Standard Operating Procedure for Rat Inhalational Pulmonary Aspergillosis

1. Purpose

   This Standard Operating Procedure (SOP) will provide information necessary for the uniform pulmonary infection of rats by *Aspergillus fumigatus* or related fungal spore inoculum preparations.

2. Scope

   This SOP and will provide sufficient information to infect rats in Acrylic inhalation chambers. These chambers are utilized for the induction of inhalational pulmonary aspergillosis. This SOP introduces the process of infection and follows it from immunosuppression, through actual infection within the chamber, through disinfection of the apparatus and, ultimately, monitoring the infected rats.

3. Definitions.

   For the purposes of this SOP, "infect" will mean to introduce into the animal a precise, quantified concentration of viable *Aspergillus fumigatus* conidia in a diluent suitable for suspending and stabilizing the same.

4. Responsibilities

   This SOP shall be utilized by employees of Research assistant status or higher without additional training. Research technicians may perform this work upon receipt of training.

5. Equipment and Materials

- Drugs
  - Depo-Medrone, (Methylprednisolone acetate. Pharmacia)
  - Cortisone acetate, (Sigma catalog #C3130, supplied as a 25 gram vial)
  - Cyclophosphamide (Endoxana, Pharmacia) or (Cytoxan, Mead Johnson supplied as a 500 milligram vial
  - Baytril (Bayer)
- Inhalation Chambers
  - Acrylic chamber (2ft.2in x 1ft.2in x1ft 6 in.) (Scott Filler, MD, Harbor-UCLA, Figure 1**)**
    - Inhalation chamber in laminar flow hood
    - Nebulizer – Hudson Micromist (Hudson RCI, Cat #1883)
      - Acceptable equivalent: Hudson Micromist, # HU41892, Southern Syringe Services Ltd Enfield UK (European Union)
    - Compressed air cylinder – medical grade air is not required
- Rats – male Sprague Dawley, 225-250 g, Charles River.
- Amphyl (Revco) [Acceptable equivalent: Vesphene (Steris) or Decon (Decon Labs)]
  - Acceptable equivalent: Virkon to wash the chamber and afterward fumigate with 4% formaldehyde.
- Sterile water [Acceptable equivalent: Sterile normal saline]

- Micropipette EDTA Tubes (Fisher)
- BD Unopette System for leukocyte enumeration (Fisher Biomedical Cat # 02-687-40).
- 70% ethanol
- 23, 25, 27, 30 gauge needles
- 5 ml, 1 ml and 0.3 ml syringes
- Potato Dextrose Agar plates (PDA)

6. Procedure
   - Preparation of Inoculum
     - *Refer to Standard Operating Procedure for Preparation of Aspergillus fumigatus Test Strains for Inhalational Aspergillus Animal Pulmonary Aspergillosis Studies.*
   - Rats
     - Use male Sprague Dawley between 225 and 250 grams. Rats smaller than this do not tolerate the immunosuppression well (larger rats up to 325g are acceptable). There will be a total of 6 rats per infected group plus an additional group of 4 which will remain uninfected. To confirm the inoculum of test strain delivered to the rats, 2-3 rats from each run of the chamber will be euthanized 1 hour post infection.

   - Immunosuppression – **Neutropenic model**
     - Immunosuppressive drugs are made and used at the following concentrations:
       - Depo-medrone 40 mg/ml [Treat with 16 mg/kg]: Depo-medrone is supplied ready to administer but MUST BE ADMINISTERED IM (this is best administered using a 0.3 or 0.5 ml syringe). Optional, according to local regulations: this can be achieved by temporarily anaesthetizing rats with inhaled isofluorane (2.5% in oxygen 1500ml/minute flow).
       - Cyclophosphamide , 75 mg/kg. Immunosuppress rats with 75 mg/kg of cyclophosphamide I.P.

       - At day –2 prior to inoculation, administer Depo-medrone [16mg/kg] IM (approximately 0.1 ml / rat) and cyclophosphamide [75 mg/kg] intraperitoneally (approximately 1 ml/ rat) to all the rats. A 30 or 27 gauge needle will work for the both compounds but small volume syringes are more accurate (0.3 or 0.5ml)

       - On day +2 post infection, the immunosuppression regimen using cyclophosphamide only 75 mg/kg should be repeated
       - At day -3 rats begin receiving a **daily dose** of the antibiotic Baytril [50 ppm] in drinking water to prevent bacterial infections due to immunosuppression that is induced for the duration of the study.

- It is possible to treat with 10 mg/kg S.C. daily (this is best administered using a 1.0 ml syringe). Injection sites should be alternated with subsequent injections.

- Immunosuppression – **Steroid model**
  - Immunosuppressive drugs are made and used at the following concentrations:
    - Day -4
      Cortisone acetate [Treat with 200 mg/kg S.C.] Prepare cortisone acetate in a 100mg/ml suspension in sterile PBS containing 0.05% Tween 80. Vortex the suspension vigorously for 1 minute and sonicate for 30 minutes to disrupt aggregates. Vortex for 10s before use. Administer using a 23G needle and 1 ml syringe.
      *Cortisone acetate will also settle rapidly, and it should be vortexed multiple times during injection.

    - Day -2, 0, +2, +4
      Cortisone acetate [Treat with 150 mg/kg S.C.]: Prepare cortisone acetate in a 100 mg/ml suspension in sterile PBS containing 0.05% Tween 80. Vortex the suspension vigorously for 1 minute and sonicate for 30 minutes to disrupt aggregates. Vortex for 10s before use. Administer using a 23 gauge needle and 1 ml syringe.
      *Cortisone acetate will also settle rapidly, and it should be vortexed multiple times during injection.

    - At day -3 rats begin receiving a **daily dose** of the antibiotic Baytril [50 ppm] in drinking water to prevent bacterial infections due to immunosuppression that is induced for the duration of the study.

      - It is possible to treat with 10 mg/kg S.C. daily (this is best administered using a 1.0 ml syringe). Injection sites should be alternated with subsequent injections.

- Inoculation of rats
  - Optional: On the morning of inoculation verify that the rats are leukopenic by tail vein phlebotomizing the control rats (10 µl volume per mouse, one half of capillary tube) and counting neutrophils using the Unopette® system. Do not bleed rats to be infected – this could affect mortality. The leukocyte count should be <1000.
  - Acrylic Chamber (optional inhalational infection chamber)
    - Place chamber in the Class II safety hood. Place all rats (maximum of 12 per run) to be infected in the inhalation chamber. Tape along the edge of the door facing out, and the top to avoid directing exiting conidia towards the hood opening. Also, plug in hole in the center of door with parafilm.

- The Micro Mist® nebulizer package comes with 5 parts: the tee, tubing, mouthpiece, jar with jet and cap, and reservoir. The mouthpiece and reservoir are simply discarded and not used. The tubing is connected to the bottom of the jar and then to the air tank. The tee (it is shaped in a "T") has 3 openings. The bottom of the tee connects to the cap of the jar. The smaller opening of the tee is the one which is connected to the chamber. This opening is smaller than the hole on the side of the chamber so it must be wrapped with parafilm to ensure a tight fit into the chamber. Do not cover the opening of the tee just the outer part of the opening so that the mist is expressed into the chamber and not being released outside of the chamber. The 3rd opening of the tee, which is the larger opening, should be completely sealed off. This may be done with a rubber stopper or it may be wrapped in parafilm. (This opening is sealed off so that the mist is directed to go into the chamber and not allowed to escape through this larger opening).
- Add 6 ml of the conidial suspension to the Micro Mist® nebulizer (or acceptable equivalent) and connect the nebulizer to the inhalation chamber. Connect the nebulizer to a tank of compressed air (medical air is not necessary).
- Run air through nebulizer at 100 kPa until the nebulizer begins to splutter, usually about 13-15 minutes.
- Turn off the compressed air and refill the nebulizer with another 6ml of suspension. While the apparatus is disconnected, gently agitate the chamber to redistribute the rats. (The rats will tend to huddle in the chamber).
- Reconnect the nebulizer and run at 100 kPa until it splutters (usually about minute 30-35) and stops delivering aerosol. Turn off compressed air at this point and leave the rats for a total exposure time of 1 hour.
- After 1 hour, open cage and transfer rats from chamber to their cages, placing the rats into individually vented cages.
- One hour later, euthanize the 2-3 rats to confirm the conidial delivery.
- Euthanize rats using an overdose of pentabarbitone delivered IP as per local regulations. Utilizing sterile technique, harvest the lungs, weigh the tissue and homogenize in 2 ml of sterile saline.
- Assess CFU (*Refer to Standard Operating Protocol for Animal Tissue Homogenization*). Prepare 1:10 and 1:100 dilutions of the homogenate and streak 100 µl of each onto PDA plates in

duplicate. Incubate overnight at 37°C and count the colonies the next day.

- Disinfection of the chamber
  - **Acrylic Chamber**
    - Add 6 ml of Amphyl to the Micro Mist® nebulizer, turn air on (as done previously) and run the nebulizer for 12 - 15 minutes.
    - Thoroughly clean the inside of the chamber with Amphyl® (or acceptable equivalent), then de-ionized water. If more experiments are planned in the next 48 hours with the same inocula then the chamber can remain in the hood until then (leave hood on). **WARNING: Do not turn on the UV light as this will damage the chamber.**
    - If another strain is to be used, or if the chamber is to be stored, then the chamber should be disinfected with 10% bleach, and 6 ml of 10% bleach should be nebulized to disinfect the channel which is not accessible for cleaning directly. The chamber (and channel) should then be extensively rinsed out with de-ionized water to remove bleach residue and dead conidia. WARNING: Do not use alcohol to clean as this will damage the chamber.
      - Optional:The chamber (and channel) should then be extensively rinsed out with Virkon followed by 70% industrial methylated spirit to remove bleach residue and dead conidia. Sterilize the chamber using a Formaldehyde vaporiser and carbon filtration unit. After disinfection wash the complete chamber with soapy water to remove all residues.

- Monitoring of rats

  - Starting on day -3 weigh rats – immunosuppression causes 10-15% weight loss. Any rat with greater than 25% weight loss should be euthanized
  - Monitor rats daily for signs of distress, such as:
    - Rapid breathing with chest indrawing
    - Breathing very slow, shallow and labored (preceded by rapid breathing)
    - Rapid weight loss due to dehydration/ renal failure
    - Ruffled fur
    - Hunched posture
    - Body temperature less than 33°C.
    - Impaired ambulation (unable to reach food or water easily)

        - Evidence of muscle atrophy or other signs of emaciation (body weight is not always appropriate).
        - Bloody nasal discharge – often seen on front paws
        - Extensive ulcerative dermatitis and infected tumors.
        - Any obvious illness such as signs of lethargy (drowsiness, aversion to activity, physical or mental alertness, anorexia (loss of appetite, especially when prolonged), bleeding, difficulty breathing, CNS disturbance and chronic diarrhea
        - Observe for urine staining as thick sticky urine is an indicator of renal failure
    - Rats that are moribund should be euthanized humanely using approved methods such as pentabarbitone overdose or inhaled anesthetic (Isofluorane). The goal should be to have all rats die by euthanasia rather than by infection. N.b. the clinical condition of rats can deteriorate rapidly following >25% weight loss or bloody nasal discharge.
    - The normal course of infection is severe disease requiring euthanasia 6-7 days post infection.

7. Deliverables
    - Analysis and interpretation of results
    - Use the log-rank test for the statistical comparisons of survival between animal groups
    - P values < 0.05 will be considered significant with adjustment for multiple comparisons.
    - Conidial delivery should be between 1000 and 10000 per animal (usually 2000-4000), although results can vary depending on homogenization technique.
    - Leukocyte count <1000
8. References

Sheppard DC, Rieg G, Chiang LY, Filler SG, Edwards JE Jr, Ibrahim AS.
Novel inhalational murine model of invasive pulmonary aspergillosis.
Antimicrob Agents Chemother. 2004 May;48(5):1908-11.

Zhao Y, Park S, Warn P, Shrief R, Harrison E, Perlin DS.
Detection of Aspergillus fumigatus in a rat model of invasive pulmonary aspergillosis by real-time nucleic acid sequence-based amplification.
J Clin Microbiol. 2010 Apr;48(4):1378-83

9. History

    Version 1.00.

10. Examples of Deliverables

    N/A

Figure 1. Acrylic Inhalation Chamber Apparatus for Aspergillosis