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# Mouse models for the study of fungal pneumonia

## A collection of detailed experimental protocols for the study of Coccidioides, Cryptococcus, Fusarium, Histoplasma and combined infection due to Aspergillus-Rhizopus

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**Mouse models have facilitated the study of fungal pneumonia. In this report, we present the working protocols of groups that are working on the following pathogens: Aspergillus, Coccidioides, Cryptococcus, Fusarium, Histoplasma and Rhizopus. We describe the experimental procedures and the detailed methods that have been followed in the experienced laboratories to study pulmonary fungal infection; we also discuss the anticipated results and technical notes, and provide the practical advices that will help the users of these models.**

**Keywords:** animal models, Aspergillus, Coccidioides, Cryptococcus, fungal infection, Fusarium, Histoplasma, pulmonary infection, Rhizopus

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### Introduction

Fungal pathogens cause infections to immunocompromised, as well as immunocompetent individuals.[1] The increasing incidence of fungal infections is due to an expanding number of immunocompromised patients, such as transplant recipients, patients with acquired immune deficiency syndrome (AIDS) and patients undergoing immunosuppressive therapy.[2-5] Moreover, there has been an increase in fungal strains that are resistant to available antifungal agents.[6,7] These changes in the epidemiology and management of fungal infections highlight the need to study fungal pathogenesis and antifungal therapies models. Because most fungal pathogens cause lung infection or use the lung as a route to disseminate to other organs,[4,7] mammalian pulmonary infection models are particularly relevant to the study of fungal pathogenesis. In this article, we describe the detailed methods to study of pulmonary fungal infections, and we cover the following pathogens: Coccidioides, Cryptococcus, Fusarium, Histoplasma and combined infection due to Aspergillus-Rhizopus.

### Mouse Model for Lung Infection with Coccidioides

*Coccidioides immitis* and *Coccidioides posadasii* are recent species designations of the dimorphic, endemic fungus formerly known as the single species, *C. immitis*.[8] The disease, coccidioidomycosis or Valley Fever, applies equally to either species and there are no currently documented differences in infection progression or outcome between the two species.

The most well-developed animal model system for respiratory coccidioidomycosis is the mouse model. Strains of mice have variable susceptibility[9,10] and a strain may be selected for its specific immune response, the genetic background or for genes that are knocked out, depending on the information desired. Outbred strains of mice, such as CD-1, Swiss-Webster and CF-1, are also susceptible and are suitable and economical for drug efficacy studies.[11]

Swiss-Webster mice have also been found useful as a resistant strain compared with several inbred strains (including C57BL/6, DBA/2n, beige and nude strains).[10,12] The most susceptible, common, immunocompetent strains of mice are BALB/c and C57BL/6 mice,[9] while DBA/2n are considered more resistant.[13,14] Lower doses of arthroconidia are fatal to mice when given by the respiratory route compared with when given intraperitoneally (i.p.),[15,16] though i.p. infections will migrate to the lungs and studies comparing lung fungal burden can be accomplished by this infection route.[17]

Infection by the respiratory route is most easily and safely accomplished by intranasal insufflation of a saline suspension containing arthroconidia (spores), which are the infectious units of Coccidioides that would be inhaled from the environment. Alternative methods of inoculation include intratracheal infection with a suspension, which is a surgical procedure, or inhalation of dry spores through an inhalation exposure system (Glas-Col).

**Animals.** *Mice.* Naïve, 8-week-old of the desired strain/gender; vaccinated; usually start with 6-week-old animals of desired strain depending on purpose of study.

**Reagents.** (1) Fungal strain: *Coccidioides immitis* or *Coccidioides posadasii*, arthroconidial suspensions.

(2) 0.9% saline-for-injection for dilutions (pharmaceutical grade).

(3) Ketamine (pharmaceutical grade), 100 mg/mL.

(4) Xylazine (pharmaceutical grade), 20 mg/mL.

(5) 70% ethanol.

(6) Vesphene (Steris, VWR).

**Equipment.** (1) Biosafety Level 3 laboratory with class III biosafety cabinet or PPE to prevent respiratory exposure, e.g., PAPR (Powered Air Purifying Respirator).

(2) Mice housed in barrier caging: minimally under microisolator lids, maximally within HEPA-filtered ventilated caging.

(3) Micropipettor.

(4) Barrier pipette tips 200 µL.

(5) Sterile screw cap tubes.

(6) Syringes 1 mL.

(7) Needles 27 gauge.

(8) Sharps disposal containers: non-reusable, autoclavable, seal shut and cannot be opened once closed.

(9) Autoclave bags.

(10) Plastic backed underpads.

(11) Kimwipes.

(12) Paper towels.

## Procedure

**Fungal preparation.** (1) *Coccidioides* spp are grown on glucose-yeast extract (GYE) agar until arthroconidia are mature.

(2) Arthroconidia are collected in an aqueous suspension and enumerated with a hemacytometer and a viable count on GYE plates to determine concentration of initial suspension.

**Intratranasal inoculation.** (1) Ketamine and xylazine are diluted in a sterile tube to be able to deliver 80 mg/kg ketamine and 8 mg/kg xylazine in 0.1 mL solution.

*Dilution is prepared with 0.9% saline for injection immediately before use. If mice are large (e.g., outbred mice), weigh mice before anesthesia and increase by 0.025 mL volume if 23–25 g, 0.05 mL if > 25 g.*

(2) The anethestic is injected using a 1 mL tuberculin syringe and a 27 gauge needle and delivered into the lower left quadrant of the peritoneal space.

(3) The arthroconidial suspension is first diluted in isotonic, sterile saline to deliver the desired dose in a 30 µL volume.

(4) Anesthetized mice are weighed and the starting weight of each animal is recorded.

(5) The mouse is held in dorsal recumbency at an angle of approximately 45 degrees to flat in the hand.

(6) Using a micropipettor, 30 µL of suspension containing the desired number of arthroconidia is introduced to the surface of the nares in small droplets.

(7) The suspension will bead up on the nose of the mouse until it inhaled.

(8) The droplets are placed on the nose until the entire volume has been inhaled.

(9) A small amount of the suspension may be exhaled or bubble back out of the nose, contaminating the face or whiskers of mouse. Use a Kimwipe wet with 70% alcohol to carefully wipe the whiskers and face of the mouse to minimize spore contamination of the bedding and cage.

(10) Place the sleeping mouse in dorsal or lateral recumbency after infection.

*If the mouse appears to have slow or shallow breathing, lateral recumbency is better for the animal.*

(11) Animals will move approximately 30–45 min after induction of anesthesia.

For successful intranasal insufflation, mice need to be anesthetized. Otherwise the animals resist inhaling the suspension that is dropped onto the nares. Options include isoflurane, short-acting barbiturates or ketamine-xylazine. The drawback to the latter two injectable products is that the barbiturates and ketamine are DEA controlled substances, which require a permit and record keeping. Ketamine-xylazine is a good combination for this procedure, however, because it provides sufficient depth of anesthesia to allow the mice to inhale the suspension, a low rate of anesthetic deaths and minimal respiratory depression can occur. The ketamine is used at 80 mg/kg and the xylazine at 8 mg/kg and delivered i.p. It requires about 10–15 min for full effect.

To verify the actual number of arthroconidia given to the mice for each experiment, the infecting suspension is either plated with the infecting inoculum in 30 µL directly onto four plates, or the inoculum is diluted to a level which can be counted, e.g., 50 colony forming units, and the appropriate volume delivered to 100 mm Petri dishes (GYE) agar. The inoculum is evenly distributed with a cell spreader and the plates are taped around the edge. Plates are incubated at 35–37°C for three days and colonies are enumerated. The average is taken to determine the number of arthroconidia the mice received at inoculation.

To quantify the tissue (lung) burden, tissue is homogenized in 1 mL of saline or PBS and serial 10-fold dilutions are performed, and 100 µL is plated per 100 mm Petri dish with GYE agar.[11] Triton X-100 and possibly other detergents should be avoided in the homogenization procedure, especially if lungs are removed in the first few days following infection, because of interference with recovering growth from first-generation spherules. Plates are incubated for 3 d at 35–37°C and colonies enumerated. If 50–100% of the lung tissue is abnormal at

the time of sacrifice, at least four dilutions will be needed to obtain a countable plate. If a little disease appears in the lung, the entire homogenate may need to be plated to obtain a count or determine cultural negativity. For these lungs, a 1:10 dilution is made of the homogenate. One hundred microliters of the dilution is placed on a plate, and the remaining 900 µL is placed on a second plate. The undiluted homogenate is divided between two additional plates. If the 100 µL of 1:10 dilution has a low count, the additional plates can be used to determine entire lung fungal burden.

All surfaces should be sprayed and wiped with Vesphene, followed by 70% ethanol. All waste should be placed in autoclave bags and the bags tied and decontaminated before removal from the biosafety cabinet. Waste must be autoclaved before disposal, and reusable items should be decontaminated on the surface or autoclaved prior to cleaning. Animal carcasses must be autoclaved prior to disposal for incineration.

## Anticipated Results

Approximately two weeks without waiting for mice to succumb to infection is sufficient to finalize the study. These studies have the advantage of being short-term and producing less animal suffering. The infectious dose can be adjusted for the strain, desired endpoint and observations needed. For these studies, the fewer mice that die prior to scheduled sacrifice, the better the results. At the scheduled endpoint, mice are sacrificed and the entire lungs harvested. The experimenter could elect to do only one lung if the other is needed for another purpose, but infections are often unequal between left and right lungs and results are more consistent if the entire lung is cultured. Place lungs in Whirlpak bags until ready to homogenize. Spleens may also be removed and cultured if it is desired to estimate the level of dissemination for a given study.

Survival studies may be required during the final stages of assessing the virulence of the strain, or the efficacy of the drug or vaccine to extend survival. Mice are inoculated the same way but observed daily with predicted signs of illness starting between day 7 and day 9 post-infection, depending on the initial inoculum. Weight loss is a very good early indicator of mice that are ill and need to be watched closely, which means every mouse should be weighed at the time of infection and on the day when the signs of illness begin. They will begin to lose weight before they show signs of ruffled fur or inactivity. As the illness progresses, mice will become thinner and have hunched postures and obviously increased respiratory rates. There may be mucopurulent discharge from the eyes. The mice will be inactive and will not be observed eating or drinking. During rapid progression toward death, mice may lose 1 g per day. Animals in this state need to be evaluated at least once daily. Currently, moribundity rather than death is the accepted endpoint in survival studies unless there is significant scientific justification for the actual death of the animal. There is no such justification with untreated mice infected with Coccidioides, because once they show consistent deterioration, they never spontaneously recover. Signs of moribundity include weight loss greater than 30%, elevated respiratory rate, hunched posture, weakness, lack of eating and drinking and dehydration of 10%. Mice in a moribund condition are euthanized with an overdose of inhalant anesthetic and the organs harvested and scored. The day of euthanasia is recorded as the day of death, though some may record the following day as the day of death. For statistical purposes, it makes no difference as long as it is the same for the entire study. Organs can be quantitated if needed at the end of the study. Cage deaths are minimized or eliminated by daily observation of animals and conscientious application of the criteria of moribundity.

## Mouse Model for Lung Infection with Cryptococcus

*C. neoformans* grows as a yeast during human and murine infection, but can also reproduce sexually, producing blastospores, or through monokaryotic fruiting producing basidiospores.[18] Because of the difficulty in isolating pure cultures of spores, yeast are commonly used as the inoculum for infection in experimental models. One way to deliver the yeast into the lungs (which are considered the primary site of infection in human disease) of the animals is intratracheal (i.t.) inoculation. The benefit of intratracheal inoculation is that the fungal inoculum is delivered into the lungs directly. In other models of primary pulmonary disease, such as intranasal infection models, most of the inoculum does not reach the lungs directly. The disadvantage of intratracheal inoculation is the need for more invasive surgery, which additionally requires careful maintenance of body temperature during the recovery from anesthesia. However, the use of shorter acting anesthetic agents lessens this concern.

**Animals.** *Mice.* The most commonly used mouse strains are relatively resistant to pulmonary infection with *C. neoformans*.[19] A/J mice, which do not secrete C5,[20] and other complement deficient mouse strains are inherently more susceptible. It is worth while to mention that immunosuppressed mice can also be used.[21,22] Within a given experiment, all mice should be of the same sex and age-matched. We most frequently use mice that are 6–8 weeks of age.

**Reagents.** (1) Fungal strains: widely used strains include H99 (*C. neoformans* var *grubii*; serotype A) and ATCC strain 24067 (*C. neoformans* var *neoformans*; serotype D).

(2) Sabouraud's dextrose broth and agar.

(3) Sterile phosphate buffered saline (1X PBS).

(4) 95% ethanol.

(5) Ketamine/xylazine: The solution is made containing ketamine and xylazine at concentrations of 12.5 mg/mL and 1 mg/mL, respectively, so that 0.1 mL can be administered per 10 g of mouse weight (e.g., a 20 g mouse will receive 0.20 mL).

(6) Tape

(7) Liquid topical tissue adhesive (e.g., Nexaband; Abbott Laboratories)

**Equipment.** (1) Shaking platform incubator.

(2) Centrifuge.

(3) Hemacytometer.

(4) Inverted microscope.

(5) Tuberculin syringes.

(6) Needles 26 and 23 gauge.

(7) Scale with beaker to weigh mice.

(8) Repetitive dispensing pipette (e.g., Tridak Stepper™; DYMAX Corporation).

(9) Mouse "operating table": Cover Styrofoam board with aluminum foil. Stretch thin rubber band between two needles embedded in the board. Tape is used to restrain paws.

(10) Surgical instruments: scissors, straight forceps, curved forceps.

(11) Glass bead sterilizer.

## Procedure

**Fungal preparation.** (1) Cultures are started by scraping an ice chip from stock frozen at −80°C and then grown in the desired liquid media at the appropriate temperature.

*Typical conditions include growth in Sabouraud's dextrose broth at 30°C with shaking at 200 rpm for ~48 h.*

(2) Yeast are then collected into a conical tube, centrifuged and washed in sterile PBS three times.

(3) Yeast are suspended in PBS, quantified using a hemacytometer and used immediately.

(4) Suspensions used for inoculation are prepared so that the desired inoculum is in a volume of 50 µL or less.

(5) Quantification of the inocula. Serial dilution aliquots of the inoculum are plated on Sabauraud's dextrose agar to determine viability and accuracy of preparation.

**Other approach for fungal preparation.** A loop full of culture was taken from the frozen stock using a sterile inoculating loop. The loop full of cryptococcal cells is placed on Sabouraud dextrose agar and incubated in a 30°C incubator for 48 h. Then the yeast cells are collected using sterile PBS and a sterile glass rod. The suspension is placed in a sterile conical tube, spun down in a centrifuge at 3,000 rpm and washed three times with sterile PBS.

**Intratracheal inoculation.** (1) Mice are weighed and then anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine (125 mg/kg) and xylazine (10 mg/kg) using a 23 gauge needle attached to a 1 mL syringe.

(2) When fully anesthetized, the animal is placed in the supine position and the upper extremities extended by taping the paws to the board.

(3) The neck is held in extension by placing the rubber band in between the upper incisors and lower teeth and then sterilely prepared by scrubbing with povidone iodine, followed by wiping with 95% ethanol.

(4) A small incision is made in the neck overlying the thyroid cartilage using scissors.

(5) The soft tissues are bluntly dissected very carefully using forceps until the trachea is visualized.

(6) The trachea is immobilized by passing curved forceps underneath the trachea and separating the blades, while being careful not to pull upward on the trachea.

(7) The inoculum is deposited into the trachea just below the cricothyroid membrane using the Stepper™ to control the inoculum volume that is fitted with a bent 26 gauge needle attached to a tuberculin syringe.

(8) The needle is then withdrawn and the incision is closed by apposing the two skin edges with liquid topical tissue adhesive.

(9) Mice are kept under a heating lamp or on a warmed surface until they regain the ability to shiver.

(10) They are then returned to their cages and monitored until they resume activity.

(11) Instruments are sterilized between mice using a glass bead sterilizer.

Once they have fully recovered from anesthesia, mice are monitored twice daily for survival and signs of illness.

## Anticipated Results

A/J mice that receive an inoculum of $10^4$ yeast of ATCC strain 24067 begin to die at about one month after infection and have a mortality of about 75% at 100 d after infection.[23] In C57BL/6 mice, which have a propensity to develop an inflammatory infiltrate that is more eosinophil-rich, mortality following an inoculum is ~60% following infection with $10^4$ yeast of ATCC strain 24067. However, organisms can still be cultured from mice surviving 200 d after infection, and histopathological study after that time has shown the presence of inflammatory infiltrates that consist mainly of lymphocytes.[24] A study looking at different susceptibility among mouse strains using higher inocula found that BALB/c mice were the most resistant, with low long-term mortality following receipt of $10^6$ yeast, while CBA/J mice were the most susceptible, with 100% mortality after receiving of $10^6$ yeast at 40 d.[25] A/J and C57BL/6 mice had intermediate susceptibility.

Potential problems during surgery include those associated with anesthesia or bleeding. Occasionally, mice are relatively more resistant or susceptible to anesthesia and, if more resistant, achieving adequate anesthesia without overdosing the mouse can be tricky. Additional small doses should not be given any more frequently than every 5–10 min, and the amount (typically no more than an extra half dose) should be judged by the degree of alertness of the individual animal. The second problem, bleeding, may result when dissecting the trachea, as small blood vessels are present in the overlying tissue and around the salivary glands that are immediately anterior. If bleeding occurs, gentle pressure can be applied using sterile gauze until the bleeding stops.

## Mouse Model of Lung Infection with Fusarium

Fusarium is a filamentous fungus that is ubiquitous in the environment. It is a major plant pathogen and an important emerging opportunistic fungal pathogen in patients with compromised immunity. Neutropenia and corticosteroid use are the major risk factors for development of fusariosis and mortality after Fusarium infection is typically very high.[26,27] There are two major entry points for Fusarium infection in patients with invasive fusariosis; the sinopulmonary route is by far the most common, followed by the skin, primarily in the setting of intravenous catheters or onychomycosis.[7,28,29]

Two mouse models of fusariosis have been established to study the infection and mimic the aforementioned entry points of mold inoculation. The inoculation of a large conidial inoculum via the lateral tail vein has been extensively used but does not mimic the pathophysiology of human fusariosis, which typically entails

introduction of Fusarium conidia via the sinopulmonary route, with subsequent necrotizing pneumonia and hematogenous dissemination in the setting of neutropenia.[30] To that end, intranasal inoculation of Fusarium conidia in mice, which have been rendered neutropenic by cyclophosphamide injection, has been more recently employed to better portray the human infection pathogenesis.[31]

Although better mimicking the pathogenesis of the human infection than the intravenous model, intranasal inoculation is an acute model of fusariosis that delivers a large conidial inoculum to mice. Instead, in humans who develop pulmonary fusariosis, conidial inoculation is subacute, with smaller numbers of conidia inhaled on a more constant basis instead of a large bolus inoculum. Hence, an alternative approach that may be used to overcome this limitation is to deliver Fusarium by aerosol inoculation, as previously described in aspergillosis.[32]

Herein we present a detailed description of the protocol used to develop a reproducible murine model of acute pulmonary fusariosis through intranasal inoculation of Fusarium conidia in neutropenic mice.

**Animals.** *Mice.* For all experiments, female BALB/c mice (Harlan Sprague-Dawley) weighting 20 to 25 g each are used and maintained in microisolators that have controlled temperature and humidity. The mice have unrestricted access to food and water and were exposed to equal cycles of dark/light (12 h/12 h).

**Reagents.** (1) Fungal strain: *F. oxysporum* isolate, which was isolated from a patient with acute myelogenous leukemia who died of fusariosis.

(2) Sterile phosphate buffered saline (1X PBS).

(3) Potato dextrose agar (PDA) slants

(4) Cyclophosphamide: Cyclophosphamide (Sigma Aldrich; cat. no. C0768) dissolved in 1X PBS to a final concentration of 15 mg/mL (stored at 4°C).

(5) Ketamine and xylazine mixture: To obtain the ketamine/xylazine anesthesia solution, mix 1.25 mL of Ketamine HCl (100 mg/mL, Hospira, Inc.), with 4.15 mL of sterile 1X PBS, and 5 mg of xylazine (Sigma Aldrich, X1251).

(6) Ethanol 95%.

(7) Bleach 20%.

**Equipment.** (1) Syringe 1 mL (Becton Dickinson).

(2) Needle 26 1/2 gauge (Becton Dickinson).

(3) Pipette.

(4) Surgical equipment (scissors, blade, forceps).

(5) Aluminum foil.

## Procedure

**Fungal preparation.** (1) PDA slants are inoculated with Fusarium conidia (from growing stock plate at room temperature) by using a cell spreader, and left at 37°C for 5 d.

(2) After 5 d, 0.1% Tween 80 in 1X PBS (10 mL) are used for washing the surface growth.

(3) The suspension is collected in a 50 mL tube. Steps 2 and 3 are repeated twice to maximize the amount of conidia obtained.

(4) The suspension is filtered through two layers of commercially available autoclaved cheesecloth. This helps exclude the agar pieces and filter the conidia through.

(5) The conidial suspension is spun down for 10 min at 4°C at 4,000x *g*.

(6) The supernatant is discarded and the pellet is re-suspended in 10 mL of sterile 1X PBS, vortexed and re-spun for 10 min at 4°C at 4,000x *g* for 10 min. This wash step is repeated twice.

(7) The supernatant is again discarded and the pellet is suspended in 1 mL of sterile PBS.

(8) The number of Fusarium conidia in the suspension is counted by a hemocytometer.

(9) The suspension is diluted to the desired conidial concentration.

*To confirm the conidial concentration, serially dilute and plate the final suspension onto PDA plates. Count the colony forming units after 48 h of inoculation at 30°C or room temperature.*

**Intranasal inoculation.** (1) The mouse is held gently inside the palm with the abdomen facing up and the head tilted down for intraperitoneal (i.p.) injection.

*This body orientation is used to prevent injury to the liver, spleen and intestines.*

(2) Two hundred to two hundred and fifty microliters of cyclophosphamide from the stock solution (dose of ~150 mg/kg mouse) is administered i.p. to induce neutropenia at day 3 prior to infection. The same injection is repeated 4 d later (i.e., at day 1 post-infection) in order to maintain low neutrophil counts.

*This regimen has been shown to render mice neutropenic (i.e., absolute neutrophil counts of $< 100\ mm^3$) within 3–4 d of the first cyclophosphamide injection, and neutropenia lasts for 4 d after the last booster injection.*[33]

(3) Three days following the first cyclophosphamide injection, a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) is injected i.p. to induce anesthesia.

(4) The mouse is placed back inside the cage for 5–10 min until anesthesia is established.

(5) Then the mouse is held inside the palm in the supine position with the head tilted up at approximately 60 degrees.

*This orientation is critical in order for the mice to inhale the inoculum into the lower respiratory tract.*

(6) A pipette is used to inoculate 35 µL of the conidial suspension (concentration: $2 \times 10^8$ conidia/mL) on the surface of the nares in small droplets.

*It is critical to slowly inoculate the conidial suspension, a few microliters at a time into the nares of the mouse. After each few microliters inoculations, allow time for the mouse to snort the inoculum into the lungs. Ensure that the inoculum is not erroneously aspirated into the oral cavity by checking the mouse mouth. After each few microliters are inhaled into the lower respiratory tract, proceed with inoculating a few more microliters until all 35 µL of the inoculum has been delivered.*

*Ketamine/xylazine anesthesia is preferred over isoflurane anesthesia because a deeper and more sustained level of anesthesia is obtained, which results in more consistent and reliable conidial inoculation.*

(7) Then the mice are monitored for recovery and any sign of distress from the anesthesia or conidial inoculation.

*Caution is needed to keep mice warm during the 20–30 min of recovery from anesthesia in order to avoid hypothermia. Caution should also be used to not exceed the aforementioned dose of ketamine/xylazine because death may ensue.*

(8) Twenty-four hours after infection, repeat step 2 with i.p. cyclophosphamide injection to maintain neutropenia in mice.

## Anticipated Results

It is important to mention that, in addition to the strain, age, gender and weight of the animals, the immunosuppressant regimen and different fungal strain might impact the anticipated results.[21,22]

The described model leads to dose-dependent mortality.[31] Using a $10^7$ conidia/mL solution leads to mortality of 30% at the day 4 post infection, and using a $10^9$ conidia/mL solution leads to mortality of 90% at the day 1 post infection. Also, this model can be employed for comparative analysis of virulence of different Fusarium strains, in addition to the pharmacology studies that can be conducted using this model.[31] Moreover, study of the immune responses against fusariosis, which are poorly defined can be investigated using this model.

Serum, whole blood, bronchoalveolar lavage fluid and/or lung tissue can be harvested from infected mice at various time points post-infection for: (1) assessment of tissue fungal burden using CFUs or qPCR (whole lung homogenates), (2) histopathological or immunohistochemical analysis (formalin-treated lung tissue sections), (3) measurement of cytokines, chemokines and other immunomodulatory molecules (serum, bronchoalveolar lavage, whole lung homogenates) and (4) characterization of cellular immunological correlates of infection using flow cytometry (whole blood, bronchoalveolar lavage, whole lung digested tissue).

Markers of severity of infection are ruffled fur, reduced grooming, reduced level of activity, weight loss, hunched posture, reduced food/water intake. Mice are euthanized humanely ($CO_2$ asphyxiation) when the end points are reached.

## Mouse Model for Lung Infection with *Histoplasma capsulatum*

In some geographic areas, *Histoplasma capsulatum* is thought to be the most common cause of fungal respiratory infections in immunocompetent hosts, and is a major cause of morbidity in immunocompetent patients.[34] *H. capsulatum* is one of a group of thermally dimorphic fungi that display a specialized developmental program that is triggered in response to mammalian body temperature.[35,36] In the soil, the cells grow in a filamentous form that produces vegetative spores (conidia). When the soil is disrupted, fragments of the filaments and the spores become aerosolized and are inhaled by mammalian hosts. At 37°C, the cells shift their morphology to a yeast form that expresses virulence factors and is capable of colonizing macrophages. Thus temperature is a key signal that determines cell morphology and specialization. Notably, this developmental switch can be recapitulated in culture by changing the temperature. Room temperature growth triggers the soil form of the organism whereas growth at 37°C triggers the host form of the organism,[37,38] thus facilitating experimental analysis of this phenomenon. Since *H. capsulatum* is a virulent fungal capable of causing disease in healthy hosts, the filamentous and spore forms are studied under biosafety level 3 (BSL3) conditions. The yeast form, which does not spontaneously aerosolize, is studied under modified BSL2 conditions.

The mouse is considered an excellent model to study *H. capsulatum* pathogenesis: rodents are a natural host for Histoplasma and disease progression mimics that in humans.[39] Animals develop a primary respiratory infection that disseminates through the reticular endothelial system to the spleen and liver. Although the natural route of infection involves inhalation of conidia and/or hyphal fragments, virulence studies in the mouse are performed with either conidia or yeast. The production of yeast cells is straightforward, but the production of conidia is more laborious (and not all laboratory-passaged strains sporulate efficiently). To generate conidia, a lawn of yeast cells is plated on sporulation medium and incubated at room temperature in a BSL3 facility for a period of weeks (the length of incubation depends on the strain background). Conidia are then purified as previously described[40] and stored in a nutrient-limited medium such as PBS to prevent germination. For long-term storage of conidia, PBS plus antibiotics such as penicillin/streptomycin is recommended. If the infection is to be performed with yeast cells, these cells are grown under standard BSL2 laboratory conditions [e.g., in Histoplasma-macrophage medium (HMM)] as previously described.[41]

**Animals.** *Mice.* WT (C57BL/6) mice for infections are used. Of note, wild-type C57BL/6 mice from different vendors (e.g., Charles River, Jackson Laboratories, Taconic) may exhibit differential susceptibility to wild-type *H. capsulatum*. Thus, it may be important to titer the lethal dose at the start of an experimental series.

**Reagents.** (1) Fungal strains: Common laboratory strains for infections include G217B and G186AR.[40] Spores are purified before the start of the experiment and stored in a BSL3 facility. The concentration of viable infectious particles should be titered prior to the mouse experiment by plating spores on BHI agar plates (below). Mouse infections with spores are performed in an animal-BSL3 (ABSL3) facility. Yeast cells are grown in HMM medium under BSL2 conditions.

(2) Synthetic medium[40] or Bird agar (www.fgsc.net/fgn51/fgn51metz.html) supplemented with 0.05% cysteine-HCl and penicillin-streptomycin (Pen/Strep) for sporulation.

(3) Brain heart infusion (BHI) agar plates supplemented with 10% sheep blood, 0.05% cysteine-HCl, 0.5% conditioned medium (made from 3 day cultures of *H. capsulatum* grown in HMM) and 10 μg/mL gentamicin for plating colony-forming units.

(4) Isoflurane inhalation 1–4%.

(5) Histoplasma-macrophage medium (HMM).[41]

(6) Phosphate-buffered saline (PBS).

**Equipment.** (1) Centrifuge

(2) Hemacytometer

(3) Tuberculin syringes

(4) Stainless steel feeding tube (intratracheal infections only)

(5) Scale

(6) Surgical instruments

(7) Sterilized glass beads

(8) Sterile glass wool

(9) Tissue-Tearor Homogenizers (BioSpec)

## Procedure

**Fungal preparation.** (1) Frozen stocks of Histoplasma strains are thawed onto Histoplasma-macrophage medium (HMM) at 37°C with 5% CO2.

(2) Five milliliters of liquid HMM medium is inoculated with yeast cells and grown for 3 d. The culture is passaged and grown for 2 d before being passaged into 10 mL HMM at a dilution appropriate to give an early or mid-log phase culture after overnight growth. For G186AR, cells are grown to late-log phase, washed and re-suspended in 50 mL PBS. For G217B, 3 mL of culture is washed in 25 mL PBS and then resuspended in 3–6 mL PBS.

(3) The 50 mL cell suspension is subjected to a low-speed spin (50x *g* for 5 min) to pellet large clumps of cells. The top 10 mL, which is enriched for single cells, doublets and triplets, is collected. Alternatively, for the G217B strain, which is much less clumpy than G186AR, cells are subjected to a quick sonication.

(4) Cells are counted on an improved-Neubauer-phase hemacytometer, and diluted in PBS to the desired concentration for an infecting dose of ~25 µL (up to maximum of 50 µL).

*Spore/conidial cells.* (1) Conidia in the G217B strain background are obtained by plating the yeast cells on 15-cm Petri plates containing synthetic medium or Bird agar. The G186AR strain grows poorly on synthetic medium and is grown on Sabouraud-dextrose agar to produce conidia.

(2) Plates are sealed with parafilm and kept at room temperature for 4 to 10 weeks in a BSL3 facility.

(3) Conidia are collected by adding a small volume of PBS to the surface of the plates and dislodging the conidia with a bent glass rod. The resultant suspension is pipetted off the plate and collected in a conical tube.

(4) The suspension is filtered through sterile glass wool to remove any mycelial fragments.

(5) The suspension is centrifuged at 2,000x *g*, at 4°C for 10 min, washed, re-suspended in PBS with Pen/Strep and stored at 4°C in a BSL3 facility until use.

(6) Conidial viability is confirmed by plating serial dilutions on brain heart infusion (BHI) agar plates and incubating for at least 10 d at 30°C in a BSL3 facility.

**Intranasal inoculation.** (1) An aliquot of the inoculum should be diluted and plated just prior to infecting the mice to confirm the dose of infectious organisms in the inoculum.

(2) Eight-week-old C57BL/6 mice are anesthetized with 1–4% isoflurane gas for 3 to 5 min. The dose and time of anesthesia can vary based on age, weight and strain of mouse used.

(3) The mouse is held in dorsal recumbency at an angle of approximately 45 degrees to flat in the hand. The mouth is gently, but firmly, held closed to ensure breathing only takes place through the nose.

(4) The infecting dose is applied to the nares. A few microliters at a time, waiting for the mouse to inhale it each time before adding the next few microliters. After the mouse has taken up the complete dose, it is maintained in the recumbent position with mouth held closed for a few more breaths, to prevent the mouse from exhaling the inoculum. Maximum dose of *Histoplasma capsulatum* should not exceed $1 \times 10^8$ CFU/mouse.

(5) The mice are weighed and then monitored until signs of recovery from anesthesia are apparent.

(6) Since it is critical to know that the viability of the inoculum was not adversely affected during the infection procedure, an aliquot of the inoculum is diluted and plated after infecting the mice.

**Intratracheal inoculation.** (1) An aliquot of the inoculum should be diluted and plated just prior to infecting the mice to confirm the dose of infectious organisms in the inoculum.

(2) Eight-week-old C57BL/6 mice are weighed and then anesthetized with 1–4% isoflurane gas.

(3) Stainless steel feeding tube (22 gauge) with a rounded tip is inserted into the trachea.

(4) Up to 50 µL of *H. capsulatum* organisms are injected into the trachea.

(5) The feeding tube is removed.

(6) The mice are monitored until signs of recovery are apparent.

(7) Since it is critical to know that the viability of the inoculum was not adversely affected during the infection procedure, an aliquot of the inoculum is plated after infecting the mice.

## Anticipated Results

The criteria for euthanasia should be determined with input from the Institutional Animal Care and Use Committee. At UCSF, when infecting with a known lethal dose, the criteria for euthanasia are a sustained three-day weight loss of > 15% from top weight. The sustained weight loss is critical to identify those mice that truly show symptoms of illness, such as hunching, panting, or lack of grooming. At a lethal dose, euthanasia usually occurs between days 6–10 after infection. Mice infected at a known sub-lethal dose are monitored every other day. If they experience > 15% weight loss, they are monitored daily and euthanized after a sustained > 15% weight loss over three days and the appearance of illness (e.g., panting, hunching or lack of grooming). Of note, mice infected with a known sub-lethal dose can experience a sustained three-day weight loss of > 15% from top weight with no other symptoms of illness; these mice then go on to recover. Therefore, for low inoculum, the criteria for euthanasia is sustained three-day weight loss of > 15% top weight with the addition of a second symptom of illness, such as hunching, panting, or lack of grooming. Mice are euthanized no more than four weeks after infection by $CO_2$ narcosis and cervical dislocation. The lungs, livers and spleens are removed and homogenized in PBS with Tissue-Tearor homogenizers. Dilution series are plated on brain-heart infusion (BHI) agar with 10% sheep blood, 0.05% cysteine, 0.5% conditioned medium and 10 g/mL gentamicin at 30°C for 10 to 14 d before enumeration of CFU.

Mice are expected to show signs of disease (hunching, panting, poor grooming, weight loss) between days 4–10 after infection, depending on dose and susceptibility. Those animals that do not succumb to infection will completely recover by day 21. Supportive measures such as a high-fat rodent food supplement and high-water gel to facilitate nutrient acquisition can be used.

## Mouse Model for Lung Infection with the Mixture of Aspergillus-Rhizopus

Mucormycosis is a fungal infection that has been described in immunocompromised patients. Treating an immunocompromised

patient for possible aspergillosis using antifungal agents might increase the probability of acquiring mucormycosis.[42] To investigate this phenomenon, a mouse model of mixed fungal (*Aspergillus fumigatus-Rhizopus oryzae)* pneumonia has been developed. Please note that the mouse model for lung infection with Aspergillus is not covered and the readers are referred to recent publications.[43,44]

Tissue burden quantification of filamentous fungus by quantifying the traditional colony forming units (CFUs) may not correctly estimate the real tissue burden. This is because traditional tissue burden quantification requires the targeted organ to be homogenized. Homogenization may lead to filaments tearing, and decrease the actual number of viable cells. In this brief section, we will describe the intra-nasal fungal co-inoculation methods and the real-time PCR assay for fungal quantification to quantify the tissue burden of these two fungal pathogens.

**Animals.** *Mice.* BALB/c 8-week-old females are used for all experiments.

**Reagents.** (1) Fungal strains: the clinical isolate of *Rhizopus oryzae* 557969 from a patient with mucormycosis[45] and the reference strain *Aspergillus fumigatus* 293 are used in all experiments. All strains are maintained at –80°C. Yeast extract agar glucose plates (YAG) are used for growing all strains.

(2) Phosphate buffered saline (1X PBS).

(3) Supplemented yeast extract agar glucose plates (YAG) (0.5% yeast extract, 1% dextrose, 0.2% vitamin mix, 0.1% trace elements, 1.5% agar and 1% $MgSO_4$).

(4) Cortisone acetate (300 mg/kg).

(5) Isoflurane.

(6) Ethanol 95%.

(7) Bleach 20%.

(8) Cyclophosphamide

**Equipment.** (1) Syringe 1 mL.

(2) Needle 26 gauge.

(3) Pipette.

(4) Surgical equipment (scissors, blade, forceps, etc.).

(5) Aluminum foil.

## Procedure

**Fungal preparation.** (1) The suspension of the spores in PBS is passed through a 40 µm-pore filter.

(2) Then the suspension is centrifuged and re-suspended to the final concentration.

(3) Then the two fungal pathogens are combined to the desired concentration.

**Intranasal co-inoculation of mixed fungal pathogens.** (1) A 10:1 spore suspension is prepared in PBS with the final concentration of *A. fumigatus* $5 \times 10^6$ spores/mL and *R. oryzae* $5 \times 10^5$ spores/mL.

(2) The mice are immunosuppressed with corticosteroids (intraperitoneal injections of cortisone acetate (300 mg/kg) at 2 d intervals starting 4 d before fungal inoculation until 4 d post fungal infection). And cyclophosphamide (200 µL with dose of 100 mg/kg) administered at 4 d, 1 d prior to infection and 3 d after infection.

(3) The water is supplied with 5% dextrose and 200 µg/mL doxycycline for prophylaxis.

(4) The mice are anesthetized by isoflurane by vaporizer.

(5) Then the mouse is held in an upright position and 35 µL of the mixed fungal suspension is introduced to the surface of the nares in small droplets, allowing inhalation of the droplets between inhalation

(6) Then the animals are monitored until the anesthesia effect had vanished.

**Fungal burden determination.** (1) The lungs from infected animals were isolated, weighed and homogenized in 1 mL of PBS.[46,47]

(2) DNA was extracted from aliquots of 180 µL of the homogenized lung.

(3) Then the tissue burden is quantified using real time PCR with PCR primers and a dually-labeled fluorescent hybridization probe specific for *A. fumigatus* and *R. oryzae*.[45]

(4) The conidial equivalent (CE) fungal concentration for each fungal species is obtained from two separate six-point standard curves prepared by spiking the uninfected lungs with known concentrations of either *A. fumigatus* or *R. oryzae.*

(5) PCR reaction is performed in both sets of spiked lungs using both *A. fumigatus* and *R. oryzae* probe-primers pairs to ensure no cross reactivity.

## Anticipated Results

The best sub-lethal infection capable of being monitored within the range of the PCR assay is achieved by the combination of 10:1 inoculum of *A. fumigatus* ($1.75 \times 10^6$ conidia) and *R. oryzaye* ($1.75 \times 10^6$ conidia).[45] The optimal inoculum ratio of Aspergillus:Rhizopus may vary, depending on the isolates studied. In monomicrobial pulmonary infection models, the lethal inoculum of *Rhizopus oryzae* is 50–100-fold less than *A. fumigatus*. The lethality of *Rhizopus oryzae* is also affected by the growth media used to harvest fungal spores. We have observed much lower mortality for animals infected with Rhizopus sporangiospores harvested from less nutrient rich media (i.e., RPMI) compared with infections that were induced with sporangiospores harvested on nutrient-rich media (i.e., supplemented yeast extract agar glucose, YAG) that supported rapid growth of Mucorales. After inoculation, the mean increase of the conidia equivalent DNA (the fungal burden) within 5 d is 1.3 $\log_{10}$ for *A. fumigatus* and 1.8 $\log_{10}$ for *R. oryzaye.*


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