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Overview and Approaches for Handling of Animal Models of Leishmaniasis

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Leishmaniasis, a disease of global relevance, results from infection with the protozoan parasite, *Leishmania*, which is transmitted to susceptible hosts through the bite of sand flies. Multiple forms of leishmaniasis may occur, including cutaneous, mucocutaneous, and visceral. Research with animal models remains an important approach to help define basic pathophysiologic processes associated with infection and disease. In this regard, mice and hamsters represent the most commonly used models. The severity of leishmaniasis in animal models depends on several factors, including genotype of the host and parasite and the dose and route of administration of the parasite to the host, and severity of outcome may range from subclinical to severe illness. This review provides basic background on leishmaniasis, relevant animal models, the pathophysiology and clinical signs in animals used as models of leishmaniasis, and general approaches to mitigate risk to personnel.

Abbreviations and Acronyms: CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis

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Introduction

Human leishmaniasis is caused by one of more than 20 obligate intracellular protozoa of the genus *Leishmania* spp., transmitted by infected female phlebotomine flies, known as the "sand fly."[43] It is worth noting that sand flies are not the same thing as sand fleas. There are approximately 1,000 sand fly species and subspecies, with 35 proven, and an additional 63 incriminated, as vectors of *Leishmania* to humans.[17,44] The earliest known evidence for the presence of *Leishmania* organisms appears in Burmese amber dating to around 100 million years ago.[53] The presence of leishmanial life stages within the alimentary tract of a blood-filled sand fly preserved in amber accompanied by the nucleated red blood cells of reptiles suggests that the complicated host-vector-parasite relationship is ancient. This might account for the broad dispersal and high variability of both sand flies and *Leishmania* organisms having had millions of years to evolve in various environments and hosts. Leishmaniasis exists contemporaneously in tropical regions of the Americas, East Africa, North Africa, and Western and Southeast Asia.

The term, leishmaniasis, is reserved for illness associated with *Leishmania* infection. Although the World Health Organization (WHO) indicates that more than 1 billion people live in areas endemic for leishmaniasis, it is believed that a very small proportion of individuals infected with the parasite will become clinically ill.[73] Globally, an estimated 2 million patients are diagnosed with leishmaniasis each year, with an overall prevalence of 12 million.[24] Although progress has been made with respect to efforts directed at medical surveillance for leishmaniasis, the reporting rates may not be optimal, thus complicating efforts to identify actual rates of infection, and without the benefit of routine medical surveillance, the true incidence is difficult to discern.[56]

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Leishmaniasis, while often lumped into one entity, represents a constellation of disease manifestations, ranging from self-limiting cutaneous lesions to fatal visceral disease. There are 3 commonly recognized clinical presentations of the disease in humans: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). In Assamese, a language native to some areas of northeastern India, VL may be referred to as *kala-azar*, which translates into English as "black disease," a term coined in the 1880s to describe the discoloration of the skin that occurs during infection.[67] VL has also been referred to as Dumdum Fever, named by Dr. William Boog Leishman for the location where the parasite was first observed in smears from the spleen of a patient who succumbed to the disease, Dumdum, Calcutta, India.[70] Post-kala-azar dermal leishmaniasis is a condition that develops in some patients, sometimes years, after recovery from VL, and manifests as dermal lesions.[75] Individuals affected by CL typically have self-limiting cutaneous ulcerations that gradually heal and are replaced by scar tissue. In contrast, lesions associated with MCL are generally not self-limiting and may involve the destruction of mucous membranes of the throat, mouth, and nose. The CL and MCL versions of the disease, while lacking the colorful nomenclature applied to VL, are of great clinical significance due to the potential for disfiguring scarring and destruction of normal anatomy that can occur secondary to the infection. Further, individuals so affected may experience social isolation, as the lesions are sometimes confused with leprosy and fungal dermatitis.[12,62]

Overview of *Leishmania*

It is difficult not to admire the elegant evolutionary choreography that exists between parasites, hosts, and vectors. *Leishmania* spp. is no exception. A stylized depiction of the *Leishmania* life cycle is presented in Figure 1. The female sand fly must take a blood meal to obtain the energy for egg development.[74] If the sand fly is infected with *Leishmania*, promastigotes can be transferred to the host.[41] Of note, sand fly saliva facilitates blood meal acquisition through anticoagulant

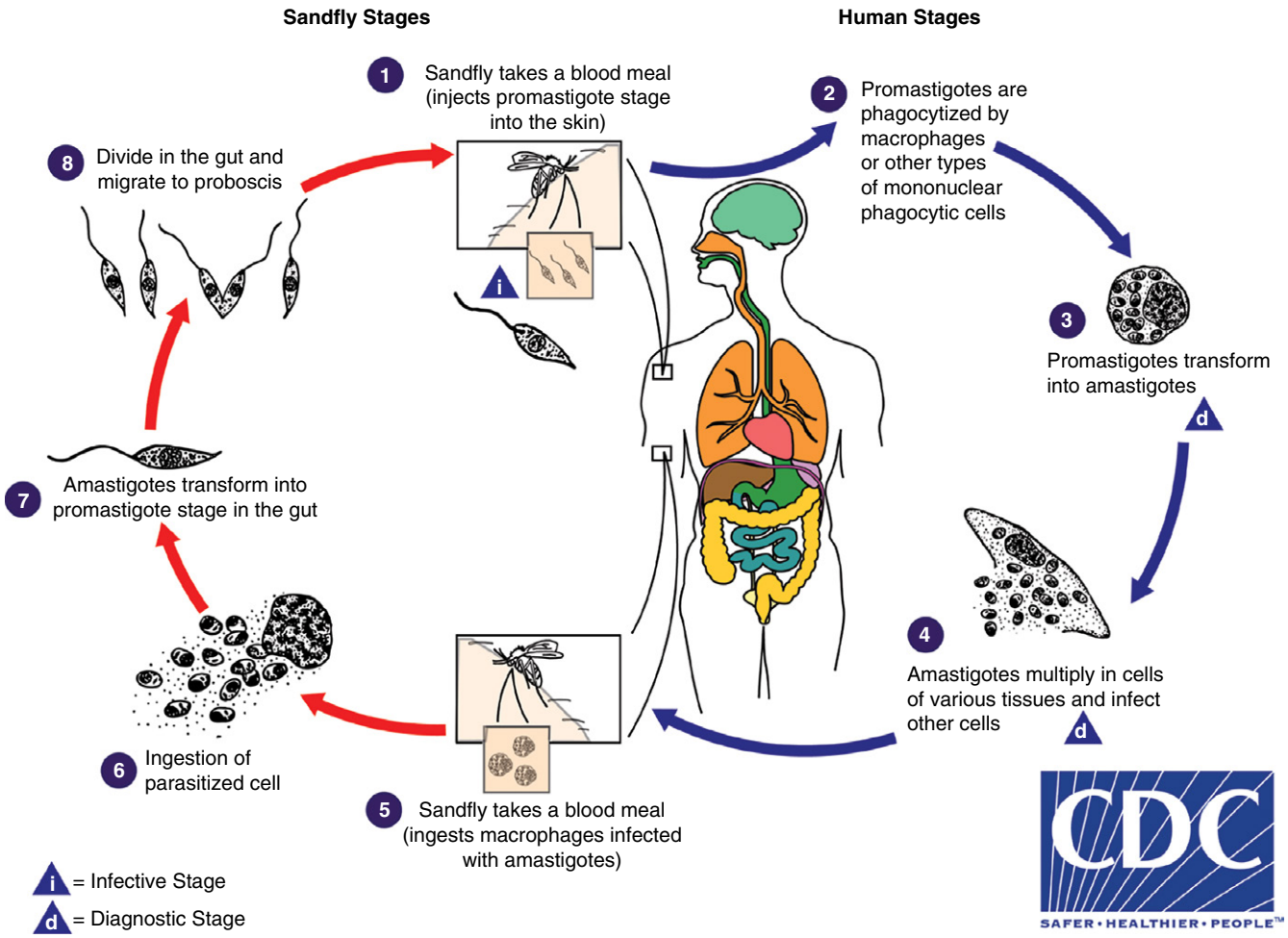

Figure 1. Life cycle of *Leishmania*. Image courtesy of DPDx, Centers for Disease Control and Prevention (https://www.cdc.gov/dpdx.). Infected sand flies transmit promastigotes to a host when taking a blood meal. Following internalization by host phagocytic cells, the amastigote form of the parasite multiplies and can infect other cells. A subsequent blood meal taken by another sand fly results in transmission of the parasite to the vector where the amastigotes transform in the gut to promastigotes, which then migrate to the proboscis and prepare to infect other hosts during ingestion of a blood meal, thus completing the cycle.

and immunomodulatory factors and can influence establishment of *Leishmania* parasites within the host.[39] Sand fly salivary components vary among phlebotomine flies (sand flies) and contribute to the highly variable manifestation and presentation of leishmaniasis.[39] Given the numerous *Leishmania* and sand fly species, the highly variable presentation of the disease entity is not surprising.

The vector-parasite-host interaction is largely the same across more than 189 vertebrate host species, including the orders Carnivora, Chiroptera, Cingulata, Didelphimorphia, Diprotodontia, Lagomorpha, Eulipotyphla, Pilosa, Primates, and Rodentia.[9] Once transferred from the vector to the host, the *Leishmania* promastigote is internalized by antigen presenting cells of the host, generally macrophages. The promastigote (Figure 2A) transforms into the amastigote form (Figure 2B) that is able to perpetuate within the host.[36] By simple binary fission, *Leishmania* amastigotes multiply and are subsequently released either through cell rupture or exocytosis. Liberated amastigotes can then be phagocytized by host cells, thus perpetuating the infection. When the female sand fly takes a blood meal from an infected host, amastigotes within macrophages may also pass to the vector. Once the amastigotes reach the gut of the sand fly, the parasite transforms to its promastigote phase and continues to divide, eventually differentiating and migrating to, and blocking, the stomodeal valve, resulting in regurgitation of *Leishmania* infectious stage promastigotes into the host, thus repeating the life cycle.[58,71]

Promastigotes are elongated with a large central nucleus and a long, motile, anterior flagellum. The promastigote is found within the midgut of the sand fly and would not be expected to be present in the infected host animal. The amastigote, defined as a protozoan that lacks visible functional external flagella or cilia, is round to oval and can be recovered from macrophages throughout the body of the host. Leishmania amastigotes exhibit a rudimentary, nonmotile flagella compared with the promastigote phase.

Commonly Used Animal Models

A great need remains for greater insight toward the full understanding of *Leishmania* pathophysiology and therapeutic approaches to leishmaniasis. Animal models, while often lacking a direct correlation with disease in humans, offer the opportunity to study various steps in the pathogenesis of leishmaniasis.[42] Further, current therapeutic approaches include treatment with compounds such as meglumine antimoniate or amphotericin B, both of which may result in serious adverse clinical effects; thus, investigations with animal models are needed to develop new clinical options that offer improved patient outcomes.[16] Animals

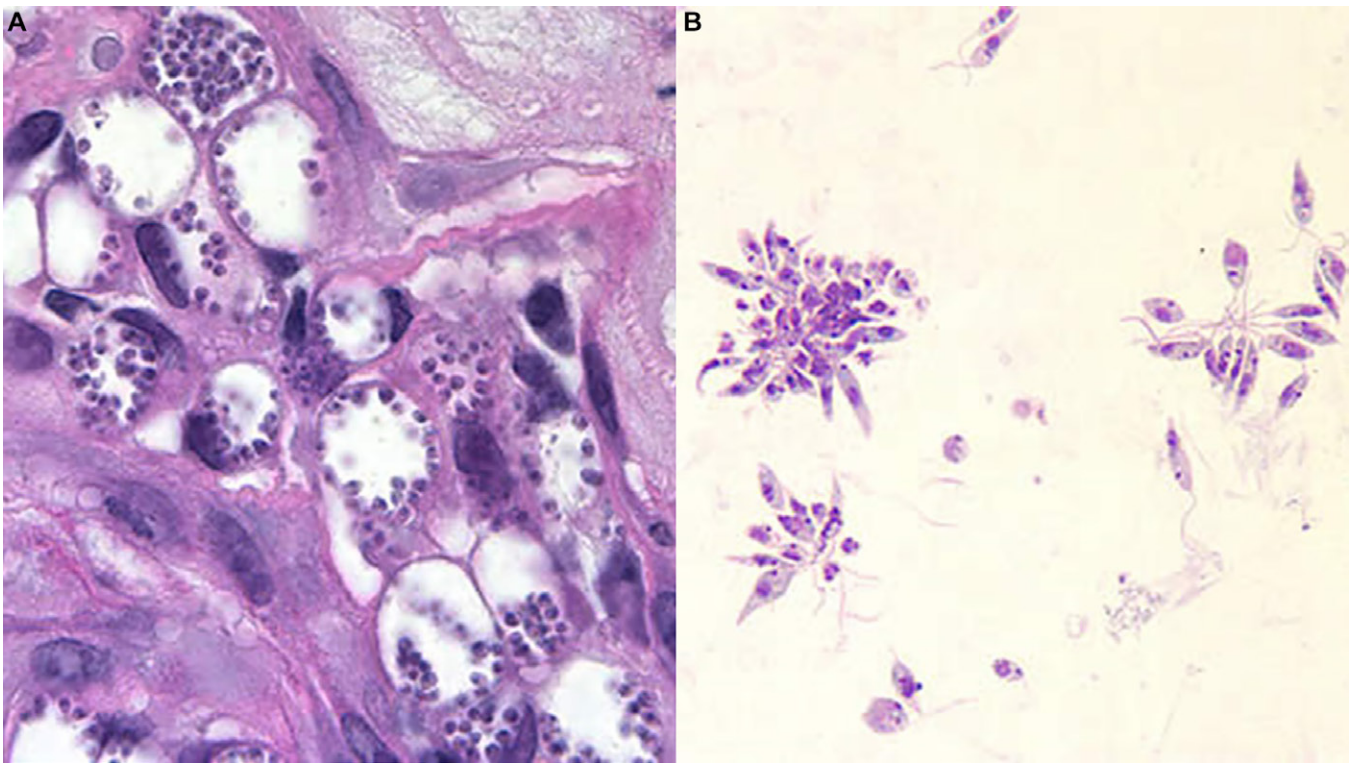

Figure 2. Amastigotes of *Leishmania* sp. (A) In a biopsy specimen from a skin lesion, stained with hematoxylin and eosin. *Leishmania* sp. (B) Promastigotes from culture. Images courtesy of DPDx, Centers for Disease Control and Prevention (https://www.cdc.gov/dpdx.).

are primarily used to model CL and VL and there are no reported standardized models specific to MCL.

Nonhuman primates, particularly *Macaca* spp., acquire infection and develop clinical disease approximating that found in humans, making them a logical model for the study of leishmaniasis.[29] Further, New World Monkeys have been used specifically for the study of VL.[5] However, the significant challenges associated with using nonhuman primates as models of leishmaniasis remain their relative expense and availability for biomedical research. Questions remain regarding the existence of immunity and cross-immunity to previous exposure to *Leishmania* spp. further complicating the use of nonhuman primates sourced from the wild, given that many species are sourced from areas of the world where *Leishmania* spp. are endemic.[54] These factors make the nonhuman primate a challenging model for leishmaniasis research except for the most critical of studies; thus, they are not commonly used.

Dogs may serve as a reservoir of leishmaniasis in endemic areas and exhibit some disease progression similar to other animals, including humans. Various factors including climate change, resulting in the expansion of vector habitats, and international travel have the potential to increase the importance of leishmaniasis in companion animals, including horses, representing a "One Health" opportunity to further the understanding of leishmaniasis epidemiology.[46] In addition, companion animals, such as dogs, may serve as useful sentinels for leishmaniasis in the human population.

Dogs have been used for modeling of leishmaniasis because they are easy to handle, are well-characterized immunologically, and have reasonable pathophysiologic homology with human leishmaniasis. With the demonstration and subsequent confirmation of vertical transmission in dogs, and the more recent evidence of vertical transmission in mice, the use of the dog model may be useful in gaining a better understanding of the adaptability of *Leishmania* organisms.[13,20,26,45,65]

Rodents are the most widely used models of leishmaniasis, as they are well-characterized, and they provide the opportunity to explore very specific and nuanced processes in parasite transmission, disease pathogenesis, and immune response.[42] Of interest, the Syrian golden hamster (*Mesocricetus auratus*) is often regarded as the best experimental model of VL.[41,57] Early research using hamster cells in vitro helped explain the activity of *Leishmania* spp. amastigotes within macrophages, and ongoing work serves to highlight the immunomodulation that occurs in *Leishmania* spp. infections that allow the disease to develop.[18,47] The hamster remains an important model for immunopathogenesis, drug discovery, and vaccine development for VL but has application in the study of CL as well.[27,57]

As with other types of biomedical research, the power of murine models for leishmaniasis benefits from the availability of genetically standardized and genetically modified animals. Importantly, this ready access allows researchers to perform controlled experiments without the confounding variable of conducting research on subjects that are genetically heterogeneous. Further, the relative ease of maintaining the animals in standardized conditions that are controlled for environmental variables adds value to the use rodent models. As well, distinct differences between mouse strains with respect to resistance and susceptibility to infection with *Leishmania* have allowed scientists to identify fundamental characteristics that confer resistance.[2,40,50] Consequently, mouse models of leishmaniasis remain extremely important to sorting out the remaining questions surrounding disease development and potential interventions, but selection of the appropriate mouse model is of paramount importance.

The Pathophysiology and Clinical Signs in Animals Used as Models of Leishmaniasis

A basic precept for an animal model of infectious disease is that it should reasonably parallel the pathophysiology and clinical manifestations of the disease in humans; therefore, illness and adverse outcomes are often part of the experimental paradigm. In the case of *Leishmania*, impacts on animals depend to a great extent on whether the infection is visceral compared with cutaneous and on the species/isolate and dose of *Leishmania* used to initiate infection, the route of administration, as well as on host factors such as the genetic background of the host being used Here, descriptions are focused on mice, hamsters, and dogs as they are the most commonly used species for the study of leishmaniasis.

Models of CL. Models of CL typically involve inoculation of live parasite organisms, usually promastigotes, subcutaneously or intradermally. Mice are the primary model for CL, although hamsters have been used in some cases. In mice, infections are usually initiated by subcutaneous administration of parasites in either the ear pinna or the rear footpad. Infections of the pinna may result in induration of the site and development of an ulcer that typically heals with time (Figure 3). Foot pad infections result in swelling of the foot (Figure 4), with assessments of treatment or other variables made by comparison of the degree of swelling, as determined by measurement of foot pack thickness with calipers or by plethysmometry, to the contralateral noninoculated foot pad.[6,8,16,33] In resistant mouse strains, swelling is typically self-limiting and rarely results in lameness; in susceptible mouse strains, lesions progress with subsequent necrosis of pedal tissues and spontaneous amputation of the foot.[3] The impact of mouse and parasite genotype on the outcome of cutaneous infections can be substantial. While BALB/c mice exhibit greater susceptibility to *Leishmania major* infection with development of large cutaneous ulcers leading to the spread of parasites to visceral sites, C3H/He, CBA, C57BL/6, and 129Sv/Ev mice are resistant to infection and develop small lesions, which typically heal in 10 to 12 wk.[4,7,10,23] In contrast, although BALB/C mice are susceptible to infection with *L. braziliensis*, the resulting lesions are neither severe nor persistent, thereby demonstrating the impact of *Leishmania* species on outcome.[22]

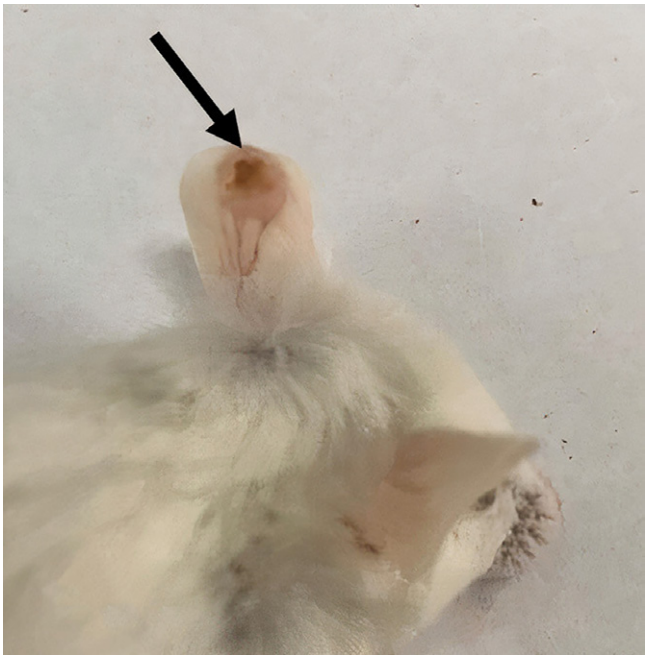

Figure 3. Induration and ulceration (arrow) of the pinna in a BALB/c mouse experimentally infected with *Leishmania*. Photo courtesy of Dr. Mary Ann McDowell.

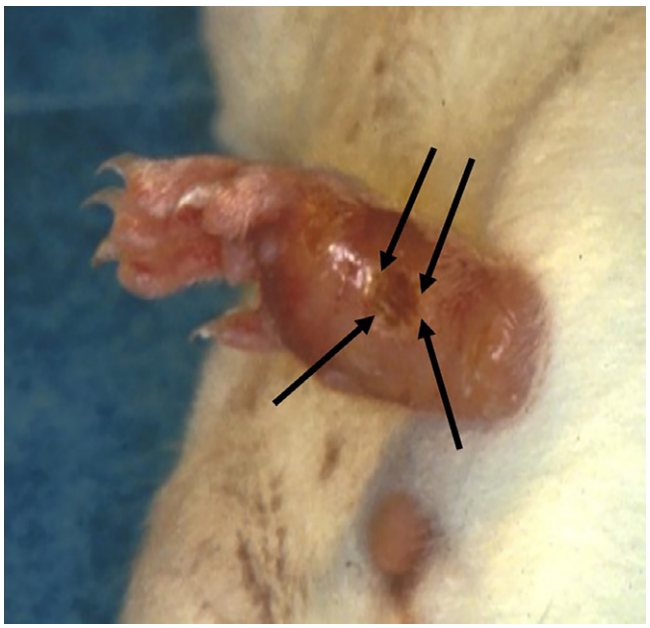

Figure 4. Induration and ulceration of the foot pad of a mouse experimentally infected with *Leishmania*. The arrows outline the area of cutaneous ulceration. Photo courtesy of Dr. Mary Ann McDowell.

CL has been modeled in golden hamsters (*Mesocricetus auratus)*, with infections established following intradermal inoculation of *Leishmania* promastigotes at the lumbosacral area.[28] In hamsters, pathologic changes and clinical illness depend, to at least some extent, on the route of inoculation and the genotype of the *Leishmania* organism being evaluated.[48]

Models of VL. Mice, hamsters, and dogs have been used as models of VL, with parasites usually being introduced via either intradermal or intravenous administration.[23] Intravenous infection of susceptible mouse strains (BALB/c and C57BL/10) with *L. donovani* and *L. infantum* generally resulted in granulomatous inflammation in the spleen and liver with an initial increased parasite burden, followed by a spontaneous decline and, ultimately, clearance as the animals mounted a cellular response against the parasite.[61]

Like humans, golden hamsters are exceptionally susceptible to infection with *L. donovani* and may develop clinical illness characterized by cachexia and weight loss, similar to human infection. Following intracardiac inoculation of parasites, hamsters will become anemic and may develop hepatosplenomegaly and proliferative glomerulonephritis.[1,48] The hamster has also been used to maintain isolates of *Leishmania* by intracardiac injection of parasites and subsequent harvesting of tissues to provide a renewable source of parasites.[15] Hamsters infected with *L. infantum* exhibit elevated blood cortisol levels that correlate with increasing severity of infection as determined by splenic and hepatic parasite burden; consequently, one can speculate that the animals experienced increased distress as the severity of infection increased.[11]

The clinical presentation of natural *Leishmania* infections varies greatly in dogs and can range from the absence of clinical symptoms to weight loss, cutaneous lesions, and protein-losing nephropathy.[21,60] Experimentally, dogs have primarily been used to study the visceral disease. Similar to other species, severity of disease in dogs can vary with several factors, including the species of *Leishmania.* Dogs infected intravenously with

L. mexicanum developed disseminated disease that included cutaneous ulcerations and, in some dogs, nephritis and hepatic necrosis.[19] Intravenous inoculation of dogs with *L. donovani* produced persistent infection that was characterized by weight loss, splenomegaly, lymphadenomegaly, and normocytic, normochromic anemia.[34] In contrast, intravenous infection of dogs with *L. infantum* can produce asymptomatic infections, as PCR evaluation of blood and liver samples demonstrated persistence of *Leishmania* through at least 7 mo following infection.[37]

Sand flies are sometimes maintained to either specifically study interactions of the *Leishmania* parasite with the vector or, in some cases, to study transmission of the parasite from infected sand flies to animal hosts, as this represents a more natural route of infection.[51,52] For both, sand flies are often maintained by providing a blood meal through access to heparinized mouse blood via an artificial membrane; however, feeding of sand flies on live animals, including mice, hamsters, rats, and rabbits, has also been used for purposes of colony maintenance and for studies involving transmission of *Leishmania* to animals.[14,55] Because sand fly bites may cause discomfort, mice or hamsters are first anesthetized and then placed into a container with sand flies, which can then take a blood meal over 30 to 60 min (Figure 5).[72] Feeding of sand flies on restrained rabbits within a specially constructed feeding cage over the course of 1 to 3 h has been described.[64]

Humane Considerations

A systematic review of literature describing research using animal models of leishmaniasis published between 2000 and 2020 demonstrated a general lack of provisions to enhance animal welfare.[69] For example, approximately 10% of studies using mice or hamsters described the use of individual, rather than group, housing; approximately 5% of studies described any attempt to introduce principles related to the principles of replacement, reduction, and refinement (3Rs); and humane endpoints were not reported in any of the reviewed studies.

As described earlier, infection with *Leishmania* may have an impact on animal well-being depending on factors such as host and parasite genotype, parasite dose, and route of administration. Infection may result in outcomes that range from absence of clinical disease to severe disease, even death. In this regard, it is essential that potential adverse impacts on animals be clearly defined and articulated to the IACUC and research staff and that endpoints are defined at which animals will be treated, removed from the study, or euthanized.[32] Typically, treatment for foot pad induration or open wounds is contraindicated due to the experimental paradigm, as data related to measurement of cutaneous lesion size, increased thickness of the foot pad, and blood inflammatory markers could be impacted. Although seldom described in published literature, endpoints that result in euthanasia of the animal should be included as part of the experimental paradigm for models of leishmaniasis and might include loss of body weight, presence of cutaneous ulceration, body condition, nonresponsiveness to external stimuli, and presence of ascites, as these are all possible clinical outcomes with CL or VL and have, in some instances, been applied as endpoint criteria.[23,31,48] Importantly, if humane endpoint criteria are to be used, it is essential that personnel be trained to evaluate animals vis-à-vis such criteria and that they be empowered to actuate euthanasia when endpoints are met.

Although nonanimal models of leishmaniasis have not been described, one interesting approach has been applied to reduce the number of animals used in longitudinal studies. Specifically, the use of *Leishmania* organisms engineered to express luciferase for infection of mice and subsequent imaging of bioluminescence were shown to allow for repeated assessment of the progression of infection using fewer animals, compared with the need to euthanize animals at multiple time points.[63]

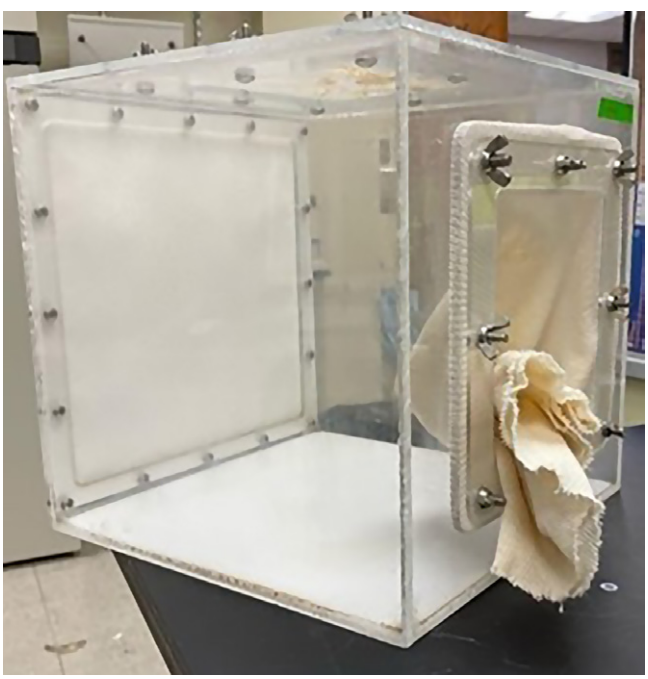

Figure 5. An adult sand fly containment system designed to facilitate blood meals on live, anesthetized animals. Sand flies are housed within the polycarbonate chamber, and anesthetized animals are placed in the cloth sleeve that is then inverted into the box through which sand flies can take a blood meal from the animal. Photo courtesy of Dr. Mary Ann McDowell.

Management Approaches to Working with Animal Models of Leishmaniasis

Leishmania spp., as an agent that is associated with human disease and poses moderate risk to personnel, requires management practices consistent with BSL2 and animal management practices consistent with animal BSL2 (ABSL2).[68] Laboratory-acquired *Leishmania* spp. infections have been associated with parenteral, mucous membranes, nonintact skin, animal bites, and "no known accident" exposures.[30] In the research setting personnel can be exposed to *Leishmania* spp. promastigotes by exposure to infected sand flies or other infectious inocula. For example, *L. infantum* has been reported in cases of venereal transmission in dogs suggesting the possibility of mucous membrane transmission to personnel who work with infected dogs.[49,59] Compliance with BSL2 standards requires that laboratory personnel receive specific training and are supervised by personnel competent in handling *Leishmania* spp.; that access to the laboratory or animal room is restricted when work is being conducted; and that procedures that may result in aerosol generation or splashes are conducted in a biosafety cabinet.[68] Housing of rodents can be safely accomplished by using individually ventilated cages of the appropriate size for the species, but work with larger species requires increased reliance on room or facility engineering controls. Risk assessment of personnel should consider the specific tasks that will be performed and the likelihood of personnel exposure to infectious Leishmania organisms. Common personal protective equipment to be worn

when handling animals or materials potentially containing *Leishmania* include eye and respiratory protection.

Importantly, laboratories conducting BSL2 or ABSL2 work must have a means of decontamination of all waste. Autoclaving is often used except in the case where chemical components of research may prove hazardous under heat and pressure. Alternatively, incineration may be a consideration based on hazard assessment and risk management. In addition, personnel should have access to occupational medical services based on workplace hazards, risk assessment, and awareness of risks based on personal health status.[68]

Consideration should be given to both vector and animal hosts with respect to management of work involving *Leishmania*. Methods for initiating and maintaining sand fly colonies and record-keeping strategies to optimize the health of the sand fly have been published elsewhere.[38] Sand fly colonies require appropriate housing and nutrition for all stages of their life cycle and are susceptible to a range of pathogens, including parasites, microsporidians, mites, fungus, bacteria, and nematodes. Currently, viruses that adversely affect sand flies are not described.

Sand flies may be provided a sugar solution or moist fruit as a food source except that the female sand flies must take a blood meal to lay eggs. Blood can be provided through access to heparinized blood via an artificial membrane; however, feeding success is generally lower than when live vertebrates are used as a blood source.[25,38] Commonly, live mice are used for purposes of colony maintenance, although other species have been sometimes used for studies involving transmission of *Leishmania* to animals.[14,55] Because sand fly bites may cause discomfort, mice are first anesthetized and then placed into a container with sand flies, which can then take a blood meal over 30 to 60 min (Figure 5).[72] Feeding of sand flies on restrained rabbits within a specially constructed feeding cage over the course of 1 to 3 h has been described.[64] Sand flies are typically sugar deprived for 24 h to encourage feeding. Blood donor animals are often euthanized after feeding to prevent any pain or distress secondary to blood feeding.

Sand flies have been characterized as weak fliers, traveling through short hopping flights.[38] While sand flies appear to have the ability to fly further than previously appreciated, they are not reported to fly fast, with top speeds of 2.52 km/h or 1.566 mph.[35] A recent study evaluating the flight behavior of sand flies demonstrated flights of ~65 m/213 ft.[66] Sand fly colonies should be maintained in facilities that can contain escaped flies with appropriately sized screens or seals over any possible egress. Double-door entry as well as air curtains may effectively contain escaped flies and prevent the research staff from accidentally carrying escaped flies beyond the containment barrier.

With respect to the management of vertebrate animals used in research on leishmaniasis, some animals may be maintained solely as a source of a blood meal. These animals should be free of infection with *Leishmania* spp. and housed under conditions appropriate for their species.[48] Animals that are infected with *Leishmania* spp., including those that serve as the source of amastigotes to infect sand flies, should be held under containment conditions. Appropriate management should consider not only risks associated with *Leishmania* but also those inherent to the host species (for example, nonhuman primates).

Summary

Critical to the advancement of treatment and prevention of leishmaniasis are strategies that can only be developed through the use of animal models. While all models are but an approximation for humans, they remain an essential feature of both basic and translational work. The impact of *Leishmania* infection on animals varies with genotype of both parasite and animal, dose of parasite, and route of administration. Key to properly conducted studies are steps taken to enhance animal welfare, including IACUC oversight and review of procedures, clearly stated and understood humane endpoints, and training of personnel with respect to proper animal handling and recognition of endpoints.

Conflict of Interest

The authors have no conflicts of interest to declare.

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