Standard Operating Procedure for Guinea Pig Inhalational Pulmonary Aspergillosis

1. Purpose

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

2. Scope

   This SOP and will provide sufficient information to infect guinea pigs in either the Madison or 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 either of the two chambers, through disinfection of the apparatus and, ultimately, monitoring the infected guinea pigs.

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
   - Drugs
     - Cortisone acetate, Sigma cat #C3130
     - Cyclophosphamide, (Cytoxan, Mead Johnson) supplied as 500 gram vials
     - Ceftazidime (Tazicef, Glaxo Smithkline, supplied as1 gram vial)
   - Inhalation chambers
     - Acrylic chamber (2ft.2in x 1ft.2in x1ft 6 in.) (Scott Filler, MD, Harbor-UCLA, Attachment 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
     - Madison chamber (University of Wisconsin at Madison, Attachment 2a)
       - This is a self contained, HEPA filtered unit.
   - Guinea Pigs – Male Hartley guinea pigs 0.450-0.500 kg, Charles River
   - Microisolators
   - Bleach, 10%
   - Sterile water [Acceptable equivalent: Sterile normal saline]
   - 70% ethanol [for cleaning Madison chamber]

- Certek Formaldehyde Generator/Neutralizer (Certek) [for decontaminating Madison Chamber]
- paraformaldehyde prills (JT Baker Cat # S898-07) [for decontaminating Madison chamber]
- ammonium carbonate (Sigma-Aldrich Cat # A9516) [for decontaminating Madison Chamber]
- Amphyl (Revco) [Acceptable equivalent: Vesphene (Steris) or Decon (Decon Labs)]
- Micropipette EDTA Tubes (Infolab)
- BD Unopette System for leukocyte enumeration (Fisher Biomedical Cat # 02-687-40).
- Needles 23,25 gauge needles
- Syringes, 10 ml, 3ml, 1ml

6. Procedure
   - Preparation of Inoculum
     - *Refer to Standard Operating Procedure for Preparation of Aspergillus fumigatus Test Strains for Inhalational Aspergillus Animal Pulmonary Aspergillosis Studies.*
   - Guinea Pigs
     - Use male Hartley guinea pigs 0.450-0.500 kg. Each experiment should include a total of at least 24-36 total guinea pigs, including 4 to 8 guinea pigs per treatment or control group and a comparable group of uninfected controls. An additional 1-2 guinea pigs will be sacrificed 1hr post infection from each individual run of the chamber to confirm the delivered inoculum.
   - Immunosuppression Regimen
     - Immunosuppressive drugs are made and used at the following concentrations:
       - Cortisone acetate [25mg/ml]: Weigh out the necessary amount of cortisone acetate and add sterile PBS containing 0.05% Tween 80. Vortex this suspension vigorously and sonicate for 10 seconds before using. (Note: This drug should be prepared the same day of use).
       - Cyclophosphamide [25mg/ml] should be dissolved by the addition of sterile water at a 25mg/ml concentration in the vial. **(Note: the concentration of this drug will change in the second round of Immunosuppression of the animals to 20mg/ml, thereby changing the amount of sterile water added to the vial).** Store at 4ºC.
       - Antibiotic Ceftazidime [50mg/ml] dissolve by addition of sterile saline (20ml) to 1g vial. Store at 4ºC.
       - At day –2 prior to inoculation, administer cortisone acetate [250 mg/kg] subcutaneously (approximately 5 ml / guinea pig) and cyclophosphamide [250 mg/kg] intra-peritoneally (approximately

5ml/guinea pig) in to all the guinea pigs. A 25 gauge needle will work for the cyclophosphamide, but cortisone may require a 23 gauge needle. Cortisone acetate will also settle rapidly, and it should be vortexed multiple times during injection.

- In addition, guinea pigs will begin receiving a daily dose of the antibiotic ceftazidime [50 mg/kg] intramuscularly (0.5ml/guinea pig) to prevent bacterial infections due to neutropenia that is induced for the duration of the study.
- On day +3 post infection, the immunosuppression regimen should be repeated using the same concentration of cortisone acetate [250mg/kg]. However, the concentration of cyclophosphamide is 200mg/kg (prepare a 20mg/ml stock to aid in calculating doses).

- Inoculation of Guinea Pigs
  - Optional: On the morning of inoculation verify that the guinea pigs are leukopenic by saphenous vein phlebotomizing control animals (0.7ml per guinea pig, one half of micropipette capillary tube) and counting neutrophils using the Unopette® system. Do not bleed guinea pigs to be infected – this increases mortality. The leukocyte count should be <1000.
  - Acrylic Chamber (optional inhalational infection chamber)
    - Place the inhalational chamber in the laminar flow hood and the compressed air cylinder to the Micro Mist® nebulizer which in turn is connected to the inhalation chamber by tygon tubing. Make sure to seal all connections with several layers of parafilm so as to avoid any leaks. Place up to 6-7 guinea pigs (depending on size) into the chamber per run. Seal the chamber with tape along the edge of the door facing out and the top to avoid directing exiting conidia towards the hood opening.
    - Add 6 ml of the conidial suspension to the Micro Mist® nebulizer reservoir (or acceptable equivalent) close and begin to run air through the nebulizer at 100 kPa until the nebulizer begins to splutter, usually about 13-15 minutes.
    - Turn off the compressed air and refill the nebulizer reservoir with an additional 6ml of the conidial suspension. At this time gently rock chamber to redistribute the guinea pigs. Guinea pigs may cluster due to the noise.
    - Reconnect the nebulizer and run at 100 kPa until it splutters (approximately 30-35 min.) and stops delivering aerosol. Turn off compressed air at this point and leave the guinea pigs in the chamber for a total exposure time of 1 hour from the beginning of the run.
    - After 1 hour, open chamber and transfer guinea pigs to their cages with 1-2 guinea pigs placed in a temporary cage.
    - One hour later, sacrifice the 1-2 guinea pigs to confirm the conidial delivery for that particular run.
    - Harvest lungs and homogenize 1 gram of tissue in 9 ml of sterile saline (refer to Standard Operating Procedure for Animal Tissue

Homogenization). Prepare a 1:10 and 1:100 dilution and spread 0.2ml of the undiluted, 1:10 and 1:1000 homogenates on Sabouraud dextrose (or potato dextrose) plates. Incubate overnight at 37°C and count the colonies the next day.

- Madison Chamber (optional inhalational infection chamber)
  - Place one guinea pig in each individual housing cage, within the cage rack, then place the rack into the chamber. The Madison Chamber will hold a maximum of 18 guinea pigs. Seal chamber door using the attached latching system.
  - Add 13-15 ml of the conidial suspension to the air-glass impinger
  - Run air through impinger at 40 l/min for 1 h. This shall be followed by a 10 minute air wash, with NO input of conidia from the impinger.
  - After 70 minutes (from beginning of run), open chamber door and transfer guinea pigs from chamber to their housing cages, placing microisolators on each cage.
  - Within 1 hour, sacrifice 1-2 guinea pigs to confirm the conidial delivery.
  - Harvest lungs (or other organs if needed) and homogenize in 9 ml of sterile saline (refer to Standard Operating Procedure for Animal Tissue Homogenization) Prepare a 1:10 and 1:100 dilution and spread 0.2ml of the undiluted, 1:10 and 1:1000 homogenates on Sabouraud dextrose (or potato dextrose) plates. Incubate overnight and count the colonies the next day.

- Disinfection of the chambers
  - Acrylic Chamber
    - Thoroughly clean the inside of the chamber with Amphyl® (or acceptable equivalent), then 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 6ml of 10% bleach should be nebulized to disinfect the channel which is not accessible for cleaning directly. The cage should then be extensively rinsed out with water to remove bleach residue and dead conidia. **WARNING: Do not use alcohol to clean as this will damage the chamber.**
  - Madison Chamber
    - Thoroughly clean the inside of the air-glass impinger with 70% ethanol, followed by a similar cleaning with sterile water.
    - Place 15 ml of 70 % ethanol into the air-glass impinger and run air through impinger at 40 l/min for 10 min.
    - Discard and repeat step 2 using sterile water.

- Spray external surfaces of the cage rack and internal housing cages and the inside of the Madison chamber with amphyl and soak for 10 minutes. Wipe dry and replace cage rack (and internal cages) into Madison chamber.
- Seal Madison chamber using the attached latch system.
- Begin final paraformaldehyde disinfection (attachment 2b).

- Monitoring of Guinea Pigs
  - Monitor guinea pigs daily for signs of distress
    - Rapid breathing
    - Breathing very slow, shallow and labored (preceded by rapid breathing)
    - Rapid weight loss due to dehydration
    - Ruffled fur
    - Hunched posture
    - Body temperature less than 30°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).
    - 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.
  - Guinea Pigs that are moribund should be euthanized humanely using approved methods such as pentobarbital overdose or CO_2 asphyxiation. The goal should be to have virtually all guinea pigs die by euthanasia rather than by infection.
  - The experiment should be continued for at least 14 days after inoculation or until all the guinea pigs are dead, which may occur at an earlier time point.

7. Attachments

   "Appendix 1 and 2a (source documents) and 2b, Madison Chamber disinfection "

8. 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 counts <1000.

   - This model consistently produces 100% mortality utilizing either the acrylic or Madison chamber aerosol challenge. For validity, the length of

survival of control infected guinea pigs utilizing this protocol should fall within the indicated ranges:
6.76 ± .18 (n=17) Acrylic chamber (Mean Day of Death ± SE)
8.09 ± .23 (n=23) Madison chamber (Mean Day of Death ± SE)

9. 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.

10. History

Version 1.00.

11. Examples of Deliverables

N/A