

Standard Operating Procedure (SOP)
Pulmonary Mucormycosis Mouse Model via Intratracheal Injection (IT)
NIH/NIAID Task Order A13
Isolates:

Clinical strains used to establish infection include:

- *Rhizopus delemar* 99-880. Genome sequence is available from the Broad Institute (<http://fungidb.org/fungidb/getDataset.do?question=GeneQuestions.GenesByTaxon&display=detail#80>)

Rhizopus oryzae 99-8920 (the genome was sequenced by Institute for Genome Sciences, University of Maryland School of Medicine). However, not yet publicly available

Mice:

Outbred ICR female mice

Suppliers for this strain that have been used include Taconic (www.Taconic.com), Harlan (www.harlan.com) and Charles River (www.criver.com). The weight and age range limits are in the Table below:

Strain	Weight	Age
ICR (CD-1)	25 - 30 grams	5 - 6 weeks

Detentions

- **Day 0** is defined as the day of infection. All days below are relative to Day 0

Diabetic Ketoacidotic (DKA) mouse model:

- **On Day -10**, make mice diabetic by administering 210 mg/kg of streptozotocin by intraperitoneal [ip] injection.
- **On Day-3** (or 7 days after streptozotocin treatment), determine the degree of glycosuria and ketonuria with keto-Diastix reagent strips (Bayer, Elkhart, Ind.).
- **On Day-2 and +3**, treat mice with 250 mg/kg cortisone acetate (CA) subcutaneously (SQ) in 0.1ml of 0.05% Tween 80 (sonicated for 30-60 sec before use) (Figure 1).



Figure 1. Timeline of the DKA animal model.

Neutropenic mouse model

- **On day -2, +3, and + 8 (relative to Day of infection which is Day 0),** immunosuppress mice with cyclophosphamide (200 mg/kg administered ip) and cortisone acetate (500 mg/kg in 0.1 ml of 0.05% Tween 80, administered SQ) (Figure 2).

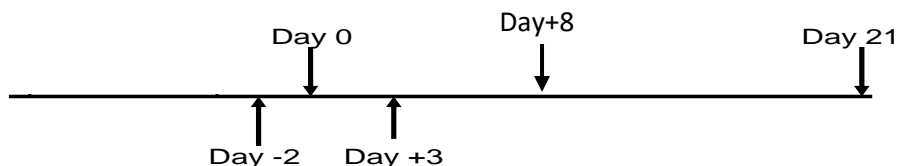


Figure 2. Timeline of the neutropenic animal model

Inoculum Preparation and Quantification:

- **Day -4**, subculture the *Rhizopus* strain from spores absorbed on silica beads on potato dextrose agar (PDA; BD Biosciences — Diagnostic Systems), incubate at 37°C for 4 hours.
- **Day 0**, collect the sporangiospores of *Rhizopus* in endotoxin-free Dulbecco's Phosphate buffered saline (PBS) containing 0.01% Tween 80.
 - Discard the supernatant and wash the spores by suspending in 5 mL sterile with 5 ml PBS.
 - Repeat the step above then resuspend the cells in 3 mL sterile PBS.
 - Prepare dilutions of the cells in sterile PBS (e.g., 1:100, 1:200, 1:400 or 1:800) and determine the number of cells/mL using a hemocytometer.
 - Adjust the inoculum to 1×10^7 cells/ml (or 2.5×10^5 cells/25 μ l)
 - Confirm the inoculum viability by serially diluting an aliquot of the inoculum in sterile PBS. Prepare dilutions (1:100,000) of the stock, and plate 100 μ L of the dilutions onto YPD or SDA agar containing 0.1% triton in triplicate.
 - Incubate the plates at 37°C and count the number of colony forming unit (CFU) the next day (CFU should be ~100 /plate).

Preparations for intratracheal infection:

- **Day -1**, put heating pads (Fisher Scientific Product # NC9411230) in a water bath heated to 60°C for overnight (alternatively, use electrically heated pads that are set to 37°C on <http://www.hygienesuppliesdirect.com/products/prod110283>).
- **Day 0** (infection day), transfer the heating pads to another water bath at 37°C 2-3 h prior to use in infection procedure

Intratracheal Inoculation:

- It is essential to regularly resuspend the *Rhizopus* suspension during the infection by vortexing.
- Sedate mice by intraperitoneal or intramuscular injection of 0.2 mL (i.e. 8 mL/kg) of a mixture of ketamine 82.5 mg/kg (prepared from a stock solution of 100

mg/mL) and xylazine 6 mg/Kg (prepared from stock solution of 100 mg/mL) (the diluent is sterile PBS). This dose will deliver full anesthesia to the mouse for ~15-30 min. See below for details on how to prepare the anesthesia.

- Put the mice on their backs on the heating pads under heating lamps (at arm's length) while waiting for infection so the mice do not get hypothermia. Alternatively, thermostatically controlled warm air boxes can be used set at 38°C.
- Lift sedated mice While pulling the tongue anteriorly and to the side with forceps, twenty five µl of fungal spores (2.5×10^5 cells) in PBS will be injected through the vocal cords into the trachea with a Fisher brand Gel-loading tip (Cat # 02-707-138).
- Place the mice on their backs on the heating pads.
- Monitor the mice and as they wake up and randomly sort into different treatment groups while transferring to cages.
- To confirm the inoculum delivered to the lungs, sacrifice two to three mice immediately after the infection and dissect the lungs, homogenize, and quantitatively culture on PDA plates plus 0.1% triton. Enumerated Colony forming units (CFU) after incubating at 37°C for 24 h.
- For uninfected control mice, deliver twenty five µl of PBS alone intratracheally in the same manner.
- Monitor the mice and as they awaken and transfer back to their cages.

Monitoring of Animals Post-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 will be identified by the following criteria:
 1. Ruffled and/or matted fur
 2. Hypothermia (cool to touch)
 4. Decreased activity
 5. Hunched posture
 6. Inability to eat or drink
 7. Excessive vaginal discharge indicative of bacterial vaginosis
 - 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 antifungal treatment, start therapy 16 hours post infection with liposomal amphotericin B (LAmB) at 15 mg/kg/d (from a stock of 4 mg/ml) or Posaconazole (POS) at 60 mg/kg/d or 30 mg/kg given twice daily (bid) (from an oral suspension).
- LAmB is given iv while POS is given by oral gavage.
- For survival studies continue treatment until day +7 while for tissue fungal burden continue treatment until day +3.

- For the tissue fungal burden, lungs (primary target organ) and brains (secondary target organs) will be collected and CFU (by qPCR) (Ibrahim et al. 2005 AAC; 49; 721) will be determined.
- Histopathological examination of severity of infection can also be assessed by fixing in 10% Zinc-buffered formalin followed by Periodic acid-Schiff (PAS) or H&E staining

Anesthesia Preparation

Stock solutions:

- 1) Ketamine (100 mg/mL)
- 2) Xylazine (100 mg/mL)

To prepare 82.5 mg/kg Ketamine + 6 mg/kg Xylazine cocktail delivered at 8 mL/kg dilute as follows:

- 5mL anaesthetic cocktail mix = 1.03 mL of NEAT ketamine + 0.375 mL of NEAT Xylazine + 3.595 mL sterile PBS
- Administer at 8 mL/kg (i.e. 0.2 mL / 25g mouse) IP or IM to achieve 82.5 mg/kg Ketamine/6 mg/kg Xylazine.

- **Calculation for Cortisone acetate (CA)-DKA:**

- 25 ICR (30 gram each)
- 250 mg/kg will be used. So per dose is $0.03 \text{ kg} \times 250 \text{ mg/kg} = 7.5 \text{ mg}$.
- 30 doses are prepared, which needs $30 \times 7.5 \text{ mg} = 225 \text{ mg}$
- Dissolve 225 mg CA in 3 ml of 0.05% Tween 20
- Sonicate and give 0.1 ml/dose through SQ.

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- **Calculation for Cortisone acetate (CA)-neutropenic:**

- 25 ICR (30 gram each)
- 500 mg/kg will be used. So per dose is $0.03 \text{ kg} \times 500 \text{ mg/kg} = 15 \text{ mg}$.
- 30 doses are prepared, which needs $30 \times 15 \text{ mg} = 450 \text{ mg}$
- Dissolve 450 mg CA in 3 ml of 0.05% Tween 20
- Sonicate and give 0.1 ml/dose through SQ.

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- **Calculation for Cyclophosphamide (Cyclo)-neutropenic:**

- 25 ICR (30 gram each)
- 200 mg/kg will be used. So per dose is $0.03 \text{ kg} \times 200 \text{ mg/kg} = 6 \text{ mg}$.
- 30 doses are prepared, which needs $30 \times 6 \text{ mg} = 180 \text{ mg}$
- Dissolve 500 mg cyclo in 16.7 ml of irrigation water
- Vortex and given 0.2 ml/dose through ip.