Standard Operating Procedure (SOP)
***Candida glabrata* Murine Invasive Candidiasis Model**
NIH/NIAID Task Order A13

Isolates:
Various groups have used different strains of *C. glabrata* in their murine models of invasive candidiasis. Our group has used clinical strains in order to establish infection. These include:

- *C. glabrata* 05-761 [1]
- *C. glabrata* 05-62 [1]
- *C. glabrata* A3 [2]
- *C. glabrata* 04-1748 [2]

Mice:
Outbred ICR mice have been evaluated and used in this model of invasive candidiasis.

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

Strain	Weight	Age
ICR (CD-1)	22 - 30 grams	4 - 5 weeks

Immunosuppression:
This murine model may be performed using neutropenic mice. A single dose of 5-fluorouracil may be used to render the mice neutropenic.

- 5-fluorouracil (50 mg/mL vial) is used without further dilutions.
- Administer a 0.1 mL dose per mouse intravenously one day prior to inoculation.
- This single dose results in profound and prolonged neutropenia (< 100 neutrophils/mm^3 for >10 days) [33].

Inoculum Preparation and Quantification:

- Subculture the *C. glabrata* strain onto two Sabouraud dextrose agar (SDA) plates for the first subculture and incubate at 37°C for 48 hours.
- From one of the first subculture plates, prepare a second subculture onto two SDA plates and incubate at 37°C for 48 hours. Reserve the other plate as backup.
- From the second subculture plates, prepare a third subculture onto at least five SDA plates and incubate at 37°C for 48 hours.
- Scrape the surface of each of the third subculture plates with a 10 µl inocula loop to collect all of the colonies from the third subculture. Suspend in 50 mL of physiologic saline with 0.1% Tween 20 in a 50 mL conical vial.
- Collect the cells by centrifugation (~2,000 rpm for 10 minutes) and wash in sterile physiologic saline with 0.1% Tween 20. Remove the supernatant and repeat the wash 2 additional times in 50 mL sterile physiologic saline with 0.1% Tween 20. After the second wash collect a loopful (10 ul) of cells and place into 2 mL of sterile saline with 0.1% Tween 20. Spin in a microcentrifuge at high speed for 3 minutes.

Discard the supernatant and then resuspend the cells in 2 mL of sterile saline.

- Prepare dilutions of the cells in sterile saline (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 (e.g., 5 x 10^8 cells/mL).
  - The inoculum should be used to infect the mice within 2 hours of preparation.
    NOTE: 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.

Intravenous Inoculation:

- Inoculate each mouse by injecting 0.2 mL of the desired inoculum in sterile saline or PBS via the lateral tail vein.
- If necessary, a heat lamp, a heated box, and/or alcohol wipe may be used to dilate the vein for better visualization.
- If necessary, briefly apply slight pressure over injection site to prevent bleeding
- Following successful inoculation, return the mice to their cages.

Monitoring of Animals Post-Inoculation:

- Although this is a nonlethal infection model, mice should be monitored at least twice daily throughout the course of the experiment to prevent and minimize unnecessary pain and distress. Moribund animals 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).

Antifungal Therapy:

- To evaluate the effects of therapy, initiate antifungal treatment after intravenous inoculation. In order to allow for the establishment of disease, begin therapy the day after inoculation (~24 hours later).
- Treatment groups typically consist of the following:

1. Placebo controls (either saline or an excipient used to dissolve or suspend one of the positive comparators)
2. Test compound
3. Positive control (e.g., fluconazole, caspofungin, amphotericin B formulation)

- Examples of doses and dosing calculations for fluconazole and caspofungin are given at the end of this SOP.

Outcome Measures: Reductions in tissue fungal burden is the primary outcome measure of antifungal therapy that is commonly used in this model.

Fungal Burden. Fungal burden should be measured at a pre-specified time point 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 (e.g., day 6 if therapy is on days 1 - 5, or day 8 if therapy is on days 1 - 7).

- At the pre-specified time point, aseptically collect the desired target organ(s) (e.g., kidneys, liver, spleen, and brains as needed), 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 (e.g., chloramphenicol at 0.05 mg/mL and gentamicin at 0.8 µg/mL) or added to the plates onto which the homogenates are plated (e.g., chloramphenicol at 0.05 mg/mL).
- 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

Examples of Doses and Dosing Calculations for Caspofungin and Fluconazole

Caspofungin (Cancidas, Merck)

Reconstitute vial of caspofungin acetate powder for injection

- Aseptically add 10.8 mL of 0.9% sodium chloride or sterile water for injection to the 50 mg caspofungin vial
- Gently mix vial until a clear solution is obtained (do not vortex)
- Reconstituted 50 mg vial = 5 mg/mL

Multiply average weight of mice by the dose to determine the amount of drug to administer to each animal (e.g. 1 mg/kg x 0.025 kg = 0.025 mg)

Divide the amount of drug to be administered to each mouse by the volume that will be administered (e.g., 0.025 mg/0.2 mL = 0.125 mg/mL)

Calculate the total volume needed to dose all of the mice (e.g., 4 mL for 20 mice; plus 1 mL overage = 5 mL)

To calculate the volume to remove from the reconstituted vial and the volume needed for the dilution use the formula C1V1 = C2V2

- C1 = concentration of reconstituted vial
- V1 = volume to remove from reconstituted vial
- C2 = concentration of solution to be administered to mice
- V2 = total volume needed to dose all mice

For example:

(5 mg/mL)(V1) = (0.125 mg/mL)(5 mL)
V1 = [(0.125 mg/mL)(5 mL)]/5 mg/mL = 0.125 mL

Remove 0.125 mL from reconstituted vial and add to 4.875 mL of 0.9% sodium chloride or sterile water for injection (total volume = 0.125 mL + 4.875 mL = 5 mL)

Gently mix and administer by intraperitoneal injection

Note: We refrigerate the reconstituted vial for up to 1 week and use the same vial for an entire week of dosing removing daily the needed volume for the day's dosing.

Fluconazole (Diflucan, Pfizer)

- Use fluconazole for injection (100 mg/50 mL vial = 2 mg/mL concentration)
- Various manufacturers make this product (e.g. Bedford Laboratories; NDC 55390-194-01)
- Store either refrigerated or at room temperature (do not freeze)

Multiply average weight of mice by the dose to determine the amount of drug to administer to each animal (e.g. 10 mg/kg x 0.025 kg = 0.25 mg)

Divide the amount of drug to be administered to each mouse by the volume that will be administered (e.g., 0.25 mg/0.2 mL = 1.25 mg/mL)

Calculate the total volume needed to dose all of the mice (e.g., 20 mL for 20 mice; plus 10 mL overage = 30 mL)

- For fluconazole, we prepare enough for the entire dosing period (e.g., 5 to 7 days of once daily dosing)

To calculate the volume to remove from the reconstituted vial and the volume needed for the dilution use the formula C1V1 = C2V2

- C1 = concentration of reconstituted vial
- V1 = volume to remove from reconstituted vial
- C2 = concentration of solution to be administered to mice
- V2 = total volume needed to dose all mice

For example:

(2 mg/mL)(V1) = (1.25 mg/mL)(30 mL)
V1 = [(1.25 mg/mL)(30 mL)]/2 mg/mL = 18.75 mL

Remove 18.75 mL from reconstituted vial and add to 31.25 mL of 0.9% sodium chloride or sterile water for injection (total volume = 31.25 mL + 18.75 mL = 50 mL)

Gently mix and administer by oral gavage

We prepare enough fluconazole to dose all of the mice for the entire treatment period and store the preparation in the refrigerator.
Note: 10 mg/kg is the largest dose of fluconazole that can be given by oral gavage using a volume of 0.2 mL. This is due to the limited concentration of the IV formulation of fluconazole (2 mg/mL). If higher doses need to be administered (e.g., 20 mg/kg), then the volume of the oral gavage must be increased (e.g., 0.3 - 0.4 mL).

- Alternatively, empty the contents of a 200 mg fluconazole capsule into 10 mL of 0.03% agar (reconstituted in water and autoclaved) to make a suspension of 20 mg/mL. This concentration is sufficient to treat mice at up to 200 mg/kg based on dosing at 10 mL/kg.

REFERENCES

1. Wiederhold NP, Najvar LK, Bocanegra R, Molina D, Olivo M, Graybill JR. In vivo efficacy of anidulafungin and caspofungin against Candida glabrata and association with in vitro potency in the presence of sera. Antimicrob Agents Chemother **2007**; 51:1616-20.
2. Brzankalski GE, Najvar LK, Wiederhold NP, et al. Evaluation of aminocandin and caspofungin against Candida glabrata including isolates with reduced caspofungin susceptibility. J Antimicrob Chemother **2008**; 62:1094-100.
3. Graybill JR, Najvar LK, Holmberg JD, Luther MF. Fluconazole, D0870, and flucytosine treatment of disseminated Candida tropicalis infections in mice. Antimicrob Agents Chemother **1995**; 39:924-9.