Standard Operating Procedure (SOP)
***Candida albicans* Murine Gastrointestinal Model for Disseminated Candidiasis (dietary 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:
One strain of mouse has been extensively evaluated and used in this model of gastrointestinal candidiasis:

- Inbred BALB/c strain [3, 4]

Suppliers for this strain that have been used include Harlan (www.harlan.com), Charles River (www.criver.com). The typical weight and age range used for each mouse strain is shown in the table below:

Strain	Weight	Age
Male BALB/c	21 - 23 grams	4 - 6 weeks

Immunosuppression:
This murine model is performed using immunocompromised mice. Immunosuppression is achieved by:

- Administration of cyclophosphamide (Cytoxan NDC# 10019-955-01) at 150 mg/kg intraperitoneally (IP) and prednisolone sodium succinate (Solu-Delta-Cortef NADA# 011-593) at 50 mg/kg subcutaneously (SC) on Days 14 and 17 post-inoculation.

Diet:

- Place mice on a purified rodent diet, AIN-93G [5] (Dyets Inc , Cat# 110700) for 14 days prior to inoculation. Mice will stay on this diet for the remainder of the experiment.
- Place mice on drinking water with Baytril (Enrofloxacin NADA# 141-068) at 50 ppM (or 50 mcg/ml) starting on Day 14 post-infection (day of first immunosuppression).
- Place mice on wired bottom cages with paper liners (Lab Supply deosorb pan liners) and collect feces immediately prior to inoculation (Day 0) and every 7 days through the remainder of the experiment.

Food & Water;

- Remove food approximately 16 hours prior to infection.
- Remove water approximately 4 hours prior to infection.

Inoculum Preparation and Quantification:

- Subculture the *C. albicans* strain at 30°C for 48 hours on yeast extract peptone dextrose (YEPD) agar prior to *in vivo* use.
- Place isolates taken from the subculture into 5 mL of YEPD and allow to grow overnight (~16-24 hours) with orbital shaking at 200 rpm in a 30°C incubator.
- 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. After the second wash collect a loopful (10 µl) of cells and place into 2 mL of sterile saline or PBS. Spin in a microcentrifuge at high speed for 3 minutes. Discard the supernatant and then resuspend the cells in 2 mL of sterile saline or 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 hemocytometer.
  - Adjust the desired infecting inoculum to 1 X 10^8 cells/mL (5 x10^7 cells/mouse).
  - The inoculum should be used to infect the mice within 2 hours of preparation. It is essential to regularly resuspend the suspension during the infection.
- 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 Sabouraud 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 0.5 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 and cervical dislocation).

Timed Sacrifice of Animals:

- As controls, animals will be sacrificed immediately prior to inoculation (Day 0) and immediately prior to immunosuppression (Day 14).
- Mice in all experimental groups, including uninfected and uninfected plus drug control groups, will be sacrificed at Day 21 post-infection.
- Livers, kidneys, spleens, forestomachs and small intestines will be harvested from all sacrificed animals and used to assess fungal burden, as described below.

Antifungal Therapy:

- To evaluate the effects of pre-immunosuppression therapy, initiate antifungal treatment beginning 3 days prior to immunosuppression.
- To evaluate the effects of post-immunosuppression therapy, initiate antifungal treatment beginning 4 days following immunosuppression.
- Antifungals will include:
    1. Test compound
    2. Positive control: fluconazole (Diflucan) at 5 mg/kg/day, IP
    3. Positive control: caspofungin (Cancidas) at 1 mg/kg/day, IP

Outcome Measures: Outcome measures of antifungal therapy that are commonly used include reductions in tissue fungal burdens in various organs at a pre-specified time point (Day 21). Daily or weekly weights may be recorded as well.

Fungal Burden. Fungal burden should be measured at a pre-specified time point prior to inoculation, prior to 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., kidney, liver, spleen, forestomach, small intestine), and record the weight of each organ for each animal.
- Place the organs 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 - 0.2 mL) of each onto Sabouraud dextrose agar. This may be done in duplicate.
    - Antibiotics may be included to prevent bacterial contamination. These may be added to either the sterile saline or PBS used to prepare the homogenates or added to the plates onto which the homogenates are plated (e.g., penicillin at 100 kU/L and streptomycin at 100 mg/L)
- 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

References:

1. Rahman D, Mistry M, Thavaraj S, et al. Murine model of concurrent oral and vaginal *Candida albicans* colonization to study epithelial host-pathogen interactions. Microbes and Infection **2007**; 9:615-622
2. Jones T, Federspiel NA, Chibana H, et al. The diploid genome sequence of *Candida albicans.* Proceedings of the National Academy of Sciences of the United States of America **2004**; 101:7329-34.
3. Spellberg BJ, Ibrahim AS, Avanesian V, et al. Efficacy of the anti-*Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. J Infect Dis **2006**; 194:256-60.
4. Spellberg BJ, Ibrahim AS, Avenissian V, et al. The anti-*Candida albicans* vaccine composed of the recombinant N terminus of Als1p reduces fungal burden and improves survival in both immunocompetent and immunocompromised mice. Infect Immun **2005**; 73:6191-3.
5. Reeves, P. G., Nielsen, F. H. & Fahey, G. C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. **1993**;123: 1939–1951.