**Endpoint Assessment in Rabbit Models of Invasive Pulmonary Aspergillosis and Mucormycosis**

Running head: Pulmonary Aspergillosis and Mucormycosis

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**Abstract**

Multiple animal models have been developed to study the pathogenesis of invasive pulmonary aspergillosis, as well as to evaluate the efficacy, pharmacokinetics, and pharmacodynamics of various antifungal agents and vaccines. Each model is beneficial depending on the questions that are asked. In this chapter we will discuss the endpoints assessment of the persistently neutropenic rabbit models of invasive pulmonary aspergillosis and invasive pulmonary mucormycosis.

1. **Introduction**

Invasive pulmonary aspergillosis (IPA) and invasive pulmonary mucormycosis (IPM) are life-threatening infections in immunosuppressed patients, particularly in those with severe and prolonged neutropenia as a consequence of aplastic anemia, myelotoxic chemotherapy for the treatment of cancer, and in those receiving immunosuppressive medication for rejection prophylaxis after organ transplantation or treatment of graft-versus-host disease in allogeneic bone marrow transplantation [1-4]. Eleven new antifungal agents have been developed in different classes including triazoles, echinocandins, and lipid formulations of amphotericin B through these model systems from bench to bedside. Several new compounds are now in the path of development. These models also provide a platform for the study of antifungal vaccines. Endpoint interpretation allows better understanding of the pathogenesis of IPA and IPM, as well as the evaluation of efficacy, pharmacokinetics, and pharmacodynamics of new antifungal agents and novel vaccines.

This chapter herein will describe a panel of endpoint variables in the persistently neutropenic rabbit model of IPA and IPM, including residual fungal burden (quantitative cultures), organism mediated pulmonary injury (lung weights, pulmonary infarct scores, and ultrafast computerized tomography), survival, serum galactomannan antigenemia (detected by the double sandwich enzyme-linked immunosorbent assay (ELISA)), (1→3)-β-D-glucan levels (detected by *Limulus* amebocyte lysate assay), and pharmacokinetic-pharmacodynamic endpoints. Further discussion is provided in describing the use of quantitative real time PCR as an endpoint in IPM.

**2.**

**2.1 Quantitative fungal cultures:**

1. Sterile polyethylene bag
2. 0.9% sterile normal saline
3. Homogenizer (Stomacher 80; Tekmar® Corp., Cincinnati, OH)
4. Sabouraud Glucose Agar (SGA) plates
5. Incubator 37°C

**2.2 Organism mediated pulmonary injury**

1. Surgical tools and instruments
2. Sedation solution: ketamine and xylazine.
3. Ultrafast electron-beam CT scanner (model CE 0459 HiSpeed CT/I; GE Medical Systems, Milwaukee, Wis.)

**2.3 Survival**

pentobarbital

**2.4 (1→3)-β-D-Glucan assay:**

The assay for detection of (1→3)-β-D-glucan (Fungitell, Associates of Cape Cod)

**2.5 Galactomannan assay**

1. Platelia *Aspergillus*, Immunoenzymatic detection of galactomannan antigen of *Aspergillus* in serum (Sanofi Diagnostics Pasteur or now Bio-Rad, Marnes La Coquette, France)
2. Microplate spectrophotometer equipped with 450- and 620-nm filters
	1. **PCR studies of IPA and IPM**

**2.6.1 *PCR for Sera***

1. MagNA Pure LC system
2. DNA Isolation Kit III for bacteria and fungi.
3. Bacteria Lysis Buffer
4. Proteinase K (Sigma Aldrich)
5. Eppendorf thermomixer (Eppendorf).
6. Phosphate-buffered saline (PBS)
7. qPCR LightCycler Instrument (Roche Applied Sciences, Indianapolis, IN).
8. Master mix for MqPCR-1: 0.25 μM of each primer, 1X PCR buffer (Invitrogen Corp., Carlsbad, CA), 3 mM MgCl2, 0.025% BSA (Sigma-Aldrich Corp., St. Louis, MO), 0.025 U Platinum® *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA), 0.2 mM PCR Nucleotide MixPlus (1 dATP, dCTP, dGTP and 3 dUTP) (Roche Applied Sciences, Indianapolis, IN), 0.1 μM of each fluorescein (FITC) and LC Red-640 probes (RD640) (Idaho Technology, Inc., Salt Lake City, UT), and 0.002 U/µL HK-UNG (uracil N-glycosylase) (Epicentre, Madison, WI).
9. Master mix for MqPCR-2: 0.5 µM of each of the primers, 1X PCR buffer (Invitrogen Corp., Carlsbad, CA), 4mM MgCl2, 0.025% BSA (Sigma-Aldrich Corp., St. Louis, MO), 0.025 U/ml Platinum® Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA), 0.2 mM PCR Nucleotide Mix PLUS (Roche Applied Sciences, Indianapolis, IN), and 0.2 µM of each FITC and RD640 probes (Idaho Technology, Inc., Salt Lake City, UT).

**2.6.2 PCR for BAL**

1. Spheroplast buffer: 1.0 M sorbitol, 50.0 mM sodium phosphate monobasic, 0.1% 2-mercaptoethanol, 10 mg of lyticase/ mL [Sigma]
2. Rocking platform
3. AP1 buffer (DNeasy plant kit; Qiagen, Valencia, CA)
4. Lysing matrix D tubes (Fastprep sample preparation system; QBIOgene/MP Biomedical, Irvine, CA)
5. FastPrep instrument (QBIOgene/MP Biomedical).
6. 100 mg/ml RNase A
7. Eppendorf thermomixer (Eppendorf, Westbury, NY)
8. DNeasy plant kit
9. AE buffer (Qiagen, Valencia, CA)
10. Sterile water
11. Oligo software (Molecular Biology Insights, Cascade, CO).
12. Probes (Idaho Technologies, Salt Lake City, UT).
13. Sequencher software package (Gene Codes Corp., Ann Arbor, MI).
14. The NCBI BLAST database search program
15. The PCR master mix: 0.5 μM of each of the primers, 5 mM MgCl2, 0.025% bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO), 0.05 U/mL Platinum *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA), 10μL PCR buffer (Invitrogen Corp., Carlsbad, CA), 0.2 mM PCR Nucleotide Mix Plus (dATP, dCTP, and dGTP at 10 mM each and dUTP at 30 mM; Roche Molecular Biochemicals, Indianapolis, IN), and 0.1 μM each of the fluorescein and LC Red-640 probes.
16. HK-UNG thermostable uracil *N*-glycosylase (Epicentre, Madison, WI)
	1. **Histopathology**
17. 10% neutral buffered formalin
18. Paraffin
19. Periodic acid-Schiff (PAS) stain kit
20. Grocott-Gomori methenamine silver (GMS) stain kit
21. Microscope
22. Collect the blood via the indwelling Silastic catheter [32] at 24 h post-inoculation, just prior to initiation of antifungal treatment or vaccine and every other day thereafter for determination of the serum galactomannan levels.
23. Determine the concentrations as described above by one-stage immunoenzymatic sandwich microplate assay method.
	1. **Pharmacokinetics and Pharmacodynamics**
24. Indwelling Silastic catheter
25. Heparinized syringes
26. HPLC or LC-MS/MS
27. PmetricsTM software (v1.3.2, University of Southern CA, Los Angeles, CA, USA)
28. ADAPT 5 (<https://bmsr.usc.edu/software/adapt/> Biomedical Simulations Resource, Los Angeles, CA, USA)

***3. Methods***

* 1. **Quantitative fungal cultures**

Quantitative lung cultures facilitate the determination of the burden of organisms within tissue. Measured in units of colony forming units per gram of tissue (CFU/g), changes in quantitative lung cultures reflect the growth of organisms in untreated controls or the effect of an antifungal agent or vaccine that may augment pulmonary host defenses.

1. Sample lung tissue from each rabbit and culture by a standard excision of tissue from each lobe.
2. Weigh each fragment is individually, place in a sterile polyethylene bag and homogenize with 0.9% sterile normal saline for 30 s per tissue sample.
3. Prepare lung homogenate dilutions (10-1 and 10-2) in 0.9% sterile normal saline.
4. Plate aliquots (100 µL) from homogenates and homogenate dilutions onto SGA plates and incubate at 37°C for 6-8 h in IPM model and for 24 h in IPA model.
5. Count the number of CFU of *Aspergillus fumigatus* or *Mucorales* for each lobe, and calculate the CFU/g (see Note 1 and 2).

**3.2 Organism mediated pulmonary injury:**

***3.2.1 Pulmonary lesion scores and lung weights***

Patients who develop IPA and IPM suffer serious morbidity and high mortality as the result of organism-mediated pulmonary injury. *Aspergillus* spp. and Mucorales mediate pulmonary injury through several mechanisms, the most striking of which is angioinvasion. Through the mechanism of angioinvasion, the damaged blood vessels and endothelium promote vascular thrombosis with ensuing tissue ischemia, infarction, and necrosis. In thrombocytopenic hosts, the infarction is hemorrhagic resulting in impaired ventilation of the damaged lung. Assessment of this process is assessed by pulmonary infarct scores, lung weights, and computerized tomographic scans.

1. For assessment of pulmonary lesion scores and lung weights, resect carefully the entire heart-lung block at necropsy.
2. Dissect the heart away from lungs, leaving tracheobronchial tree and lungs intact.
3. Weigh the lungs and inspect by at least two observers, who are blinded to the treatment group, and record hemorrhagic infarct lesions (if any) in each individual lobe.
4. Define hemorrhagic infarcts as dark red consolidated lesions that correspond histologically to coagulative necrosis and intra-alveolar hemorrhage.
5. Add the number of positive lobes together, and calculate the mean value of all positive lobes for each treatment group [5-10].

**3.2.2 Ultrafast computerized tomography (UFCT):**

Two-dimensional ultrafast computerized tomography is an accurate tool in measuring pulmonary infiltrates in real time in experimental IPA. Pulmonary infiltrates in neutropenic hosts with invasive pulmonary aspergillosis are caused by vascular invasion, hemorrhagic infarction, and tissue necrosis. Monitoring the dynamics of pulmonary injury of invasive aspergillosis is an important tool for assessing response to antifungal therapy and vaccines. Serial CT scans of the lungs are obtained during all experiments in order to monitor the effects of antifungal or vaccine therapy on organism-mediated pulmonary injury during the course of infection.

1. Sedate the rabbits with ketamine and xylazine and place prone, head first, on the scanning couch.
2. Perform CT with an ultrafast electron-beam CT scanner (see Note 3).
3. Use a small scan circle and a 9.6-cm-diameter reconstruction circle with a matrix of 512 by 512 (see Note 4).
4. Scan parameters are 80 kV and 120 mA, and the scan duration is 0.8 s (see Note 5).
5. Photograph the images by using lung windows with a level of −600 Hounsfield units and a width of 1,800 Hounsfield units.
6. Establish a mean 2-dimensional CT scan lesion score by evaluating the infiltrate in each lobe (see Note 6 and 7).

***3.2.3 Multidimensional volumetric imaging (MDVI)***

We further developed a method for multidimensional volumetric imaging (MDVI) of pulmonary infiltrates for measuring therapeutic response to antifungal or vaccine therapy in IPA in persistently neutropenic rabbits [12]. We developed a semiautomatic method to measure the volume of lung lesions, which is implemented as an extension of the MEDx visualization and analysis software using UFCT. The steps used in the technique are described below. A point is placed within the lung on each CT section.

1. Perform seeded region growing with an inclusion threshold range of (1,023 to 775) to segment the lung.
2. A contour of the segmented lung boundary is automatically created.
3. If necessary, select the option to semiautomatically exclude non-lung tissue or include nonsegmented lung tissue and to re-perform region growing.
4. If necessary, also make fine manual adjustments of the lung boundary contour.
5. Compute the area of all pixels inside the lung boundary contour that are above a threshold of 500 Hounsfield units. This is the lung lesion area. Then determine the sum of the lesion areas from all relevant CT sections and multiplied it by the slice thickness. This is the total lesion volume. Implement a common strategy of first segmenting the lung image and then look inside the lung tissue for lesions (see Note 8 and 9).
6. Compare volumetric infiltrate measures with UFCT reading, histopathological resolution of lesions, microbiological clearance of *Aspergillus fumigatus*, and galactomannan index (GMI)[12].
7. Perform these steps for each CT section.

**3.3. Survival:**

Although measurement of survival is a time-honored but relatively insensitive endpoint in assessing the effects of antifungal or vaccine therapy, immunomodulation, or virulence in IPA or IPM, a difference in survival provides reassuring data supporting potential clinical efficacy. Survival in IPA or IPM is ultimately determined by organism-mediated pulmonary injury.

1. Record duration of survival in days post-inoculation for each rabbit.
2. Euthanize the rabbits upon reaching prespecified humane endpoints approved by Animal Care and Use Committee by intravenous administration of pentobarbital as described above at the end of each experiment.
3. Euthanize surviving rabbits on day 14 of study.
4. Analyze and plot the data by log-rank test by Kaplan-Meier methods [6,13,8,9].

**3.4 (1→3)-β-D-Glucan assay:**

(1**→**3)-β-D-glucan in serum, bronchoalveolar lavage (BAL) fluid or cerebrospinal fluid (CSF) also is used as a sensitive biomarker for antifungal or vaccine therapeutic response. (1**→**3)-β-D-glucan in serum or BAL fluid also demonstrates a strong correction between therapeutic outcome and biological outcome variables, including quantitative cultures, organism-mediated pulmonary injury (lung weights, pulmonary infarct score, and CT scan lesion scores), and survival.

1. Collect blood samples from each rabbit every other day for determination of plasma (1→3)-β-D-glucan levels.
2. Use the Fungitell assay for detection of (1→3)-β-D-glucan which is licensed for diagnosis of invasive fungal infections.
3. Detection of (1→3)-β-D-glucanis possible using a colorimetric assay read at 405 nm (with 490 nm background subtraction), based upon *para*-nitroanilide absorption at that wavelength. Lipopolysaccharide and (1→3)-β-D-glucaninitiate the coagulation cascade in the horseshoe crab (*Limulus polyphemus* or *Tachypleus* *tridentatus*) by activating different serine protease zymogens, factors C and G. Lipopolysaccharide specifically activates factor C, while (1→3)-β-D-glucanactivates factor G. Specificity of (1→3)-β-D-glucanis ensured by using a *Limulus polyphemus* amebocyte lysate that is depleted of factor C. The activated factor G converts the inactive proclotting enzyme to the active clotting enzyme, which in turn cleaves pNA from the chromogenic peptide substrate, Boc-Leu-Gly-Arg-pNA, creating a chromophore that absorbs at 405 nm. The reagent is used in the Fungitell kinetic assay to detect the rate of optical density increase in a sample. This rate is interpreted against a standard curve to produce estimates of (1→3)-β-D-glucanlevels in the sample.
4. Interpretation of (1→3)-β-D-glucanvalues, according to manufacture’s instructions for use: < 60 pg/mL, negative; 60 to 79 pg/mL, indeterminate; ≥ 80 pg/mL, positive. The correlation coefficient of the standard curve is r≥ 0.9992 (range from 0.9982 to 0.9998) [14,15,9] (see Notes 10 and 11).

**3.5 Galactomannan assay:**

Galactomannan is a cell wall carbohydrate found in *Aspergillus* spp. Our studies have consistently demonstrated a strong correlation between therapeutic outcome and biological outcome variables, including quantitative cultures, organism-medicated pulmonary injury (lung weights, pulmonary infarct score, and CT scan lesion scores), and survival [16,5,17,14,18,15,19,8-10].

1. Collect the blood every other day from each rabbit for determination of serum galactomannan antigen levels.
2. Perform the Platelia *Aspergillus* one-stage immunoenzymatic double-sandwich microplate assay (EIA) to determine galactomannan antigen levels according to manufacturer’s directions (see Note 12).
3. Briefly, mix 300 µL of each sample (serum or BAL fluid) with 100 µL of treatment solution and subsequently heat the mixture at 100°C for 3 min with a solution of EDTA in order to dissociate the immune complexes and to precipitate serum proteins that could interfere with the ELISA (see Note 13).
4. Centrifuge the specimens at 10,000 rpm for 10 min.
5. After centrifugation, use the supernatant for further testing.
6. Add an aliquot of 50 µL of conjugate (horseradish peroxidase–labeled monoclonal antibody EBA-2) to each well of the monoclonal antibody EBA-2–coated microplate, followed by the addition of 50 µL of the supernatant and incubate for 90 min at 37°C.
7. After incubation wash the plates five times and add 100 µL of substrate-chromogen solution containing tetramethylbenzidine to each well, and incubate the plates for 30 min at room temperature in darkness (see Note 14).
8. Stop the enzyme reaction by the addition of a 1 N sulfuric acid solution and the blue color in the wells will change to yellow.
9. Determine optical absorbance of specimens and controls with a spectrophotometer set at 450/620 nm wavelengths.
10. Calculate the galactomannan index (GMI) for each test sample by dividing its optical density (OD) by the mean cutoff value of the threshold control serum provided in the test kit (titrated at 1 ng/mL).
11. For validation, each assay contained one negative control (no galactomannan), one positive control containing 5 ng/mL of galactomannan, and a duplicate threshold controlserum containing 1 ng/mL galactomannan for calibration and conversion of the measured absorbances into indexes.
12. The optical density of the threshold control (cut-off control serum) is recommended by the manufacturer to be between 0.300 and 0.800 (≥0.300 and ≤0.800), the ratio between the negative control and the threshold control is recommended to be below 0.40 (<0.40), and that of the positive control and the threshold control is recommended to be greater than 1.50 (>1.50).
13. Calculate the ratio between the optical density of the test sample and that of the threshold control for each sample.
14. According to the manufacturer’s recommendations, a ratio of less than 0.50 (<0.50) is considered negative, sera/BAL fluid with an index greater than or equal to 0.50 (≥0.50) are considered positive for galactomannan antigen. For convenience, the term galactomannan index (GMI) simply refers to EIA test results.
15. Plot serial serum or BAL fluid galactomannan levels over time of administration of antifungal compound or vaccine and time of initiation of study drug treatment.

**3.6 PCR studies of IPA and IPM:**

Genomic DNA of Mucorales or *Aspergillus* spp., which can be detected in BAL fluid and in serum or plasma, can be used as endpoint biomarkers for therapeutic response to antifungal agents or vaccines and as indicators of virulence. We will exemplify the utility of fungal PCR through our studies of experimental IPM.

***3.6.1***

1. apply
2. (see Note 15)

***3.6.2 PCR for Sera***

Circulating genomic DNA from Mucorales test species is measured in plasma every other day by a quantitative real time Light Cycler based PCR assay using primers and probes designed from 28s rRNA gene sequences.

1. Extract p
2. Add Vortex thes incubate
3. centrifugethe mix Equilibrate the s
4. Process aStore the s
5. Perform qPCR assays with a LightCycler. Include a “kit blank” (water processed through extraction protocol) and a negative master mix control (water) in each PCR run.
6. Prepare master mixes in a laminar flow hood that is located in a room separate from that where DNA extractions are performed.
7. For both assays, add 5 µL of extracted plasma to 15 µL of master mix. Just prior to adding 5 µL of extracted specimen to the master mix, briefly vortex all extracted specimens then centrifuge for 45 s at 4,500 × g.
8. Uracil is released by incubating at 37°C for 15 min, followed by enzyme inactivation at 95°C for 3 min.
9. Perform touch-down PCR cycling as follows: 95°C denaturation for 0 s, followed by annealing in 1°C incremental steps between 68°C and 54°C for 5 s each with a 72°C extension of 15 s for each cycle.
10. Follow touchdown cycling with 35 cycles of 95°C for 0 s, 54°C for 5 s and 72°C for 15 s.
11. Following amplification, perform melt curve analysis by cooling from 96°C to 40°C for 30 s, followed by a gradual increase in temperature (2°C/s) to 75°C.
12. In case of the second master mix (MqPCR-2), as with qPCR-1, HK-UNG thermostabile uracil N-Glycosylase is utilized as recommended by the manufacturer to prevent potential amplicon carryover.
13. The cycling conditions are as follows: uracil activation, 37°C, 15 min (slope 20°C/s), uracil heat-inactivation, 95°C, 3 min (slope 20°C/s) for 1 cycle.
14. Amplification cycles: denaturation 95°C, 0 s (slope 20°C/s), annealing 57°C, 5 s (slope 10°C/s), extension 72°C, 15 s (slope 3°C/s) for 50 cycles. A melt cycle (96°C, 0 s (slope 20°C/s), 40°C, 30s (slope (2°C/s), 75°C, 0 s (slope 0.2°C/s)) is performed at the end of each run to confirm that the correct amplicon is generated via PCR product melt temperature analysis.
15. DNA copy number/mL of serum is used for quantification of circulating DNA.
16. The area under the concentration time curve (AUC) is then calculated.
17. The AUC0–∞ is calculated from amplicon plasma concentration profiles over time using the linear trapezoidal rule with extrapolation to infinity by standard techniques.

***3.6.3 PCR for BAL***

1. Thaw frozen BAL fluid samples before extraction is performed.
2. As previously described [16], vortex the samples for 1 min before a 500-μL aliquot is taken for processing.
3. Following centrifugation for 10 min at 16,000 *g*, discard the supernatant, and gently resuspend the pellet in 100 μL spheroplast buffer and 10 μL of lysing enzymes and incubated at 37°C on a rocking platform for 60 min.
4. After centrifugation for 20 min at 400 μ*g*, resuspend the spheroplast- BAL fluid pellet in 400 μL AP1 buffer.
5. Add the sample to lysing matrix D tubes and process using a FastPrep instrument.
6. Centrifuge specimens at 16,000 *g* for 30 to 60 s and then vortex gently.
7. Transfer an aliquot of 300 μL of the specimen to a 1.5-mL tube and adjust to a volume of 400 μL with AP1 buffer.
8. Add an aliquot of 4 μl of RNase A (100 mg/ml), vortex vigorously, and incubate for 10 min at 65°C in an Eppendorf thermomixer at 1,200 rpm.
9. Follow the DNeasy plant kit protocol with the following modification: after the 200 μL of preheated (65°C) AE buffer is applied to the column, heat the entire apparatus (column and collection tube) at 65°C in an Eppendorf thermomixer for 5 min.
10. Also, process an aliquot of 100 μL of sterile water as described above as a control for any contamination from the DNA extraction kit components.
11. Design a real-time quantitative PCR assay targeting the ITS1, 5.8S, and ITS2 regions of the rRNA gene complex.
12. Design the primers and probes using Oligo software based on a multiple sequence alignment of rRNA sequences from GenBank, utilizing the Sequencher software package. Use the NCBI BLAST database search program to determine the uniqueness of the primers and probes for *A. fumigatus* (see Note 16).
13. Utilize HK-UNG thermostable uracil *N*-glycosylase as recommended by the manufacturer to prevent potential amplicon carryover.
14. Each reaction mix containes an aliquot of 5 µL of extracted specimen, together with 15 μL of the master mix.
15. The cycling conditions are as follows: uracil activation (37°C, 180 s), uracil heat inactivation (95°C, 60 s for 1 cycle), and denaturation (95°C, 0 s), annealing (58°C, 5 s), and extension (72°C, 15 s) for 50 cycles.
16. Perform quantitation standards in conjunction with each set of samples.
17. Screen the BAL fluid samples from each rabbit with the following controls: (i) DNA extracted from normal BAL fluid and (ii) a negative control master mix (sterile water).
18. Use tenfold serial dilutions of genomic DNA extracted from *A. fumigatus* isolate 4215 as external quantitation standards for all quantitative PCRs.
19. In order to confirm the lack of PCR inhibitors in BAL fluid samples, perform a separate set of PCR/fluorescent resonance energy transfer reactions which specifically did not target *A. fumigatus* (or mammalian) DNA (see Note 17).
20. Test the presence of inhibitors, by comparing the amplification efficiency of this reaction with the extracted samples against those performed with sterile water (see Note 18).

**3.7. Histopathology:**

Histopathology of IPA and IPM provides important insight into the relative burden of organism, morphology of organisms, and degree of organism-mediated pulmonary injury.

1. Excise the lesions and fix in 10% neutral buffered formalin.
2. Stain the paraffin-embedded tissue sections with periodic acid-Schiff (PAS) and Grocott-Gomori methenamine silver stains (GMS).
3. Examine the tissues microscopically for histologic indicators of pulmonary injury [15,7,22,10].

**3.8 Pharmacokinetics and Pharmacodynamics:**

The rabbit models of IPA and IPM are also used to determine the immunopharmacology, pharmacokinetics (PK) and pharmacodynamics (PD) of the exposure-response relationship of antifungal agents and vaccines [23-29,6,19,7,22,9,30,31]. We will describe herein the use of serum galactomannan suppression to characterize the PK/PD relationship [18].

***3.8.1 Blood collection***

1. Collect the blood via the indwelling Silastic catheter [32] at 24 h post-inoculation, just prior to initiation of antifungal treatment or vaccine and every other day thereafter for determination of the serum galactomannan levels.
2. Determine the concentrations as described above by one-stage immunoenzymatic sandwich microplate assay method.

***3.8.2 Single and multiple-dose PK analyses***

1. Conduct single-dose PK analyses in four non-infected rabbits per each dosage group.
2. Time points, depending upon the antifungal agent or vaccine, for example may include pre-dose, 1, 2, 4, 8, 12, 18, 24, and 48 h post-dosing.
3. Determine multiple-dose PKs in infected animals 7 days post-inoculation.
4. Draw blood samples in this population pre-dose and at 1, 4, 8, and 24 h post-dosing.
5. Collect each blood sample for PK assay in heparinized syringes.
6. Separate plasma samples immediately by centrifugation at 400 × g and store at -80°C until assayed.
7. Measure the antifungal agent or vaccine in serum by a validated HPLC or LC-MS/MS assay.

***3.8.3 Pharmacokinetic and pharmacodynamic mathematical modeling***

Perform mathematical modeling in a step-wise manner as follows.

1. First, construct a population pharmacokinetic (PPK) model using non-parametric estimation in PmetricsTM software [33] and fitted to the rabbit plasma concentration data [33].
2. Estimate the weighting functions using a combination of ADAPT 5 and Pmetrics error estimation runs. Specifically, in ADAPT 5, estimate slope and intercept values using maximum likelihood estimation for each individual rabbit.
3. In Pmetrics, use the error estimation run script (i.e. ERRrun) to estimate the assay error polynomial coefficients directly from the data.
4. After establishing a model that best describes the PK data, add the galactomannan concentrations to the dataset for each individual rabbit.
5. Build linked PK-PD model using the following set of differential equations:

Eq. 1 $dXP/dt\left(1\right)= -Ka∙X\left(1\right)$

Eq. 2 $dXP/dtXP\left(2\right)= Ka∙X\left(1\right)-\left(\left(\frac{Cl}{V}\right)∙X\left(2\right)\right)-Kcp∙X\left(2\right)+Kpc∙X(3)$

Eq. 3 $dXP/dtXP\left(3\right)= Kcp∙X\left(2\right)-Kpc∙X(3)$

Eq. 4: $dXP/dt\left(4\right)= Kpmax∙\left(1-\left(\frac{\left(\frac{X\left(2\right)}{V}\right)^{Hp}}{C50p^{Hp}+\left(\frac{X\left(2\right)}{V}\right)^{Hp}}\right)\right)∙X\left(4\right)∙^{}$

 $\left(1-\left(\frac{X\left(4\right)}{popmax}\right)\right)-ksmax∙X\left(4\right)∙(\frac{\left(\frac{X\left(2\right)}{V}\right)^{Hs}}{C50s^{Hs}+\left(\frac{X\left(2\right)}{V}\right)^{Hs}})$

The first three equations describe the pharmacokinetics of the drug, (compartment 1, theoretical absorptive compartment for oral administration; 2, central compartment, and 3, peripheral compartment). Cl is the clearance defined, as the amount of drug being cleared from the central compartment over time and V is the volume of the central compartment. Ka is the first order absorption constant, Kcp is the rate of drug moving from the central to peripheral compartment and Kpc is the rate of drug moving from the peripheral to central compartment. Equation 4 describes the rate of change of the serum galactomannan values. Kpmax represents the maximum rate of production, Hp is the slope function for production, C50p is the amount of drug where there is half maximal production, popmax is the theoretical maximum density of galactomannan, ksmax is the maximum rate of galactomannan suppression, C50s is the amount of drug where there is half maximal suppression, and Hs is the slope function for the galactomannan suppression.

1. Evaluate an acceptance of the final model by visual inspection of the observed-versus-predicted values plotted over time after the Bayesian step, the coefficient of determination (r2) from the linear regression of the observed versus predicted values, as well as, evaluation of the estimated bias and precision.
2. After fitting the model to the PK-PD data, use the Bayesian posterior estimates for each rabbit to estimate the concentration-time profiles for antifungal agent or vaccine and GMI for each rabbit. Perform the simulations in ADAPT 5.
3. Calculate area under the plasma concentration-time curves (AUCs) by the linear trapezoidal rule from the simulated concentration-time profiles on the last day of dosing (at steady-state) in ADAPT 5.
4. Using simulated AUC values of the antifungal agent or vaccine and the GMI at the end of the dosing period for each rabbit, construct an inhibitory sigmoid Emax model to establish the pharmacodynamic relationship between exposure (AUC) over MIC and response (GMI). Use the model equation 5 described below:

Eq. 5: $Effect=E0-(\frac{Emax∙\left(\frac{AUC}{MIC}\right)^{H}}{EC50^{H}+\left(\frac{AUC}{MIC}\right)^{H}})$

E0 is the baseline level of galactomannan prior to exposure to drug, Emax is the maximum galactomannan value, EC50 is a measure of drug potency, and H is the slope factor.

***3.8.4 Bridging to humans***

To bridge the exposure-response results from the rabbits to humans, a PPK model may be constructed using the antifungal agent or vaccine plasma concentration data from one or more clinical trials.

1. Develop a PPK model using non-parametric estimation in Pmetrics software [33] (see Note 19).
2. Use Mean Bayesian parameter estimates from the fitted human model to perform Monte Carlo simulations of 1000 patients using Pmetrics.
3. Use the simulations to calculate steady state AUCs within Pmetrics by the trapezoidal rule for each simulated patient.
4. Using equation 5 above, calculate the effect of AUC/MIC at various MIC values representative of the MIC distribution and epidemiological cutoff value for the investigational antifungal agent for the organism.

**3.9 Statistical analysis:**

1. Perform the comparisons between the groups by analysis of variance (ANOVA) with Bonferroni’s correction for multiple comparisons or the Mann-Whitney U test, as appropriate. A two-tailed P value of ≤0.05 is considered to be statistically significant.
2. Plot survival by Kaplan-Meier analysis. Analyze differences in survival of treatment groups and untreated controls by log-rank test.
3. Express the values as means ± standard errors of the means (SEMs).
4. Compare pharmacokinetic parameters using ANOVA or Student’s *t* test, as appropriate.
5. Perform correlation between AUC0–24 and outcome variables using Pearson’s correlation method.

**4. Notes:**

1. A finding of one colony of *Aspergillus fumigatus* or Mucorales is considered positive culture.
2. Before implementing these comparative experiments, we conducted preliminary studies of the methodology of quantitative cultures of infected tissues [5-10]. Among the methods studied are cultures of lung sections, minced tissue, and gently homogenized tissue. No loss of viability is found using these techniques. As there are no significant differences among these methods, the aforementioned method is selected for all subsequent experiments.
3. These parameters resulted in a pixel size of less than 1 mm.
4. The use of an automatic method to segment the lung, such as seeded region growing, provides for a robust technique with less inter/intraoperator variability than manual tracing. The threshold range (1,023 to 775) is determined empirically. If it is necessary to include/exclude tissue that is not properly segmented in the region-growing step, an option exists for the operator to trace the targeted tissue to include/ exclude. For inclusion, the pixel values within the targeted tissue will be modified to be within the threshold range. For exclusion, the pixel values will be modified to be outside the threshold range. Perform the region-growing algorithm again, and the targeted tissue is now included/excluded as desired.
5. The threshold value of 500 Hounsfield units for lesion determination is based on previous literature and empirical evidence. Selecting a threshold value of 500 Hounsfield units excludes the air in the lungs but might include blood vessels, which have similar density to the lung lesions. Inclusion of these small vessels may reduce the sensitivity of detecting a lesion volume change over time.
6. The substrate solution will react with the complexes bound to the well to form a blue color reaction.
7. The assays are specific for the target genera and did not amplify mammalian genes or those found in other fungal genera, including *Candida* spp. and *Aspergillus* spp. that are likely to be present in the same host [16,20,13,21].
8. The amplicon generated is 253 bp long.
9. The primers and probes are designed to target the common plasmid cloning vector pBR322. The PCR master mix included a specific amount of target DNA (pBR322).
10. Equivalent amplification efficiencies compared to reactions performed with water reflected the lack of inhibitors.
11. The model fitting process includes evaluating both two- and three-compartment models with and without lag-time and oral bioavailability terms.

**References**

1. Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, Ito J, Andes DR, Baddley JW, Brown JM, Brumble LM, Freifeld AG, Hadley S, Herwaldt LA, Kauffman CA, Knapp K, Lyon GM, Morrison VA, Papanicolaou G, Patterson TF, Perl TM, Schuster MG, Walker R, Wannemuehler KA, Wingard JR, Chiller TM, Pappas PG (2010) Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis 50 (8):1091-1100. doi:10.1086/651263

2. Kosmidis C, Denning DW (2015) The clinical spectrum of pulmonary aspergillosis. Thorax 70 (3):270-277. doi:10.1136/thoraxjnl-2014-206291

3. Patterson TF (2009) Risk stratification for invasive aspergillosis: early assessment of host susceptibility. Med Mycol 47 Suppl 1:S255-260. doi:10.1080/13693780902718339

4. Rubio PM, Sevilla J, Gonzalez-Vicent M, Lassaletta A, Cuenca-Estrella M, Diaz MA, Riesco S, Madero L (2009) Increasing incidence of invasive aspergillosis in pediatric hematology oncology patients over the last decade: a retrospective single centre study. J Pediatr Hematol Oncol 31 (9):642-646. doi:10.1097/MPH.0b013e3181acd956

5. Francis P, Lee JW, Hoffman A, Peter J, Francesconi A, Bacher J, Shelhamer J, Pizzo PA, Walsh TJ (1994) Efficacy of unilamellar liposomal amphotericin B in treatment of pulmonary aspergillosis in persistently granulocytopenic rabbits: the potential role of bronchoalveolar D-mannitol and serum galactomannan as markers of infection. J Infect Dis 169 (2):356-368

6. Petraitiene R, Petraitis V, Groll AH, Sein T, Piscitelli S, Candelario M, Field-Ridley A, Avila N, Bacher J, Walsh TJ (2001) Antifungal activity and pharmacokinetics of posaconazole (SCH 56592) in treatment and prevention of experimental invasive pulmonary aspergillosis: correlation with galactomannan antigenemia. Antimicrobial agents and chemotherapy 45 (3):857-869. doi:10.1128/AAC.45.3.857-869.2001

7. Petraitis V, Petraitiene R, Groll AH, Bell A, Callender DP, Sein T, Schaufele RL, McMillian CL, Bacher J, Walsh TJ (1998) Antifungal efficacy, safety, and single-dose pharmacokinetics of LY303366, a novel echinocandin B, in experimental pulmonary aspergillosis in persistently neutropenic rabbits. Antimicrobial agents and chemotherapy 42 (11):2898-2905

8. Petraitis V, Petraitiene R, Hope WW, Meletiadis J, Mickiene D, Hughes JE, Cotton MP, Stergiopoulou T, Kasai M, Francesconi A, Schaufele RL, Sein T, Avila NA, Bacher J, Walsh TJ (2009) Combination therapy in treatment of experimental pulmonary aspergillosis: in vitro and in vivo correlations of the concentration- and dose- dependent interactions between anidulafungin and voriconazole by Bliss independence drug interaction analysis. Antimicrobial agents and chemotherapy 53 (6):2382-2391. doi:10.1128/AAC.00329-09

9. Petraitis V, Petraitiene R, Moradi PW, Strauss GE, Katragkou A, Kovanda LL, Hope WW, Walsh TJ (2016) Pharmacokinetics and Concentration-Dependent Efficacy of Isavuconazole for Treatment of Experimental Invasive Pulmonary Aspergillosis. Antimicrob Agents Chemother 60 (5):2718-2726. doi:10.1128/AAC.02665-15

10. Petraitis V, Petraitiene R, Sarafandi AA, Kelaher AM, Lyman CA, Casler HE, Sein T, Groll AH, Bacher J, Avila NA, Walsh TJ (2003) Combination therapy in treatment of experimental pulmonary aspergillosis: synergistic interaction between an antifungal triazole and an echinocandin. The Journal of infectious diseases 187 (12):1834-1843. doi:10.1086/375420

11. Walsh TJ, Garrett K, Feurerstein E, Girton M, Allende M, Bacher J, Francesconi A, Schaufele R, Pizzo PA (1995) Therapeutic monitoring of experimental invasive pulmonary aspergillosis by ultrafast computerized tomography, a novel, noninvasive method for measuring responses to antifungal therapy. Antimicrob Agents Chemother 39 (5):1065-1069

12. Petraitis V, Petraitiene R, Solomon J, Kelaher AM, Murray HA, Mya-San C, Bhandary AK, Sein T, Avila NA, Basevicius A, Bacher J, Walsh TJ (2006) Multidimensional volumetric imaging of pulmonary infiltrates for measuring therapeutic response to antifungal therapy in experimental invasive pulmonary aspergillosis. Antimicrob Agents Chemother 50 (4):1510-1517. doi:10.1128/AAC.50.4.1510-1517.2006

13. Petraitis V, Petraitiene R, Antachopoulos C, Hughes JE, Cotton MP, Kasai M, Harrington S, Gamaletsou MN, Bacher JD, Kontoyiannis DP, Roilides E, Walsh TJ (2013) Increased virulence of Cunninghamella bertholletiae in experimental pulmonary mucormycosis: correlation with circulating molecular biomarkers, sporangiospore germination and hyphal metabolism. Med Mycol 51 (1):72-82. doi:10.3109/13693786.2012.690107

14. Hope WW, Petraitis V, Petraitiene R, Aghamolla T, Bacher J, Walsh TJ (2010) The initial 96 hours of invasive pulmonary aspergillosis: histopathology, comparative kinetics of galactomannan and (1->3) beta-d-glucan and consequences of delayed antifungal therapy. Antimicrob Agents Chemother 54 (11):4879-4886. doi:10.1128/AAC.00673-10

15. Petraitiene R, Petraitis V, Bacher JD, Finkelman MA, Walsh TJ (2015) Effects of host response and antifungal therapy on serum and BAL levels of galactomannan and (1-->3)-beta-D-glucan in experimental invasive pulmonary aspergillosis. Med Mycol 53 (6):558-568. doi:10.1093/mmy/myv034

16. Francesconi A, Kasai M, Petraitiene R, Petraitis V, Kelaher AM, Schaufele R, Hope WW, Shea YR, Bacher J, Walsh TJ (2006) Characterization and comparison of galactomannan enzyme immunoassay and quantitative real-time PCR assay for detection of Aspergillus fumigatus in bronchoalveolar lavage fluid from experimental invasive pulmonary aspergillosis. J Clin Microbiol 44 (7):2475-2480. doi:10.1128/JCM.02693-05

17. Hope WW, Kruhlak MJ, Lyman CA, Petraitiene R, Petraitis V, Francesconi A, Kasai M, Mickiene D, Sein T, Peter J, Kelaher AM, Hughes JE, Cotton MP, Cotten CJ, Bacher J, Tripathi S, Bermudez L, Maugel TK, Zerfas PM, Wingard JR, Drusano GL, Walsh TJ (2007) Pathogenesis of Aspergillus fumigatus and the kinetics of galactomannan in an in vitro model of early invasive pulmonary aspergillosis: implications for antifungal therapy. J Infect Dis 195 (3):455-466. doi:10.1086/510535

18. Kovanda LL, Petraitiene R, Petraitis V, Walsh TJ, Desai A, Bonate P, Hope WW (2016) Pharmacodynamics of isavuconazole in experimental invasive pulmonary aspergillosis: implications for clinical breakpoints. J Antimicrob Chemother 71 (7):1885-1891. doi:10.1093/jac/dkw098

19. Petraitiene R, Petraitis V, Lyman CA, Groll AH, Mickiene D, Peter J, Bacher J, Roussillon K, Hemmings M, Armstrong D, Avila NA, Walsh TJ (2004) Efficacy, safety, and plasma pharmacokinetics of escalating dosages of intravenously administered ravuconazole lysine phosphoester for treatment of experimental pulmonary aspergillosis in persistently neutropenic rabbits. Antimicrob Agents Chemother 48 (4):1188-1196

20. Kasai M, Harrington SM, Francesconi A, Petraitis V, Petraitiene R, Beveridge MG, Knudsen T, Milanovich J, Cotton MP, Hughes J, Schaufele RL, Sein T, Bacher J, Murray PR, Kontoyiannis DP, Walsh TJ (2008) Detection of a molecular biomarker for zygomycetes by quantitative PCR assays of plasma, bronchoalveolar lavage, and lung tissue in a rabbit model of experimental pulmonary zygomycosis. J Clin Microbiol 46 (11):3690-3702. doi:10.1128/JCM.00917-08

21. Walsh TJ, Wissel MC, Grantham KJ, Petraitiene R, Petraitis V, Kasai M, Francesconi A, Cotton MP, Hughes JE, Greene L, Bacher JD, Manna P, Salomoni M, Kleiboeker SB, Reddy SK (2011) Molecular detection and species-specific identification of medically important Aspergillus species by real-time PCR in experimental invasive pulmonary aspergillosis. J Clin Microbiol 49 (12):4150-4157. doi:10.1128/JCM.00570-11

22. Petraitis V, Petraitiene R, Groll AH, Roussillon K, Hemmings M, Lyman CA, Sein T, Bacher J, Bekersky I, Walsh TJ (2002) Comparative antifungal activities and plasma pharmacokinetics of micafungin (FK463) against disseminated candidiasis and invasive pulmonary aspergillosis in persistently neutropenic rabbits. Antimicrob Agents Chemother 46 (6):1857-1869

23. Groll AH, Gullick BM, Petraitiene R, Petraitis V, Candelario M, Piscitelli SC, Walsh TJ (2001) Compartmental pharmacokinetics of the antifungal echinocandin caspofungin (MK-0991) in rabbits. Antimicrob Agents Chemother 45 (2):596-600. doi:10.1128/AAC.45.2.596-600.2001

24. Groll AH, Lyman CA, Petraitis V, Petraitiene R, Armstrong D, Mickiene D, Alfaro RM, Schaufele RL, Sein T, Bacher J, Walsh TJ (2006) Compartmentalized intrapulmonary pharmacokinetics of amphotericin B and its lipid formulations. Antimicrob Agents Chemother 50 (10):3418-3423. doi:10.1128/AAC.00241-06

25. Groll AH, Mickiene D, Petraitis V, Petraitiene R, Kelaher A, Sarafandi A, Wuerthwein G, Bacher J, Walsh TJ (2005) Compartmental pharmacokinetics and tissue distribution of the antifungal triazole ravuconazole following intravenous administration of its di-lysine phosphoester prodrug (BMS-379224) in rabbits. J Antimicrob Chemother 56 (5):899-907. doi:10.1093/jac/dki287

26. Groll AH, Mickiene D, Piscitelli SC, Walsh TJ (2000) Distribution of lipid formulations of amphotericin B into bone marrow and fat tissue in rabbits. Antimicrob Agents Chemother 44 (2):408-410

27. Groll AH, Mickiene D, Werner K, Piscitelli SC, Walsh TJ (1999) High-performance liquid chromatographic determination of liposomal nystatin in plasma and tissues for pharmacokinetic and tissue distribution studies. J Chromatogr B Biomed Sci Appl 735 (1):51-62

28. Groll AH, Sein T, Petraitis V, Petraitiene R, Callender D, Gonzalez CE, Giri N, Bacher J, Piscitelli S, Walsh TJ (1998) Compartmental pharmacokinetics and tissue drug distribution of the pradimicin derivative BMS 181184 in rabbits. Antimicrob Agents Chemother 42 (10):2700-2705

29. Lee JW, Amantea MA, Francis PA, Navarro EE, Bacher J, Pizzo PA, Walsh TJ (1994) Pharmacokinetics and safety of a unilamellar liposomal formulation of amphotericin B (AmBisome) in rabbits. Antimicrob Agents Chemother 38 (4):713-718

30. Walsh TJ, Foulds G, Pizzo PA (1989) Pharmacokinetics and tissue penetration of fluconazole in rabbits. Antimicrob Agents Chemother 33 (4):467-469

31. Walsh TJ, Jackson AJ, Lee JW, Amantea M, Sein T, Bacher J, Zech L (2000) Dose-dependent pharmacokinetics of amphotericin B lipid complex in rabbits. Antimicrob Agents Chemother 44 (8):2068-2076

32. Walsh TJ, Bacher J, Pizzo PA (**1988**) Chronic Silastic central venous catheterization for induction, maintenance and support of persistent granulocytopenia in rabbits. Lab Anim Sci 38 (4):467-471.

33. Neely MN, van Guilder MG, Yamada WM, Schumitzky A, Jelliffe RW (2012) Accurate detection of outliers and subpopulations with Pmetrics, a nonparametric and parametric pharmacometric modeling and simulation package for R. Therapeutic drug monitoring 34 (4):467-476. doi:10.1097/FTD.0b013e31825c4ba6