disease progression both systemically and in specific organs[5]. However, there are significant limitations to this host model, including maintenance costs, small number of offspring, and decreased power and reproducibility associated with small sample sizes[5]. Therefore, other, more simple model organisms such as zebrafish (*Danio rerio*), fruit fly (*Drosophila melanogaster*), wax moth (*Galleria mellonella*), and nematode (*Caenorhabditis elegans*) have been developed. These non-mammalian model organisms are smaller, require less laboratory maintenance and larger sample sizes allow for greater power and reproducibility compared to murine models[6]. Each of these models have specific advantages and disadvantages that need to be considered when choosing an infection model. *G. mellonella* offers the most physiologically similar environment to humans as it can be grown at 37 °C and has various phagocytic cells[7]. Furthermore, this model allows for the direct injection of a specific inoculum[7]. However, there is no fully sequenced genome, and no established method of creating mutant strains. Similar to *G. mellonella*, the *D. rerio* model allows for direct injection of a specific inoculum[5,7]. It also has both adaptive and innate immune systems[5], which is unique to this non-mammalian model, yet requires aquatic breeding tanks to maintain. *D. melanogaster* and *C. elegans* have similar advantages and disadvantages, which include fully sequenced genomes that are easy to manipulate and generate mutant strains[7] but do not have adaptive immunity or cytokines[7]. Of all these non-mammalian models*, C. elegans* has the most rapid life cycle, self-fertilize to generate large numbers of genetically identical offspring, and are the most amenable to large-scale screens[6–8]. *C. elegans* has been extremely powerful for high-throughput screening of antifungal drugs[9,10], characterizing virulence factors[7], and identifying *C. albicans*-specific host defense networks[11]. The innate immune system in *C. elegans* has multiple components that are highly conserved with humans[12]. Host innate defenses include production of antimicrobial peptides[13] (AMPs) and reactive oxygen species[14–16].

The severity of *C. albicans* infection is predominantly measured by host survival but cannot capture non-lethal virulence phenotypes. An often-overlooked aspect of host fitness is reproduction, but several studies suggest that *C. albicans* impacts reproduction by reducing sperm viability[17,18], suggesting that this may be an important aspect of host fitness to study.

Therefore, the impact of *C. albicans* infection on host fecundity is a useful way to study non-lethal virulence phenotypes. We have developed two infection assays using *C. elegans* to investigate both survival and reproduction phenotypes in healthy hosts[19,20]. Here we describe both the fecundity and lineage expansion assays. Fecundity measures both progeny produced and survival of single hosts, and lineage expansion assesses the consequences of infection over three host generations. We demonstrate how these assays can be utilized to screen *C. albicans* deletion mutants to capture both dramatic and subtle differences in lethal and non-lethal virulence phenotypes.

PROTOCOL

1. Preparatory steps for the experiments

1.1. Preparing *C. albicans* and *Escherichia coli* cultures

NOTE: The strains used in this study are listed in **Table 1**.

1.1.1. Maintain *C. albicans* and *E. coli* strains as glycerol stocks at −80 °C.

1.1.2. Using a sterile toothpick, streak desired *C. albicans* strain onto solid yeast peptone dextrose (YPD) (1% yeast extract, 2% bactopeptone, 2% glucose, 1.5% agar, 0.004% adenine, 0.008% uridine) and grow overnight at 30 °C.

NOTE: If the *C. albicans* strain is auxotrophic, supplement the media with the necessary amino acids.

1.1.3. Using a sterile loop or toothpick, inoculate a single *C. albicans* colony into 2 mL of liquid YPD. Incubate at 30 °C with shaking for 24 h.

1.1.4. Using a sterile toothpick, streak *E. coli* (OP50) onto Luria Broth (LB; 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar) agar plates. Incubate overnight at 30 °C.

1.1.5. Inoculate a single OP50 colony into 50 mL of LB. Incubate at 30 °C with shaking overnight.

1.2. Nematode growth media preparation

1.2.1. In a 2 L flask, add 29 g of nematode growth media powder (NGM; 17.5 g/L agar, 3.0 g/L Sodium Chloride, 2.5 g/L Peptone, .005 g/L cholesterol) to 1L of water and mix with a stir bar.

1.2.2. To inhibit *E. coli* overgrowth and allow *C. albicans* proliferation supplement NGM with 0.2 g/L streptomycin sulfate after autoclaving.

NOTE: If using NGM for nematode maintenance, streptomycin is not required.

1.3. Maintaining nematode populations

1.3.1. Spread 300 µL of overnight *E. coli* culture onto prepared NGM agar plates using a metal spreader. This technique will be referred to as seeding.

1.3.2. Allow plates to dry at room temperature (RT). Grow plates overnight at 30 °C.

1.3.3. Maintain nematode population by chunking every 3-4 days onto a newly seeded NGM plate with *E. coli*. Store at 20 °C. Chunking is a technique used to quickly transfer a random population of nematodes to a new fresh plate. To do this, use a sterile spatula to cut a small square (1 x 1 inch) out of the NGM agar plate. Carefully transfer the square to a new seeded NGM plate with the side with the nematodes facing down on the new agar[8].

NOTE: *C. elegans* maintained at 20 °C will produce offspring ~48 h later and useful to consider when synchronizing a population of nematodes. *C. elegans* maintained at 25 °C will develop and reproduce faster and populations maintained at 15 °C will have slower growth.

1.4. Nematode population synchronization

1.4.1. Begin with an existing nematode population maintained on NGM/OP50.

1.4.2. Pipette ~3 mL of M9 buffer onto the NGM plate containing nematodes. Wash nematodes eggs off the plate and gently use the tip of the pipette to scrape eggs off the agar (they tend to stick). Using a P1000 pipette, transfer the liquid containing both eggs and worms to a 15 mL conical tube. To assess the number of eggs still on the plate, use a dissecting microscope to look at the agar.

1.4.3. Centrifuge conical for 2 min at 279 x g and RT.

1.4.4. Remove the supernatant, being careful not to disturb the nematode pellet.

1.4.5. Add 3 mL of 25% bleach solution (“CAUTION” when handling).

1.4.6. Invert tube for 2 minutes. Check that the nematodes are dead using the dissecting microscope - they will be stick-straight and non-motile.

NOTE: This will only kill the existing nematodes. The integrity of the eggs will not be affected.

1.4.7. Centrifuge for 2 min at 279 x g and RT.

1.4.8. Remove the supernatant and resuspend the pellet in 3 mL M9.

1.4.9. Centrifuge for 2 min at 279 x g and RT.

1.4.10. Remove the supernatant and resuspend the pellet in 300 µL of M9.

1.4.11. Using a dissecting microscope, check the concentration of eggs by pipetting 5 µL of eggs onto a small Petri plate. The ideal concentration should be between 20-100 eggs. If the culture is too dilute, concentrate the solution by centrifugation and removal of excess liquid. If the culture is too concentrated, add more M9 the desired concentration is reached.

1.5. Prepare *C. albicans* and *E. coli* cultures for nematode infection (seeding).

1.5.1. Prepare a blank solution. In a cuvette, combine 900 µL of ddH_2O and 100 µL of liquid YPD.

1.5.2. Insert the cuvette into the spectrophotometer. Set the wavelength to 600 nanometers using the up arrow. Click on the button "0 ABS 100% T" to set the blank solution.

1.5.3. In a new cuvette, combine 900 µL of ddH_2O and 100 µL of overnight yeast culture. Take the blank solution out of the spectrophotometer and add the cuvette containing the yeast solution. Record the optical density shown on the screen (do not press any buttons). Multiply the reading by 10 (the yeast solution measured was a 1 in 10 dilution).

1.5.4. Normalize culture to 3.0 OD_{600}/mL with ddH_2O in a 1.5 mL microcentrifuge tube. 1 OD_{600} is approximately 3 x 10^{-7} CFU/mL[21].

NOTE: If the OD_{600} reading is 6.7, 3 OD/6.7 OD= 0.447 mL, add 447 µL of *C. albicans* culture to the microcentrifuge tube. Centrifuge at maximum speed (16, 873 x g) for 30 s. Remove supernatant and resuspend in 1 mL of ddH_2O.

1.5.5. Transfer the overnight *E. coli* culture to a 50 mL conical tube.

1.5.6. Centrifuge the culture at 279 x g for 2 min at RT.

1.5.7. Aspirate a majority of the supernatant, leaving ~1 mL.

1.5.8. Resuspend the pellet in the remaining supernatant and transfer to a pre-weighed 1.5 mL microcentrifuge tube.

1.5.9. Spin down the microcentrifuge tube at maximum speed for 30 s.

1.5.10. Using a p1000 pipette, remove the supernatant and weigh the final pellet.

1.5.11. Dilute *E. coli* to 200 mg/mL in ddH_2O.

1.5.12. Use master mix calculations (Table 2) and scale appropriately.

2. Fecundity assay

NOTE: Representative data in shown in **Supplementary Table 1** and a schematic in shown in **Figure 1A**.

2.1. Obtain or prepare the following: 35 mm x 10 mm Petri plates, NGM supplemented with 0.2 g/L streptomycin sulfate, *E. coli* OP50 culture, LB, *C. albicans* culture, YPD, Wire Pick, M9 buffer (3.0 g/L KH_2PO_4, 6.0 g/L Na_2HPO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl), 15 mL conical tubes.

Two Days Prior to Experiment

2.2. Inoculate *C. albicans* strains in 2 mL of YPD and *E. coli* (OP50) in 50 mL of LB and grow overnight at 30 °C.

2.3. Prepare 35 mm x 10 mm Petri plates with NGM supplemented with 0.2 g/L streptomycin sulfate. The number of plates prepared should last the whole experiment. The recommended number of replicates is 10 per treatment. For 10 replicates, 70 plates will be used. 1 L of NGM will make ~250 plates.

One Day Prior to Experiment

2.4. Seed 35 mm x 10 mm NGM agar plates supplemented with streptomycin for Day 0, Day 2 & Day 3 according to the seeding protocol described above with the mastermix concentration (**Table 2**). Pipette the appropriate amount of mastermix onto the center of the plate. Spreading the culture is not necessary because a single spot of microbial growth is sufficient for host feeding and allows for us to easily identify hosts outside of the seed. Incubate the plates overnight at RT.

NOTE: The Day 0 mastermix contains 50 µL of "seed" per replicate for each experimental treatment. Days 2 -7 include 10 µL of "seed" per replicate for each experimental treatment. There is no Day 1 plate because nematodes will reach the L4 stage 48 h after being synchronized onto a Day 0 plate. Once they reach L4, individual nematodes will be transferred to Day 2 plates.

[Place Table 2 here].

Day of Experiment (Day 0)

2.5. Synchronize nematodes and plate ~50 eggs onto each Day 0 replicate of the control plates (OP50 only) and treatment plates (*C. albicans* + OP50). Incubate at 20 °C for 48 h.

Day 2

2.6. Transfer a single L4 nematode by picking[8] from the Day 0 plate to each of the replicate Day 2 seeded plates. L4 hosts can be identified by a small pocket in the middle of the dorsal side of their body[22]. Transfer the nematodes hatched and matured from the same type of seeded plate (i.e., L4 nematodes from D0 control plates must be transferred to Day 2 control plates).

2.7. Inoculate *C. albicans* cultures needed in 2 mL of YPD and *E. coli* (OP50 strain) in 50 mL of LB.

Day 3

2.8. Transfer nematodes from Day 2 plates to Day 3 seeded plates, keeping track of each replicate (i.e., Replicate A from Day 2 must be moved to the Replicate A Day 3 plate).

2.9. Incubate Day 2 (only containing eggs) and Day 3 (containing the single adult) plates at 20 °C for 24 h.

2.10. Seed 35 mm x 10 mm NGM supplemented with streptomycin plates for Days 4 & 5 using 10 µL of mastermix per plate (**Table 2**) and incubate plates at RT for 24 h.

Day 4

2.11. Transfer nematodes from Day 3 plates to Day 4 seeded plates.

2.12. Incubate Day 3 and Day 4 plates at 20 °C for 24 h.

2.13. Using a dissecting scope, count the viable progeny for each Day 2 plate. Note any replicates that died or are no longer on the plate (censored). Once the number of progeny are recorded, discard Day 2 plates.

NOTE: Censored refers to nematodes that disappear on the plate. This can occur when nematodes crawl off the plate. Although less common during the 24 h window, dead nematodes' carcasses disintegrate into the agar also resulting in censorship. Censored data is not included in the final progeny and survival data analysis.

2.14. Inoculate new cultures of *C. albicans* strains in 2 mL of YPD and *E. coli* (OP50) in 50 mL of LB.

Day 5

2.15. Transfer nematodes from Day 4 plates to Day 5 seeded plates.

2.16. Incubate Day 4 and Day 5 plates at 20 °C for 24 h.

2.17. Count the viable progeny for each Day 3 plate. Note any replicates that died or are no longer on the plate (censored). Once the number of progeny are recorded, discard Day 3 plates.

2.18. Seed 35 mm x 10 mm NGM supplemented with streptomycin plates for Days 6 & 7 using 10 µL of mastermix per plate (**Table 2**) and incubate plates at room temperature for 24 h.

Day 6

2.19. Transfer nematodes from Day 5 plates to Day 6 seeded plates.

2.20. Incubate Day 5 and Day 6 plates at 20 °C for 24 h.

2.21. Count the viable progeny for each Day 4 plate. Note any replicates that died or are no longer on the plate (censored). Once the number of progeny are recorded, discard Day 4 plates.

Day 7

2.22. Transfer nematodes from Day 6 plates to Day 7 seeded plates.

2.23. Incubate Day 6 and Day 7 plates at 20 °C for 24 h.

2.24. Count the viable progeny for each Day 5 plate. Note any replicates that died or are no longer on the plate (censored). Once the number of progeny are recorded, discard Day 5 plates.

Day 8

2.25. Count the viable progeny for each Day 6 plate. Note any replicates that died or are no longer on the plate (censored). Once the number of progeny are recorded, discard Day 6 plates.

Day 9

2.26. Count the viable progeny for each Day 7 plate. Do not count the largest nematode (parent). Note any replicates that died or are no longer on the plate (censored). Once the number of progeny are recorded, discard Day 7 plates.

NOTE: This assay can also be used to assess survival. Record when each nematode died. At the end of the experiment, the percentage of nematodes that survived over the seven-day experiment can be compared for each treatment. Nematodes will sometimes crawl away from the food/pathogen source and try to climb the slides of the Petri plate. Check all areas of the plate before moving on. Dead nematodes will generally leave behind a carcass. Censor any nematode that cannot be located and do not count that nematode as dead. Do not include censored data in the final analysis of progeny and survival.

2.27. Analyze data for brood size and late reproduction using either one-way ANOVA or Kruskal-Wallis, depending on the normality of the data sets, as well as post-hoc Tukey/Dunn's multiple testing to identify significant differences between the treatment groups using GraphPad Prism software. Detect differences between survival curves using the Wilcoxon log-rank test.

3. Lineage Expansion Assay

NOTE: Representative data in shown in **Supplementary Table 2** and a schematic is shown in **Figure 2A**.

3.1. Obtain or prepare the following: 100 mm x 15 mm Petri plates containing NGM agar supplemented with 0.2 g/L streptomycin sulfate, *E. coli* OP50 cultures, LB*, C. albicans* cultures, YPD, M9 buffer, Wire Pick, 15 mL conical tubes.

Day -2

3.2. Inoculate *C. albicans* strains in 2 mL of YPD and *E. coli* (OP50) in 50 mL of LB and grow overnight at 30 °C.

Day -1

3.3. Seed 100 mm x 15 mm NGM agar plates supplemented with streptomycin with 300 µL of mastermix per plate (**Table 3**). Spread the mastermix onto the plate using a sterile metal spreader. Incubate the plates overnight at 30 °C. Six replicates per treatment is recommended. Thus, prepare seven plates (One plate for synchronized nematodes, six plates for each adult nematode) per treatment.

[Place Table 3 here.]

Nematode population growth: Day 0

3.4. Synchronize nematodes and plate 10-25 eggs on a single plate for each control (OP50 only) and treatment (*C. albicans* + OP50) and incubate at 20 °C for 48 h.

Day 2:

3.5. Transfer a single L4 worm from each control and treatment Day 0 plate to seeded plates of the same treatment. L4 hosts can be identified by a small pocket in the middle of the dorsal side of their body[22].

3.6. Incubate at 20 °C for 5 days (a total of one week following synchronization).

Day 7:

3.7. Using a p1000 pipette, wash entire nematode population from each plate using 5 mL of M9 buffer and transfer to a 15 mL conical tube.

3.8. Store at 4 °C for 1 h to allow the nematodes to settle for easier counting.

3.9. Dilute each conical to final volume of 10 mL with M9 buffer.

3.10. For each biological replicate, count the number of nematodes in a 20 µL aliquot. Repeat this to obtain 6 technical replicates for each biological replicate. Back calculate to determine the total population size. If samples are too dilute (i.e., fewer than 10 nematodes per sample), concentrate the population in a smaller volume of M9 buffer.

NOTE: Centrifugation of live nematodes will not harm the nematodes.

Example calculation: $\frac{70 \text{ Hosts}}{20 \text{ µL (aliquot)}} = \frac{X \text{ (Total hosts)}}{10{,}000 \text{µL (Total Volume)}}$

3.11. Analyze data for lineage expansion using either one-way ANOVA or Kruskal-Wallis, depending on the normality of the data sets, as well as post-hoc Tukey/Dunn's multiple testing to identify significant differences between the treatment groups using GraphPad Prism software.

Representative Results:

Here we present two assays that measure *C. albicans* virulence as a non-lethal phenotype using *C. elegans* as an infection model. The first assay, fecundity, monitors how *C. albicans* infection impacts single hosts for progeny production and survival. The second assay, lineage expansion, measures how *C. albicans* infection impacts population growth over multiple generations.

The fecundity assay has multiple measures of host fitness during C. albicans infection. To assess how *C. albicans* infection impacts two distinct measures of host fitness, survival, and reproduction, we developed the fecundity assay[19], which monitors individual hosts. Briefly, single hosts are isolated to control plates (uninfected) or *C. albicans* treatment plates and monitored every day for seven days for survival, and the number of daily progeny produced (**Figure 1A**). As this assay encompasses multiple measures of host fitness, it is an efficient way to assess *C. albicans* virulence. First, we evaluated total brood size by calculating the sum of daily progeny per host. Hosts infected with wildtype *C. albicans* produce a significantly smaller brood size on average compared to uninfected hosts (**Figure 1B**, black vs grey bars, $p < 0.0001$, Kuskal-Wallis test, and post-hoc Dunn's multiple comparison test). By monitoring daily progeny production, we can also detect differences in the timing of reproduction. We previously demonstrated that *C. albicans* infected hosts have delayed reproduction, with a large fraction of their progeny produced later in their adulthood compared to uninfected hosts[19]. The fraction of late reproduction is calculated by dividing the progeny produced on Days 4-7 by the total number of progeny produced. Uninfected hosts produce ~20% of their progeny during this late reproduction window whereas wildtype *C. albicans* infected hosts have 60% of their total offspring in this late reproduction window (**Figure 1C**).

The fecundity assay not only provides data to assess non-lethal virulence phenotypes, the data can also be used to assess host survival in a seven-day period. We plotted host survival (Fig**ure 1D**) and observed a decrease in survival for wildtype *C. albicans* infected hosts compared to uninfected hosts (grey vs. black lines, **Figure 1D**), but this difference was not statistically significant ($p = 0.687$, Log-rank test). This can be attributed to the short seven-day window of time this experiment covers. We previously showed it takes eight days for *C. albicans* infected hosts to reach 50% mortality[19]. Thus, this assay can only detect difference in host survival at very early time points.

Fecundity assays capture how *C. albicans* infection alters host fitness during early adulthood. We used this assay to screen for differences in virulence across reproductive and survival phenotypes using two *C. albicans* deletion strains (**Table 1**) previously identified to decrease virulence in murine, *D. melanogaster,* and *C. elegans* infection models[11, 19, 23–25]. *CAS5* is a gene that encodes a transcription factor that regulates cell wall homeostasis, adherence, and stress response[26]. Here, hosts infected with *C. albicans cas5Δ/Δ* have significantly larger brood sizes and a smaller fraction of late reproduction compared to wildtype *C. albicans* infected hosts (**Figure 1B,C**, orange bars, $p < 0.0001$, Kruskal-Wallis test, and post-hoc Dunn's multiple comparison test). Additionally, hosts infected with *C. albicans cas5Δ/Δ* do not have significantly different brood sizes or fraction of late reproduction compared to uninfected hosts ($p > 0.9999$, Kruskal-Wallis

test, and post-hoc Dunn's multiple comparison test), indicating that *cas5Δ/Δ* strains are avirulent. Furthermore, we found that *cas5Δ/Δ* reduces host mortality compared to wildtype *C. albicans* (**Figure 1D**, orange), although this difference is not statistically significant (p = 0.687, Log-rank test), likely due to the short timeframe in which host fitness is evaluated. *RIM101* is a gene that encodes a transcription factor required for alkaline-induced hyphal growth[27]. Hosts infected with *rim101Δ/Δ C. albicans* had a significantly smaller fraction of late reproduction compared to wildtype *C. albicans* (**Figure 1C**, blue bar, p = 0.0184, Kruskal-Wallis test, and post-hoc Dunn's multiple comparison test) infected hosts, despite having similar total brood sizes (**Figure 1B**, blue bar, p = 0.6979, Kruskal-Wallis test, and post-hoc Dunn's multiple comparison test). Additionally, *rim101Δ/Δ* infected hosts had similar mortality to hosts infected with wildtype *C. albicans* (**Figure 1D**, blue, p = 0.687, Log-rank test). Taken together, we demonstrated the utility of this assay to distinguish subtle difference between *C. albicans* strains.

The lineage expansion assay measure the virulence phenotype combining costs to host survival and reproduction. To quickly assess how *C. albicans* infection impacts host fecundity and survival over three generations, we developed the lineage expansion assay[19], which monitors the progeny production of individual hosts. Briefly, single hosts are isolated to control plates (uninfected) or *C. albicans* treatment plates and after seven days the total number of viable progeny in the F1 and F2 generations are counted (**Figure 2A**). Uninfected hosts produced a progeny population ~35,000 in this timeframe (**Figure 2B**), compared to wildtype *C. albicans* infected hosts which produced a progeny population size of ~25,000, nearly a 30% reduction (**Figure 2B**). This simple assay can be used to rapidly screen through mutant strains of *C. albicans*. Both *cas5Δ/Δ* and *rim101Δ/Δ C. albicans* infected hosts produced progeny populations that were significantly larger than wildtype *C. albicans* infected hosts (**Figure 2B**, $p < 0.0001$ & p = 0.0185 respectively, Tukey's multiple comparisons test). Furthermore, *cas5Δ/Δ C. albicans* infected hosts produced progeny populations that were comparable to uninfected hosts, suggesting that this *C. albicans* strain is avirulent, and the *rim101Δ/Δ C. albicans* strain has reduced virulence for this virulence phenotype.

FIGURE AND TABLE LEGENDS:

Figure 1: Fecundity and survival assessed in single hosts infected with different C. albicans strains. A) Experimental schematic of the fecundity assay. **B)** Total brood size for uninfected (OP50) hosts (n = 23), and hosts infected with wildtype (WT; SN250, n = 21), *cas5Δ/Δ* (n = 26), and *rim101Δ/Δ* (n = 26) *C. albicans* strains. The box represents the interquartile range, the midline indicates the median, and the whiskers represent the range. Error bars are the normalized range of the data. Treatments that share letters are not significantly differ, whereas treatments with differing letters are statistically significant, Kruskal-Wallis test and post-hoc Dunn's multiple comparison test. **C)** Fraction of late reproduction for uninfected hosts, and hosts infected with wildtype, *cas5Δ/Δ*, and *rim101Δ/Δ C. albicans* strains. Bars represent the mean, error bars represent +/- 1 SD, and symbols represent individuals hosts. Treatments that share letters are not statistically significantly different, whereas treatments with differing letters are significant, Kruskal-Wallis test and post-hoc Dunn's multiple comparison test. **D)** Survival curves of uninfected hosts, and hosts infected with wildtype, *cas5Δ/Δ*, and *rim101Δ/Δ C. albicans* strains

for the first seven days of adulthood. Error bars represent ± 1 SD. Data from B, C, and D were collected from the same experiment and host sample sizes are the same in each panel.

Figure 2: Virulence of *C. albicans* strains measured by host reproduction and death over multiple generations A) Experimental schematic of the lineage expansion assay. **B)** Box and whiskers plot of the population size (representing the number of F1 and F2 progeny) produced within 7 days from a single founder host exposed to OP50 food source alone (uninfected n=10, black), WT C. albicans (SN250, n=12, grey) or *C. albicans cas5ΔΔ* (n=12, orange), and *rim101ΔΔ* (n=12, teal) mutant strains (pink). Boxes indicate the 25-75th quartiles with median indicated. Error bars are the normalized range of the data. Treatments that share letters are not significantly different, whereas treatments with differing letters are statistically significant, one-way ANOVA and post-hoc Tukey multiple comparison test. Data was initially published in Feistel et al. 2019[16].

Table 1: *C. albicans* strains used in this study

Table 2: Mastermix volumes of *E. coli* and *C. albicans* cultures needed to infect nematodes for the fecundity assay.

Table 3: Mastermix volumes of *E. coli* and *C. albicans* cultures needed to infect nematodes for the lineage expansion assay.

Supplemental Table 1: Sample Fecundity Data

Supplemental Table 2: Sample Lineage Expansion Data

DISCUSSION

Here, we present two simple assays that measure fungal virulence. Both assays leverage *C. elegans* as a host system that includes monitoring for both lethal and non-lethal host phenotypes. For example, fecundity assays investigate the reproductive success of individual infected hosts while also measuring individual survival. The daily monitoring provides not only total brood size, but also reproductive timing, and time of death. The lineage expansion assay was developed as a simplified version of the fecundity assay that is less cumbersome as it requires fewer host transfers and daily counting. The lineage expansion assay provides a multigenerational and quantitative measure that combines multiple aspects of host fitness. Together, these assays are a powerful way to quickly screen the virulence of *C. albicans* strains, including mutant strains and diverse clinical isolates. Furthermore, given the ease of the infection assay, where hosts are reared on the pathogen as the food source, this makes it easy to apply to other microbial pathogens to assess their virulence.

There are three main technical considerations to be mindful of. First, when synchronizing host populations to collect eggs, the timing of the bleach step needs to be closely monitored, by assessing host movement and/or host shape (dead hosts will no longer be sinusoidal) under a microscope. Once L1-adult hosts are dead, quickly centrifuge and remove the bleach to ensure the integrity of the eggs. Second, for both fecundity and lineage expansion it is critical that L4

hosts are isolated and transferred 48 hours following synchronization (Day 2), as this is the last developmental stage prior to reproductive maturity and non-L4 hosts can shift the reproductive timing. While 48 hours is typically when *C. elegans* reach L4, this can vary slightly. Third, when counting the final progeny population for lineage expansion, the concentration of the sample is important. If the sample is too dilute, the counts will be too low and cause significant technical variation. The sample concentration can be adjusted by centrifugation and removing excess buffer. If the sample is too concentrated, it will be difficult to count every host in that aliquot and cause significant technical variation. The sample concentration can be adjusted by adding more buffer. Keeping these considerations in mind will ensure the success of these relatively simple assays.

The assays we describe have some distinct differences from other *C. elegans* infection assays, including the use of solid NGM media and using OP50 as a food source for both uninfected and infected treatments. When hosts are reared only in the presence of *C. albicans*, their development is slower than when reared in conjunction with *E. coli*[28,29]. During our experiments, hosts are exposed to *C. albicans* throughout development and early larval stages cannot consume large yeast cells[28,29].

While this is a highly adoptable system that allows for high replication and quantification, there are a few limitations when using *C. elegans* to study host-pathogen interactions. First, interactions between the pathogen and host immune function are limited as *C. elegans* lack an adaptive immune system and pro-inflammatory cytokines and chemokines[30]. Second, since pathogens are introduced via the *C. elegans* diet, it is difficult to control the inoculum and ensure that all nematodes ingest the same amount[7]. However, with fluorescently tagged pathogens, and recent methods to extract yeast[31] and bacteria[32] from the *C. elegans* gut, we can enumerate the number of colonies ingested. Finally, *C. elegans* cannot survive at temperatures similar to the human body, instead are grown at temperatures ≥ 5°C physiologically relevant[33]. Thus, growth and proliferation of the pathogen will be different in *C. elegans* compared to humans.

Fungal virulence is predominantly assessed using murine models and is often restricted to monitoring survival. However, using *C. elegans* as a simple host model for fungal infection offers three unique advantages: First, the ease of infectivity and laboratory handling makes it amenable to scientists of all training levels. Second, the large number of hosts that can be infected and monitored more reliably and confidently captures the phenotypic variation in and between treatments. Third, in *C. elegans*, the innate immune pathways genes have highly conserved mammalian homologues, including the p38 MAP kinase PMK-1[13], the TIR-1 (SARM) protein which functions to activate the PMK-1 pathway in *C. elegans* immunity[34], and the dual oxidase BLI-3, which generates reactive oxygen species in response to pathogen infection in *C. elegans*[14–16]. The conservation of immunity and the availability of different immune mutants at the Caenorhabditis Genome Center, makes it easy to investigate the impact of host immunity on the pathogenicity of *C. albicans* and other pathogens. We recently showed that these assays can be used with *sek-1* hosts to demonstrate that immunocompromised are highly susceptible to fungal infection[19,20]. Together, these assays our results and the other applications described here offer many reasons to use *C. elegans* to investigate the virulence of *C. albicans*.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have no competing interests to disclose.

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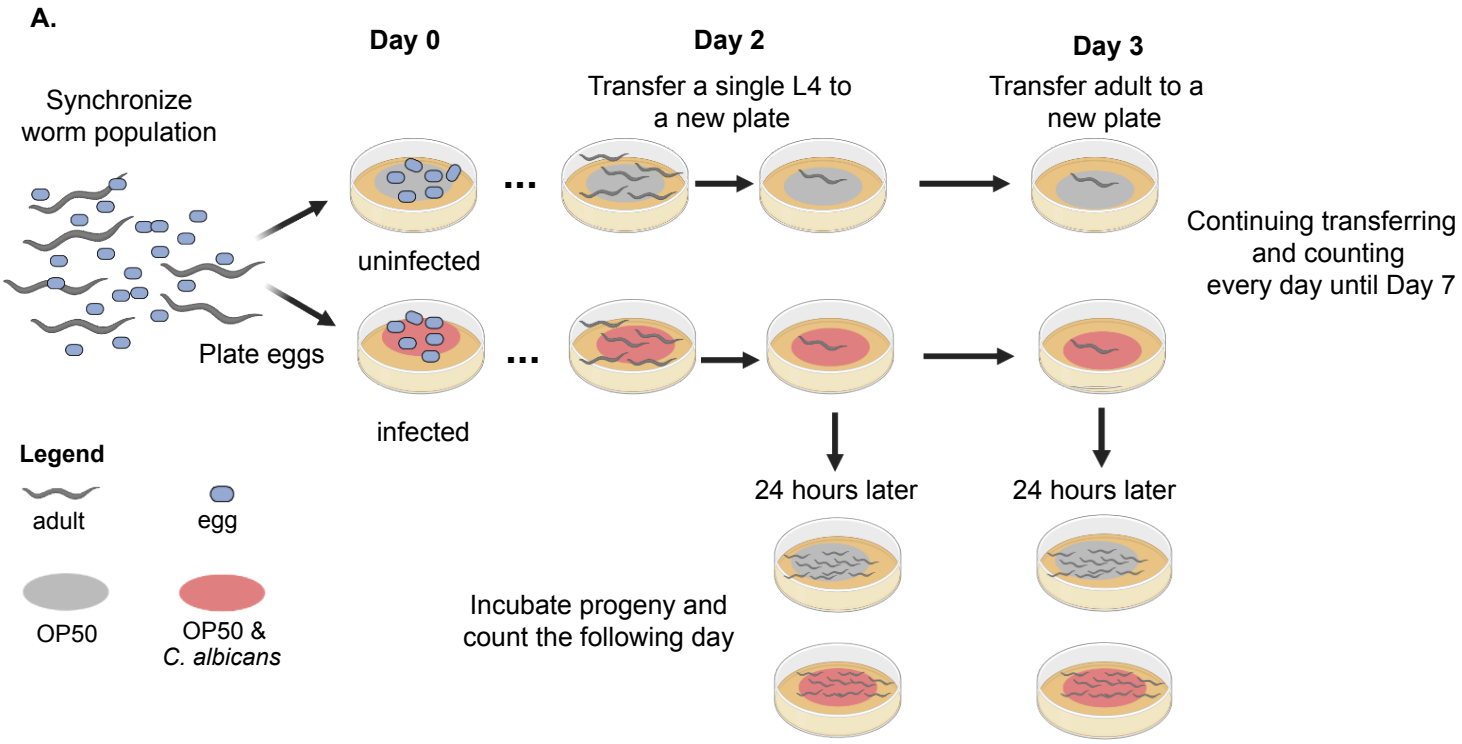
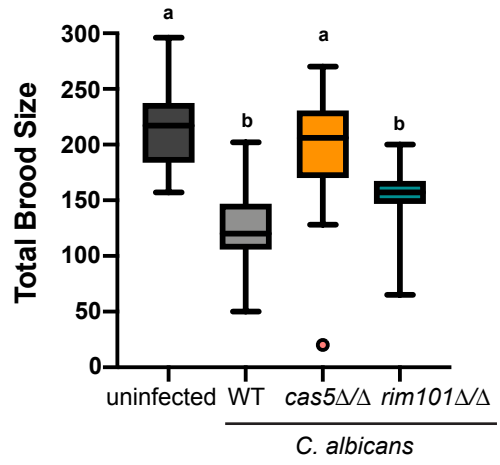
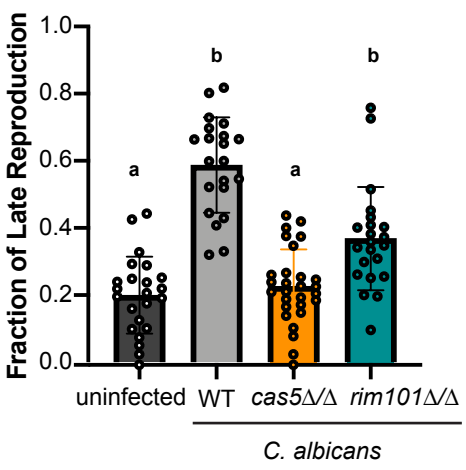
Figure 1

Click here to access/download;Figure;2020.10.06 Fig. 1 Fecundity.pdf







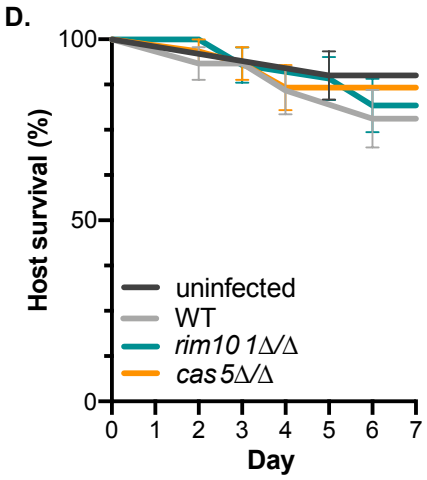


Figure 2

Click here to access/download;Figure;2020.10.06 Fig. 2 Lineage Expansion.pdf

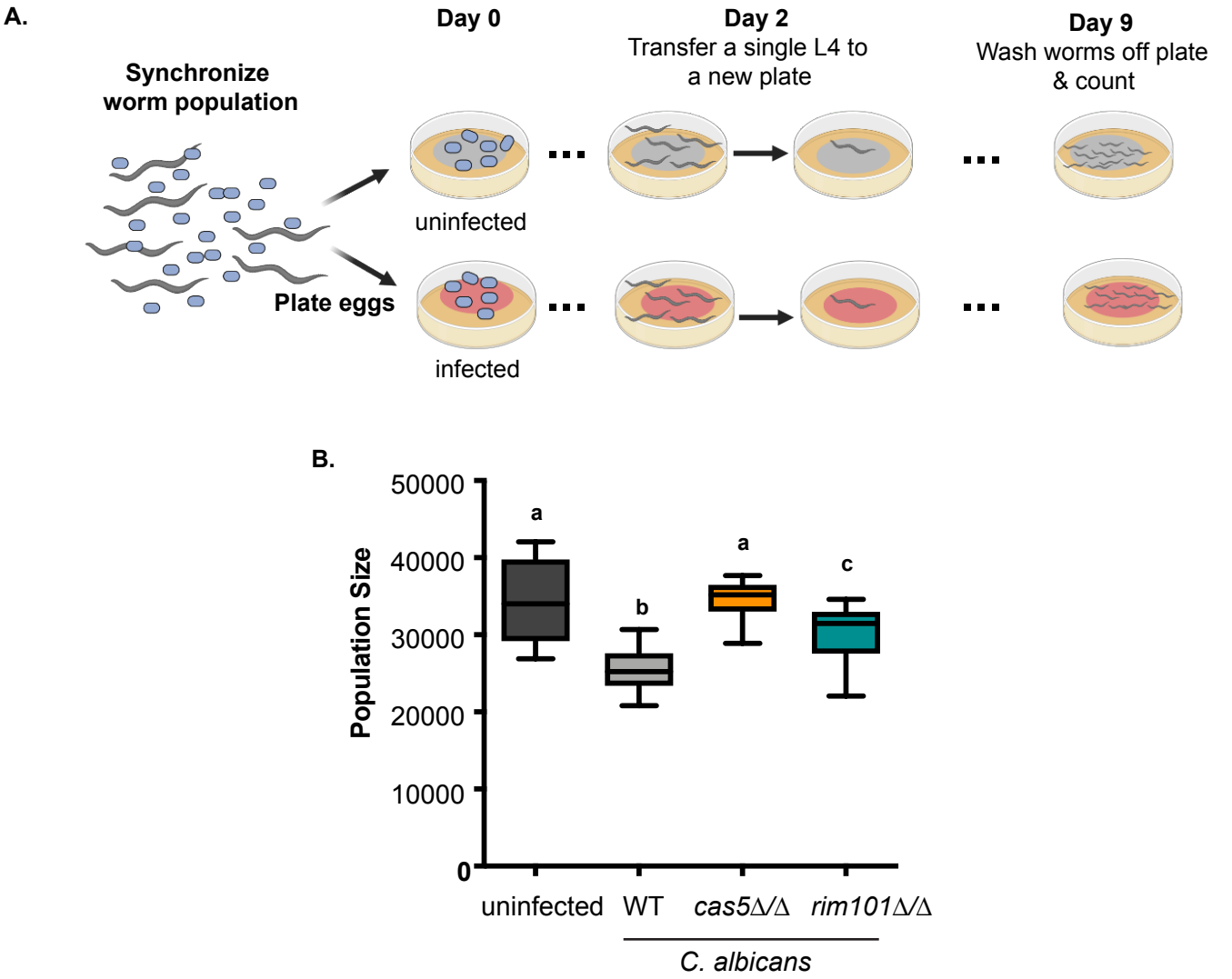


Table 1

Strain	Genotype	Source
SN250 (WT)	his1Δ/his1Δ, leu2Δ::C.dubliniensis HIS1/leu2Δ::C.maltosa LEU2, arg4Δ /arg4Δ, URA3/ura3Δ::imm434, IRO1/iro1Δ::imm434	Noble et al. (2010)
rim101Δ/Δ	his1Δ/his1Δ, leu2Δ::C.dubliniensis HIS1/leu2Δ::C.maltosa LEU2, arg4Δ /arg4Δ, URA3/ura3Δ::imm434, IRO1/iro1Δ::imm434 orf19.7247Δ::C.dubliniensisHIS1/orf19.7247Δ::C.maltosaLEU2	Noble et al. (2010)
cas5Δ/Δ	his1Δ/his1Δ, leu2Δ::C.dubliniensis HIS1/leu2Δ::C.maltosa LEU2, arg4Δ /arg4Δ, URA3/ura3Δ::imm434, IRO1/iro1Δ::imm434 orf19.4670Δ::C.dubliniensisHIS1/orf19.4670Δ::C.maltosaLEU2	Noble et al. (2010)

Table 2

For 1 replicate	*E. coli* (OP50) control condition			*C. albicans* & *E. coli* (OP50) treatment condition			
	OP50	H2O	Total	OP50	*C. albicans*	H2O	Total
Day 0	6.25 ul	43.75 ul	50 ul	6.25 ul	1.25 ul	42.5 ul	50 ul
Days 2-7	1.25 ul	8.75 ul	10 ul	1.25 ul	.25 ul	8.5 ul	10 ul

Table 3

	E. coli (OP50) control condition			*C. albicans* & *E. coli* (OP50). treatment condition			
For 1 replicate	**OP50**	**H2O**	**Total**	**OP50**	***C. albicans***	**H2O**	**Total**
	1.25 ul	8.75 ul	10 ul	37.5 ul	7.5 ul	255 ul	300 ul

Table of Materials

Click here to access/download;Table of Materials;20201008JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL eppendorf microtubes 3810X	Millipore Sigma	Z606340	
100 mm x 15 mm petri plates	Sigma-Aldrich	P5856-500EA	
15 mL Falcon Conicals	Fisher Scientific	14-959-70C	
50 mL Falcon Conicals	Fisher Scientific	14-432-22	
Adenine	Millipore Sigma	A8626	
Agar (granulated, bacterilogical grade)	Apex BioResearch Produces	20-248	
Aluminum Wire (95% Pt, 32 Gauge)	Genesee Scientific	59-1M32P	
Ammonium Chloride	Millipore Sigma	254134	
Bacterial Cell Spreader	SP Scienceware	21TP50	
BactoPeptone	Fisher BioReagants	BP1420-500	
Disposable Culture Tubes (20 x 150 mm)	FIsherBrand	14-961-33	
Dissection Microscope (NI-150 High Intensity Illuminato	Nikon Instrument Inc.		
E. coli	Caenorhabditis Genetics Center	OP50	
Glucose	Millipore Sigma	50-99-7	
Medium Petri Dishes (35 X 10 mm)	Falcon	353001	
Metal Spatula	SP Scienceware	8TL24	
Nematode Growth Media (NGM)	Dot Scientific	DSN81800-500	
Potassium Phosphate monobasic	Sigma	P0662-500G	
Sodium Chloride	Fisher Scientific	BP358-1	
Sodium Phosphate	Fisher Scientific	BP332-500	
Streptomycin Sulfate	Thermo-Fisher Scientific	11860038	
Tryptone	Millipore Sigma	91079-40-2	
Uridine	Millipore Sigma	U3750	
Wildtype *C. elegans*	Caenorhabditis Genetics Center	N2	
Yeast Extract	Millipore Sigma	8013-01-2	

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We fixed all spelling mistakes and grammatical errors.

2. Please provide an email address for each author.

We included email address for all authors.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: US Biological, Eppendorf, Falcon etc

We removed all use of commercial product names and replaced them with more general materials.

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We removed all of the personal pronouns throughout the text.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Thank you for this suggestion. We made sure all of the protocols were written in imperative tense and removed any phrases that were not.

6. Please consider providing solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text. Reagents and equipment needed can be listed assay-wise in the Table of Materials.

We appreciate this suggestion yet choose to leave the solution compositions (LB, YPD, M9, etc). within the text. We think that listing the reagents needed at the beginning of each assay protocol makes it easier for the reader to know what is needed. Given the overlap in the materials used for each assay, we thought it would be unnecessarily redundant to list materials needed for each assay.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol

action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We appreciate this note. We added additional specific steps to measure the optical density of yeast cultures. We also recommended the number of replicates to use and were as explicit as possible when explaining each step.

8. Lines 379-380: To make things clearer, please bring 35,000 nematodes in the population after 500, add a semicolon after 500, and then add 10,000 µL (total volume)/20 µL (sample volume) = 500.

We modified this example calculation to be clearer (Lines 785-6).

9. Please move the figure and table legends section to come between representative results and discussion sections.

We have moved the figure and table legends accordingly.

10. As we are a methods journal, please add limitations to the Discussion.

We added a limitations section to the discussion lines 1023-33.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. “This figure has been modified from [citation].

mSphere's **policy regarding authors' copyright permission can be found here: https://journals.asm.org/content/statement-author-rights. We have cited the appropriate publication in the figure legend.**

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
In this protocol manuscript, Smith et al describe two novel infection assays using C. elegans as a model host to study virulence phenotypes of the fungal pathogen Candida albicans. This manuscript describes both a fecundity assay that measures worm reproduction and survival, as well as a lineage expansion assay, which measures infected worm populations are able to proliferate over multiple generations. This is a clearly written manuscript describing novel and recently described protocols that will be of interest to many in the field of microbial pathogenesis and host-pathogen interactions. The figures are exceptionally clear and well presented. A few minor points will further improve this protocol.

Major Concerns:
1. In the introduction, could the authors expand on some of the other benefits to the C. elegans model compared to other non-mammalian animal models (drosophila, zebrafish etc).

We thank the reviewers for this helpful feedback and now include some of the benefits of using *C. elegans* compared to the other non-mammalian model systems in the introduction (Lines 151-74).

2. Could the authors also expand (in the introduction or conclusion) on some of the limitations of the C. elegans system? Differences between human and C. elegans immune system, infection temperature, any technical limitations, etc?

This was a common suggestion and we have now added a section in the discussion discussing the limitations of the model system (Lines 1023-33).

3. Throughout the protocol, remove references to brand names where unnecessary (i.e. Eppendorf tubes > microcentrifuge tubes, Falcon tube > conical tube)

Please see our response to the editorial comments.

4. Can the authors define and describe L4 larva earlier in the text so that it is clear to readers going through the protocol? Could an image be included for clarity (or maybe this will be included in the video?).

Thank you for this helpful suggestion. After introducing L4 larva in the fecundity assay, we have added a description of this larval stage and reference a source that includes pictures of the *C. elegans* life stages that might find helpful to readers (Lines 572-3).

5. If possible, it would help to cite the figures throughout the protocol, in addition to in the results section.

Thank you for this useful suggestion. We now cite the figures at the start of each protocol.

6. Can the authors spend some time in the protocol describing how the data is analyzed, plotted, and statistical analysis. The statistical tests used should also be described in the results, as the authors describe how some results are significant or not significant, but do not define the test. The authors should also describe how many biological/technical replicates are typically included in the assay.

We included a brief description of the stastics used to determine significant differences between treatment groups (Lines 677-81 & 776-9).

7. In the paragraph starting on line 414, is it accurate to describe the cas5 mutant strain as 'avirulent', as opposed to having reduced virulence? How can one tell definitively that it has no level of virulence?

The cas5 deletion strain is described as avirulent since it does not cause any detectable harm to the host, as determined by comparing it to the uninfected treatment. We clarified this statement in lines 857-9.

8. Could the authors describe in the discussion how this system might be applied to the study of other microbial pathogens?

***C. elegans* are used to study many different pathogens, both fungal and bacterial. We added a sentence to the discussion that clarifies that *C. albicans* can be substituted for the readers' pathogen of choice (Lines 985-7).**

Minor Concerns:
1. The two sentences on lines 84 and 85 both begin with 'therefore' - could one be changed?

We have removed the second 'therefore'.

2. Perhaps include a reference to another recent, simple model host for fungal pathogenesis studies in paragraph two of the introduction: PMID: 32842847

Thank you for the suggestion. This was an interesting paper and definitely sounds like a very useful model system. However, we wanted to focus more on the more common models for fungal pathogenesis.

3. Line 187 - can the authors describe how the desired egg concentration is determined and what the range is?

We expanded on the desired range in lines 471-5.

4. Line 209, can the authors define the maximum speed specifically?

We added the maximum speed (Line 493).

5. Line 224 culture shouldn't be in italics

We changed this text to regular font.

6. It is not clear throughout the protocol what is meant when "(censored)" is included in some parts. Can this be described?

We have included a description of what is meant by censored in Lines 608-11.

Reviewer #2:

Manuscript Summary:
The authors share the novel development of non-lethal assays to assess C. albicans infection in the nematode C. elegans over different generations of animals to try to further understand how a C. albicans infection may affect downstream reproduction and survival in populations as a whole. Using a fecundity assay and a lineage assay, the authors are able to show that worms infected with wildtype *C. albicans* have lower overall levels of reproduction over generations, and those worms that do reproduce do so at later generation stages. However, worms that are left uninfected or infected with mutant, avirulent strains of C. albicans fare better in the context of reproduction and overall brood size over generations. The authors suggest that these studies can be repeated with numerous other fungal strains and mutants to further understand the impact of C. albicans infections on reproductive health and overall fitness of hosts over time.

Overall, the article is fairly well written and the experiments suggested do show the importance of non-lethal consequences of C. albicans infections in nematode generations. However, there are some concerns that I have, and would like to have addressed as follows:

Major Concerns:
-Line 213 of methods, what is the importance of this E. coli concentration? How does this affect the infection or the feeding of the worms? Can the worms be fed at a different concentration?

We used standardized concentrations of *C. albicans* and *E. coli* that are based on several previous studies (Feistel et al 2019; Jain et al 2009; Jain et al 2013; Issi et al 2017). The nature of this assay does not allow us to know the specific number of bacteria being consumed by the nematodes. This concentration allows for the worms to survive during the whole course of the experiments. Without the *E. coli* the nematodes will likely starve and go into dauer as their mouths are too small in early larval stages to consume the larger yeast colonies (Lines 1018-21).

-Why do you choose the temperatures that you use for these experiments? Why is E. coli grown at 30C and not 37C? If the E. coli and C. albicans are grown at 30C, why are the experimental infections then grown at 20C?

***E. coli* can grow at a range of temperatures (30C – 42C) (Doyle & Schoeni 1984, Applied and Environmental Microbiology) and *C. albicans* can be grown at a range of temperatures including 30C (Lemos-Carolina M. et al. 1982). Therefore, growth at 30C is appropriate for incubating cultures of *E. coli* and *C. albicans*. We are limited to the temperature of the model system. As 20C is an optimal temperature for *C. elegans* growth and thus all of our experiments are performed at this temperature.**

-It is well known that Candida grows differently, including morphology, at different temperatures…as your infections are mainly at 20C, have you tried infection at higher temperatures? Especially with a WT strain, the infection is mainly going to be yeast at 20C…what about the impact of hyphae during the infections? A wildtype strain that is able to grow both yeast and hyphae during the infection may be important to showcase here as well - can worms grow at higher temperatures?

***C. elegans* have a maximum growth temperature of 25C. While 20C is too low to reflect human body temperature, we chose to perform these assays at 20C, as it is the optimal temperature for *C. elegans* growth and development and did not want to confound virulence phenotypes with those associated with higher temperatures. However, in liquid media it has been shown that *C. albicans* can still form hyphae and actually penetrate the nematode cuticle at these lower temperatures (Pukkila-Worley et al. 2009, Eukaryotic cell). This has not yet been demonstrated on solid media.**

-It might be good to add in some background on fecundity/reproduction in the context of animals models as well as human infections with C. albicans in the introduction. How relevant are these assays/results to what we see in human infections? Is there data out there on how Candida infections impact reproduction or downstream generations in humans?

As *C. albicans* colonizes many niches in the human body, including the urogenital tract, it is important to study the impact of *C. albicans* on fecundity and reproduction. Furthermore, reproduction is an important measure of host fitness that is often overlooked. Several studies suggest that *C. albicans* can actually impact reproduction and male fertility (Castrillón-Duque et al. 2018, J Reprod. Infertil.) Furthermore, another study demonstrated that vaginal colonization of *C. albicans* can cause infertility in a

mouse model (Vander & Prabha, 2015, Journal of Medical Microbiology. We discuss these points in the introduction (Lines 181-3).

-In the fecundity assay, please expand on each point where you state that you are inoculating Candida and E.coli strains to state that they are growing overnight and being mixed the next day for the infection seeding - this gets a little confusing if you read it and only see that you need to grow E.coli and Candida overnight but no other instructions **.**

Thank you for this suggestion. After inoculation we added a line that states they need to be grown overnight at 30C. Lines 552-3 & 709-10.

-Line 239 of methods, why is spreading of the cultures not necessary?

Spreading the culture in this case is not necessary because we are using small plates and a single spot of microbial growth is sufficient for host feeding and allows for us to easily identify hosts outside of the seed (Lines 564-6).

-Authors suggest that L4 stage nematodes are used for experiments, but there is no indication of what L4 means in the rest of the text - please expand on the developmental stages here that are needed

Please see our response to reviewer 1.

-What is the impact of centrifuging on live worms to change the volume of media they are resuspended in? Does centrifuging impacting their survival?

We centrifuge the live worms to pellet them at the bottom in the case that liquid needs to be removed to make a more concentrated solution of nematodes. Centrifugation does not impact their survival. The nematodes are fully intact after centrifugation and continue to move when pipetted onto a plate.

-How do you make sure that each nematode is getting infected similarly? Are worms expected to eat the Candida? If they are not expected to, how do they get infected? Are there worms that are not infected on Candida plates? How do you account for these?

Since *C. elegans* ingest the pathogens, we cannot ensure that all host are infected to the same degree. In previous work we show that we can extract viable colonies (Smith & Hickman, 2020, mSphere), thus demonstrating that *C. albicans* inhabits host guts. Other studies have used fluorescently tagged yeast strains to indicate host colonization (Elkabti et al. 2018, J. Fungi).

Minor Concerns:
-Absolutely appreciate the schematics in figures 1 and 2

Thank you ☺

-Add C. albicans to the list of keywords

Great suggestion, we added it!

-Line 82 in introduction, remove the second "and"

We removed the second ‘and’.

-Lines 94-96 in introduction, sentence is fragment, please change/add to sentence to make it a complete sentence

We since modified the sentence (Line 151).

-Line 136 of methods, remove "US Biological"

We removed this, along with other commercial product names.

-Line 146 of methods, please add in when you add nematodes to the plates alongside the infections.

We clarified when nematodes were synchronized and added to the seeded plates for each assay (Lines 566-8).

-Line 172 of methods, is centrifugation at room temperature?

We have indicated “RT” for room temperature after each step that describes centrifugation.

-Line 176 of methods, is the bleach treatment to only kill worms that are not inside eggs? Do the eggs fare well after bleach treatment?

The bleach treatment kills live worms but not eggs. Eggs are left fully intact unless exposed to bleach for periods longer than 5 minutes (Sulston and Hodkin 1988 CSHL 587-606). We added a note after this step to make this clear to readers. (Line 450).

-Line 187 of methods, what is a common egg/uL concentration that one would usually use? Are there different amounts of eggs for different studies? Can you please expand on this?

We expanded on the desired range in lines 471-5.

-Line 189 of methods, do you physically count the eggs that you have within those 5 uL? What do you do with this concentration afterward?

You physically count the eggs, which is described in lines 471-5. “The ideal concentration should be between 20-100 eggs. If the culture is too dilute, concentrate the solution by centrifugation and removal of excess liquid. If the culture is too concentrated, add more M9 the desired concentration is reached.”

-Line 236 of methods, change 15 mm to 10 mm

We have revised this.

-Line 233 of methods, approximately how many plates should one prepare for duplicate/triplicate experiments?

At the start of each assay protocol, we described how many biological replicates are typically used and the number of reagents needed for that recommended amount (Lines 544-6 & 702-4).

-Line 270 of methods, first time using word "embryos" - is this the developmental stage?

We have revised the text to consistently use the term eggs to avoid unnecessary confusion.

-Line 329 of methods, for the nematodes that "disappear"…do they escape? Where do they go? How do you account for them?

We discuss censorship in lines 608-11. "Censored refers to the nematodes that disappeared on the plate. This can occur when nematodes crawl off of the plate. Although less common during the 24 h window, dead nematodes' carcasses disintegrate into the agar also resulting in censorship. Censored data is not included in the final progeny and survival data analysis."

-Line 422 of results, change "morality" to "mortality"

We have revised this.

-Line 498 of discussion, confusion with the "4)" sentence…this does not make sense, please fix.

We have revised this.

-Table S1: what is MH88? This is not discussed at any point during the study.

MH88 is our internal name for the C. albicans reference strain. We have changed the text to refer to this as SC5314, the standard name to avoid confusion.

-Table of materials: Add M9 buffer, wire pick, add strain type of E. coli used

These are included in the materials list.

Reviewer #3:

Manuscript Summary:
The manuscript describes a well-detailed protocol to assess Candida albicans virulence in C. elegans. There are protocols already published on this subject, but the novelty is that it addresses the study of non-lethal phenotypes.

Major Concerns:
I think the information given in lines 191-193 is imprecise, after an O.D. 1.0 the linearity of the readings is lost. How is it possible to measure an O.D. 3.O with precision? I suggest changing this to cell countings per milliliter**.**

Yeast cultures are typically measured by optical density. We have included an approximation for the number cells in 1 mL when OD_{600} is 1.0 to further clarify (Lines 489-90).

The justification for the use of this model is that the immune response is closer to that observed in mammals, but no effort to analyze immunological parameters is included in the study. Therefore, other invertebrate models might be as good as C. elegans to address the non-lethal phenotypes. Please discuss this point.

The purpose of this study is not to necessarily investigate immunological parameters, rather to use *C. elegans* as a host system to screen for virulence phenotypes, which can be accomplished in both healthy and immunocompromised host genotypes. In the discussion, we discuss the conservation of *C. elegans* immune system with mammals and how this can be leveraged with these assays (lines 1052-61).

There is no explanation for the inclusion of Escherichia coli in some experiments, please elaborate.

We have included a brief justification for including *E. coli* in these types of experiments (Lines 1004-9). Essentially, nematodes mouths are too small in early larval stages to consume large yeast cells.

Minor Concerns:
Although the manuscript is well written, there is room for improvement.
In some parts liters are abbreviated with L, and in others with l, please use the appropriate one throughout the manuscript (L).

We have fixed these issues throughout the manuscript text.

			Day								Total	% Late
		Rep	0	1	2	3	4	5	6	7		
Treatement - OP50	Date	A	0	0	76	201	2	0	0	0	279	0.01
		B	0	0	110	159	3	0	1	1	274	0.02
		C	0	0	107	136	9	1	0	0	253	0.04
		D	0	0	33	63					96	0.00
		E	0	0	56	233	20	1	0	0	310	0.07
		F	0	0	66	213	10	0	0	0	289	0.03
		G	0	0	75	189	17	3	1	1	286	0.08
		H	0	0	108	186	10	2	0	0	306	0.04
		I	0	0	112	141	4				257	0.02
		J	0	0	78	143	22	5	0	0	248	0.11
		Mean	**0**	**0**	**82**	**166**	**11**	**2**	**0**	**0**	**260**	0.05
		SEM	*0*	*0*	*8*	*15*	*2*	*1*	*0*	*0*	*19*	0.18
		death	0	0	0	1	0	0	0	0	**1**	**10**
Treatment - MH88	Date	A	0	0	59	69	79	23	1	0	231	0.45
		B	0	0	58	96	121	4	0	0	279	0.45
		C	0	0	82	158	28	0	0	0	268	0.10
		D	0	0	51	134	34	0	0	0	219	0.16
		E	0	0	96	111					207	0.00
		F	0	0	36	88	112	37	1	1	275	0.55
		G	0	0	65	100	78	28	10	1	282	0.41
		H	0	0	41	155	38	1	0	0	235	0.17
		I	0	0	56	126	62	17	2	0	263	0.31
		J	0	0	34	101	50	40	0	1	226	0.40
		MEAN	**0**	**0**	**58**	**114**	**67**	**17**	**2**	**0**	**249**	0.34
		SEM	*0*	*0*	*6*	*9*	*11*	*5*	*1*	*0*	*9*	2.03
		death	0	0	0	1	0	0	0	0	**1**	**10**

Population size in 20 ul			
	Biological Replicate		
Technical Replicate	A	B	C
1	34	44	41
2	45	46	34
3	33	41	35
4	38	39	38
5	39	33	39
6	42	37	40
Average Count	39	40	38
Dilution Factor	10,000 uL (Diluted sample) / 20 ul (aliquot) = **500**		
Population Size (Av. Count * Dilution Factor)	19250	20000	18917