

Standard Operating Procedure (SOP)

***Candida albicans* Murine Gastrointestinal Model for Disseminated Candidiasis (antibiotic approach)**

NIH/NIAID Task Order A13

Isolates:

The murine model of gastrointestinal candidiasis described below has been validated with two separate wild-type isolates of *Candida albicans* that are susceptible to clinically available antifungal agents. These include:

- 529L (1)
- SC5314 for which the genome has been sequenced and is available (www.candidagenome.org) (2)

Mice:

The typical weight and age range used for each mouse strain is shown in the table below:

Strain	Weight	Age
Male Balb/c	20 - 22 grams	4 - 6 weeks
Female C3H/HeN(3)	20 – 22 grams	4 - 6 weeks
Male C57BL/6(4)	20 – 22 grams	4 - 6 weeks

One supplier for these strains is Taconic Biosciences (www.taconic.com).

Food & Water:

- Place mice, up to 5 animals per cage, on PicoLab Vac Lab rodent chow, (Newco Distributors, Cat# 15061). Mice will stay on this diet for the remainder of the experiment.
- Initiate antibiotic treatment beginning 4 days prior to oral gavage inoculation. Place mice on drinking water containing streptomycin (Sigma-Aldrich, Cat# S9137) 2mg/ml, penicillin G (Sigma-Aldrich, Cat# P3032) at 1500 U/ml, and fluconazole (Western Medical Supplies, Cat# 530845) at 0.25 mg/ml for 3 days then switched to the same concentrations of streptomycin and penicillin (without fluconazole) in their drinking water for one more day.
- Mice are supplied with sterile bedding, sterile water and sterile chow and maintained under pathogen-free conditions.
- Mice that are treated with oral antibiotics are extremely susceptible to gastrointestinal colonization with *C. albicans*, which apparently can be spread from cage to cage by aerosolization. If it is desirable to have a group of mice that receive oral antibiotics but are not colonized with *C. albicans* or to groups of antibiotic-treated mice that are colonized with different strains of *C. albicans*, these mice should be housed in separate rooms, if at all possible. Also, fecal pellets from the mice should be collected and analyzed weekly to verify the colonization status of the animals.
- Collect feces immediately prior to inoculation (Day 0) and every 7 days through the remainder of the experiment to assess fecal fungal burden, as described in outcome measures.

Inoculum Preparation and Quantification:

- Subculture the *C. albicans* strain at 30°C for 48 hours on yeast extract peptone dextrose (YEPD) agar.
- Place isolates taken from the subculture into 10 mL of YEPD broth and allow to grow overnight (~16-24 hours) with orbital shaking at 200 rpm in a 30°C incubator. Inoculate 100 µl of the overnight culture into 10 mL of YEPD broth and allow to grow overnight with orbital shaking at 200 rpm. Repeat 1 more time.

- Collect the cells by centrifugation (~2,000 rpm for 10 minutes) and wash in sterile physiologic saline or sterile phosphate buffered saline (PBS). Remove the supernatant and repeat the wash 2 additional times in sterile physiologic saline or sterile PBS.
- Prepare dilutions of the cells in sterile saline or PBS (e.g., 1:200 to 1:400 or 1:1000 to 1: 10,000) and determine the number of cells/mL using a hemacytometer.
 - For inoculation by oral gavage, adjust the desired infecting inoculum to 1×10^6 cells/mL (1×10^5 cells/mouse) in sterile saline.
 - The inoculum should be used to infect the mice within 2 hours of preparation. It is essential to regularly vortex the suspension during the infection to prevent settling.
- Confirm the inoculum viability by serially diluting an aliquot of the inoculum in sterile saline or phosphate buffered saline. Prepare serial dilutions (e.g., 1:1000, 1:10,000, and 1:100,000) of the stock, and plate 100 microliters of the dilutions onto Sabaurou dextrose agar (may be done in duplicate for each dilution). Incubate the plates at 37°C and count the number of colonies the next day.

Oral Gavage Inoculation:

- Inoculate each mouse with 0.1 mL of the desired inoculum in sterile saline or PBS by oral gavage.
- Following successful inoculation, return the mice to their cages.

Monitoring of Animals Post-Inoculation:

- Following inoculation, mice should be monitored at least twice daily throughout the course of the experiment to prevent and minimize unnecessary pain and distress. Moribund animals, if any, will be identified by the following criteria:

1. Ruffled and/or matted fur
2. Weight loss (e.g., >20%)
3. Hypothermia (cool to touch)
4. Decreased activity
5. Hunched posture
6. Inability to eat or drink
7. Torticollis or barrel rolling

Any animal displaying more than one of these criteria should be humanely euthanized using two forms of approved euthanasia (e.g., 5% isoflurane or pentobarbital anesthesia followed by exsanguination via cardiac puncture or cervical dislocation).

Immunosuppressive-induced Candidiasis:

- To induce hematogenously disseminated candidiasis, treat mice with cyclophosphamide 14 days after exposure to *C. albicans*. Cyclophosphamide induces susceptibility to disseminated candidiasis because it causes leukopenia and disrupts the GI mucosa.
- Cyclophosphamide (Western Medical Supply, Inc.) should be administered by subcutaneous injection: 250mg/kg on day 0 and 200mg/kg on day 4 (Balb/c mice) or 150mg/kg every other day for 3 doses starting on day 0 (C3H/HeN mice). Note that we have been unable to identify a non-toxic immunosuppressive regimen that causes C57Bl/6 mice to become susceptible to disseminated candidiasis.

Antifungal Therapy:

- To evaluate the effects of therapy, initiate antifungal treatment 1 day after starting immunosuppression and continue for a total of 5 days.

- Antifungals may include:

1. Test compound
2. Positive control: fluconazole (Diflucan) at 10 mg/kg/day by gavage
3. Positive control: caspofungin (Cancidas) at 1 mg/kg/day by IP injection

Timed Sacrifice of Animals:

- Mice will be sacrificed one day after fungal therapy is stopped (day 6 if therapy is on days 1-5).
- Mice will be sacrificed 6 days after the first dose of the immunosuppressive agent.
- Livers and kidneys will be harvested from all sacrificed animals and used to assess fungal burden, as described below.

Outcome Measures: Fecal fungal burden is sustained during the duration of the experiment as long as antibiotics are maintained in the drinking water. Fungal dissemination occurs in all mice with a high level of mortality following the administration of the immunosuppressive agent. The outcome of disseminated candidiasis can be monitored by:

- Survival
- Tissue fungal burden. When disseminated candidiasis is initiated by invasion from the gut, the target organ is the liver.

Fungal Burden. Fungal burden should be measured at a pre-specified time point following inoculation, immunosuppression and following the initiation of antifungal therapy. To help control for antifungal carry-over, this time point should be at least one day after antifungal therapy is stopped.

- At the pre-specified time point, aseptically collect the desired target organ(s) (e.g., liver, kidney, small intestine), and record the weight of each organ for each animal.
- Fecal pellets can also be collected serially without sacrificing the mice. To collect the pellets, place individual mice into separate 600 ml beakers. The mice will usually produce fecal pellets within 5 min.
- Place the organs or fecal pellets into an appropriate volume of sterile saline or PBS (e.g., range of 1 - 5 mL) and homogenize using either a tissue grinder or tissue homogenizer.
- Prepare appropriate dilutions (e.g., 1:10, 1:100, 1:1000) in sterile water or PBS and plate an appropriate volume (e.g., 0.1 mL) of each onto Sabaurou dextrose agar.
 - Antibiotics to prevent bacterial contamination (e.g., chloramphenicol 0.08 mg/ml and streptomycin at 100 mg/L) should be added to the agar before it is poured into the petri dishes.
 - Incubate the plates at 37°C for at least 24 hours and count the number of colonies for each dilution. Calculate the number of colony-forming units (CFU)/gram of tissue.
 - A longer period of incubation may be used (48 - 72 hours) to allow for the growth of cells damaged but not killed by antifungals

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2. **Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, Davis RW, Scherer S.** 2004. The diploid genome sequence of *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101**:7329-7334.
3. **Koh AY, Kohler JR, Coggshall KT, Van Rooijen N, Pier GB.** 2008. Mucosal damage and neutropenia are required for *Candida albicans* dissemination. *PLoS Pathog.* **4**:e35.
4. **Shankar J, Solis NV, Mounaud S, Szpakowski S, Liu H, Losada L, Nierman WC, Filler SG.** 2015. Using Bayesian modelling to investigate factors governing antibiotic-induced *Candida albicans* colonization of the GI tract. *Sci Rep* **5**:8131.