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*Original Research*

# Animal Models of Aspergillosis

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**Aspergillosis is an airborne fungal disease caused by *Aspergillus* spp., a group of ubiquitous molds. This disease causes high morbidity and mortality in both humans and animals. The growing importance of this infection over recent decades has created a need for practical and reproducible models of aspergillosis. The use of laboratory animals provides a platform to understand fungal virulence and pathophysiology, assess diagnostic tools, and evaluate new antifungal drugs. In this review, we describe the fungus, various *Aspergillus*-related diseases in humans and animals and various experimental animal models. Overall, we highlight the advantages and limitations of the animal models, the experimental variables that can affect the course of the disease and the reproducibility of infection, and the critical need for standardization of the species, immunosuppressive drugs, route of infection, and diagnostic criteria to use.**

Aspergillosis is an opportunistic fungal airborne infection caused by ubiquitous saprophytic molds that belong to the *Aspergillus* genus.[69] Infection has been documented in humans and animals and usually occurs through the inhalation of unicellular spores—known as conidia[127]—or, more rarely, after ingestion or wound contamination. In humans, *Aspergillus*-related diseases show a broad clinical spectrum,[186] ranging from chronic localized aspergilloma to acute invasive aspergillosis, and are dependent on the patient's underlying medical condition.[118] Similarly, *Aspergillus*-related diseases are quite variable in animals, and every species has susceptibility for developing aspergillosis.[215]

Further progress regarding the pathophysiologic process and virulence of *Aspergillus* spp. is expected,[42] but recent major advances include the identification of underlying genetic risk factors based on single-nucleotide polymorphisms in genes affecting the host immune response.[61] However, the diagnosis of aspergillosis remains difficult because tools are inaccurate or nonstandardized.[69] Moreover, treatment is limited by the unavailability of antifungal drugs, their high cost, and pharmacodynamics or pharmacokinetic properties that are difficult to manage.[236] Animal models of aspergillosis can be used to address all of the pending issues.[56,97,185]

In this overview, we discuss *Aspergillus* fungus and its related diseases as well as the technical parameters, benefits, and limitations of various animal models of aspergillosis.[71]

**Description of *Aspergillus* fungus.** *Aspergillus* spp. belong to the *Ascomycotina* phylogenetic clade and to the *Eurotiales* order.[68] These organisms are very prevalent in the environment and are commonly found in soil and on plants, in decaying organic matter, and in humid places.[186] Overall, *Aspergillus* spp. represent 1% to 7% of the environmental fungi.[192]

Approximately 185 ubiquitous *Aspergillus* species have been described thus far.[69] Microscopically, all have a filamentous, hyaline, and septate mycelium. In humans, *Aspergillus fumigatus* is isolated from more than 80% of clinical samples positive for *Aspergillus* spp., regardless of the medical context and the nature of the biologic specimen.[69] In animals, *A. fumigatus* infection has been reported primarily in birds and, more rarely, in honey bees, dogs, cats, horses, cetaceans, and monkeys.[215] In addition to the small size of *A. fumigatus* spores (diameter, approximately 2 to 3μm)[186] and its ability to rapidly grow at 37 °C,[30] several virulence factors are involved in its pathogenic power: production of the pigment melanin;[240] discharge of proteolytic enzymes, including elastases[60] and lipases,[1] and toxins including gliotoxin;[60,176] and the possession of adhesion factors including hydrophobins.[60] Notably, *A. fumigatus* is often misidentified as other species of the same *Fumigati* section, including *Neosartorya udagawae*, *A. lentulus*, *N. pseudofischerii*, and *A. viridinutans*, which all have very similar phenotypic characteristics.[125] Among the other *Aspergillus* sections, *A. niger*, *A. flavus*, *A. terreus*, and *A. nidulans* are sometimes reported as pathogens, but each of these species is generally less virulent than *A. fumigatus*.[186]

**Difficulties in managing *Aspergillus*-related diseases in humans.** Innate immunity and bronchotracheal mechanical defenses aid in clearing *Aspergillus* conidia from the airways. In immunocompetent patients, macrophages and neutrophils play a critical role in this process, together with mucociliary clearance.[47] Soluble molecules including lysozyme, defensins, and surfactant proteins help in controlling infection and deleterious inflammation.[250] When these means of defense are entirely or partially abolished, *Aspergillus* spores initiate a filamentous expansion, which marks the beginning of the pathogenic process for aspergillosis.[47]

In humans, *Aspergillus*-related diseases are varied, and their clinical spectrum depends on the host's immune status and underlying medical condition. The *Aspergillus* syndromes are primarily classified according to their degree of invasiveness and their anatomic locations. Invasive aspergillosis is the most severe form of *Aspergillus* disease and is usually encountered in highly immunocompromised patients;[142] its prevalence is highest in neutropenic patients undergoing chemotherapy for acute myeloid leukemia.[31,142] In bone-marrow transplant patients, the incidence of invasive aspergillosis has been estimated as 7% to 13%.[130] Invasive aspergillosis occurs in 1% to 6% of patients with solid-organ transplants and more often in those with lung

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transplants than kidney or liver transplants.[87] The symptoms of invasive aspergillosis first involve the lungs but can be generalized with dissemination, especially in the brain.[117] Symptoms usually include antibiotic resistant fever ≥38.5 °C for more than 48 h, cough, chest pain, and difficulty in breathing.[69] Invasive aspergillosis continues to be associated with high morbidity, and mortality rates range from 30% to 70%.[110] Many other syndromes caused by *Aspergillus* spp. have been described in humans, including allergic bronchopulmonary aspergillosis, which affects patients with respiratory diseases such as asthma, cystic fibrosis, and sinusitis.[154] In allergic bronchopulmonary aspergillosis, persistent colonization with *Aspergillus* conidia induces an excessive immune reaction toward hyphae, which evolves in situ[5] and leads to the formation of an aspergilloma (a 'fungus- ball'), which can develop within preexisting cavities created by previous diseases, such as tuberculosis and sarcoidosis.[229] In addition, aspergillomas can occur in natural cavities, including the maxillary sinus or the sphenoid and ethmoid sinuses. Involvement of the frontal sinus remains extremely rare.[29] Eye infections including keratitis are usually superficial and limited and are the consequence of trauma with contaminated plants.[124]

Low sensitivity and specificity limit current diagnostic methods for aspergillosis.[7] Therefore, to increase the chance for accurate diagnosis, recent guidelines recommend to use several tests at a time concomitantly.[11,67] Histopathology remains the reference standard for confirmation of the aspergillosis diagnosis. When positive, biopsy samples from lung or other infected tissues show septate hyphae. However, the septa are not always apparent and, in such cases, filaments may be mistaken for zygomycota. *Aspergillus* spp. filaments usually range in diameter from 2.5 to 4.5 µm and are dichotomously branched primarily at acute angles of approximately 45°.[69] Use of silver stains, including Gomori methenamine–silver staining, give the fungal walls a gray–black color and makes them easier to observe on a slide preparation. However, because invasive procedures to collect biopsy samples are sometimes difficult to perform in weakened patients, other diagnostic alternatives are important. Computed tomography is a readily available imaging approach that may be highly suggestive of invasive aspergillosis when a halo sign and, at later stages of infection, an air-crescent sign are observed in the lungs.[35]

Mycologic cultures obtained from respiratory samples allow the identification of the *Aspergillus* section according to the specific phenotypic characteristics of the growing colony, including color, size, and microscopic features;[69] from that point, DNA sequencing is needed to confirm the species. Importantly, a positive culture does not establish the diagnosis of a true infection; it might merely reflect simple colonization of the upper airways, given that *Aspergillus* spores are ubiquitous.[186] In neutropenic patients with invasive aspergillosis, detection of the *Aspergillus* galactomannan antigen (a specific cell-wall carbohydrate component of *Aspergillus*) can aid in diagnosis. However, false-positive *Aspergillus* galactomannan tests have been obtained from non-neutropenic patients[148] and in persons who undergo intravenous antibiotic treatment, are injected with fluids containing gluconate or citric acid, or receive parenteral nutrition.[130,151] In addition, tests using pan-fungal cell-wall biomarkers including (1→3) β-D-glucans in blood are available, but they do not allow differentiation between invasive aspergillosis and other fungal diseases. Consequently, these tests should be considered as a way to exclude fungal infection when negative.[126,130] Quantitative PCR analysis still lacks standardization for detecting *Aspergillus* DNA in blood or respiratory samples and thus has not been included in the pivotal criteria for definitive diagnosis yet.[69,130,148] Anti*Aspergillus* antibody detection is only valuable for the diagnosis of chronic aspergillosis,[187] including aspergilloma and allergic bronchopulmonary aspergillosis. Overall, most serologic assays still are hampered by a lack of reproducibility due to poor standardization.[177] Such limitations delay the initiation of antifungal therapy.

The current treatments for aspergillosis are based on antifungal drugs and surgery, used either alone or in combination. The choice is made according to the clinical presentation, the anatomic location of the disease, and the underlying medical condition. Voriconazole (an azole drug) and liposomal amphotericin B (a polyene) are the 2 most commonly used medications in cases of invasive aspergillosis. As monotherapy, they are prescribed for at least 12 wk and often for several months.[236] Posaconazole is now preferred for prophylaxis.[146] For allergic bronchopulmonary aspergillosis, which is less aggressive and a more chronic process compared with invasive aspergillosis, findings suggest the use of oral corticosteroids for 6 to 9 mo.[163] Because itraconazole is considered to have a 'steroid-sparing' effect, which increases their efficacy and thus allowing decreased doses, it is often given with corticosteroids.[75] Surgical debridement or lobectomy are recommended for aspergilloma when the lesion is small and easily accessible,[65] and long-term oral antifungal therapy is sometimes needed for cases of complicated aspergilloma, which can cause hemorrhage when the masses are too close to large blood vessels.

**High variability of *Aspergillus*-related veterinary diseases.** *Aspergillus* spp. has been found worldwide in almost all domestic and wild animals, ranging from insects and corals to NHP.[215] Compared with its incidence in humans, aspergillosis is a common infection in birds, particularly pet parrots, penguins, captive raptors, mallards and other ducks, turkeys, and Japanese quail, in which it causes pulmonary and air sac infection.[80] Aspergillosis has also been described in cats and in dolichocephalic and mesocephalic dogs in which it remains an uncommon disease affecting only the nasal passages.[215] In large animals, *Aspergillus* infection is assumed to be rare but has been reported with increasing frequency.[2] It can lead to various diseases like mycotic abortion and gland infection in cows as well as guttural pouch involvement in horses.[215] In marine mammals, airways are usually initially affected leading to pneumonia, but other organs including the brain may also be infected following fatal dissemination.[2]

In all of the animal species just mentioned, the diagnosis of aspergillosis remains very challenging. The methods are similar to those described earlier for the human disease. Overall, veterinary diagnostic tools are less developed and have infrequently been validated in large trials.[39,62] Additional difficulties exist regarding the definition of an appropriate cutoff value and disease staging in animals. Briefly, measurement of galactomannan antigen in blood was shown to have inconsistent reliability in raptors, waterfowl, and falcons but was more valuable in turkeys and chickens.[80,82] The ubiquitous presence of similar carbohydrates in the environment can result in false positives;[14] therefore repeated assessments are usually encouraged. Detection of anti*Aspergillus* antibody is almost always positive in some birds, including penguins, even when the animals are clinically normal.[58] Furthermore, advanced diagnostics including medical imaging are not readily available in every veterinary facility. The use of quantitative PCR analysis for *Aspergillus* has not been validated for use in animals.

Antifungal treatment for animals is often based on low-cost azoles, including clotrimazole, enilconazole, and itraconazole,

delivered through the topical, oral, or nebulization route. Polyenes, such as desoxycholate amphotericin B and nystatin, and allylamines like terbinafine are potential alternatives in *Aspergillus*-infected animals.[26]

**Goals and contributions of animal models of aspergillosis.** The development of animal infection models can help to address the pathophysiologic processes of aspergillosis[22] as well as the appreciation of the fungus virulence, the assessment of diagnostic tools,[21,24,252] and therapeutic effects of antifungal drugs.[23] These complex questions demand different technologies but can clearly benefit from the use of valid animal models. In spite of some inherent limitations that are detailed below, models have been designed to reproduce the clinical course and the signs of the disease as observed in human patients and potentially provide reliable answers to scientific questions, while being more reproducible and cost effective than clinical trials.[56,181]

Thus far, animal models have already provided relevant answers regarding aspergillosis. A vast majority of all reported work has brought accurate information about immunopathology, for example, disease transmission, innate and acquired host-response, genes and proteins involved in fungal invasion, susceptibility to infection.[71] For example, genetic knockout models have revealed that IL6, IL12, and IFNγ were protective factors against *A. fumigatus*[40,51,63] and that IL17, TLR4, and TLR2 are greatly important in the innate response.[79,233] *Aspergillus*-infected TLR2 knockout mice have low TNFα and IL12 levels as well as lower survival and higher tissue fungal burden than infected immunocompetent mice.[19,27] Other studies have focused on preclinical therapy (pharmacology, pharmacokinetics, toxicology),[83,190,213] and on diagnostic and imaging approaches.[252] For example, the therapeutic potential of posaconazole against aspergillosis has been demonstrated in mice.[34] In addition, a mouse model of cerebral aspergillosis was used to show that combined therapy comprising either caspofungin and amphotericin B lipid complexes or caspofungin and liposomal amphotericin B has not enhanced first-line treatment.[50,102,144] Similarly, using an endocarditis model in guinea pigs,[152] investigators demonstrated the superiority of voriconazole over itraconazole to cure aspergillosis. Furthermore, a few studies described the interesting use of several mouse infection models that offered the opportunity to study aspergillosis in specific contexts, including solid-organ transplantation,[96] bacterial superinfection,[253] and concurrent with chronic granulomatous disease.[66,165] Key articles regarding animal models of aspergillosis are grouped by topic in Figure 1.

**General description of the various available animal models of aspergillosis.** Several mammalian species have been used as models for aspergillosis,[71] most frequently mice,[44,56,136] rats[48,70,86,129] guinea pigs,[113] and rabbits.[9,44,46] In addition, some investigators have described experimental aspergillosis in nonconventional laboratory species, including NHP[147] and cows.[106]

***Mice.*** Mouse models are the focus of more than 85% of the publications on experimental aspergillosis.[56,136] The first publication was in 1967.[81] Mice and humans show similarities in organs and systems, biochemistry, and pathology. In addition, this species is relevant as an animal model because the mouse and human genomes are less than 1% different.[169] Moreover, mice are inexpensive, and their body size allows the use of a relatively large number of animals to be tested simultaneously under identical conditions, which can enhance the power of statistical analysis. Laboratory reagents dedicated to mouse models are readily available especially those for addressing the disease–host response. Young mice with lower weight are assumed to be more susceptible to infection because they require a lower fungal inoculum for the infectious challenge. However, compared with rabbits and other rodents, the small size of this species permits only small volumes of blood to be collected. Moreover, repeated sampling is difficult, especially from animals with decreased clinical condition. In addition, the small lung size of mice may contribute to the different kinetics compared with humans. At the same speed of fungal growth, the much smaller murine lung will be overwhelmed much faster than the human lung, with possible consequences regarding the likelihood of hematogenous dissemination.[3,101,230,248]

More than 20 distinct mouse strains have been used to study aspergillosis in studies, and they all display substantial differences of susceptibility to infection.[66,131,132] Outbred mouse strains like Albino Swiss Webster[140,178,184,224] and CD1[20,145,201,214,218,231,242] primarily have been used especially for therapeutic assays. Outbred Swiss OF1[172,199,209] and NMRI[12,13] mice were tested in pharmacologic–pharmacokinetic and toxicologic studies. The DBA2 inbred strain, which is deficient in C5 complement, was shown to be highly susceptible to experimental aspergillosis,[41,95,254] whereas C57BL/6, BALB/c, and CD2F1 inbred mice were less likely to develop invasive aspergillosis, although they provided a permissive background for maximal expression of most mutations.[95] Targeted mutations in innate and adaptive immunity are ideal models for forward screening of genes that have a role in the susceptibility or resistance to invasive aspergillosis and have provided new insights into pathophysiology. For example, 129/Sv mice have been used for this purpose.[78,88,143,227,247] C57BL/6 mice have been useful for inducing mutations in $gp^{91phox}$ or $gp^{47phox}$ genes to generate defective oxidative burst in phagocytic cells, to address chronic granulomatous disease.[64,76,111,196,206,248] In this model, subacute aspergillosis developed even with a very low fungal inoculum.[10] Other studies in infected mice demonstrated that IL6, IL12, IL4, IL10, IL12, IL17, IFNγ, TLR4, and TLR2 play important roles in regulating the immune response against *Aspergillus*.[40,51,63]

Mice have also been successfully used to address other forms of aspergillosis, such as cerebral infection[45] and allergic diseases, especially *Aspergillus* bronchitis.[198] Unlike traditional murine models of allergic airway disease, which use ovalbumin, models of fungus-induced inflammation and atopy do not require additional adjuvants,[123,156] because fungal allergen proteases alone elicit adjuvant effects to usually innocuous proteins.[179] Thus, such models in C57BL/6 mouse strains iteratively exposed to *Aspergillus* are excellent tools for the study of airway hyperreactivity and allergic inflammation in response to conidia and specific hyphal antigens. For example, these models demonstrated that protease allergens (for example, Asp f13 and Asp f5) may be more important than nonprotease allergens for atopy and inflammation, although their specific roles in airway hyperreactivity—that is, Th2 cytokine induction manifested by increased levels of IL4 and elevated IgE levels in serum—and airway wall remodeling are only partially elucidated to date.[77] The use of relatively Th2-dominant mouse strains, such as BALB/c, or mice with inducible $T_{reg}$ deficiency results in a model that more closely resembles allergic bronchopulmonary aspergillosis rather than bronchitis.[160,249] Studies using these animal models have provided important insights into evaluating the activity of therapeutic agents for diverse allergic conditions,[153,197] innate immunity,[197,198] and B-cell, T-cell, cytokine, and chemokine responses against *A. fumigatus*.[79,164,170]

***Rats.*** Rats are a viable model species, given their ease of use and the availability of immunologic reagents.[43,70] These animals accommodate large volumes of blood and other biologic

| Topics addressed | References |
|---|---|
| General topics by species | 115 |
| Mice | 74, 195 |
| Rats | 43, 70, 92, 159, 161 |
| Guinea pigs | 15, 152 |
| Rabbits | 28, 112, 116, 122, 141, 182, 183, 188, 189, 205, 245 |
| Route of inoculation | 17 |
| Intravenous injection | 113, 184, 185, 211, 216 |
| Inhalational chamber nebulization | 6, 220, 221, 225, 226 |
| Intranasal inoculation | 17, 84, 133–135 |
| Oro-pharyngeal aspiration | 8, 219, 228 |
| Intratracheal administration | 43, 70 |
| Intracerebral injection | 45, 50, 53, 102, 223 |

**Figure 1.** Selected articles addressing important topics regarding animal models of aspergillosis

specimens, including bronchoalveolar lavage fluid.[72] In addition, rats are amenable to repeated sampling, and the animals are relatively inexpensive in terms of housing and purchase.

Rat models have primarily been used to address invasive aspergillosis with an initial pulmonary course.[70,72,92] Studies in rats have addressed new treatments[23,121,171] and diagnostic methods[21,24,252] as well description of disease.[22] In addition, these rodents are relevant models for studying cerebral aspergillosis: their median survival time (around 3 d) was consistent with the course of the human disease; the animals developed similar histopathologic patterns, with high numbers of cerebral abscesses containing abundant fungal hyphae and neutrophils, to those in humans; and the infection spread to peripheral organs in more than 80% of challenged rats.[257]

Male adult rats have frequently been used in models of invasive aspergillosis.[203,246,257] Challenging young rats (that is, 6 to 8 wk old) is critical to being able to use a reduced fungal inoculum and increasing their susceptibility to infection.[43,70] Outbred strains including Sprague–Dawley, Wistar albino, and albino×CD rats have typically been used,[43,48,70] because they were judged as excellent multipurpose models for safety and efficacy testing. Inbred strains including RP, Lewis, Dark Agouti, and albino×Oxford strains have been used in studies relating to immunology and inflammatory responses.[158,159,191,243]

***Other rodents and small mammals.*** Guinea pigs and rabbits have shown good correlation with humans regarding specific aspects of invasive aspergillosis.[121,181] For example, guinea pigs have been used to study fungal endocarditis,[152] and rabbits have been used in a model of fungal keratitis.[116] Rabbits emerged as a relevant animal model because they are naturally highly susceptible to infection.[211] In addition, their larger size than mice and rats accommodates serial sampling including repeated blood collection and bronchoalveolar lavage. Rabbits do not manifest an acute infection pattern; studies have addressed therapeutic questions linked to clinical efficacy and pharmacodynamics–pharmacokinetics of antifungal drugs.[36,202] Moreover, rabbits are considered to have a comparable in vivo metabolism to humans.[90] However, despite all of their valuables features, rabbits and guinea pigs have been used infrequently to study aspergillosis, perhaps due to expense, husbandry concerns, and lack of immunologic reagents as compared with mice and rats.

**Key technical parameters for animal models of aspergillosis.** ***Generating optimal conditions for experimental infection.*** An example experimental invasive infection protocol for aspergillosis in rats is illustrated in Figure 2.[43] All animal models except birds[14] require immunosuppression to generate a reproducible invasive infection.[71] Animals are usually rendered neutropenic through repeated injections of alkylating drugs, including intraperitoneal cyclophosphamide,[70,129] or immunomodulated through injections of subcutaneous steroids.[212] Alkylating drugs bind to DNA during cellular replication and thus induce profound leukopenia.[43,70,108] The histologic and radiologic features of animal models treated with alkylating drugs were highly similar to those of profoundly neutropenic infected patients, such as those undergoing leukemia.[43] However, the leukopenic animal models are currently becoming less relevant, because the characteristics of human patients infected with *A. fumigatus* have been progressively changing over time and are now at decreased risk for invasive disease, especially because of the systematic use of antifungal prophylaxis.[142,238] Steroid usage yielded a pathogenesis pattern distinct from that of neutrophil-depleting drugs, which affect alveolar macrophage function and thus reduce the first barrier to pulmonary infection. Steroid-treated animal models have been used to mimic patients with solid-organ transplants or those with stem cell transplants undergoing graft-versus-host disease. Hydrocortisone and triamcinolone both affect both T- and B-cell lymphocytes and decrease the production of cytokines, thus compromising the adaptive immune response against invasive aspergillosis.[150,232] Overall, steroid-treated models manifest massive inflammation with excessive neutrophil recruitment that results in insufficient fungal clearance, whereas leukopenic models induced by alkylating drugs enable unrestricted fungal growth.

Steroid-treated animal models mimic a different human patient group than those treated with alkylating agents; thus the 2 types of models provide different information. For example, gliotoxin acts as a virulence factor in corticosteroid-treated but not in leukopenic mice. Use of alkylating drugs or steroids is not expensive but quite reliable, provided that the protocol of immunosuppression is consistently applied. Many doses have been described, including single or repeated applications. Overall, cortisone at 100 to 200 mg/kg SC 3 times a week for the 2 wk before experimental infection and cyclophosphamide at a 150 mg/kg IP 3 times during the week before infection have been used most commonly in mice. Injections can be continued after the infectious challenge. Some investigators have used lower dosages to minimize other infections.[43] Other immunosuppressive medications, including tacrolimus and cyclosporine A, have been tested but with less success.[43,46,180] Some investigators have used monoclonal antibodies, such as Ly6 (Gr1) rat $IgG_{2b}$ MAb57 (clone RB6-8C5), to achieve neutrophil depletion. This strategy yields a rodent model that specifically reflects aspects of invasive aspergillosis during immunoreconstitution in humans.[8,25,98,109,207,213,222,248] In addition to the various conventional immunosuppressive protocols, some investigators have used protein deficiency to reproduce the clinical conditions in which aspergillosis preferentially affects weakened, malnourished patients.[43,212] Given the risk of concomitant bacterial infection in immunocompromised rodents, germ-free housing

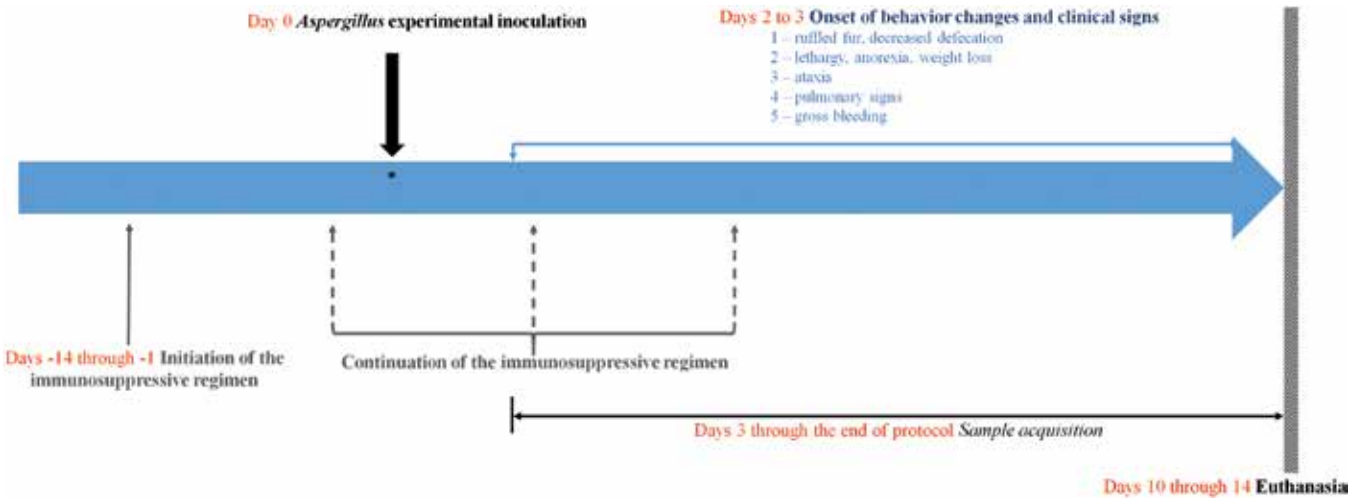


**Figure 2.** General protocol for *Aspergillus* experimental infection in animal models.[43,70] This flowchart indicates the estimated timing for major events, based on a model involving neutropenic rats challenged by intratracheal nebulization of *A. fumigatus* conidia. The duration of a protocol depends on the experimental design but lasts 15 to 30 d on average. In most invasive models, immunosuppression is needed to make the animals susceptible to experimental infection. Unique or repeated administration of alkylating drugs or steroids has been mostly described in the published literature. The regimen starts a few days to 2 wk before the *Aspergillus* challenge. Animals are then inoculated with *Aspergillus* conidia (day 0). Several routes of infection have been reported, including intravenous injection, nebulization, and intranasal or intratracheal deposition. Generally, the onset of clinical signs occurs 48 to 72 h after experimental inoculation (days 2 to 3). After that time, the animals become moribund from aspergillosis. At the end of protocol, surviving animals and controls are euthanized and examined through necropsy, histopathology, and mycologic culture, among other methods.[173]

and prophylactic antibiotics are recommended.[208] Tetracyclines, β-lactamins, and fluoroquinolones can be provided in the food or drinking water or by injection.[208]

Because other *Aspergillus*-diseases are not directly dependent on the host immunologic status, immunocompromise need not be introduced in corresponding animal models.

***Choosing the best route for experimental infection.*** Several routes of infection have been described to induce experimental aspergillosis in animal models.[71] Nebulization of *Aspergillus* conidia within a sealed plastic inhalational chamber has been used to reproduce the natural pathophysiology of aspergillosis.[221,225,235] Controlling the infection is difficult in such devices,[235] because animals are free to passively breathe at their own pace (Figure 3 A). Standardizing nebulization protocols is difficult because the number of fungal elements inoculated into lung tissue is variable, and animals do not react consistently.[221,225] In the inhalational model, the fungal inoculum that is effectively deposited is estimated as $1.0 \times 10^4$ spores per mouse, but sometimes with great variability, such that a large volume of fungal suspension typically is needed to initially generate the *Aspergillus* cloud inside the device.[99,100] In contrast, intravenous inoculation leads to homogenous infection that is readily reproducible. This route is likely the easiest to standardize and can require fewer animals than inhalational models. A mean inoculum size of $1.0–2.0 \times 10^7$ conidia is sufficient, regardless of the animal model. In general, the first target organs are the kidneys and brain, followed by the spleen and liver.[105] However, as with intraperitoneal inoculation,[211] intravenously induced infection does not mimic natural aspergillosis, which is usually not a blood-inoculated disease in humans and animals.[104] Alternatively the deposition of a few droplets of spore suspension into the nares can be considered close to natural infection (Figure 3 B), but the development of aspergillosis was quite variable in challenged immunocompetent mice, due to their upper mucociliary clearance which can expel as many as $1.0 \times 10^8$ conidia daily.[73] However the deposition of fungal solution into the nares deposited only approximately 10% of the dose into the lungs,[149] fungal burdens were smaller with higher standard deviations, and generated less homogenous pneumonia.[225] Pulmonary aspiration after intranasal deposition was suggested to drive the spore suspension toward the lung alveoli.[133–135] In addition, dissemination typically is limited to the liver when using rats,[24,43] whereas CNS involvement occurs in many mouse models.[136,194]

Some investigators have used bronchotracheal inoculation as an alternative method, in which the spore suspension is deposited through a tracheotomy in anesthetized animals (Figure 3 C).[16] With this method, the fungal inoculum is tightly controlled and deposited in a sinopulmonary organ for aspergillosis development.[121,212] In addition, surgery is minimal and bypasses only the upper airways and their putative defenses.[14] Noninvasive procedures, such as deposition into the caudal oropharynx of anesthetized rodents, in which normal breathing results in fluid aspiration into the lungs,[138,139,175,206,219,228] and novel devices (for example, Microsprayer Aerosolizer [PennCentury, Wyndmoor, PA]) achieve reproducible bronchotracheal inoculation without surgery.[43,70] As shown in Figure 3 D, administration of the fungal inoculum is relatively easy.[43] General anesthesia of 4 to 5 min per animal is sufficient to complete serial inoculations on several subjects. In addition, Microsprayer devices have been tested successfully in mice and rats.[43,70,72] Overall, disseminated models are used commonly for therapeutic studies. In contrast, the vast majority of studies that address the host response and fungal factors contributing to invasive aspergillosis are performed with pulmonary models.

In models of allergic aspergillosis, sensitization is achieved through repeated administration of a low-dose fungal inoculum, delivered by using similar devices as for bronchotracheal inoculation.[32] In contrast, alternative routes of experimental infection have been chosen to address some very specific forms of invasive aspergillosis; these routes include local eye invasion during endophthalmitis or ulcerative keratitis;[49,107,137,251,255] cerebral infection[55,166] after abrasion or removal of the corneal


**Figure 3.** Examples of different routes for experimental infection. (A) Hermetically sealed inhalational chamber in which the *Aspergillus* spp. spore suspension is nebulized. Contact of the animals (here, rats) with the spore 'cloud' must be prolonged, and the amount of inoculum is variable. (B) Intranasal deposition of *Aspergillus* spp. conidia suspension into mouse nares. (C) Instillation of the *Aspergillus* spp. spore suspension directly into the rat trachea by using a tuberculin syringe. This protocol requires that animals are anesthetized and tracheotomized. (D) Instillation of *Aspergillus* spp. spore suspension directly into the trachea by using the Microsprayer Aerosolizer (PennCentury) device. The Microsprayer Aerosolizer is composed of a metal elbow with a screw-on syringe adaptor; the device sprays a liquid solute in fine droplets due to a prism placed at its end. The anesthetized animal is positioned on a work stand, which is then tilted at 45° to allow for intubation. An otoscope is used as a laryngoscope. Spores are deposited directly at the bottom of the trachea, without surgery, because the device has the same curvature as the airways of the animal. Reprinted with permission from Guillaume Desoubeaux and Centre d'Etude des Pathologies Respiratoires, Tours, France.

epithelium;[37,200] and direct injection through the central area of the frontal bone[45,155] or inside the cisterna magna.[257]

***Selecting the* Aspergillus *strain.*** A review of the literature revealed great variability regarding the *Aspergillus* strains used in experimental animal models.[71] This choice might influence conclusions from the studies,[204] although only a few reports thoroughly addressed variations in virulence among distinct *Aspergillus* strains.[94] The decision regarding which strain to use depends on the scientific question. For example, hypovirulent strain that induces lower mortality is more useful for studying the benefits of various diagnostic tools during early-stage disease and throughout the course of the disease. Conversely, a hypervirulent strain is more suited to assess overall survival in preclinical therapeutic assays, when mortality rates are almost 100% without intervention. In the light of its widespread historical use,[174] AF293 (also known as ATCC MYA4609 and CBS 101355) is the strain used most often, although it is known to be less virulent than others. Dal/CEA10 (that is, ATCC MYA1163 or CBS 144.89) has also been used. CEA10 is the parental strain for a strain deficient for nonhomologous recombination, a feature that makes the construction of genetically modified fungal strains significantly more efficient. Other *Aspergillus* species including *A. flavus*, *A. terreus*, *A. niger* and *A. nidulans* have been less studied.[108,168]

The size of the *Aspergillus* inoculum for the challenge dose is a matter of debate, although dose-dependent correlation between the number of conidia and the severity of infection is assumed.[45,51,52] However, the infectious dose depends not only on the strain but also on the mode of immunosuppression. For the CEA10 strain, as few as $5.0 \times 10^4$ conidia were sufficient to induce lethal aspergillosis in leukopenic mice, whereas the dose lethal to 90% of corticosteroid-treated mice was 10- to 20-fold higher. In addition, broad dose ranges have been noted depending on the inoculation route: for example, from $1.0 \times 10^2$ to $1.0 \times 10^9$ for mice infected through intranasal route.[89,244] In contrast, the intravenous route has demonstrated an excellent infection:dose-to-mortality ratio[211] and requires a lower *Aspergillus* inoculum than do nasal and pulmonary routes as well as the inhalational model, for which $1.0 \times 10^9$ conidia/mL in 12 mL of suspension are usually needed for nebulization inside a chamber.[99,100,225] Intratracheal challenge is generally achieved with 1.0 to $2.0 \times 10^7$ conidia.[33,85,162] It should be noted that standardized culture conditions and preinfection technical steps (incubation temperature and humidity, age of culture, diluent, and method for conidia counting) are mandatory to ensure reproducibility. Therefore, providing this necessary information in publications is critical.

**Refinement and endpoints to relevantly assess the outcomes.** ***Refinement: attempts to improve the animal welfare.*** Investigators should thoroughly consider opportunities for refinement before initiating animal protocols. When assessing therapeutic effect or diagnostic performance, researchers must use a statistical power analysis to justify that they have sufficient (but not excessive) numbers of animals to estimate accurately to an appropriate level of precision. As with human clinical trials, estimation of the number of subjects to include should take into account several pivotal parameters: the primary outcome, method of comparison, and the type of variable measured (for example, binary [mortality, success rate of a procedure, and so forth] or continuous [weight, number of neutrophil leukocytes, and so forth] or discrete [number of respiratory episodes and so forth]).

Once challenged, animals should be checked twice daily, given the rapid onset of clinical signs and morbidity in most models. Thorough monitoring is suggested and should start from the second day after experimental inoculation when clinical status begins to deteriorate. Ideally, the experiments should be designed in such a way as to minimize mortality, except for preclinical therapeutic assays aimed at evaluating effects on mortality.[54] However, animal models of invasive aspergillosis usually develop according to an acute pattern, and avoiding death as protocol endpoint can be difficult.[72,119] To improve animal welfare and to enable the collection of sufficient biologic specimens (blood, tissue, and so forth) prior to sudden death, euthanasia should be decided on the basis of clinical scores, such as those obtained by using validated grids assessing variable endpoints and visible criteria.[38,167] For example, one scoring system evaluates twice daily the discomfort level according to a scale that scores the animal from 1 to 6 on the basis of appearance changes (dirty nose, red-rimmed eyes, ruffled fur, extreme pallor, and so forth), physiologic behavior changes (gasping, wheezing, bleeding, respiratory distress, icteric urine, prostration, instability, lethargy, and so forth),[22,43,70] reaction to stimuli, body temperatures changes,[4] and variation of body weight loss ≥20% of baseline. An empirical example of such a scale might be: score 1, no discomfort; 2, minor discomfort; 3, marked discomfort; score 4, serious discomfort; score 5, severe discomfort; score 6, death.[43,72,167] As an alternative, in vivo imaging is a noninvasive method to monitor disease progression and fungal

burden in leukopenic mice; this approach has the potential to significantly reduce the number of animals needed in therapeutic studies. Other imaging techniques have also been shown to be helpful.[193] In addition, the detection of galactomannan antigen has been reported to be useful for defining endpoints for euthanasia.[43]

***Methods for validating the development of experimental infection and assessing fungal load.*** Assessments are needed to ensure that the disease develops correctly and that the animal model reliably mimics aspergillosis.[71] Serum measures of BUN, creatinine, ALT, and AST have been reported as indirect nonspecific biomarkers that reflect the effects of invasive aspergillosis.[224] Necropsy confirmation of infection is highly recommended.[120] When positive, histopathology provides unquestionable evidence of aspergillosis.[91] Mycologic cultures from lungs or other organs enable relative quantification of the fungal burden through colony counting, but overall culture-based methods are hampered by insufficient interlab reproducibility and are time-consuming. However, because of dead fungus and dormant spores remaining in the lung, these methods underestimate assessments of the fungal load.[211] In addition, mechanical homogenization, particularly of lung parenchyma, can result in possible underestimation due to the multicellular filamentous nature of *Aspergillus* spp., because a single disrupted filament can give rise to an unknown number of colonies.[57,217] Furthermore, differentiating simple colonization from actual invasive infection can be difficult, especially in guinea pigs.[114] Therefore, nonculture-based methods, used alone or in combination, can provide more precise monitoring of the experimental disease.[43,132,241,256] The chitin assay, which measures a specific cell-wall fungal component, makes it possible to quantify the infection in lung and other tissues.[23] However, such tests can only be performed after the animal has been euthanized and may be time-consuming when large numbers of samples must be analyzed.[18] Determination of galactomannan antigen in blood has been used often because it reflects the progressive increase in mycelium load due to tip extension of hyphae[57] and thus the extent of infection in challenged animals.[241] Variable sensitivity or specificity occurred depending on the species tested.[59,80] Although promising, the application of (1→3) β-D-glucans measurement and quantitative PCR analysis needs to be thoroughly validated in animal models.[157] For example, PCR analysis is assumed very specific and sensitive, especially when targeting multicopy genes,[132,157] but it does not indicate the viability of the fungus, and some false-negative issues due to the limitations of the DNA extraction method can arise.[157,220,256] Measurement of surrogate endpoint biomarkers alone is insufficient to diagnose the actual extent of disease[119] and therefore should be coupled with the aforementioned criteria.

**Several pending problems and the urgent need for standardization in animals models of aspergillosis.** The knowledge accumulated over decades from animal models of aspergillosis demonstrates their utility for reproducing clinical infection in humans and animals. However, there is no consensus currently regarding the best experimental methodology. Overall, choices regarding the optimal animal species, strain, sex, and weight to use vary widely. In addition to invasive models, high variability occurs regarding immunosuppressive regimen, fungal strain, level of inoculum, and route for infectious challenge. Several standardization initiatives for invasive models of aspergillosis are available.[210] Furthermore, methods-based publications should be cited in studies, to provide an important resource for facilitating standardization and comparability[93,136,234] and to improve the foundation for evaluating novel diagnostic tools and treatment regimens.[115,237]

In addition to the current lack of standardization, animal models might be criticized for poorly paralleling actual human disease. For example, in allergic models, repeated challenge with antigens, extracts, or conidia does not result in airway colonization with live hyphae, bronchiectasis, or mucus plugging—all of which hallmarks of allergic bronchopulmonary aspergillosis in human patients. Therefore, these models likely more accurately reproduce *Aspergillus*-induced asthma or severe asthma with fungal sensitization rather than allergic bronchopulmonary aspergillosis or *Aspergillus* bronchitis.[128,229] Overall, the availability of an animal model in which hyphae actively grow within the airways and produce a wide range of immunoreactive secondary metabolites would be a useful tool for studying host–pathogen interactions during allergic bronchopulmonary aspergillosis and therapeutics. For invasive models, experimental aspergillosis typically develops in challenged animals according to a hyperacute process that follows a unique and massive exposure. For example, infected rabbits die within an average of 5 to 10 d, rats between 3 and 7 d, and mice 4 to 7 after infection. In contrast, aspergillosis develops according to an indolent pattern in humans and naturally infected animals, due to continuous or repeated exposure to a much smaller number of conidia.

To address this limitation, some investigators suggest decreasing the infectious inoculum or reducing the immunosuppressive regimen slightly.[168] Others propose a model involving agarose beads coated with *Aspergillus* spores so that the fungus remains alive for several weeks before the initiation of the immunosuppressive regimen, thus mimicking continuous exposure to molds as might be encountered in the environment.[173] Mice infected for 6 wk with conidia-containing beads first developed chronic noninvasive airway infection with *A. fumigatus*. By day 7 after challenge, intraluminal leukocyte infiltration was already accompanied by peribronchial inflammation, composed of both neutrophils and mononuclear cells, and persisted in the lungs of mice for as long as 1 mo.[239] Subsequent treatment with cortisone acetate 2 wk after the beginning of the colonization led to the development of invasive aspergillosis, suggesting that this model may be excellent for studying the pathogenesis that occurs in patients with underlying chronic lung disease.

No single model can answer all of the questions regarding *Aspergillus*, and each has its own limitations. However, the development and use of animal models suitable for basic and translational studies remains a key to aid in future advances against aspergillosis.

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