Characterization of Canine Leishmaniasis in the United States: Pathogenesis, Immunological Responses, and Transmission of an American Isolate of *Leishmania infantum*

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(ABSTRACT)

Leishmania infantum, an etiologic agent of zoonotic visceral leishmaniasis, has recently emerged in the foxhound population in the United States and parts of Canada. *Leishmania* infections are usually spread to mammals by infected sand flies, however epidemiological data do not support a role for sand fly transmission in North America. The purpose of this work was to isolate and characterize *L. infantum* from a naturally infected foxhound from Virginia (LIVT-1 isolate). A mouse model of North American leishmaniasis was developed using immunocompetent and genetically immunodeficient mouse strains infected with LIVT-1 promastigotes by different inoculation routes. The intravenous route of infection was superior to the subcutaneous route for inducing consistent experimental infections and mice lacking interferon gamma, inducible nitric oxide synthase, or B-cells were resistant to clinical disease.

Experimental infections in dogs were performed to examine the infectivity, immune responses, and pathogenicity of LIVT-1. Experimentally infected dogs developed parasitologically proven infections and a range of clinical manifestations that were similar to those observed in naturally occurring disease. Diagnostic tests including culture and cytologic evaluation of bone marrow and lymph node aspirates, polymerase chain reaction, and serology by indirect fluorescent antibody test, and recombinant K39 (rK39) immunoassay were evaluated. Kappa statistics revealed that PCR had the highest level of agreement with culture and cytology results although the rK39 dipstick assay consistently identified more experimentally infected dogs. Flow cytometry revealed no significant differences ($p>0.05$) in CD4+ or CD8+ expression on peripheral blood lymphocytes.

Alternate transmission mechanisms in experimentally inoculated mice and dogs were investigated. PCR revealed a low level of vertical and direct transmission of LIVT-1 in inoculated BALB/c mice. *Leishmania* DNA was detectable by PCR in tissues from puppies from a LIVT-1 infected beagle.

Although the strain of *L. infantum* infecting foxhounds in North America appears to predominantly use a non-vector transmission mode, the disease it produces is similar to canine leishmaniasis in other parts of the world. Non-sand fly transmission may be responsible for maintaining infections in the foxhound population. Results from this work will lead to improvement in diagnosis, clinical management, and control of canine leishmaniasis in North America.

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DEDICATION

I dedicate this dissertation to Stanley "Shack" Shackleford, beloved uncle and godfather (1936-1997).

"You were always on my mind…"

-*Willie Nelson*

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CHAPTER 1

INTRODUCTION

Visceral leishmaniasis is an important insect-vectored zoonotic disease in many parts of the world. Dogs are major reservoir hosts for human infections with *Leishmania infantum*. Approximately 57,000 human deaths result annually from visceral leishmaniasis (Reithinger and Davies, 2002). Children and immunocompromised individuals are at highest risk of developing life-threatening disease.

Prior to 2000 most cases of canine leishmaniasis in the United States were diagnosed from dogs that had traveled abroad or originated from endemic areas. Rare cases of leishmaniasis acquired in the United States were diagnosed in dogs for over 20 years, although they were considered insignificant until the first major outbreak occurred in a New York hunt club kennel (Gaskin et. al, 2002). Beginning in the summer of 1999, foxhounds in the kennel developed clinical signs including skin lesions, wasting, kidney failure, and arthritis. Diagnostic studies of some of the dogs at North Carolina State University College of Veterinary Medicine revealed *Leishmania* infection. Overall, 42% of foxhounds from the hunt club were seropositive for antibodies to *Leishmania*. Promastigotes were cultured from aspirated and biopsy samples from the dogs. Isoenzyme analysis of the parasites documented their identity as *L. infantum* zymodeme MON1, the most common type isolated from human visceral leishmanaisis patients in the Mediterranean basin (Gaskin, 2002).

Since the first major foxhound outbreak in 2000, the Centers for Disease Control and Prevention (CDC) have conducted a large-scale serological survey testing over 12,000 dogs in North America (Schantz, 2003). The CDC considers indirect fluorescent antibody titers (IFAT) of >1:64 suggestive of active infection. Epidemiological data indicates 2% of foxhounds are seropositive, but not other breeds of hunting dogs, pet dogs, or wild canids (Schantz, 2003).

Formerly considered an exotic disease, canine leishmaniasis caused by *L. infantum* has become established in the foxhound population in parts of North America. The recognition that *L. infantum* is endemic in the North American foxhound population has raised concern that infected dogs may serve as a source of infection for the human population. Thus far, no cases of

autochthonous human visceral leishmaniasis have been reported from the United States or Canada.

In the usual life cycle, *Leishmania* infections are transmitted to mammals by phlebotomine sand flies when flagellated promastigotes are inoculated into the bite wound during blood feeding. Rare cases of non-insect transmission by sexual and direct contact, blood transfusions, and vertical transmission have been described (reviewed by Rosypal et. al, 2005). The mechanism for transmitting *Leishmania* infections in foxhounds is unclear. Sand flies are widely distributed throughout much of the United States (Young and Perkins, 1984) although epidemiological data do not support a role for sand fly transmission of *L. infantum* in the Unites States and Canada (Monti, 2000).

The strain of *L. infantum* present in North America may be more prone to non-vector transmission. Alternate transmission dynamics, such as transplacental transmission, may explain the maintenance of *L. infantum* in the North American foxhound population. Although infections have existed in foxhounds for over 20 years, the sand flies present in North America are not important biting pests of humans or domestic animals. The variant of *L. infantum* appears to have become established in the absence of sand fly transmission, making it biologically different from other strains of the parasite.

Canine leishmaniasis is a severe systemic disease although many dogs infected with *Leishmania* organisms do not develop clinical disease. Diagnosis is often difficult because clinical signs are variable. Veterinarians in the United States are unfamiliar with canine leishmaniasis since it has only recently been recognized as an endemic disease. Most of what is known about canine leishmaniasis comes from studies in the Mediterranean region using naturally and experimentally infected dogs. Little information regarding the strain of *L. infantum* infecting dogs in North America is available.

Due to the paucity of data concerning North American canine leishmaniasis, the present study was undertaken to characterize *L. infantum* from foxhounds from the United States. Pathogenesis, immunological responses, and transmission of a strain of *L. infantum* isolated from a naturally infected foxhound from the United States were investigated. There were two central hypotheses for this dissertation. First, immunity and pathogenesis of North American canine leishmaniasis will be similar to Mediterranean *L. infantum* infections in dogs and secondly, *L. infantum* from North American foxhounds can be transmitted by non-sand fly mechanisms.

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CHAPTER 2

LITERATURE REVIEW

CANINE VISCERAL LEISHMANIASIS AND ITS EMERGENCE IN THE UNITED STATES

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ABSTRACT

Leishmaniasis is an important insect vectored disease that accounts for approximately 57,000 human deaths worldwide annually (Reithinger and Davies, 2002a). Leishmaniasis is a zoonosis. Dogs are important reservoirs of human visceral leishmaniasis (HVL) (Ashford et. al., 1998) and dog ownership is considered a risk factor for HVL in most endemic areas (Gavgani et. al., 2002). *Leishmania* spp. can cause visceral, cutaneous or mucocutaneous disease in humans and animals. Members of the *Leishmania donovani* complex cause human and canine visceral leishmaniasis (CVL) in parts of Europe, the Middle East, Africa, Asia, China and the Americas (Mauricio et. al., 1999). The member of the *L. donovani* complex identified in dogs in the United States (Gaskin et. al., 2002: Rosypal et al., 2003) and most often found in the Mediterranean basin, China and the Middle East is *L. infantum* (Moreno and Alvar, 2002). A similar or identical parasite, *L. chagasi*, is recognized as a cause of HVL and CVL in Latin America (Mauricio et. al., 1999).

Until early 2000, CVL was thought by most public health officials and veterinarians to be an unimportant disease in the United States. Most reported cases were in dogs that had originated in or traveled from areas where leishmaniasis is endemic (Lindsay et. al., 2002). The recognition that *L. infantum* was actually endemic in Foxhounds in the United States in 1999 (Gaskin et. al., 2002) changed this thinking and now there is concern the disease may make its way into the human population (Enserink 2000). The mode of transmission of *L. infantum* in the U.S. is currently unknown. Recent work provides evidence that although the North American isolate of *L. infantum* appears to have adapted a non-sand fly mode of transmission, it appears that other aspects of its behavior and pathogenesis are similar to Mediterranean isolates (Rosypal et. al.,2005b).

Leishmaniasis

The leishmaniases are a group of diseases caused by infection with protozoan parasites in the genus *Leishmania*. Most species of *Leishmania* are zoonotic, with the exception of *L. tropica* and *L. donovani*. The three major clinical forms of disease in humans are cutaneous, mucocutaneous, and visceral leishmaniasis. *Leishmania* parasites are present in 88 countries worldwide with 12 million people infected (World Health Organization (WHO), http://www.who.int/inf-fs/en/fact116.html, 2003). There are approximately 2 million new cases of leishmaniasis annually including 500,000 new cases of visceral leishmaniasis (WHO, http://www.who.int/inf-fs/en/fact116.html, 2003).

Cutaneous leishmaniasis typically results from infection with *Leishmania* parasites that are restricted to the skin. Lesions can be mild to severe depending on both the infecting species of *Leishmania* and the immune response of the host. Localized cutaneous leishmaniasis is restricted to a single or a few lesions that either heal spontaneously or can be treated with anti-leishmanial drugs. Diffuse cutaneous leishmaniasis can occur if the host becomes immunosuppressed and *Leishmania* parasites disseminate over large portions of skin.

Mucocutaneous leishmaniasis usually begins as a cutaneous lesion that eventually spreads to the mucosal tissue of the nose, mouth, and pharynx. Mucosal infection causes destruction of the tissue and can produce grossly disfiguring lesions. Mucocutaneous leishmaniasis is a severe disease and is usually caused by infection with organisms in the *L. brasiliensis* complex (Handman, 2001).

Human visceral leishmaniasis (HVL) results from dissemination of *Leishmania* parasites to the internal organs. HVL is marked by hepatosplenomegaly and causes malfunction of the liver, spleen, and bone marrow. Visceral leishmaniasis is most commonly caused by infection with *L. donovani* complex, which includes *L. infantum*, but other species of *Leishmania* can visceralize (Table 1).

Leishmania life cycle

Leishmania species are flagellated protozoan parasites in the Phylum Sarcomastigophora that are transmitted by the bite of infected female sand flies.

Sand fly vector biology

Sand flies are small bloodsucking Dipteran flies in the family Psychodidae, subfamily Phlebotominae. Six genera of sand flies are recognized but only two genera are of medical

importance: *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Lane, 1993). They are found mainly in the tropics, subtropics, and temperate regions and are distributed between 50°N and 40°S in both the northern and southern hemispheres (Tables 2 and 3). Sand flies live in a wide variety of habitats including desert, rainforest, and intradomiciliary. In addition to leishmaniasis, sand flies also vector the etiologic agent of bartonellosis and several arboviruses.

Phlebotomine sand flies are small, delicate flies with long legs and mouthparts. They are covered with hair and hold their wings above their bodies in a characteristic "V" shape. Sand flies are poor fliers and exhibit a short hopping flight when approaching a host. Due to poor flight ability, it is assumed that sand flies generally stay close to their breeding sites although they can travel longer distances if assisted by the wind (Killick-Kendrick, 1999).

Sand flies are crepuscular or nocturnal in activity, but they will bite during the daylight if disturbed. Resting places are cool, humid microenvironments and include tree holes, caves, houses, and animal burrows. Both males and females feed on a sugar source, but only females blood feed and use nutrients in the blood for egg development. *Leishmania*-infected sand flies are unable to take a single full blood meal and tend to probe their host before feeding which increases the possibility of parasite transmission. Probing may be induced by damage to the cardiac valve via the activity of chitinolytic enzymes produced by promastigotes, which subsequently obstructs engorgement (Schlein et. al., 1992). Infected sand flies tend to both probe and feed more often in order to complete engorgement. While blood feeding, sand flies salivate into the bite wound of the vertebrate host. Sand fly saliva contains vasodilatory and immunomodulatory compounds that influence *Leishmania* infection in the mammalian host (Belkaid et. al., 2000; Kamhawi et. al., 2000; Rogers and Titus, 2003).

Sand flies undergo complete metamorphosis and their life cycle consists of egg, four larval instars, pupa, and adult. Most species are terrestrial for their entire lives. The life cycle of sand flies is variable and depends on the species, food availability, and environmental factors. Depending on the phlebotomine species, females mate before, during, or after blood feeding. Eggs are laid after blood feeding and the time from taking a blood meal to oviposition depends on the species and ambient temperature. Due to their small size and diverse ecological habitats, immature sand flies have proved difficult to find. As a result, most of what is known about the

sand fly lifecycle comes from observations of common laboratory reared species such as *P. papatasi* (Tesh and Guzman, 1996).

Fourteen species of sand flies (genus: *Lutzomyia*) have been recorded in North America (Young and Perkins, 1984) (Table 2). The most common species in the United States are *Lu. shannoni*, *Lu. cruciata*, *Lu. anthophora*, and *Lu. diabolica*. None of these species have been shown to transmit *Leishmania* infection to dogs. However, *Lu. shannoni* has been experimentally infected by feeding on symptomatic dogs (Travi et. al., 2002). *Lutzomyia anthophora* and *Lu. diabolica* are suspected as vectors of cutaneous leishmaniasis caused by *L. mexicana* (McHugh et. al., 2001).

Non-sand fly transmission

Evidence indicates that transmission of *Leishmania* spp. may also occur through exchange of blood or other bodily secretions (Nuwayri-Salti and Fallah-Kansa, 1985). Transmission by blood transfusion has recently been demonstrated in dogs in the United States that have received packed RBC from donor foxhounds (Owens et. al., 2001; Giger et. al., 2002). Congenital transmission of visceral leishmaniasis has been reported several times in humans (Meinecke et. al., 1999). It is less clear if congenital transmission occurs in dogs. A case of *Leishmania* infection observed in a puppy (Mancianti and Sozzi, 1995) could have occurred either during birth or transplacentally. A study of congenital transmission of leishmaniasis in dogs from Brazil found no evidence of congenital transmission in 63 puppies from 18 naturally infected dogs by histopathologic, parasitologic, and PCR evaluation of various tissues (Andrade et. al., 2002). This is strong evidence that congenital transmission is not an effective means of maintaining CVL in the Brazilian dog population. In contrast to this work, Rosypal et. al. (2005a, Rosypal et. al., 2005d) have recently shown that transplacental transmission of a North American isolate of *L. infantum* is possible in experimentally infected dogs and mice.

Developmental cycle in the host

Female sand flies inject flagellated stages (promastigotes) into the skin of the host while feeding (Figure 1). Promastigotes are elongate stages which have a single nucleus, an anterior flagellum, and a kinetoplast (Figure 2). They divide by longitudinal binary fission. The kinetoplast is an area of the parasite's mitochondrion that contains large amounts of mitochondrial DNA. The kinetoplast stains dark like the nucleus with blood stains. The flagellum originates in the vicinity of the kinetoplast. Promastigotes are motile and pull

themselves along by their anterior flagellum. The parasites are ingested by macrophages but they are not killed. Inside macrophages the promastigotes withdraw their external flagellum and become amastigotes.

Amastigotes are round to slightly oval stages (Figure 3). They have a single nucleus, a kinetoplast and a rudimentary flagellum. The amastigotes divide by binary fission until they rupture the host cell. The released amastigotes are ingested by other macrophages and new cells are infected. Infection spreads from the skin to internal organs by movement of infected macrophages or amastigotes in the vascular system. Sand flies become infected when they ingest amastigotes while feeding on an infected host. Inside the gut of the fly the amastigotes become promastigotes. The promastigotes migrate to the hypostome of the sand fly and are inoculated into the host when the fly feeds. The promastigotes are ingested by macrophages and the new host is infected.

Initial infection

Leishmania parasites exist as two developmental forms: intracellular amastigotes in mammalian macrophages and flagellated promastigotes within the sand fly vector. Promastigotes are inoculated into the bite wound of the mammalian host during sand fly blood feeding. Once in the mammal, promastigotes are opsonized with complement component C3. Mac-1, the integrin receptor for iC3b, is present on the surface of macrophages. Surface bound C3 binds to Mac-1 and is followed by phagocytosis of the promastigote (Mosser and Brittingham, 2002). Once internalized, the phagosome, which contains the promastigote, fuses with lysosomes to form a phagolysosome. The mature phagolysosome is the major site of microbicidal activity in macrophages due to its low pH and production of toxic radicals such as nitric oxide. Promastigotes slow the phagosomal maturation process during which time they are in the process of transforming to amastigotes and are sensitive to acidic pH. Forty-eight hours following phagocytosis, amastigotes are formed within the macrophage (Matlashewski, 2002). Amastigotes survive and proliferate in low pH until eventually the host macrophage lyses and releases amastigotes. Newly released amastigotes are opsonized with host IgG that binds to Fcγ receptors on macrophages (Matlashewski, 2002).

Clinical signs of visceral leishmaniasis in dogs

In endemic areas, there are no differences in the prevalence of infected male or female dogs. In general, all breeds appear to be equally susceptible, although North African Ibizian

hounds may be naturally resistant (Solano-Gallego et. al., 2000). There is some indication that short haired dogs may have a higher prevalence than long haired dogs in the same area (Franca-Silva et. al., 2003). Dogs kept outdoors or that are frequently outside are more likely to be infected because of more exposure to infected sand flies.

Clinical signs of canine leishmaniasis are variable (Ferrer, 1999; Blavier et. al., 2001). Many dogs are naturally resistant to disease and appear clinically normal despite being infected. These dogs may show only a localized nodular reaction at the site of the sand fly bite. In endemic areas it is believed that approximately 10% of infected dogs develop overt clinical disease (Ferrer, 1999). Clinical signs include local or generalized lymphadenopathy, alopecia, cutaneous lesions, splenomegaly, epistaxis, emaciation, ocular lesions, renal failure, lameness, diarrhea, and onychogrypyhosis. Body temperature is usually normal or below normal. Canine leishmaniasis is a chronic and slowly progressive disease.

Immunity to canine visceral leishmaniasis

Dogs naturally or experimentally infected with *L. infantum* develop a spectrum of disease ranging from asymptomatic or resistant dogs to oligo- or polysymptomatic dogs. The outcome of disease in CVL is largely mediated by the development of the cell-mediated immune (CMI) response. Resistance to canine leishmaniasis is associated with a strong Th1 type cellular immune response. Analysis of cytokines from peripheral blood mononuclear cells has revealed that asymptomatic dogs develop a Th1 type response marked by increased secretion of IL-12, TNF, and IFN-γ compared to symptomatic dogs (Pinelli et. al., 1994; Santos-Gomes et. al., 2002). Resistant dogs typically have low or inapparent specific antibody production and positive leishmaninin skin test (Pinelli et. al., 1999). Conversely, the lack of an appropriate CMI results in disease progression in symptomatic dogs. Symptomatic dogs develop a marked humoral response and simultaneous lack of peripheral blood mononuclear cell proliferation *in vitro* to *Leishmania* antigens (Abranches et. al., 1991).

CD4+ T cells play a key role in immunity to leishmaniasis both by influencing the production of a particular cytokine profile and by interacting with infected macrophages. Symptomatic dogs have decreased levels of CD4+ T cells compared to non-infected and asymptomatic dogs, which is responsible for the lack of CMI in susceptible dogs (Abranches et. al., 1991; Bourdoiseau et. al., 1997). Moreover, infected macrophages can alter the canine immune response because they are deficient in costimulatory molecules that are required for T

cell activation (Pinelli et. al., 1999). Thus, infected macrophages have a reduced ability to interact with T cells, which subsequently interferes with initiation of IFN-γ production and parasite destruction.

Diagnosis and antibody responses

The diagnosis of CVL is often difficult due to the variability of clinical presentation. The simplest method is to demonstrate amastigotes (Figure 4) in stained smears from skin lesions, bone marrow smears, or aspirates from enlarged lymph nodes. Unfortunately, this method will only detect about 60% (bone marrow) to 30% (lymph nodes) of cases (Ferrer, 1999). Histological examination of biopsies also can be used but suffers from low sensitivity. Parasites can be isolated in culture of clinical samples but this method is usually limited to research and diagnostic laboratories.

Serological methods can be used to detect antibodies. The indirect fluorescent antibody test (IFAT) is the "gold standard" by which other serological tests are measured. Serological examinations by IFAT are done at initial dilutions of serum at 1:16. An IFAT cutoff titer of 1:64 is usually considered positive. However, *Leishmania* parasites have been isolated from CVL dogs with IFAT titers ≤ 1:16 (Gaskin et. al., 2002).

Dogs that initially present with clinical signs of CVL demonstrate high levels of both IgG1 and IgG2 by IFAT or ELISA (Bourdoiseau et. al., 1997; Leandro et. al., 2001; Solano-Gallego et. al., 2001). Levels of these isotypes are variable in asymptomatic dogs (Solano-Gallego et. al., 2001). With treatment IgG1 levels decrease while IgG2 titers remain relatively constant. Regardless of the health status, however, the levels of IgG1 antibodies are usually lower than those of IgG2 (Bourdoiseau et. al., 1997).

Other serological diagnostic methods include complement fixation, indirect hemagglutination, latex agglutination, direct agglutination of amastigotes or promastigotes, FAST agglutination, counter-immunoelectrophoresis, whole parasite and antigen specific ELISA, colloidal gold immunoassay, Western blot and rK39 antigen dipstick assay (Ferrer, 1999; Reithinger et. al., 2002b). In areas where *Trypanosoma cruzi* is present there is a possibility of serological cross reactivity with many of these tests (Camargo and Rebonato, 1969; Chiaramonte et. al., 1999). Cross reactions do not occur with the rK39 based immunoassay because it is based on a *Leishmania* specific amastigote protein (Burns et. al., 1993). Immunoassays based on the rK39 antigen also are good indicators of active CVL and HVL

(Badaro et. al., 1996). Commercially available rK39 antigen assays are available (*Leishmania* RAPYDTEST, Intersep, Workingham, United Kingdom, Kalazar Detect™ Rapid Test, InBios International Ltd., Seattle, WA).

The Western blot test may be helpful in monitoring specific responses to treatment of CVL cases (Rierra et. al., 1999). There is a decrease in the intensity of reactive promastigote bands, especially those in the region of 12-30 kD, after treatment.

The polymerase chain reaction (PCR) can be used to detect *Leishmania* in blood, skin biopsies and lymph node and bone marrow aspirates (Reale et. al., 1999). Bone marrow is the tissue of choice followed by lymph node and blood. Prevalence of infection is usually higher using PCR methods than serological methods (Leontides et. al., 2002). Animal inoculation (usually hamsters) and in vitro culture are about 80% as sensitive as PCR using bone marrow (Ashford et. al., 1995), although a false negative test can result if few parasites are present.

Abnormal biochemical features may also be helpful in confirming a diagnosis of leishmaniasis. Table 4 lists common biochemical alterations seen in 139 dogs with leishmaniasis (Solano-Gallego et. al., 2001).

Treatment

Treatment of CVL is challenging because few dogs are parasitologically cured (Lamonthe, 1999; Baneth and Shaw, 2002). This means that many dogs will relapse within a few months when treatment is stopped because all amastigotes have not been killed. The pentavalent antimonials, meglumine antimonite (Glucantime™) or sodium stibogluconate (Pentostam™), are commonly used to treat CVL. The mode of action of pentavalent antimonials is unclear, but it likely inhibits parasitic enzymes involved in glycolysis and fatty acid oxidation (Baneth and Shaw, 2002; Ciaramella and Corona, 2003). Allopurinol (Zyloric™) is used for maintenance therapy to prevent relapses or in combination with one of the antimonials in initial treatment. There is some evidence that resistance to antimonials develops in dogs (Baneth and Shaw, 2002; Ciaramella and Corona, 2003).

In the U.S., Pentostam™ is available from the Centers for Disease Control and Prevention (CDC). Pentostam is an aqueous solution of 330 mg/ml of agent which is equivalent to 100 mg/ml pentavalent antimony. Both sodium stibogluconate and meglumine antimonate are administered on the basis of their antimony content. Antimony compounds are eliminated faster if given intramuscularly (IM) than subcutaneously (SC) or intravenously (IV) in dogs and it is

important to maintain serum levels of the compound to treat leishmaniasis (Lindsay and Blagburn, 2001). Additionally, IM injection has the potential to cause severe side effects including muscular fibrosis and abscess formation (Baneth and Shaw, 2002). For these reasons, IM injection of antimonials is not recommended. Pentavalent antimonials are relatively well tolerated. Side effects include pain at the site of injection, gastrointestinal disturbances, delayed muscle pain, and joint stiffness (Lindsay and Blagburn, 2001; Baneth and Shaw, 2002). Canine leishmaniasis is treated with 30-50 mg/kg BWT pentavalent antimony in the form of sodium stiboglucanate by IV or SC injection administered daily for 3-4 weeks (Baneth and Shaw, 2002). Relapses may occur a few months to a year after treatment and should be treated with another course of pentavalent antimony. The use of pentavalent antimonials is contraindicated in patients with myocarditis, hepatitis or nephritis.

Allopurinol is an attractive alternative to antimonial treatment of CVL (Cavaliero et. al., 1999). Allopurinol interferes with normal protein synthesis in *Leishmania* parasites. Like other *Leishmania* treatments it does not completely eradicate all parasites (Koutinas et. al., 2001). Allopurinol given orally at 10 to 30 mg/kg once or twice daily is effective in producing clinical cures but relapses are common once therapy is discontinued. Oral treatment with 20 mg/kg allopurinol for 1 week a month is highly effective in preventing relapse in dogs (Ginel et. al., 1998).

Combination therapy of antimonials plus allopurinol may be superior to either agent used alone in the initial treatment of CVL (Baneth and Shaw, 2002). The antimonial is given SC at the regular dosage levels for 3 to 4 weeks along with allopurinol twice daily at 15 to 20 mg/kg. Allopurinol is then continued for maintenance therapy.

Several other chemotherapeutic agents have been used in the treatment of CVL including pentamidine, paromomycin, and several formulations of Amphotericin B (Lamonthe, 1999; Baneth and Shaw, 2002). These may be alternatives to antimonials especially if antimonial drug resistance is suspected.

Prevention of infection in dogs by insecticide impregnanted collars and vaccination

Recent studies have indicated that the use deltamethrin impregnated collars will reduce transmission of *L. infantum* infection to dogs (Killick-Kendrick et. al., 1997, Halbig et. al., 2000; Maroli et. al., 2001, Gavgani et. al., 2002). Experimental studies indicate that the deltamethrin collars decrease sand fly bites by 80 to 96% (Killick-Kendrick et. al., 1997, Halbig et. al., 2000).

Field studies using the deltamethrin collars in dogs in an endemic area of southern Italy found a reduction in seroconversion rates from 50% to 86% (Maroli et. al., 2001). A study conducted in Iran found the use of deltamethrin collars on dogs significantly decreased the seroconversion rates in dogs and children living in villages with collared dogs (Gavgani et. al., 2002).

In addition to the use of insecticide-impregnated collars, topical insecticide treatment of dogs can also produce a reduction in sand fly bites. A study using a combination of 10% imidacloprid and 50% permethrin in a spot-on formulation showed a repellent efficacy of 55.9 to 94.6% over a 29 day period (Mencke et. al., 2003). Another study using a topical application of pyrethroid/pyriproxfen demonstrated a significant decrease in both repellency and knockdown activity (Mercier et. al., 2003) of sand flies. The use of topical insecticides on dogs shows promise in the control of sand fly feeding and subsequent transmission of leishmaniasis.

Vaccination of dogs against leishmaniasis would benefit both the canine and human populations in endemic areas, however there is currently no approved vaccine for prevention of leishmaniasis. A recent report reviewed the current knowledge and status of canine *Leishmania* vaccines and it was suggested that the main requirement for an effective *Leishmania* vaccine is the induction of a stable and long-lasting TH1 type CMI response (Gradoni, 2001). Attenuated or otherwise genetically modified live vaccines show promise in animal models (Handman, 2001) but because of safety and other limitations are more valuable as tools for defining immune responses than actual vaccines. Killed promastigote antigens with bacillus Calmette-Guérin adjuvant have shown promise in some studies but complete protection has not been achieved (Gradoni, 2001; Handman, 2001; Mohebali et. al., 2004). Defined protein, recombinant protein and DNA vaccines have been developed and tested (Gradoni, 2001; Handman, 2001). Efficacy of these vaccines depends on the antigen chosen and often on the type of TH1 type CMI inducing adjuvant administered. In a Phase III field trial in Brazil, dogs vaccinated with a *Leishmania* fucose mannose ligand (FML) vaccine showed encouraging results in that 92% of vaccinated dogs were protected and 8% developed only mild signs of CVL (da Silva et. al., 2000).

Canine culling programs for prevention of human Leishmania infection

In Brazil, culling of *Leishmania* IFAT positive dogs has been used as a means of reducing HVL in the human population (Ashford et. al., 1998). The effectiveness of culling programs has been debated for years (Dye, 1996) but current opinion is that culling IFAT

antibody positive dogs is of little value (Courtenay et. al., 2002). Two important reasons for failure of culling programs to control CVL in endemic areas and decrease HVL are lack of sensitivity of the IFAT and other serological tests and a delay in culling positive dogs which is usually between 80 and 180 days of sampling (Courtenay et. al., 2002). These flaws permit sand fly transmission to continue from infected dogs and hampers control programs. Even under optimal conditions using a sensitive ELISA and shortened time interval from diagnosis to dog removal, one study showed that dog culling did not reduce the incidence of CVL in Brazil (Moreira et. al., 2004). It has been suggested that the use of deltamethrin collars could replace dog culling because of the significant impact they have on human transmission. Reithinger et. al. (2004) demonstrated that the use of deltamethrin collars was more useful than culling in controlling CVL in endemic areas with high transmission rates.

History of endemic CVL in dogs in the United States

The first case of endemic CVL was reported in a 7 year-old female foxhound from Oklahoma with generalized alopecia associated with *Demodex folliculorum* infestation (Anderson et. al., 1980). The dog had never been outside the United States and had been confined to a 150 mile radius around Oklahoma City. This dog came from a kennel of 17 foxhounds and additional dogs in the kennel were found to be infected (Kocan et. al., 1983). The second case of endemic CVL came from an English foxhound in an Ohio research colony (Swenson et. al., 1988). A male dog developed visceral leishmaniasis and eventually died. Both its dam and sire were born in the United States. Serological examination of 25 dogs from the colony revealed that 8 had antibodies to *Leishmania*. Other isolated cases of leishmaniasis in dogs with no history of foreign travel occurred in a pet Basenji in Texas (Sellon et. al., 1993) and a pet Toy Poodle in Maryland (Eddlestone, 2000).

In early 2000, leishmaniasis was diagnosed in foxhounds from a hunt club in New York (Gaskin et. al., 2002; Schantz, 2002). In the late summer of 1999, a number of foxhounds at the hunt club developed signs of disease including bleeding, wasting, seizures, hair loss, skin lesions, kidney failure, and swollen limbs and joints. Several dogs died. Some of the affected dogs were evaluated at North Carolina State University College of Veterinary Medicine for diagnostic studies, and cytopathologic examination of joint fluid from one of the hounds revealed amastigote forms of *Leishmania* sp. This finding was confirmed at necropsy of several dogs, and promastigotes were isolated and grown in cell culture. Diagnostic studies at the New York

hunt club kennel revealed that 39 of 93 (42%) foxhounds were seropositive for antibodies to *Leishmania*. Culture of aspirates or biopsy material from lymph nodes and other tissues of 15 seropositive dogs resulted in isolation of *Leishmania* spp. promastigotes from 15 dogs. Genetic typing of the parasites at the Institute of Public Health in Rome found the organisms to belong to the *L. infantum* MON1 zymodeme, which is the most common type isolated from HVL cases in the Mediterranean area (Gaskin et. al., 2002).

The authors obtained 3 isolates of *L. infantum* from 3 naturally infected foxhounds from Virginia by culture of bone marrow and lymph nodes (Rosypal et. al., 2003) We experimentally transmitted one of these strains of *L. infantum* to mice and beagle dogs (Rosypal et. al., 2005b; Rosypal et. al., 2005c) and deposited 2 parasite cultures in the American Type Culture Collection, Manassas, Virginia (LIVT-2 strain ATTC# 5D918 and LIVT-3 ATTC strain #5D919). Because the potential exists for selection of these American isolates for non-vector transmission, we also found evidence that these parasites can be vertically transmitted in experimentally infected dogs and mice (Rosypal et. al., 2004a; Rosypal et. al., 2005d).

In view of the sporadic cases seen in foxhounds in the past 20 years in the U.S., the CDC has undertaken a large scale survey of foxhounds in the United States. Since early 2000, sera from more than 10,000 foxhounds and other hunting dogs in 35 states and Canada have been tested (Schantz, 2002). High IFAT titers of at least 1:64, indicative of active infection, have been found in approximately 2% of foxhound samples, although some culture positive dogs had lower titers. Seropositive dogs were detected in 60 kennels in 22 states and 2 Canadian provinces. Serological testing of 600 pet dogs and 300 wild canids from geographic localities close to the infected foxhounds did not yield positive animals (Schantz, 2002) and no cases of autochthonous HVL have been reported in the United States.

The means by which infection is transmitted in dogs in the U.S. is not well understood at this time. Sand flies are widely distributed in much of the U.S., although they are usually not regarded as important human or livestock pests. One species, *Lu. shannoni*, has been experimentally infected with *L. infantum* by feeding on symptomatic CVL dogs (Travi et. al., 2002). *Lu. shannoni* , is widely distributed through the eastern U.S. as far north as New Jersey and evidence indicates this species may be a competent vector (Young and Perkins, 1984) of another *Leishmania* species, *L. mexicana*. Current epidemiological data does not presently support a major role for sand flies in transmission of CVL in the U.S. The management and

patterns of movement of foxhounds have led to the suggestion that direct dog-to-dog contact may be more important than sand fly transmission in the U.S. The risk to humans of CVL in the U.S. has not been established. Cases of HVL originating in the United States have not been reported. Sand flies are not a familiar human pest and, although direct transmission of the organism from dogs is theoretically possible, there are no cases reported in the medical literature. Even in the Mediterranean region where 20% of dogs are infected, there are few clinical cases in humans. Persons most at risk of developing disease are those with weakened immune systems.

Leishmania and HVL in the immunosuppressed patient

Of concern is the possible establishment of human leishmaniasis in the U.S. is the emerging importance of leishmaniasis as an opportunistic infection in cases of HIV infection. Recently HVL has emerged as an important opportunistic infection of individuals co-infected with HIV in endemic areas. Co-infection is increasingly more common due to overlapping geographical distributions as HIV spreads to rural areas and leishmaniasis spreads from rural to more urban environments (WHO, http://www.who.int/inf-fs/en/fact116.html, 2003). Immunocompetent people are frequently bitten by *Leishmania*-infected sand flies but do not develop overt disease. However in individuals dually infected with HIV and *Leishmania*, leishmaniasis rapidly develops into a severe and life-threatening disease. Both HIV and *Leishmania* are able to infect and destroy macrophages (Wolday et. al., 1999). Co-infection produces a cumulative immune deficiency which hastens the onset and severity of both diseases (Desjeux, 1999). Following initial infection, both pathogens are able to develop latent infection (Wolday et. al., 1999), but it is unclear if the severity of leishmaniasis in co-infected patients is due to reactivation of latent infection or primary infection (Alvar et. al., 1997).

Diagnosis of HVL in HIV patients is challenging due to overall immune depression which results from dual infection. The typical clinical signs (fever, hepatosplenomegaly, lymphadenopathy) are not always present in co-infected individuals and diagnosis may be further confused by presentation of atypical sites of lesions such as the lungs and gastrointestinal tract (Alvar et. al., 1997). Impairment of the immune system also decreases antibody production. Thus, serologic diagnosis of HVL in HIV infected patients yields a high proportion of false negative test results and should be confirmed by bone marrow aspirate or culture (Pintado et. al., 2001). Also, *Leishmania*/HIV patients exhibit peripheral parasitemia due to uncontrolled

multiplication of the parasite, and as a result can act as a reservoir host for infection by sand flies or by parenteral infection by intravenous drug users (Wolday et. al., 1999; Desjeux, 1999).

Treatment of immunocompromised patients is aimed at clinical and parastiological cure, although relapses are common and more frequent in *Leishmania*/HIV cases. Treatment usually consists of pentavalent antimonials alone or in combination with amphotericin B, followed by prophylactic treatment with antimony, allopurinol, pentamidine, or amphotericin B (Kager, 2002). Effective treatment results in clinical cure, but rarely parasitological cure due to latently infected cells. Relapse of leishmaniasis occurs as the HIV patient's immune system weakens and dormant infection is reactivated (Alvar et. al., 1997). Since the introduction of highly active anti-retroviaral therapy (HAART), the incidence of most AIDS-associated opportunistic infections has decreased significantly and is likely the result of increased CD4+ T lymphocytes (Kaplan, 2000). Leishmaniasis is not currently considered an AIDS defining illness, although VL does occur in HIV patients. HAART appears to reduce the incidence of VL in co-infected individuals (de la Rosa et. al., 2002; del Guidice et. al., 2002).

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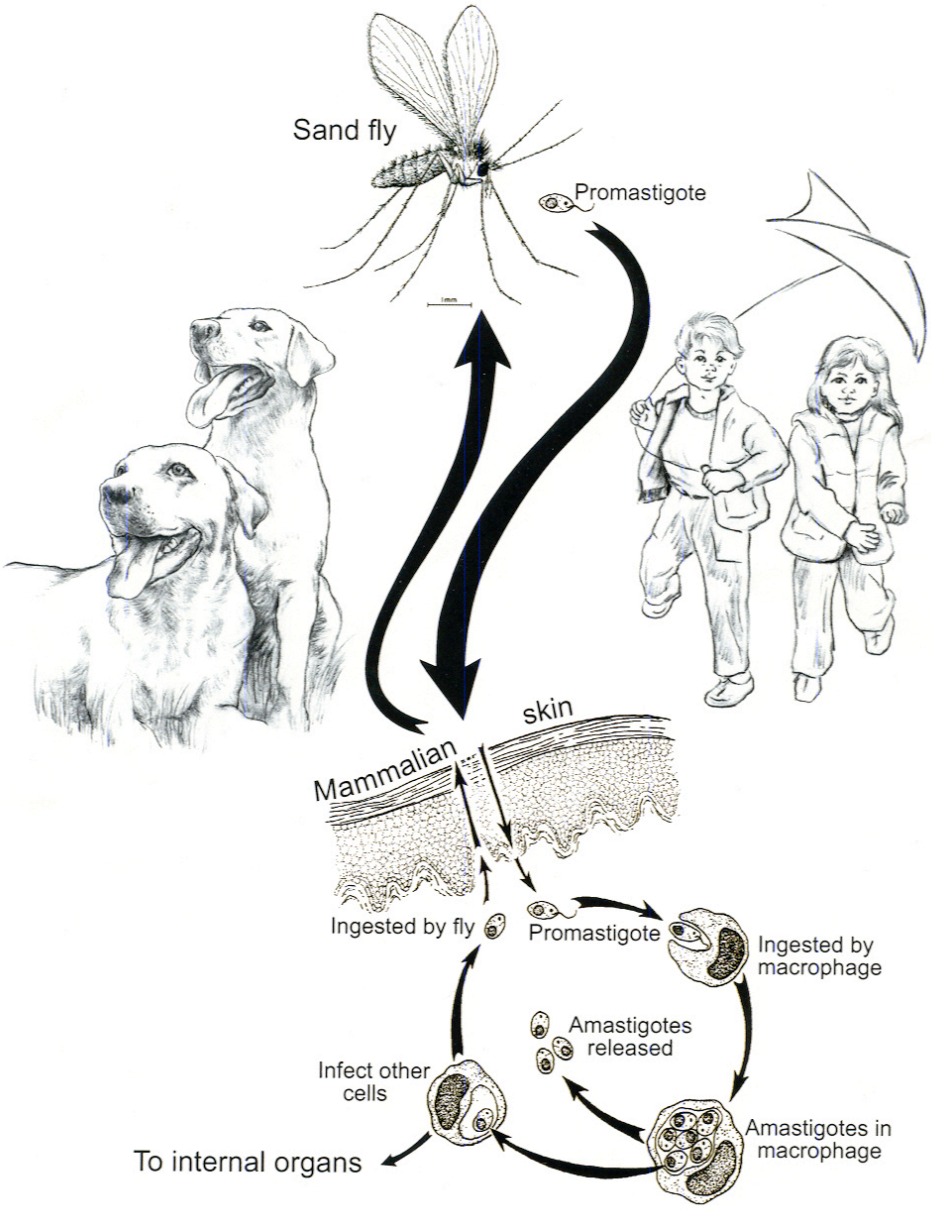

Figure 1. Life cycle of *Leishmania infantum*.

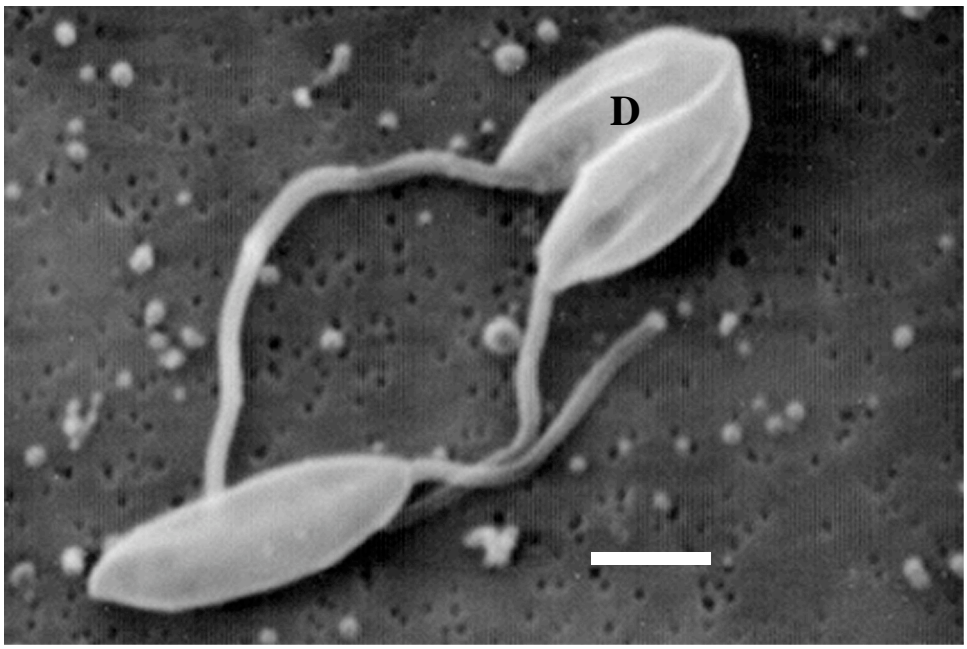

Figure 2. Scanning electron micrograph of promastigotes of the LIVT-1 strain of *Leishmania infantum* from culture. Note the dividing promastigotes (D). Bar = 2 μm.

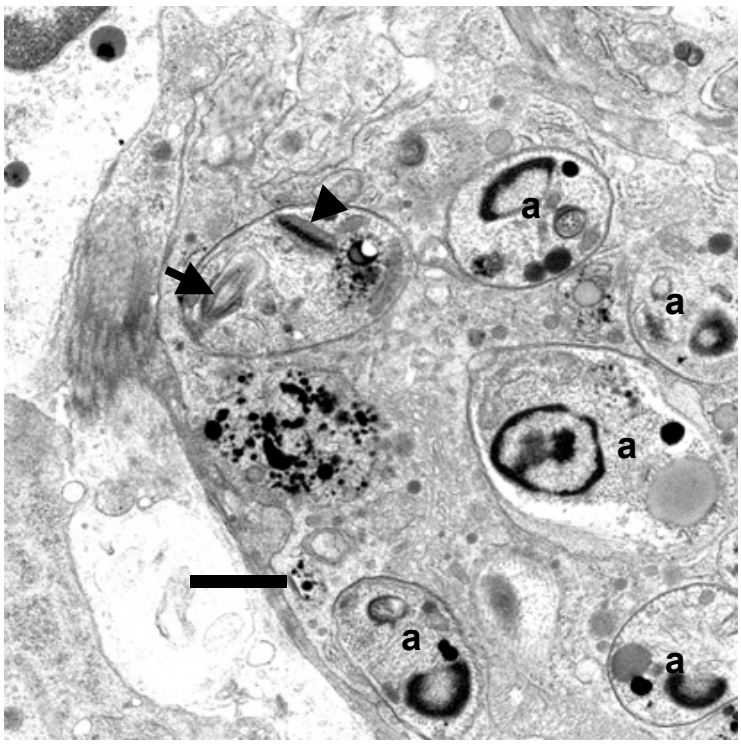

Figure 3. Transmission electron micrograph of amastigotes (a) of the LIVT-1 strain of *Leishmania infantum* in the spleen of an experimentally infected mouse. A kinetoplast (arrowhead) and rudimentary flagellum (arrow) are labeled in one amastigote. Bar = 1 μm.

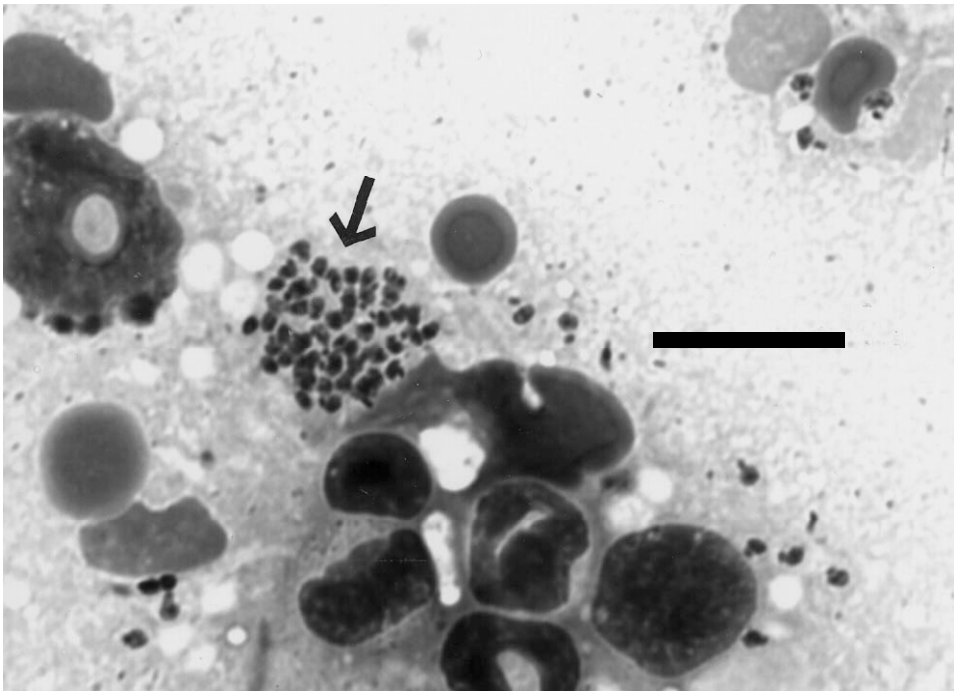

Figure 4. Impression smear of amastigotes of the LIVT-1 strain of *Leishmania infantum* in the spleen of an experimentally infected mouse. An arrow points to a group of amastigotes. Bar = 10 μm.

Table 1. Common etiologic agents of leishmaniasis and their main distribution (Data from *Handman E. 2001. Leishmaniasis: current status of vaccine development. Clin Microbiol Rev 14: 229-243.* and *Melby PC. 2002. Vaccination against cutaneous leishmaniasis. Am J Clin Dermatol 3: 557-570.*

Disease manifestation	*Leishmania* species	Distribution
Cutaneous	*L. major*	North Africa, India, Middle East, Asia
	L. tropica	North Africa, India, Middle East, Asia
	L. aethiopica	North Africa
	L. mexicana complex	Mexico, Central America, Texas
	L. brasiliensis complex	South America
Mucocutaneous	*L. brasiliensis* complex	South America
Visceral	*L. donovani* complex	Mediterranean basin, Middle East, India, Central Asia, South America

Table 2. Proven or suspected vectors of canine leishmaniasis (Data from Killick-Kendrick, 1999; Lane, 1993; Travi et. al., 2002)

Vector species	Causative organism (*Leishmania* spp.)	Distribution of sand fly
OLD WORLD VECTORS		
Phlebotomus		
P. chinensis	*L. infantum*	North and central China
P. longiductus	*L. infantum*	North Africa and central Asia
*P. perniciosus**	*L. infantum*	Mediterranean basin
*P. ariasi**	*L. infantum*	Western Mediterranean
*P. perfiliewi**	*L. infantum*	Mediterranean basin
P. longicuspis	*L. infantum*	North Africa, Spain
*P. neglectus**	*L. infantum*	Eastern Mediterranean
P. tobbi	*L. infantum*	Eastern Mediterranean
P. kandelakii	*L. infantum*	Lebanon, Turkey, Iran, Afghanistan
P. syriacus	*L. infantum*	Israel, Jordan, Syria
*P. langeroni**	*L. infantum*	North Africa, Spain
P. smirnovi	*L. infantum*	Central Asia
P. transcaucasicus	*L. infantum*	Azerbaijan
NEW WORLD VECTORS		
Lutzomyia		
*Lu. longipalpis**	*L. infantum (=L. chagasi)*	Central and South America
Lu. evansi	*L. infantum*	Columbia, Costa Rica, Venezuela
*Lu. youngi**	*L. infantum*	Central and South America
Lu. shannoni	*L. infantum*	Southeastern USA, South America

*proven vector

Table 3. Phlebotomine sand flies (Genus: *Lutzomyia*) in the United States (Data from Young and Perkins, 1984; Travi et. al., 2002)

Species	Distribution	Vector competency	Species vectored (*Leishmania* spp.)
Lu. cruciata	Florida	suspected	*L. mexicana*
Lu. diabolica	Texas	proven	*L. mexicana*
Lu. xerophila	Southern California	unknown	
Lu. anquilonia	Colorado, Washington	unknown	
Lu. anthophora	Texas	proven	*L. mexicana*
Lu. shannoni	Southeastern US	proven	*L. infantum*
Lu. tanyopsis	Arizona	unknown	
Lu. texana	Texas	unknown	
Lu. apache	Arizona, Texas	unknown	
Lu. oppidana	Texas, Colorado, Washington, Montana	unknown	
Lu. stewarti	California	unknown	
Lu. vexator	Western, southeastern US	unknown	
Lu. californica	California, Washington	unknown	
Lu. cubensis	Florida Keys	unknown	

Table 4. Biochemical features in 139 dogs with leishmaniasis (Data from Solano-Gallego et. al., 2001)

Ratio of albumin / globulins < 0.59	63.2%
Hypoalbuminemia	60.1%
Hypergammaglobulinemia	57.8%
Hypergammaglobulinemia and hypoalbuminemia	52.0%
High total serum protein	49.2%
Azotemia	24.2%
Hypercreatinemia	18.7%

CHAPTER 3

EMERGENCE OF ZOONOTIC CANINE LEISHMANIASIS IN THE UNITED STATES: ISOLATION AND IMMUNOHISTOCHEMICAL DETECTION OF *LEISHMANIA INFANTUM* FROM FOXHOUNDS FROM VIRGINIA.

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Key words: *Leishmania infantum*, dog, isolate, immunohistochemistry

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ABSTRACT

Previously considered an exotic disease, canine leishmaniasis caused by *Leishmania infantum* has recently been detected within the foxhound population in the United States and parts of Canada. *Leishmania infantum* is the etiologic agent of visceral leishmaniasis in many areas of the world and dogs are considered a major reservoir host for human *Leishmania* infections. Human visceral leishmaniasis has recently emerged as an opportunistic infection among individuals co-infected with HIV/AIDS and in persons taking immunosuppressive drugs. We report the isolation of *L. infantum* from 3 naturally infected foxhounds from Virginia by culture of popliteal lymph node and bone marrow, and the development of an immunohistochemical test to detect the parasite in tissues.

INTRODUCTION

Parasites in the genus *Leishmania* are transmitted by sand flies and can cause a spectrum of diseases. Visceral leishmaniasis (VL) is a potentially fatal vector-borne disease caused by parasites in the *L. donovani* complex. VL causes approximately 57,000 human deaths annually and children and immunocompromised individuals are most commonly affected, especially in the Mediterranean basin [9]. VL has recently emerged as an opportunistic infection among individuals co-infected with HIV/AIDS and in persons taking immunosuppressive drugs. Zoonotic VL is caused by *L. infantum* and the identical parasite *L. chagasi* [7], which are both members of the *L. donovani* complex. Dogs with canine visceral leishmaniasis (CVL) are considered to be important reservoir hosts for human infections although many dogs do not develop clinical disease. Visceral leishmaniasis caused by *L. infantum* is endemic in many areas of the world including parts of Asia, Africa, the Middle East, southern Europe, and Latin America.

Sporadic cases of endemic CVL have been diagnosed in dogs in the United States for over 20 years [10]. However, CVL was previously considered an unimportant disease in North America until 2000 when leishmaniasis was diagnosed in foxhounds in a hunt club in New York [5]. After isolation, the parasites were typed at the Institute of Public Health in Rome and determined to belong to *L. infantum* zymodeme MON-1, the most common type causing human visceral leishmaniasis in the Mediterranean area [5]. Since 2000, the U.S. Center for Disease Control and Prevention (CDC) has conducted a large-scale serological survey in the United States and Canada testing over 12,000 foxhounds, other breeds of hunting dogs, and wild canids. Sero-positive dogs have been detected overall in 2% of foxhounds, but not in other breeds of dogs or in wild canids (Schantz P.M. 2003. Emergence of canine visceral leishmaniasis in North America, 2000-2003. *Proceedings of the American Association of Veterinary Parasitologists 48th annual meeting*, p. 25-26, Abstract). The risk of transmission of *Leishmania* from infected dogs to people in the United States is unknown at this time. Sand flies are widely distributed throughout much of North America [14], however epidemiological data do not demonstrate a role for sand fly transmission of CVL in the United States. So far no cases of autochthonous human visceral leishmaniasis in the United States have been reported.

Our objectives for this study were to establish a permanent culture of a strain of *L. infantum* isolated from naturally infected foxhounds from the United States and to develop an

immunohistochemical staining procedure to detect amastigotes in tissues from American foxhounds with canine leishmaniasis.

MATERALS AND METHODS

Dogs. In May 2000, the authors obtained 3 naturally infected adult female foxhounds from a hunt club in Glen Allen, Virginia. Dog 1 was 2-years old at the time of arrival and dog 2 and dog 3 were 5-years old. The dogs had no history of foreign travel and were diagnosed with canine leishmaniasis by culture of lymph node biopsy. The dogs were housed together at the animal facilities at Virginia-Maryland Regional College of Veterinary Medicine. After several months, dog 1 began to lose weight. By December 2000, the dog had wasted severely and was euthanized and examined at necropsy. Dog 2 and dog 3 did not develop clinical signs of CL, but were euthanized and examined at necropsy in April 2001.

Culture of promastigotes and mouse inoculation. At necropsy, portions of popliteal lymph node and bone marrow tissue were cultured in vitro in *Leishmania* culture media (30% v/v fetal bovine serum, 1% penicillin/streptomycin, 2% v/v human urine, in Grace's Insect Media) at 25°C. Popliteal lymph node and bone marrow tissues from dog 1 were pooled and subcutaneously inoculated into interferon-γ-gene knockout (IFN-γ-KO) (BALB/c genetic background, *N*=3) mice. Lymph node and bone marrow tissues from dog 2 and dog 3 were individually inoculated into IFN-γ-KO mice. Mice were euthanized one month post-inoculation (PI) and their spleens were homogenized and cultured in vitro in *Leishmania* culture media at 25°C.

Immunohistochemistry. At necropsy, selected tissues from the 3 dogs were fixed in 10% formalin, embedded in paraffin, and 6 to 8-μm sections were made and left unstained for immunohistochemical analysis. Immunohistochemical staining was performed on unstained tissues using Peroxidase Rabbit IgG Vectastain® ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Serum from a rabbit hyperimmunized with *L. infantum* promastigotes (Strain LIVT -1 isolated from dog 1) was used as the primary antibody. The paraffin-embedded tissue sections were deparaffinized with Hemo-De (2 x 10 min) and rehydrated with increasingly dilute concentrations of ethanol and phosphate buffered saline (PBS). Tissues were then incubated in 3% hydrogen peroxide in 100% methanol solution for 30 min. Before staining, tissues were heated in Antigen Retrieval Citra (Biogenex, San Ramon, CA) for 20 min. Slides were washed in PBS (2 x 5 min) and incubated in normal goat serum for 20 min. Slides were rinsed in PBS and incubated in primary antibody at a 1:100 dilution overnight at 4°C. Slides were washed with

PBS (2 x 5 min) and incubated in biotinylated anti-rabbit IgG for 30 min at room temperature. Slides were washed with PBS (2 x 5 min) and incubated in avidin and biotinylated horseradish peroxidase macromolecular complex (ABC) for 30 min at room temperature. Slides were washed with PBS (2 x 5 min). Diaminobenzidine (DAB) in 0.1 M Tris was used as the enzyme substrate for peroxidase. Tissues were incubated in DAB solution for 4 min at room temperature and washed in running tap water for 5 min. Tissues were counterstained with Gill-2 Hematoxylin (Fisher Scientific, Fair Lawn, NJ) for 2 min followed by 1 min in Scott's tap water substitute (Sigma Diagnostics, St. Louis, MO). Samples were mounted on microscope slides under coverslips.

For controls, pre-immune rabbit serum was used as the primary antibody, no biotinylated antibody was added, or no ABC reagent was added. A portion of spleen from an IFN-γ-KO mouse chronically infected with *L. infantum* was stained simultaneously with the foxhound tissues and used as a positive control for immunoreactivity.

RFLP analysis of *Leishmania* N-acetylglucosamine-1-phosphate transferase gene (*nagt*). Genomic DNAs were isolated from LIVT1-3 for PCR amplification of *nagt*, as described previously [1]. The PCR products were digested according to manufacturers' procedures (New England Biolab) with *Acc*1 and *Nsi*I, which were selected on the basis of *L. infantum nagt* sequence (Acc# = AF205934) to discriminate this against those from other Old World species, e.g. *L donovani, L. major* and *L. tropica*. The PCR products and their digests were resolved by agarose gel electrophoresis, stained with ethidium bromide and photographed over a transilluminator.

RESULTS AND DISCUSSION

Promastigotes were successfully isolated from lymph node and bone marrow cultures from all 3 dogs by 1 week PI (Fig. 1). The 3 isolates were named LIVT-1 (from dog 1), LIVT-2 (from dog 2), and LIVT-3 (from dog 3). Two of the cultures were deposited in the American Type Culture Collection, Manassas, VA (LIVT-2 strain ATTC# 5D918 and LIVT-3 strain ATTC# 5D919).

The highly conserved single-copy gene encoding *N*-acetylglucosamine-1-phosphate transferase (*nagt*) was PCR-amplified readily from the genomic DNAs of all three isolates, as found previously for other *Leishmania* species [1]. PCR products appeared as a single band of expected length, i.e. ~1.4 kb (Fig. 1A). Digestion of the *nagt* PCR products with *Acc*1 (Fig. 1B) and *Nsi*1 (data not shown) produced RFLP specific to that of *L. infantum* (*chagasi*). Different patterns would be expected from the *nagt* sequences of other species known, e.g. variants of *L. major* (ACC# AF205930, AF205931, AF205932), *L. tropica* (ACC# AF205933), *L. amazonensis* (ACC# M96635). The results obtained suggest that LIVT 1-3 are *L. infantum* as found previously in infected hunt dogs elsewhere in North America.

Promastigotes were also isolated from all mice inoculated with dog tissues. Prior to euthanasia, none of the IFN-γ-KO mice developed clinical signs of leishmaniasis. Promastigotes were isolated in culture from spleens of inoculated IFN-γ-KO mice one month PI by the methods described.

In the immunohistochemically stained tissues, amastigotes were seen as brown structures in hematoxylin-stained host cells. No amastigotes were observed in the control slides. Amastigotes were present in spleen tissues from all 3 dogs. In dog 1, amastigotes were seen in the small intestine. Amastigotes were present in lymph nodes from dog 2 and dog 3. Ferrer et al. [4] described similar observations of amastigotes in canine lymph node and spleen tissues.

Endemic CVL was rarely diagnosed in dogs in the United States prior to the major outbreak in foxhounds. The first reported case was diagnosed in a foxhound originating in Oklahoma [2] and then additional dogs in the kennel were also found infected [6]. The second case of endemic CVL came from a foxhound in an Ohio research colony that had never left the United States [12]. Cases of autochthonous CVL were reported in pet dogs with no history of foreign travel [3,11].

Leishmaniasis is a protozoal disease vectored by sand flies, and species in the genus *Lutzomyia* are widespread throughout the United States [14]. None of these species have been proven to transmit *Leishmania* infection to dogs; however, *Lutzomyia shannoni*, which is present throughout much of the eastern United States, has been experimentally infected with *L. infantum* by feeding on symptomatic dogs with CVL [13]. Although dogs are the main reservoir for human visceral leishmaniasis, no cases of autochthonous human VL have been reported from the United States. Children and people who are immunocompromised are most at risk for developing this visceral disease.

Diagnosis of canine leishmaniasis can be difficult because the clinical features of disease are variable, although rK39-ELISA proves to be useful for this purpose for diagnosing CVL in Turkey [8]. Diagnosis is usually made by identification of amastigotes in skin lesions or in Giemsa-stained aspirates of lymph node, bone marrow, or spleen. Culture of organisms from biopsy material can also be used for parasitological diagnosis, but is not routinely used by veterinarians. The immunohistochemical staining procedure is a simple and useful technique for diagnosis of *Leishmania infantum* in dogs that is more sensitive than evaluation of Geimsa-stained or hematoxylin- and eosin-stained tissues.

This is the first report of infection of mice with *L. infantum* isolated from dogs that had never left the United States. We are currently defining the infection parameters of our isolates in mice.

ACKNOWLEDGMENTS

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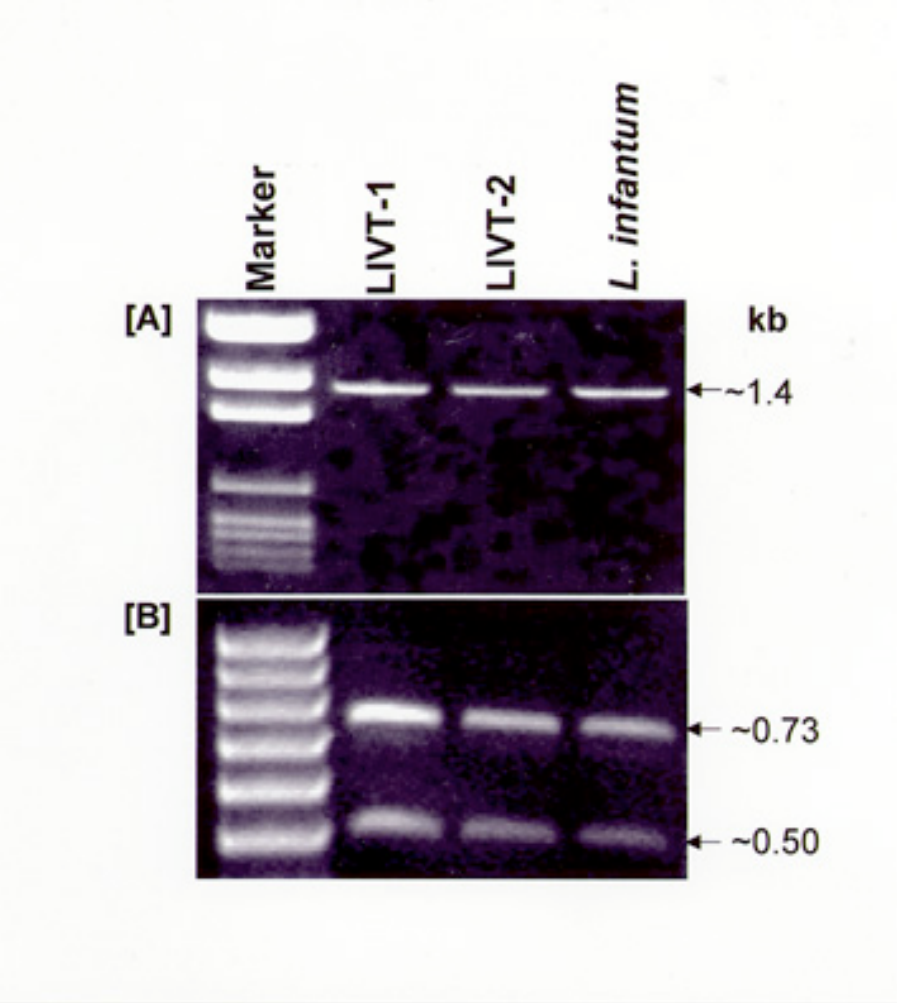

Fig. **1.** Identity of LIVT's to *Leishmania infantum (chagasi*) in RFLP of *nagt* PCR-amplified from genomic DNA. **A.** The ~1.4 kb *nagt* PCR-amplified from genomic DNAs from all *Leishmania* isolates, represented by LIVT-1 and -2. **B.** *Acc*I RFLP analysis of the PCR products from [A], producing fragments of ~730 bp, ~500 bp and ~175 bp from all samples, indicative of their identity to *L. infantum.* Note: the smallest fragment of ~175 bp is too faint to be visible in this gel.

CHAPTER 4

INFECTIONS IN IMMUNOCOMPETENT AND IMMUNE DEFICIENT MICE WITH PROMASTIGOTES OF A NORTH AMERICAN ISOLATE OF *LEISHMANIA INFANTUM*

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Key words: *Leishmania infantum*, mouse, interferon gamma, nitric oxide, B-cell-deficient

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ABSTRACT

Leishmania infantum, an etiologic agent of zoonotic visceral leishmaniasis, is widespread among foxhounds in the United States. Experimental infections with a North American isolate of *L. infantum* were evaluated using two inoculation routes in immunocompetent and immunosuppressed mouse strains. Groups of 2-5 interferon gamma gene knockout (IFN-γ-KO) (BALB/c-Ifng), inducible nitric oxide synthase (NOS) gene knockout (iNOS-KO) (C57BL/6), B-cell-deficient (μMT) (C57BL/6), and BALB/c mice were intravenously (IV) or subcutaneously (SC) inoculated with various doses of promastigotes of the LIVT-1 strain of *L. infantum*. None of the mice developed clinical signs of leishmaniasis during the 8-9 weeks of the study. Promastigotes were cultured from spleens of all IV-infected mice by 3 days post culture. Spleens from SC-infected mice infected with greater than 1 x 10^6 parasites became culture positive 3-24 days post culture, but promastigotes were not cultured from mice infected with 1 x 10^5 or 5 x 10^5 LIVT-1 promastigotes. Histological lesions were prominent in the livers of IV infected mice but were mild to nonexistent in SC infected mice. Serological responses were low and transient as determined by indirect fluorescent antibody testing in all groups. These results indicate the IV route of infection is superior to the SC route in a mouse model of North American leishmaniasis and that mice lacking INF-γ, iNOS or mice that are B-cell-deficient are not more susceptible to acute infection.

1. INTRODUCTION

Visceral leishmaniasis (VL) is a potentially fatal disease caused by infection with parasites in the *Leishmania donovani* complex. Sand flies are the insect vector of leishmaniasis. Approximately 57,000 deaths result annually from VL. Children and persons with weakened immune systems are at the highest risk for fatal infections (Reithinger and Davies, 2002). *Leishmania infantum* and *L. chagasi,* the etiologic agents of zoonotic VL (ZVL), are indistinguishable species and both are members of the *L. donovani* complex (Mauricio et. al., 1999).

Leishmaniasis caused by *L. infantum* is well established in Latin America, Africa, Asia, and the Mediterranean region of Europe (Enserink, 2000). Cases of endemic canine visceral leishmaniasis (CVL) have been occasionally reported in the United States for over 20 years (reviewed by Rosypal et. al., 2003), although the first major outbreak of *L. infantum* occurred in 1999-2000 in a foxhound hunt club kennel (Gaskin et. al., 2002). Since 2000, the Centers for Disease Control and Prevention (CDC) have tested dogs throughout much of the United States and Canada and have determined the seroprevalence of antibodies to *Leishmania* is 2% overall in foxhounds, but not in other breeds of dogs (Schantz, 2003). A study from Michigan indicates the prevalence in the U.S. dog population is less than that in foxhounds (Grosjean et. al., 2003). Transmission dynamics of *L. infantum* in U.S. foxhounds are unclear at this time. Sand flies are present in many parts of the U.S. (Young and Perkins, 1984), however *L. infantum*-infected sand flies have not been documented (Owens et. al., 2001).

Dogs with CVL are the primary reservoir host for human infections (Slappendel and Ferrer, 1998). CVL is a chronic, systemic disease characterized by both cutaneous and visceral involvement. Clinical manifestations of CVL are variable and many dogs infected with *Leishmania* parasites do not develop overt disease (Berrehal et. al., 1996). The outcome of disease with CVL is primarily mediated by the cell-mediated immune (CMI) response and resistance to CVL is associated with a strong Th1 type cellular immune response. Analysis of cytokines from peripheral blood mononuclear cells revealed that asymptomatic dogs develop a Th1 type response marked by increased secretion of interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin 2 compared to symptomatic dogs (Pinelli et. al., 1994; Santos-Gomes et. al., 2002). Symptomatic dogs develop a strong humoral response (Nieto, et. al, 1999) and a simultaneous lack of an appropriate CMI resulting in disease progression (Pinelli et. al., 1994).

Nitric oxide (NO) is a free radical derived from L-arginine in a reaction catalyzed by NO synthase (NOS). Nitric oxide synthase exists in three isoforms that are encoded by separate genes and are regulated by diverse signaling pathways (Huang and Fishman, 1996; Ma et. al., 1996). Of these isoforms, inducible NOS (iNOS) is produced in response to cytokines such as IFN-γ, TNF, and LPS. Nitric oxide is important in immunity to some protozoan parasites, including *Leishmania* (Li et. al., 1999).

Due to the lack of information on the strain of *L. infantum* infecting foxhounds in the U.S., the present study was conducted to establish a laboratory animal model of infection using promastigotes of a North American isolate of *L. infantum*. We evaluated infections in immunocompetent and immunodeficient mice that were infected with varying parasite numbers by two inoculation routes.

2. MATERIALS AND METHODS

2.1. L. infantum promastigotes

Promastigotes of *L. infantum* were isolated from popliteal lymph node and bone marrow tissues from a naturally infected foxhound from Virginia (LIVT-1 strain, Rosypal et. al., 2003). Promastigotes were subcutaneously (SC) inoculated into interferon-γ-gene knockout (IFN-γ-KO) (BALB/c genetic background) mice. Chronically infected mice were euthanized and their spleens were harvested, homogenized and cultured in vitro at 25°C in *Leishmania* culture media (30% v/v fetal bovine serum, 1% penicillin/streptomycin, 2% human urine, in Grace's Insect Media). Six-day-old promastigotes cultured from spleen tissue were used for inoculation studies. For quantitative analysis, promastigotes were harvested by removing the media and passing it through a 3 μm filter to remove splenocytes. The filtrate was pelleted by centrifugation. The number of promastigotes was determined using a hemacytometer. Promastigotes were resuspended in Hank's balanced salt solution (HBSS) for infection.

2.2. Mouse strains and inoculations

One strain of IFN-γ-KO (BALB/c-Ifng) mice, one strain of inducible nitric oxide synthase knockout (iNOS-KO C57BL/6) mice, one strain of B-cell-deficient (μMT C57BL/6) mice, and BALB/c mice were examined for susceptibility to infection to *L. infantum* promastigotes by different inoculation routes. Mice were obtained from Jackson Laboratories, Bar Harbor, ME. All mice that were intravenously (IV) inoculated were anesthetized prior to injection by intraperitoneal injection of 60 mg/kg bodyweight (BW) ketamine and 5 mg/kg BW xylazine. Mice were warmed on a heating pad to induce vasodilation prior to IV injection into the lateral tail vein.

Groups of male IFN-γ-KO mice were SC inoculated with 10 x 10^6 (*N*=5), or 1 x 10^5 (*N*=3) *L. infantum* promastigotes. One group of female IFN-γ-KO mice (*N*=2) was IV inoculated with 5 x 10^6 promastigotes.

One group of male μMT C57BL/6 mice (*N*=3) was inoculated SC with 10 x 10^6 *L. infantum* promastigotes. The μMT C57BL/6 mice were generated by deletion of the JH locus, which prevents immunoglobulin heavy chain assembly and subsequently inhibits B cell maturation (Chen et. al., 1993).

Two groups of female iNOS-KO mice were SC inoculated with 10 x 10^6 (*N*=4) or 5 x 10^6 *L. infantum* promastigotes. Three groups of male iNOS-KO mice were SC inoculated with 1 x 10^6 (*N*=4), 5 x 10^5 (*N*=4), or 1 x 10^5 (*N*=4) *L. infantum* promastigotes.

Five groups of female BALB/c mice (*N*=5) were SC inoculated with 10 x 10^6, 5 x 10^6, 1 x 10^6, 5 x 10^5, or 1 x 10^5 *L. infantum* promastigotes. Another 5 groups of female BALB/c mice (*N*=5) were IV inoculated with 10 x 10^6, 5 x 10^6, 1 x 10^6, 5 x 10^5, or 1 x 10^5 *L. infantum* promastigotes.

Control mice consisted of female BALB/c mice either SC inoculated (*N*=4) or IV inoculated (*N*=5) with 0.1 ml HBSS. Male iNOS-KO mice (*N*=4) SC inoculated with HBSS also served as controls.

Previous work has indicated that supernatants of Con-A stimulated splenic lymphocytes from the same source of IFN-γ-KO mice do not produce IFN-γ as determined by ELISA (Karpuzoglu-Sahin et al., 2001a,b). The lack of IFN-γ in these IFN-γ-KO mice confirms that the IFN-γ gene is not present. Similar cultures from iNOS-KO mice from the same source had reduced levels of IFN-γ (~2000 pg/ml) compared to wildtype C57BL/6 (10,000 pg/ml). When measured by the Griess assay (Sigma Chemical, St. Louis, MO), neither IFN-γ-KO nor iNOS-KO mice have detectable levels of NO (Ebru Karpuzoglu-Sahin and S. Ansar Ahmed, unpublished).

2.3. Culture and examinations

Spleens were used to culture *L. infantum* promastigotes from all mice. At necropsy, a portion of spleen was removed aseptically, homogenized, and cultured in vitro at 25°C in *Leishmania* culture media. Cultures were examined for parasite growth with an inverted microscope every day for 6 weeks.

At necropsy, livers were removed from all mice and fixed in 10% neutral buffered formalin. Fixed livers were embedded in paraffin and 6-8 μm sections were prepared for histological examination after hematoxylin and eosin staining. Histological sections were coded with a number and examined for lesions by one of the authors (DSL). Because only 1 section of liver was examined from each mouse and because the amount of liver present was variable liver lesion scores are presented only as estimates and were not subjected to statistical analysis.

2.4 Serology

Mice were bled from the retro-orbital plexus at approximately 2-week intervals and were euthanized 8-9 weeks after inoculation. Sera from 2 mice per group were tested for antibodies to *L. infantum* by the indirect fluorescent antibody test (IFAT). Sera were screened at a 1:25 dilution in phosphate buffered saline (PBS). Positive sera were titrated to a final dilution of 1:400 by doubling dilutions. LIVT-1 promastigotes were used as antigen. Approximately 50,000 promastigotes were placed in each well of a 12-welled IFA slide. Promastigotes were fixed onto the slides using acetone and stored at -20°C. Serum (30 µl) was incubated on the slides for 30 minutes at room temperature in a humidified box. Slides were washed twice for 5 minutes in PBS. Slides were stained and counterstained with goat anti-mouse IgG FITC conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) (30 μL per well) at a dilution of 1:10 in PBS-Evan's Blue solution incubated on the slides for 30 minutes at room temperature in a humidified box. The slides were washed twice in PBS for 5 minutes and mounted in 90% glycerin-PBS solution. Slides were viewed using an Olympus BX60 epifluorescent microscope. Positive samples exhibited a complete promastigote surface fluorescence. Negative samples exhibited background or no fluorescence. Known positive and negative samples were used as controls each time the IFAT was conducted.

3. RESULTS

None of the infected IFN-γ-KO, iNOS-KO, μMT, or BALB/c mice developed clinical signs of leishmaniasis and none developed lesions at the injection sites. One BALB/c mouse was found dead 2 days after IV injection of 10 x 10^6 *L. infantum* promastigotes. None of the control BALB/c or iNOS-KO mice developed clinical signs.

Spleens from all BALB/c and IFN-γ-KO mice that were IV inoculated with various dosages of *L. infantum* ranging from 1 x 10^5 to 10 x 10^6 promastigotes were culture positive by 3 days post culture (PC) (Table 1). IFN-γ-KO mice SC infected with 10 x 10^6 promastigotes were culture positive 12 days PC. No promastigotes were cultured from IFN-γ-KO mice SC inoculated with 1 x 10^5 parasites. Promastigotes were isolated from μMT mice 3 days PC. All infected iNOS-KO mice became culture positive 13-24 days PC. Promastigotes were cultured from spleens of BALB/c mice SC inoculated with 1 x 10^6 to 10 x 10^6 *L. infantum* promastigotes 12 days PC. Promastigotes were not cultured from BALB/c mice SC inoculated with 1 x 10^5 or 5 x 10^5 LIVT-1 promastigotes. No promastigotes were recovered from BALB/c or iNOS-KO mice inoculated with HBSS.

A small number of microscopic lesions were present in the livers of SC-infected BALB/c mice. There were 1 to 4 small foci present in most livers from these mice. Some SC-infected BALB/c mice had no visible lesions. A variable number of lesions were observed in livers from IV-inoculated BALB/c mice (Fig. 1). Livers from BALB/c mice IV-inoculated with 5 x 10^6 or 10 x 10^6 promastigotes were <5- 40% affected. Three of five livers from BALB/c mice IV-inoculated with 1 x 10^6 parasites were 10-20% affected and 2 of these mice had no lesions present. In livers from BALB/c mice IV-inoculated with1 x 10^5 and 5 x 10^5 organisms, lesions were present in 10-20% and 5% of the tissues, respectively. Microscopic lesions were not observed in uninfected BALB/c mice.

A range in the amount of liver affected was induced by experimental infections in immunodeficient mice. No lesions were present in IFN-γ-KO mice SC inoculated with 1 x 10^5 or 10 x 10^6 promastigotes. Lesions were observed in 5-10% of liver tissue examined from IFN-γ-KO mice IV inoculated with 5 x 10^6 parasites. Lesions were apparent in 5-10% of liver tissue from μMT mice. There were 1-5 small focal lesions present in most livers from SC-infected iNOS mice. Microscopic lesions were not present in some SC-inoculated iNOS mice. Livers

from two iNOS mice SC-inoculated with 10 x 10^6 promastigotes were 5 and 20% affected. A single lesion was observed in each of 2 HBSS-inoculated iNOS mice.

A variable and low level of seroconversion was observed in inoculated mice by IFAT (Table 2). IFN-γ-KO mice SC inoculated with 1 x 10^5 promastigotes had titers of 1:25 8-9 weeks PI. IFN-γ-KO mice IV inoculated with 5 x 10^6 promastigotes had titers of 1:25 to 1:50 at 8-9 weeks PI. No antibodies were detectable in μMT mice. The iNOS-KO mice SC inoculated with 1 x 10^6 promastigotes had titers of 1:25 5-6 weeks PI. All inoculated BALB/c mice had a titer of 1:25 or 1:50 sometime during the course of the study with the exception of BALB/c mice IV inoculated with 1 x 10^5 promastigotes. Control mice inoculated with HBSS did not develop antibody titers to *L. infantum*.

4. DISCUSSION

A challenge to studying CVL has been the lack of a consistent, reproducible experimental model of infection (reviewed by Moreno and Alvar, 2002). Our study demonstrates that the intravenous inoculation route of American *L. infantum* promastigotes is a more efficient method of infection than the subcutaneous route in mice. Promastigotes were isolated from the spleens of mice IV infected with1 x 10^5 to 10 x 10^6 promastigotes by 3 days post culture. This is in contrast to work by other investigators who found that infection was not detectable in mice IV inoculated with less than 10^6 *L. donovani* promastigotes (Howard et. al., 1987). Spleens from mice SC infected with 1 x 10^5 or 5 x 10^5 LIVT-1 promastigotes never developed positive cultures. Promastigotes were isolated from spleens of mice SC infected with 1 x 10^6 to 10 x 10^6 *L. infantum* promastigotes by 3-24 days PC.

Results from this work demonstrate that SC and IV inoculation routes were capable of inducing lesions in livers from mice with intact and deficient immune systems. Most mice infected via the IV route developed a higher percentage of hepatic lesions compared to SC-inoculated mice. More variability in the number of lesions was observed in IV-infected mice. This observation is in agreement with work by Rolão et. al. (2004) who described a high degree of parasite density variability in spleens from BALB/c mice IV infected with *L. infantum*. This phenomenon could be attributed to difficulty performing IV injections in mice that potentially results in leakage of inoculum into tissues in close proximity to the tail vein (Rolão et. al., 2004).

It is interesting to note that the immunodeficient mice did not develop overt leishmaniasis during the observation period studied. The type of immune response developed by the host primarily mediates the outcome of leishmaniasis. Resistance to leishmaniasis is associated with a CD4+Th1 immune response and the production of IFN-γ subsequently activates macrophages to become leishmanicidal (reviewed by Liew and O'Donnell, 1993). In patients with asymptomatic VL caused by *L. infantum*, CD4+ T cells produce high levels of IFN-γ (Mary et. al., 1999). In murine studies, Heinzel et. al. (1989) reported that susceptible BALB/c mice had no demonstrable IFN-γ mRNA in draining lymph nodes and Belosevic et. al. (1989) found that *in vivo* depletion of IFN-γ in resistant C3H/HeN mice rendered mice susceptible to leishmaniasis. Although BALB/c mice are a susceptible strain to *L. infantum*, our experimentally infected mice did not develop clinical signs of disease during the 8-9 week study period. In long-term studies

of infection from our laboratory, IFN-γ-KO mice infected for over 8 months develop severe visceral leishmaniasis (data not shown).

Nitric oxide is the principal effector mechanism involved in killing intracellular amastigotes and is generated by macrophages activated by IFN-γ or TNF-α in the presence of LPS, or both cytokines (reviewed by Liew and O'Donnell, 1993). Murray and Nathan (1999) previously demonstrated that iNOS is required *in vivo* for control of visceralizing *Leishmania donovani* infection in mice. Similarly, *in vitro* studies conducted on murine and canine macrophages have identified IFN-γ-induced NO as the major microbicidal molecule responsible for killing amastigotes (Roach et. al., 1991; Sisto et. al., 2001; Pinelli et. al., 2000; Panaro et. al., 2001). Inducible NOS is present in mouse macrophages (Stuehr et. al., 1988) and iNOS mRNA is detectable in *L. donovani*-infected tissues from BALB/c mice (Taylor et. al., 1997). Although targeted disruption of the iNOS gene leads to disease progression in *L. major*-infected mice (Wei et. al., 1995), clinical leishmaniasis was not induced in iNOS-KO mice inoculated with various dosages of LIVT-1.

Disease progression of CVL is associated with a marked humoral response and a simultaneous lack of protective CMI (Pinelli et. al., 1994; Nieto et. al., 1999; Abranches et. al., 1991). Similarly, murine models have not demonstrated a protective role for the humoral immune response in controlling leishmaniasis. Sacks et. al. (1984) found that B cell depletion rendered susceptible BALB/c mice resistant to *L. major*. The B cell-deficient mice used in the present study did not develop clinical leishmaniasis. This is in contrast with work by Scott et. al (1986) who found that B cell depletion in resistant C3H mice by treatment with α-μ antibody enhanced susceptibility to *L. major*. This could be attributable to the resistant C57BL/6 background on which the mice were generated or the short observation period.

Numerous factors impact antibody production in experimentally infected animals including parasite strain and stage, inoculum size, and inoculation route (Abranches et. al., 1991; Santos-Gomes and Abranches, 1996). Serological response to American *L. infantum* in the mouse model presented here was low and transient by IFAT. Reports of *Leishmania* antibody levels among foxhounds and other dogs in the United States have ranged from 1:16 to ≥ 1:512. (Gaskin et. al., 2002; Grosjean et. al., 2003). Serological examinations of dogs experimentally infected with LIVT-1 in our laboratory have also yielded erratic responses (data not shown).

Variable antibody responses have also been reported in dogs experimentally infected with Mediterranean isolates of *L. infantum* (Abranches et. al., 1991; Santos-Gomes et. al., 2003).

The murine model of leishmaniasis presented here could be used to test chemotherapeutic agents. The inability of immunodeficient mice to mount competent immune responses precludes their use in vaccination trials. However, the model using mice with intact immune systems could be used to evaluate the immune response to vaccines. The IV inoculation route is a potentially limiting factor in the model presented in this study because it requires technical expertise. The mouse model for studying North American *L. infantum* has an advantage over other experimental models such as dog and hamsters, because immunological reagents for mice are readily available. Inbred strains of mice are affordable, easily obtained and they also have the advantage of sharing genetic homogeneity compared to purpose-bred laboratory dogs.

This is the first report of a murine model of leishmaniasis developed using a North American strain of *L. infantum* isolated from naturally infected foxhounds with no history of foreign travel. We are currently conducting studies with our LIVT-1 isolate to determine the pathogenesis and immunological responses to CVL in experimentally infected dogs.

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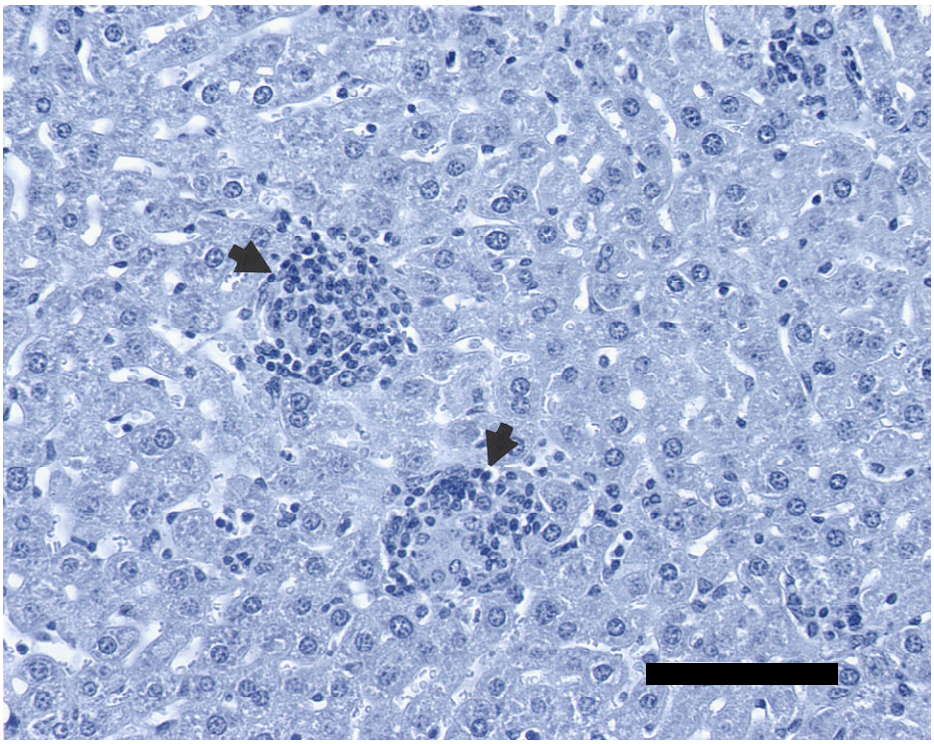

Figure 1. Liver from a BALB/c mouse that was intravenously inoculated with promastigotes of *Leishmania infantum*. Note that 2 areas of inflammation are present (arrows). Bar = 50 μm.

Table I. Promastigotes cultured from spleens of SC or IV inoculated mice

Mouse Strain (N=number of mice)	Treatment	Inoculation Route	Promastigotes isolated[a]	Days PC
IFN-γ-KO N=5	10 x 10^6 LIVT-1	SC	Yes	12
IFN-γ-KO N=3	1 x 10^5 LIVT-1	SC	No	
IFN-γ-KO N=2	5 x 10^6 LIVT-1	IV	Yes	3
μMT N=3	10 x 10^6 LIVT-1	SC	Yes	3
iNOS-KO N=4	HBSS	SC	No	
iNOS-KO N=4	10 x 10^6 LIVT-1	SC	Yes	13
iNOS-KO N=4	5 x 10^6 LIVT-1	SC	Yes	15
iNOS-KO N=4	1 x 10^6 LIVT-1	SC	Yes	14
iNOS-KO N=4	5 x 10^5 LIVT-1	SC	Yes	21
iNOS-KO N=4	1 x 10^5 LIVT-1	SC	Yes	24
BALB/c N=4	HBSS	SC	No	
BALB/c N=5	10 x 10^6 LIVT-1	SC	Yes	12
BALB/c N=5	5 x 10^6 LIVT-1	SC	Yes	12
BALB/c N=5	1 x 10^6 LIVT-1	SC	Yes	12
BALB/c N=5	5 x 10^5 LIVT-1	SC	No	
BALB/c N=5	1 x 10^5 LIVT-1	SC	No	
BALB/c N=5	HBSS	IV	No	
BALB/c N=5	10 x 10^6 LIVT-1	IV	Yes	3
BALB/c N=5	5 x 10^6 LIVT-1	IV	Yes	3
BALB/c N=5	1 x 10^6 LIVT-1	IV	Yes	3
BALB/c N=5	5 x 10^5 LIVT-1	IV	Yes	3
BALB/c N=5	1 x 10^5 LIVT-1	IV	Yes	3

[a] Yes = at least 1 mouse was positive; No = no mouse in the group was positive.

Table II. IFAT titers (2 mice per group) from SC or IV inoculated mice

Mouse Strain	Treatment	Inoculation Route	1 week PI	2-4 weeks PI	5-6 weeks PI	8-9 weeks PI
IFN-γ-KO	10 x 10^6 LIVT-1	SC	0	0	0	0
IFN-γ-KO	1 x 10^5 LIVT-1	SC	0	0	0	1:25
IFN-γ-KO	5 x 10^6 LIVT-1	IV	0	0	0	1:25-1:50
μMT	10 x 10^6 LIVT-1	SC	0	0	0	0
iNOS-KO	HBSS	SC	0	0	0	0
iNOS-KO	10 x 10^6 LIVT-1	SC	0	0	0	0
iNOS-KO	5 x 10^6 LIVT-1	SC	0	0	0	0
iNOS-KO	1 x 10^6 LIVT-1	SC	0	0	1:25	0
iNOS-KO	5 x 10^5 LIVT-1	SC	0	0	0	0
iNOS-KO	1 x 10^5 LIVT-1	SC	0	0	0	0
BALB/c	HBSS	SC	0	0	0	0
BALB/c	10 x 10^6 LIVT-1	SC	0	0	1:25	0
BALB/c	5 x 10^6 LIVT-1	SC	0	0	1:50	1:25-1:50
BALB/c	1 x 10^6 LIVT-1	SC	1:50	1:25	1:25	0
BALB/c	5 x 10^5 LIVT-1	SC	1:25	1:25	1:25	1:25-1:50
BALB/c	1 x 10^5 LIVT-1	SC	0	0	1:25	1:25-1:50
BALB/c	HBSS	IV	0	0	0	0
BALB/c	10 x 10^6 LIVT-1	IV	0	1:25-1:50	1:25-1:50	1:50
BALB/c	5 x 10^6 LIVT-1	IV	1:25	0	1:25	1:25
BALB/c	1 x 10^6 LIVT-1	IV	0	1:25	0	0
BALB/c	5 x 10^5 LIVT-1	IV	0	0	0	1:50
BALB/c	1 x 10^5 LIVT-1	IV	0	0	0	0

Table III. Examinations of livers from experimentally infected immunocompetent and immune deficient mice

Mouse Strain	Treatment	Inoculation Route	Liver lesions[a]
IFN-γ-KO	10 x 10^6 LIVT-1	SC	NVL
IFN-γ-KO	1 x 10^5 LIVT-1	SC	NVL
IFN-γ-KO	5 x 10^6 LIVT-1	IV	5-10%
μMT	10 x 10^6 LIVT-1	SC	5-10%
iNOS-KO	HBSS	SC	0-1 focus
iNOS-KO	10 x 10^6 LIVT-1	SC	1 focus-20%
iNOS-KO	5 x 10^6 LIVT-1	SC	1-5 foci
iNOS-KO	1 x 10^6 LIVT-1	SC	1-3 foci
iNOS-KO	5 x 10^5 LIVT-1	SC	1 focus-5%
iNOS-KO	1 x 10^5 LIVT-1	SC	0-1 focus
BALB/c	HBSS	SC	NVL
BALB/c	10 x 10^6 LIVT-1	SC	0-2 foci
BALB/c	5 x 10^6 LIVT-1	SC	1-3 foci
BALB/c	1 x 10^6 LIVT-1	SC	0-3 foci
BALB/c	5 x 10^5 LIVT-1	SC	0-4 foci
BALB/c	1 x 10^5 LIVT-1	SC	1-2 foci
BALB/c	HBSS	IV	NVL
BALB/c	10 x 10^6 LIVT-1	IV	20-40%
BALB/c	5 x 10^6 LIVT-1	IV	<5-40%
BALB/c	1 x 10^6 LIVT-1	IV	0-20%
BALB/c	5 x 10^5 LIVT-1	IV	10-20%
BALB/c	1 x 10^5 LIVT-1	IV	5%

[a]Liver lesions expressed as percentage of liver tissue demonstrating lesions or as individual focal lesions when minimal involvement was present, NVL: no visible lesions

CHAPTER 5

UTILITY OF DIAGNOSTIC TESTS USED IN DIAGNOSIS OF INFECTION IN DOGS EXPERIMENTALLY INOCULATED WITH A NORTH AMERICAN ISOLATE OF *LEISHMANIA INFANTUM INFANTUM*

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ABSTRACT

Eight female beagles were infected with either 1 x 10^7 (low dose, LD) or 2 x 10^8 (high dose, HD) promastigotes of a North American isolate of *Leishmania infantum infantum* (LIVT-1 strain) isolated from naturally infected foxhounds from Virginia. Two female beagles served as negative controls and two male beagles chronically infected (> 3 years) with *L. infantum chagasi* served as positive controls. Bone marrow (BM) and lymph node (LN) aspirates were collected every 6 – 8 weeks for cytologic evaluation, parasite culture and PCR. Serum samples were collected monthly for determination of serologic responses by indirect fluorescent antibody test (IFAT), and to a diagnostic rK39 antigen. Cultures of BM and LN aspirates and cytology evaluation were consistently positive in positive control dogs during the course of study. Negative control dogs were negative on BM and LN cultures, and cytologic evaluation of aspirates. Amastigotes were present on cytological examination of BM aspirates in 2 experimentally infected dogs. Cultures of LN aspirates were positive on 22 samples, while BM cultures were positive on 12 samples for all dogs. IFA titers ranged from 0 – 1:400 in experimentally infected dogs during the course of the study. Recombinant K39 immunoassay tests were consistently positive in positive control dogs and in the HD *L. i. infantum* infected dogs by approximately 8 weeks. PCR products on BM aspirates were identified more consistently in the HD *L. i. infantum* group of dogs compared to the LD *L. i. infantum* infected dogs. Kappa statistics revealed that PCR correlated better with cultures and cytology than IFAT, or the rK39 immunoassay results in the experimentally infected dogs.

INTRODUCTION

Leishmania parasites are flagellated protozoans that cause a number of disease manifestations in humans and animals and are vectored by sand flies. Members of the *L. donovani* complex are the etiologic agents of human visceral leishmaniasis (human VL) in the Middle East, Africa, Asia, the Mediterranean basin, South America, and North America.[1] The etiologic agents of zoonotic VL are *L. infantum infantum* and the conspecific organism *L. infantum chagasi*[1], which are both members of the *L. donovani* complex. Dogs with canine visceral leishmaniasis (CVL) are recognized as major reservoir hosts for human infections.

Although sporadic cases of CVL have been documented in the United States for over 20 years[2], the first major outbreak occurred in the summer of 1999, when a number of Foxhounds at a hunt club in New York were diagnosed with leishmaniasis. Dogs developed signs of disease that included bleeding diathesis, chronic wasting, seizures, hair loss, skin lesions, kidney failure, and swollen limbs and joints.[3] Diagnostic studies at the kennel revealed that 39 of 93 (42%) Foxhounds were seropositive for antibodies to *Leishmania*. Culture of aspiration or biopsy material from lymph nodes and other tissues of 15 seropositive dogs resulted in isolation of *Leishmania* sp. promastigotes from 15 dogs.[3] Genetic typing of the parasites found them to belong to the *L. i. infantum* MON1 zymodeme, which is the most common type isolated from human VL cases in the Mediterranean area.[1,a]

Since early 2000, sera from more than 12,000 Foxhounds and other hunting dogs in 35 states and parts of Canada have been tested for anti-*Leishmania* antibodies. High IFAT titers of at least 1:64, indicative of active infection, have been found in approximately 2% of Foxhound samples, although some culture positive dogs had lower titers.[3] Seropositive dogs have been detected in 60 kennels in 22 states and 2 Canadian provinces. Serological testing of 600 pet dogs and 300 wild canids from geographic localities close to the infected Foxhounds has not yielded positive animals. Serological cross-reactivity to *Trypanosoma cruzi*, a related parasite that is also endemic in the United States, has been reported.[a,4]

The mode of transmission of *L. i. infantum* in Foxhounds in the United States is currently unknown. Sand flies are found throughout many parts of the U.S.[5], however epidemiological data do not support a role for sand flies in CVL transmission in the U.S. This suggests that an alternate mode of transmission may maintain infections in U.S. foxhounds because *L. i. infantum* has existed for over 2 decades in foxhounds in the United States[2]. The variant of the parasite

may have been selected for non-sand fly vectored transmission making it different from other strains of *L. i. infantum* found in Mediterranean countries. Consequently, the strain of *L. i. infantum* present in North America may also differ in its biological behavior in dogs. Transmission of *L. i. infantum* has been documented via blood transfusions in the U.S.[6,7]

We isolated *L. i. infantum* from 3 naturally infected Foxhounds from Virginia by culture of bone marrow and lymph nodes and used this isolate of *L i. infantum* (LIVT-1 strain[8]) to experimentally infect a group of dogs. The present study was conducted to explore the behavior and pathogenesis of the strain of *L. i. infantum* infecting Foxhounds in the U.S. and to improve diagnostic testing of North American CVL. Diagnostic testing of experimentally infected dogs was conducted for > 1 year and included cytologic examination and cultures of bone marrow (BM) and lymph node (LN) aspirates, indirect fluorescent antibody test (IFAT), rK39 antigen detection in sera, and PCR product detection in BM, LN, and selected other tissues. Kappa values were calculated to determine the agreement of test results with BM and LN cultures, and cytologic examinations of BM and LN preparations in the experimentally infected dogs.

MATERIALS AND METHODS

Isolation and Infection Procedure

Isolation of infective promastigotes from naturally infected Virginia foxhounds used in this study has been previously described.[8] LIVT-1 promastigotes were subcutaneously inoculated into interferon-γ-gene knockout (IFN-γ-KO) (BALB/c genetic background) mice. Chronically infected mice were euthanized and their spleens were homogenized and cultured in vitro at 25°C in *Leishmania* culture media (30% v/v fetal bovine serum, 1% penicillin/streptomycin, 2% human urine, in Grace's Insect Media). Purpose-bred female beagles were obtained from Covance Research Products, Inc. (Cumberland, VA). Eight six-month old female beagles were injected with six-day-old LIVT-1 promastigotes cultured from murine spleen tissue. Media from spleen cultures was removed and passed through a 3 μm filter to remove splenocytes. The filtrate was pelleted by centrifugation. Promastigotes were enumerated using a hemacytometer. Promastigotes were resuspended in Hank's balanced salt solution (HBSS) for infection. Dogs were injected IV with 2 x 10^8 (N=4; high dose HD) or 1 x 10^7 promastigotes (N=4, low dose, LD) in 1 ml of HBSS. Two female beagles were inoculated with 1 ml HBSS and served as negative controls. Two male beagles chronically infected (> 3 years) with *L. infantum chagasi* served as positive controls. Dogs with *L. i. chagasi* infections were selected for positive controls because they had been verified to be persistently infected by use of culture, PCR, and serological methods for a period of 3 years prior to use in the present study.

Culture and Microscopic Evaluation of Bone Marrow and Lymph Node Aspirates

Fine needle aspirates of LN were performed with a 25 gauge needle and 12 ml syringe. Prescapular and popliteal lymph nodes were aspirated in all dogs as these lymph nodes were readily assessable at the time of sampling. Fine needle aspirates of LN and BM were cultured *in vitro* in *Leishmania* culture media at 25°C. A minimum of 2 slides was made from each fine needle aspirate. Bone marrow aspirates were conducted under heavy sedation and local anesthesia and at least 2 slides were made from each sample. Bone marrow aspirates were collected from either left or right proximal humeri in all dogs for all sampling periods. Slides were stained with a commercial Romanowsky-type stain (Dip-Quick®, Jorgensen Labs, Loveland Co). Lymph node and BM aspirates were evaluated by one investigator (GCT).

Microscopic evaluation of 200 fields (1000X) bone marrow and 100 fields (1000X) of lymph nodes were performed on each animal.

Indirect Fluorescent Antibody Test (IFAT)

Dog plasma was examined at doubling dilutions beginning at 1:25 in phosphate buffered saline (PBS) and titrated to a final dilution of 1:400. LIVT-1 promastigotes were used as antigen. Approximately 50,000 promastigotes were placed in each well of a 12-welled IFAT slide. Promastigotes were affixed to slides using acetone and stored at -20° C. Plasma (30 μL) was incubated on the slides for 30 minutes at room temperature in a humidified box. Slides were washed twice for 5 minutes in PBS. Anti-dog IgG FITC conjugate (30μL) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at a dilution of 1:10 in PBS-Evan's Blue solution was incubated on the slides for 30 minutes at room temperature in a humidified box. Slides were washed twice for 5 minutes in PBS and mounted in 90% glycerin-PBS solution. Slides were viewed using an Olympus BX60 epifluorescent microscope. Positive samples exhibited a complete promastigote surface fluorescence. Negative samples exhibited background or no fluorescence. Positive and negative control sera for the *Leishmania* IFAT were obtained from dogs serologically proven infected and uninfected, respectively. Positive and negative controls were used each time the IFAT was conducted. Randomly selected plasma samples were tested for cross-reactivity to *Trypanosoma cruzi*. The procedure was the same as described above, except amastigotes and trypomastigotes of the Brazil strain of *T. cruzi* were used as antigen. Serum from a dog with a proven *T. cruzi* infection was used as a positive control.

rK39 Antigen Immunoassay

Dog plasma was also tested using recombinant K39 antigen which is an immunodominant protein on amastigotes specific to members of the *L. donovani* complex and has the advantage of showing no cross-reactivity to *T. cruzi*.[9] The presence of anti-recombinant K39 antibodies was tested by a commercial rK39 dipstick immunoassay (Kalazar Detect™ Rapid Test, InBios International Ltd., Seattle, WA) according to the manufacturer's test procedure. Briefly, 20 µl of plasma were loaded onto the application pad of the dipstick. The dipstick was then placed in a well of a 96 well round bottom tissue culture plate containing 3 drops of buffer. Test results were read after 10 minutes. According to the manufacturer's instructions, the test was positive when red bands appeared on both the control line and the test line. The presence of a red band only on the control line indicated a negative result. The test was qualitative and the

manufacturer indicated that a faint pink band should be considered a positive result. Limit of detection was 1:32 – 1:64 for humans. The specificity of the rK39 dipstick was verified by testing known serum from a dog with a confirmed *T. cruzi* infection.

Polymerase Chain Reaction (PCR)

DNA from bone marrow material was extracted using a commercial kit (DNA Mini Kit, Quiagen®, Valencia, CA). For each 50 µl reaction, 1 µl of DNA was added to 45 µl of Platinum® PCR Supermix (Invitrogen™ Life Technologies, Carlsbad, CA) in a 0.5 ml thin-walled microcentrifuge tube. To the reaction tube, 2 µl of primers 13A and 13B were added, which amplify a conserved minicircle region of kinetoplast DNA from all species of *Leishmania*.[10] Optimal PCR amplification conditions were as follows: initial denaturation at 95° for 2 min, 38 cycles consisting of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 68°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products were electrophoresed on a 2% agarose gel and with size markers to detect the 116 bp PCR product. DNA extracted from LIVT-1 promastigotes was used a positive control and a negative control without DNA was included. To determine the sensitivity of the PCR assay, DNA was extracted from a known number of LIVT-1 promastigotes. Ten-fold serial dilutions of DNA were made and subjected to PCR. DNA detection limit was < 1 organism.

Selected tissue samples were taken at necropsy. DNA was extracted and tested by PCR. Portions of liver, spleen, LN, BM, kidney, lung, heart, intestine, skin, vagina, uterus, conjunctiva, whole blood, penis, and testes were collected for PCR testing.

Statistical Analysis

Kappa values were calculated by use of two different criteria with FREQ SAS procedure (SAS, Version 8.2, Cary, NC) to determine the level of agreement between the diagnostic tests performed. The first criteria used in test analysis considered any positive culture or cytology preparation in an animal to be compared to other tests at each period as a separate event. The other criteria used to calculate Kappa values used a positive culture or cytology preparation for the animal to be considered positive for all succeeding tests periods. The Kappa values presented here is the Kappa values calculated when one positive culture of LN or BM, or a positive cytologic preparation was considered positive for the succeeding test period. Pretest probability of approximately 0.5 was used to report values of a positive predictive value test

(PPVT), and a negative predicative value test (NPVT). Kappa values for test agreement: < 0.2 = poor; 0.21-0.40 = fair; 0.41-0.60= moderate; 0.61-0.80=good; 0.81-1.00=very good.[11]

Animal Care and Use

All procedures performed on animals were approved by the institution's University Animal Care and Use Committee. All dogs were housed in individual kennels for the duration of the study. Dogs were examined daily during the course of study. Serum samples were collected once monthly beginning at 7 weeks post-infection for 47-112 weeks.

RESULTS

Dogs in this study were followed after experimental infection for a period of 62 –112 weeks. Positive and negative control dogs did not exhibit clinical manifestations of disease that could be attributed to *Leishmania* during the study period. Body weight and activity levels for positive and negative control dogs remained normal. Both negative control dogs had normal estrous cycles during the course of study. All four dogs in the HD group of dogs exhibited clinical signs compatible with leishmaniasis by 6 months post-infection. Clinical signs included mucoid ocular discharges (FIGURE 1), facial, periocular, and aural dermatitis, alopecia, seborrhea, weight loss, draining skin lesions, lameness, polyarthritis (FIGURE 2), and generalized lymphadenopathy. Alopecia was generalized and involved the face, trunk and extremities.(FIGURE 3). One HD dog died approximately 62 weeks post-infection following complications from cesarean section surgery. All remaining dogs were humanely euthanized between 75 – 112 weeks post-infection (PI).

Three of the four HD dogs had at least one episode of polyarthritis characterized by fever, depression, lethargy, shifting leg lameness, and joint effusions. Joint effusions were characterized by an acute purulent non-septic inflammation. Joint fluid was cultured for aerobic bacteria and *Leishmania* in two dogs but no growth was detected. Dogs with polyarthritis were treated with an oral non-steroidal anti-inflammatories (NSAID) resulting in complete resolution of clinical signs within 7 days. Two dogs were maintained on oral NSAID's for 8 - 15 months. Two dogs in the HD group developed hypoalbuminemia (1.7 and 2.3 gm/dl – Normal range 2.8 – 4.0) and hyperproteinemia (Total proteins -11.2 and 9.2 gm/dl and 9.4 and 6.9 gm/dl of globulins) approximately 10 months post-infection. One HD dog developed proteinuria (3 + Bumin) with a urine protein creatinine ratio of 1.01 (Normal < 1.0 UPC). All four dogs in the LD group remained clinically normal. No significant laboratory abnormalities were present in the four LD dogs during the course of study (61 weeks).

Positive control dogs were positive on bone marrow and lymph node cytology or cultures at seven different sampling times (TABLE 1). Negative control dogs were negative on bone marrow or lymph node cytology or cultures for the duration of study. Amastigotes were observed cytologically in bone marrow and/or lymph node aspirates in 93% of samples for positive control dogs. Amastigotes were not observed in any negative control dog in bone

marrow and lymph node aspirates. Two dogs (1 LD group and 1 HD group) had amastigotes present in bone marrow samples on two separate periods.

Reciprocal IFA titers in the positive control dogs were $\geq$ 400 at the beginning of the study (TABLE 2). These dogs were infected for > 3 years and were consistently positive on cultures for *Leishmania infantum chagasi*. IFA titers did not fluctuate in positive control dogs for approximately 70-91 weeks. IFAT titers were 0 in one negative control dog for 77 weeks and the other negative control dog had titers from 0 to 1:25. The 1:25 titers were considered non-specific and not indicative of infection in this dog because all other diagnostic tests were negative. HD dogs seroconverted (reciprocal titers $\geq$ 50) by approximately 12 weeks post-infection (Figure 4). Two dogs in this group had reciprocal IFA titers that persisted at $\geq$ 400 for the majority of the sampling periods. Two dogs in the HD group had reciprocal titers that fluctuated from 0 to 400, during the course of study. Dogs in the LD group had reciprocal IFA titers that fluctuated at a low level 0 –100 during the course of study (Figure 5). Plasma samples tested by the *T. cruzi* IFAT showed some cross-reactivity. *T. cruzi* IFAT titers of 1:25 and 1:100 corresponded to *L. infantum infantum* titers of 1:25-50 and 1:400, respectively (data not shown).

The rK39 immunoassay test was positive at every evaluation period for 70-91 weeks in positive control dogs (Table 3). The rK39 immunoassay was negative in negative control dogs for 91 weeks post-infection. Three dogs in the LD group had a positive rK39 immunoassay test result sometime during the course of study. These three dogs had 52%, 25% and 0.05% of their samples positive by the rK39 test. Three of four dogs in the HD group had a positive rK39 test by 12 weeks post-infection. Dogs in the HD group had a positive rK39 immunoassay test result (86%, 93%, 95%, 100%) for 18 to 61 or 91 weeks post-infection. The rK39 immunoassay was negative on serum from a *T. cruzi*- infected dog.

PCR test results were positive for each bone marrow sample tested in both positive control dogs on 8 different sampling periods over 77 weeks (Table 4) and amplified the expected 116 bp product (FIGURE 6). Negative control dogs were negative at each sampling time for 61 weeks post-infection. Three of four HD dogs were positive by PCR on bone marrow aspirates by 12 weeks post-infection. One dog in the HD group had one positive PCR test result from bone marrow aspirate at 47 weeks post-infection and was PCR positive on bone marrow tested at necropsy. The four HD dogs were positive by PCR on bone marrow samples taken at necropsy. PCR test was positive in one LD dog at 24 weeks post-infection and remained positive until 47

weeks post-infection. Two of four LD dogs were positive on bone marrow at the time of euthanasia at 112 weeks post-infection.

Of the additional tissues sampled at necropsy, PCR was positive on all tissues tested from one positive control dog (Table 5). PCR testing was positive on all tissues except lung, kidney, testes and conjunctiva from the other positive control dog. Two of four HD dogs were PCR positive on all tissues tested. The remaining two HD dogs were positive on all tissues except uterus in one dog and uterus, heart and kidney samples were negative in the other HD dog. *Leishmania* DNA was detected in all samples except lung tissue from one LD dog. Of the remaining 3 LD dogs, some samples of liver, vagina, lymph node, kidney, spleen, and intestine were PCR positive. Spleen tissues from all infected dogs were PCR positive.

Kappa statistics (Table 6) were calculated for the IFA test (1:50 and 1:100 dilutions), PCR and rK39 immunoassay test results at weeks 7, 12, 18, 24, 31, 47 and 61 weeks post-infection. Cut-off points of 1:50 and 1:100 for IFAT were selected because these values were closest to the 1:64 titer that the Centers for Disease Control and Prevention considers indicative of infection[24]. The IFAT titer $\geq$ 50 had Kappa values that ranged from –0.02 to 0.53. Kappa value for this test was 0.15 for all weeks which showed poor agreement with cytology or culture. IFAT titer $\geq$ 100 had Kappa values that ranged from 0.11 to 0.83 and a Kappa value of 0.22 for all weeks. The use of a higher breakpoint titer with the IFAT only slightly improved the agreement of IFA tests with cultures or cytology.

Recombinant K39 immunoassay test results had Kappa values that ranged from –0.17 to 1.0. Kappa statistics for all weeks of study was 0.17 which revealed poor agreement with cultures or with demonstration of the organism on cytology preparations.

PCR test results had Kappa values that ranged from 0.33 to 0.83, with an overall Kappa value of 0.51 for all weeks. This test showed moderate agreement with lymph node and bone marrow cultures, and demonstration of the organism on cytology preparations.

Sensitivities for PCR, rK39 and IFA (1:50 & 1:100 dilutions) were 78%, 90%, 92%, 89%, respectively, while specificities were 71%, 27%, 27% and 29% (1:50 & 1:100).

DISCUSSION

Dogs experimentally infected in this study exhibited a range of clinical manifestations. Clinical features in naturally occurring infections also are variable with the phase of disease and state of immunity. Clinical signs were similar to naturally occurring infections in dogs.[3,12,13,14] Dogs in the HD group showed more severe clinical signs during the course of study, while LD dogs did not have apparent clinical signs compatible with leishmaniasis, other than a mild generalized lymphadenopathy. This is compatible with descriptions of natural infections in dogs, with some proportion of animals having clinical signs, while other dogs remain clinically asymptomatic. Clinical signs in the HD group consisted predominately of dermatological signs. Natural infections in dogs routinely result in a generalized dermatitis that begins on the head and face, and progresses to other portions of the body. In two separate studies approximately 40% of dogs with natural infections demonstrated some type of dermatologic manifestation.[12,14]

Polyarthritis was observed in 3 of 4 HD infected dogs. Lameness and polyarthritis are reported in natural infections, but the incidence of this clinical sign was < 10% of dogs in one study.[13,15] Foxhounds that were diagnosed with leishmaniasis from New York in 2000 had clinical findings of polyarthropathy.[3] This observation may suggest that this clinical manifestation may be different with different isolates of the parasite. It could be that we were more likely to observe these signs, because of close monitoring of experimentally infected dogs, as compared to observations made on naturally infected dogs.

Cytological demonstration of the organism in dogs in bone marrow samples, synovial fluid and lymph node aspirates were not efficient means to document infection. Only positive control dogs had amastigotes present in the majority of bone marrow and lymph node samples. Bone marrow samples were more likely to demonstrate the organism than lymph node aspirates in positive control dogs and in experimentally infected dogs. Two experimentally infected dogs had amastigotes present during the entire course of study. Approximately 90% of dogs were diagnosed by cytological means in two different studies.[13,15] Increasing the amount of time devoted to this task, or increasing the number of fields evaluated could have improved sensitivity of this test. Numbers of organisms are often variable in clinical specimens.

Cultures of bone marrow and lymph node aspirates were consistently positive in the HD group of dogs and positive control dogs. Cultures are negative in up to 20% of natural infections.[14] The most commonly used method to diagnose *Leishmania* infections in dogs is by

demonstration of amastigotes in stained smears of lymph node or bone marrow aspirates. This technique however suffers from low sensitivity and detects only 60% (bone marrow) and 30% (lymph node) of canine leishmaniasis cases.[16] Culture of promastigotes from biopsy material is another parasitological diagnostic method although it is not routinely used in clinical practice. Results from this work indicate that cultures of LN aspirates are more useful for identifying dogs infected with a North American isolate of *L. i. infantum* than culture of BM or cytology. This is in contrast to a study from Turkey, in which more *Leishmania*-infected dogs were detected by microscopy than by LN culture.[17]

Indirect fluorescent antibody test has been traditionally used for screening of dogs for Leishmaniasis. The Centers for Disease Control suggest that dogs with reciprocal titers > 64 are suggestive of infection. Sensitivity of the IFA to detect infected dogs is reported to range from 16.7% - 98%, and is dependent upon the cut-off points and technical considerations.[12,15,18,19] False positives and false negative IFAT results occur in natural infections. False positive IFAT would overestimate the true number of animals with infections. Several authors have suggested that the prevalence of infection in certain endemic areas of the world is approximately 10-60 %.[18,20] IFA titers may be negative early in the disease process and even persist after treatment or infection. False negative results of up to 38% have been reported in chronically infected dogs and indogs unable to mount an immune response to infection.[21] In the United States, a broad range of *Leishmania* antibody levels in Foxhounds and other dogs have been reported.[3,4] Serological examinations of dogs experimentally infected with LIVT-1 have also yielded erratic responses when tested by IFAT. This is in agreement with previous studies in which variable antibody responses were observed in dogs experimentally infected with Mediterranean isolates of *L. i. infantum.*[22,23]

Seroconversion was demonstrated in both high and LD groups of dogs. Criteria for IFAT recommended by the Centers for Disease Control is a reciprocal titer of >64.[24] IFAT in the HD group were sustained at a higher dilution and 2 of 4 dogs maintained a reciprocal titer > 400 for the duration of study. These two dogs also exhibited more significant clinical signs during the course of the study. However, Pozio suggests there is limited association between the severity of infection and antibody response.[25]

Kappa statistics for both dilutions of IFAT did not reveal a good association with culture or cytological demonstration of the organism. Sensitivity for the IFAT at 1:50 and 1:100 for all

weeks was 92% and 89%, respectively. This is similar to the performance of IFAT in previous studies of naturally infected dogs ranging from 29 – 100%.[3,13,15,26]

Although kappa statistics showed poor agreement overall with culture and cytology, the recombinant K39 dipstick assay consistently identified more experimentally infected dogs than cytology, culture, IFAT, or PCR. Previous work demonstrated superior performance of rK39 diagnostic methods compared to other serological assays.[17,26,27] All dogs that received the HD of promastigotes had positive rK39 immunoassays by 18 weeks PI during which time they were became clinically asymptomatic. Other researchers have similarly shown an increase in anti-rK39 antibodies in dogs and humans with subclinical infections months before developing clinical signs of visceral leishmaniasis.[17,27] This effect is attributed to high numbers of dividing amastigotes preceding and during the acute phase of leishmaniasis.[27]

A low number of positive rK39 immunoassays were observed in asymptomatic dogs that received the LD of LIVT-1 promastigotes. According to the manufacturer, a faint pink band should be considered a positive result. Although the dipstick is a qualitative test, the intensity of the chromagen reflects the antibody titer. These asymptomatic dogs exhibited faint bands on the test lines, which most likely indicated a low level of anti-rK39 antibodies in the absence of active disease. Variable antibody responses in dogs experimentally infected with a North American isolate could be due to differences among isolates of the viscerotropic *Leishmania* species from different geographic locations as previously described.[28,29,30]

The dipstick assay used in this work did not react with sera from a dog with a confirmed *T. cruzi* infection. This is in agreement with previous studies using rK39 antigen showing no cross-reactivity in dogs and humans with American trypanosomiasis.[9,26,27,31,32] This result was expected because cross-reactivity is less likely to occur when utilizing defined antigens such as rK39 in a serological assay.[27,29]

The overall sensitivity of the rK39 immunoassay was 90% for all weeks. Sensitivity was similar to previously reported levels for rK39 ELISA and dipstick assays ranging from 92.4% to 97% in naturally infected dogs from the Mediterranean.[17,26,28] One study reported a lower sensitivity of 72-77% using the rK39 dipstick in Brazilian dogs with natural infections[33], which may indicate variations among different isolates of *L. infantum (chagasi)* or among rK39 dipstick manufacturers. The rK39 dipstick assay is easier to interpret than IFAT although it is still subjective because the test must be visualized and faint bands can be difficult to discern. In

addition, the rK39 dipstick is a simple, rapid test that is easier to perform than IFAT and does not require sophisticated equipment or technical expertise.

PCR on bone marrow had an overall Kappa value of 0.51 for all weeks, which showed moderate agreement with demonstration of organism by culture and cytological evaluation of bone marrow and lymph node aspirates. In the HD group of dogs, 18 of 25 bone marrow samples tested by PCR were positive for *Leishmania* DNA. Infection was detectable as early as 7 weeks post-infection and all dogs in the group eventually developed clinical leishmaniasis. In contrast, the asymptomatic dogs that received the LD of LIVT-1 promastigotes had only 5 PCR positive bone marrow samples of 32 that were tested. Two dogs in the group never had a positive PCR test on bone marrow samples. The remaining 2 dogs were PCR positive on BM samples taken at necropsy, and this may have been due to the larger sample size compared to aspirates. Other researchers have previously found PCR to be useful for detecting naturally-infected dogs from the Mediterranean region with active leishmaniasis, but not with subclinical infections.[34-36] PCR performed on tissue samples collected at necropsy revealed that the parasite had disseminated throughout the bodies of most of the dogs.

Although the North American isolate of *L. i. infantum* appears to have been adapted to a different mode of transmission, it appears that other aspects of its behavior and pathogenesis are similar to Mediterranean isolates. The LIVT-1 isolate[8], like others obtained from Foxhounds in the United States, is considered to be the MON-1 zymodeme[3,a]. The isoenzyme analysis used to genotype *Leishmania* isolates is not sensitive enough to determine all genetic variation that would confer biological differences in modes of transmission or parasite infectivity. Therefore, parasites with the same isoemzyme profiles might be biologically different. Genetic variability among isolates from a single zymodeme of *Leishmania braziliensis* has been previously documented[37] and may be associated with parasite transmission. Overall, this work shows that the disease caused by a North American isolate of *L. i. infantum* in experimentally infected dogs resembles canine leishmaniasis in natural infections and in experimental canine infections using Mediterranean isolates of *L. i. infantum*. We observed overt signs of disease in some of the dogs whereas others remained subclinically affected. Cytological demonstration, culture, and IFAT did not consistently identify infected dogs. PCR showed the highest level of agreement with culture and cytology for diagnostics. Major disadvantages associated with molecular techniques are the need for specialized laboratory equipment and technical expertise. The rK39 antigen

dipstick test, however, consistently identified more experimentally infected dogs and has the advantage of being rapid, simple to use, and does not require technical skill or equipment.

This is the first report of experimental canine leishmaniasis developed using North American *L. i infantum* isolated from a naturally infected foxhound with no history of foreign travel. We are currently conducting studies with the LIVT-1 isolate to determine transmission dynamics of *L. i. infantum* in North America.

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Footnote

[a]Schantz P. Emergence of canine visceral leishmaniasis in North America, 2000-2003. (Abstract 4). In. Proceedings of the American Association of Veterinary Parasitologists 48th Annual meeting, Denver, CO, 2003, p.25-26.

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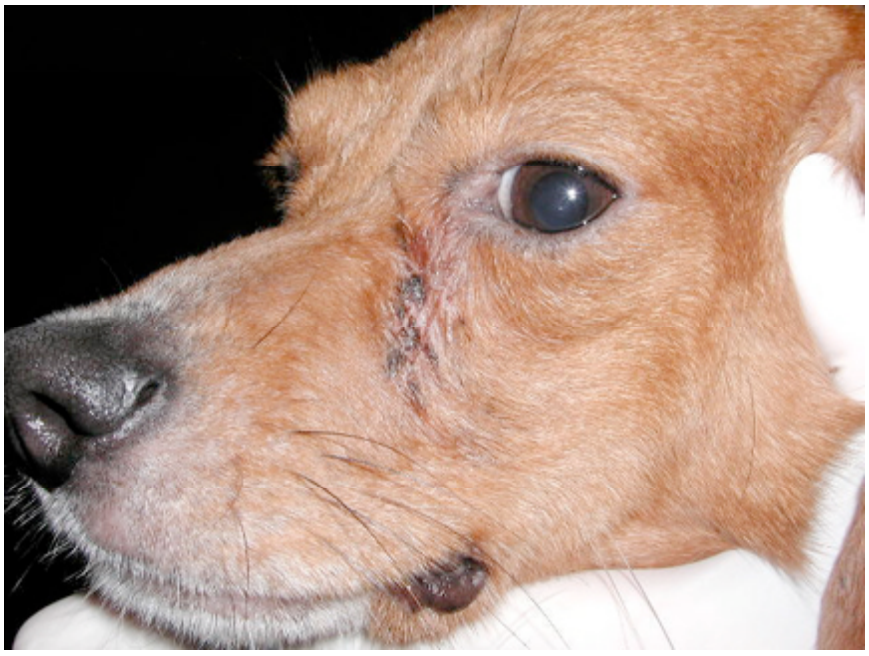

Figure 1. Ocular discharge in a dog that was experimentally infected with a high dose of LIVT-1 promastigotes.

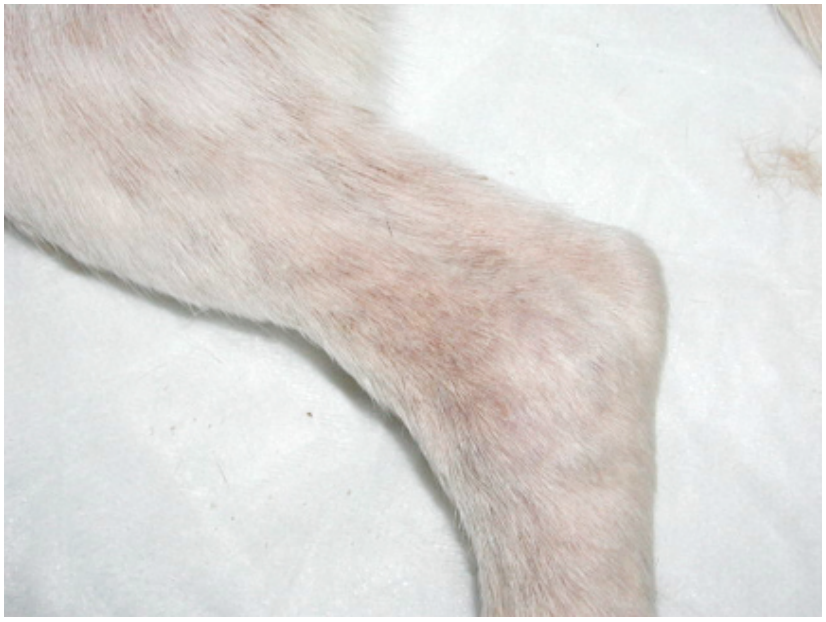

Figure 2. Inflamed hock from a dog with polyarthritis that was experimentally infected with a high dose of LIVT-1 promastigotes.

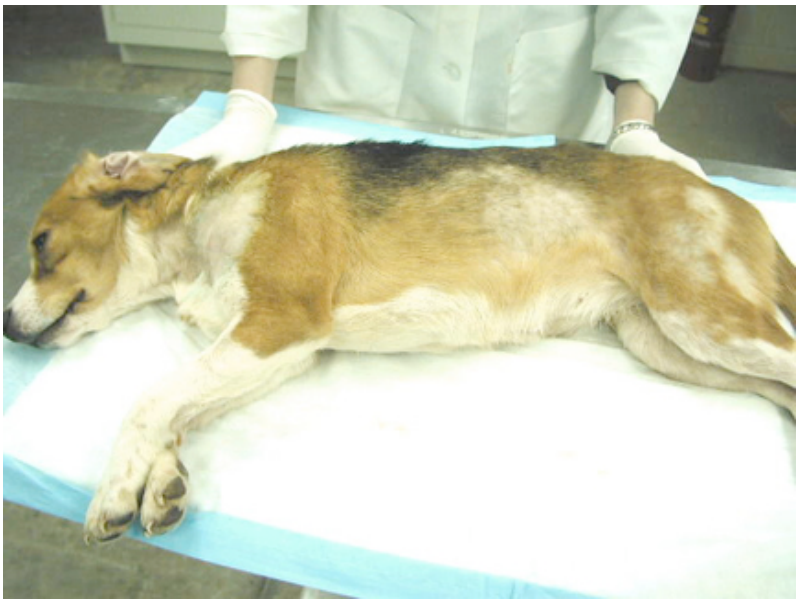

Figure 3. Generalized truncal and extremity alopecia in an experimentally infected dog with clinical leishmaniasis.

Table 1. Results of culture and cytology of lymph node and bone marrow aspirates from all dogs for 77 weeks.

Dog ID	7 weeks		12 weeks		18 weeks		24 weeks		34 weeks		47 weeks		61 weeks		77 weeks	
	Cytology	Culture	Cytology	Culture	Cytology	Culture	Cytology	Culture	Cytology	Culture	Cytology	Culture	Cytology	Culture	Cytology	Culture
Pos Cont 1	Pos-BM	Pos BM&LN	Pos-BM	Pos BM&LN	Pos-BM	Pos BM&LN	Pos-BM	Pos- LN	Pos-BM	NG	Neg	Pos- LN	Neg	NG	NP	NP
Pos Cont 2	Neg	Pos BM&LN	Neg	Pos BM&LN	Neg	Pos- LN	Pos-BM	Pos BM&LN	Pos-BM	Pos- LN	Pos BM&LN	Pos- LN	Pos BM&LN	Pos BM&LN	NP	NP
LD 1	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	Pos- LN	Neg	NG	Neg	Pos- BM
LD 2	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	Pos- LN
LD 3	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	Pos- LN	Neg	NG	Neg	NG
LD 4	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Pos-BM	NG	Neg	NG	Neg	NG	Neg	NG
Neg Cont 1	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	NP	NP
Neg Cont 2	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	NG	NP	NP
HD 1	Neg	Pos-BM	Neg	NG	Neg	NG	Neg	NG	Neg	NG	Neg	Pos- LN	NP	NP	NP	NP
HD 2	Neg	NG	Neg	Pos BM&LN	Neg	NG	Neg	NG	Neg	NG	Neg	NG	NP	NP	NP	NP
HD 3	Neg	NG	Neg	Pos BM&LN	Neg	Pos- LN	Neg	NG	Neg	Pos- LN	Neg	Pos- LN	NP	NP	NP	NP
HD 4	Neg	NG	Neg	Pos-BM	Neg	NG	Neg	Pos-BM	Neg	NG	Pos-BM	Pos- LN	NP	NP	NP	NP

Key to Table- Pos Cont= positive control dog; LD= low dose dog; Neg Cont= negative control dog; HD= high dose dog; Neg= negative; Pos= positive; NG= no growth; BM= bone marrow; LN= lymph node, NP= not performed

Table 2. IFAT results in all dogs for 91 weeks.

	Sampling Times for IFA Tests in Weeks																					
Dog ID	**7**	**12**	**16**	**18**	**24**	**28**	**32**	**34**	**38**	**42**	**47**	**50**	**54**	**58**	**61**	**66**	**70**	**74**	**77**	**83**	**87**	**91**
Pos Dog 1	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400
Pos Dog 2	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	400	NP	NP	NP	NP	NP
LD Dog 1	200	25	50	50	100	0	25	25	0	25	0	25	25	25	0	0	0	25	25	NP	NP	NP
LD Dog 2	0	25	0	50	50	100	25	0	100	50	50	0	200	0	0	0	0	0	0	NP	NP	NP
LD Dog 3	25	200	50	50	25	0	0	50	50	0	200	50	25	25	25	0	0	0	0	NP	NP	NP
LD Dog 4	50	400	25	25	100	25	25	0	25	0	25	0	0	50	0	0	0	0	0	NP	NP	NP
Neg Dog 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NP	NP	NP
Neg Dog 2	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	NP	NP	NP
HD Dog 1	0	100	100	50	50	50	0	50	25	100	400	50	25	400	0	50	25	50	25	50	25	50
HD Dog 2	25	50	50	400	50	25	25	50	100	0	50	100	50	50	50	NP	NP	NP	NP	NP	NP	NP
HD Dog 3	100	400	400	400	400	100	50	400	400	100	400	400	400	400	400	400	400	400	400	400	400	400
HD Dog 4	50	400	400	400	400	400	400	400	400	400	400	400	NP	400	400	400	400	400	400	400	400	400

Key to Table - Pos = Positive; Neg= Negative; LD = Low Dose Group; HD = High Dose Group; NP = Not performed

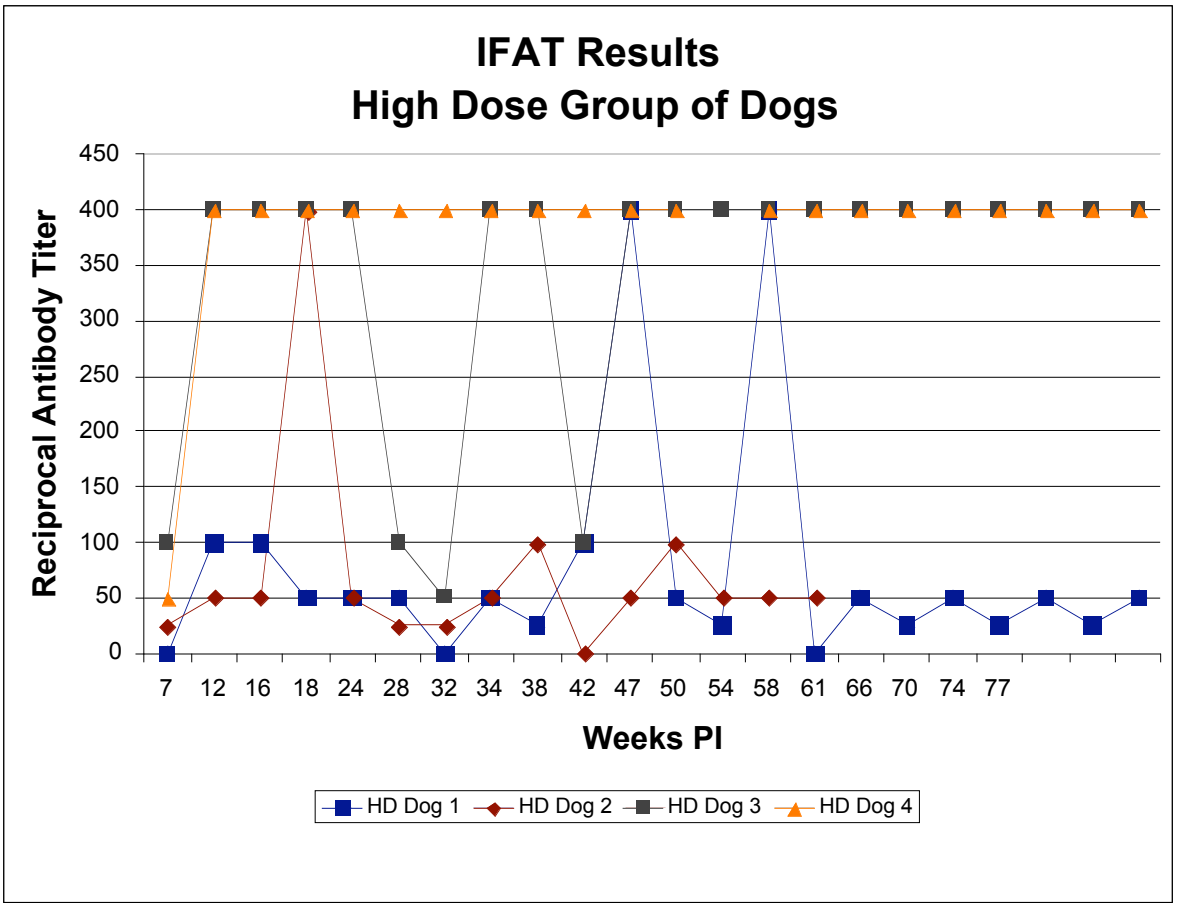

Figure 4. IFAT results in the high dose experimentally infected dogs (n=4)

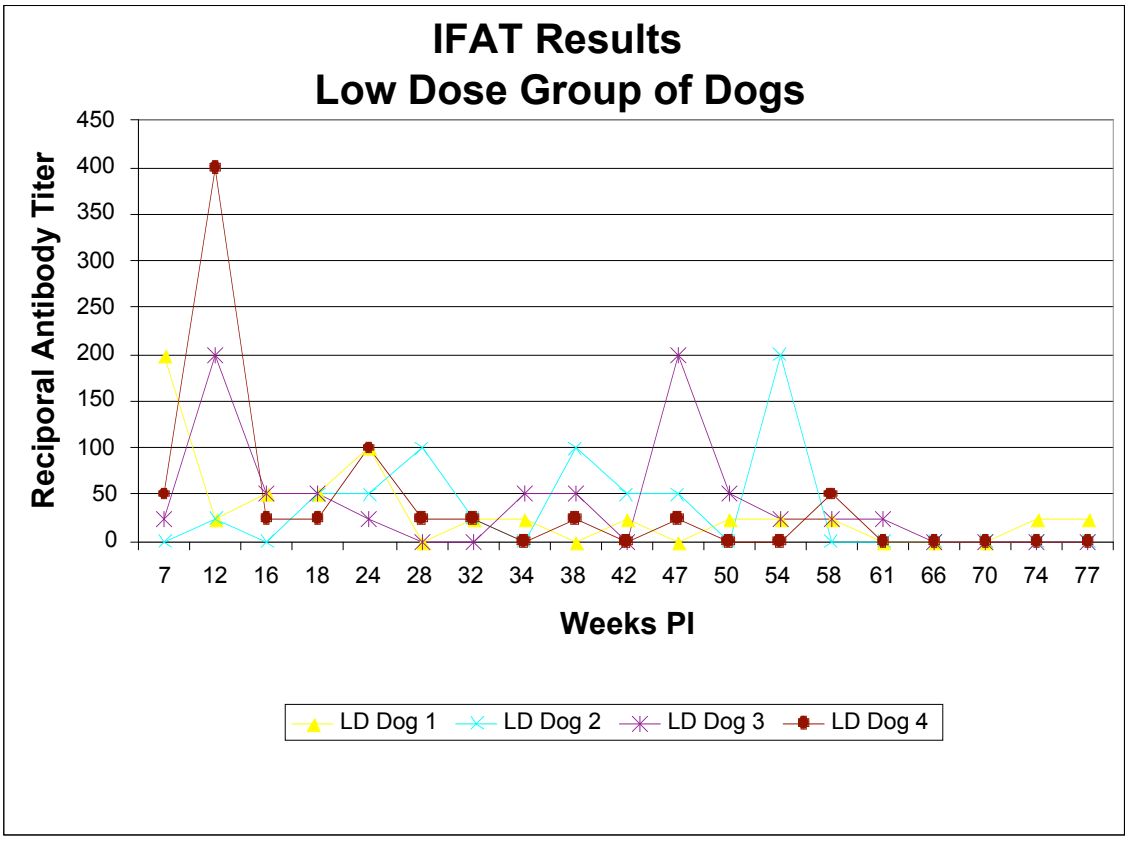

Figure 5. IFAT results in the low dose experimentally infected dogs (n=4)

Table 3. rK39 immunoassay test results in all dogs for 91 weeks.

	rK39 Antigen - Weeks Post-infection																					
Dog ID	**7**	**12**	**16**	**18**	**24**	**28**	**32**	**34**	**38**	**42**	**47**	**50**	**54**	**58**	**61**	**66**	**70**	**74**	**77**	**83**	**87**	**91**
Pos Dog 1	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Pos Dog 2	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	NP	NP	NP	NP	NP
LD Dog 1	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	NP	NP
LD Dog 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	NP	NP
LD Dog 3	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	NP	NP
LD Dog 4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NP	NP
Neg Dog 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Neg Dog 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
HD Dog 1	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
HD Dog 2	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	NP	NP	NP	NP	NP	NP	NP
HD Dog 3	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
HD Dog 4	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	NP	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Key to Table - Pos = Positive; Neg= Negative; LD = Low Dose Group; HD = High Dose Group; NP = Not performed

Table 4. PCR test results on bone marrow samples in all dogs.

Dog ID	7	12	18	24	34	47	61	77	Post
Pos Dog 1	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
Pos Dog 2	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
LD Dog 1	Neg	Neg	Neg	Pos	Pos	Pos	Neg	NP	Pos
LD Dog 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NP	Pos
LD Dog 3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NP	Neg
LD Dog 3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NP	Neg
Neg Dog 1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NP	Neg
Neg Dog 2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NP	Neg
HD Dog 1	Neg	Pos	Pos	Pos	Pos	Pos	NP	NP	Pos
HD Dog 2	Neg	Neg	Neg	Neg	Neg	Pos	NP	NP	Pos
HD Dog 3	Neg	Pos	Pos	Pos	Pos	Pos	NP	NP	Pos
HD Dog 4	Pos	Pos	Pos	Pos	Pos	Pos	NP	NP	Pos

Key to Table - Pos = Positive; Neg= Negative; LD = Low Dose Group; HD = High Dose Group; NP = Not performed

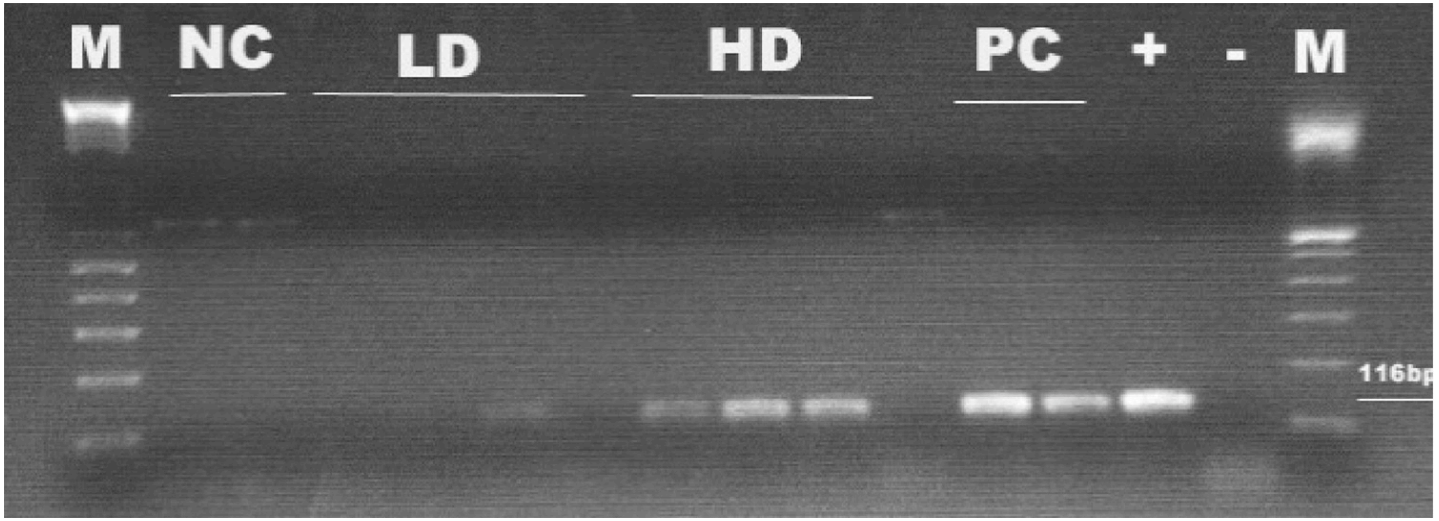

Figure 6. 2% agarose gel showing PCR results on bone marrow samples from all dogs 24 weeks post inoculation. M, molecular weight marker; NC, negative control dogs; PC, positive control dogs; +, positive control; -, negative control; bp, base pair

Table 5. Results of PCR performed on selected tissues taken at necropsy from experimentally infected dogs.

Groups	Dogs	heart	liver	conjunctiva	vagina or penis	lymph node	kidney	lung	skin	spleen	intestine	uterus or testes
Low Dose												
Group	1	+	+	+	+	+	+	-	+	+	+	+
	2	-	+	-	-	+	-	-	-	+	-	-
	3	-	-	-	-	+	+	-	-	+	+	-
	4	-	-	-	+	-	-	-	-	+	-	-
High Dose												
Group	1	-	+	+	+	+	-	+	+	+	+	-
	2	NP	+	NP	NP	+	+	+	+	+	+	-
	3	+	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+
Positive Control												
Group	1	+	+	-	+	+	-	-	+	+	+	-
	2	+	+	+	NP	+	+	+	+	+	+	+

NP- not performed

Table 6. Kappa statistics for IFAT, PCR and rK39 tests for all dogs.

IFAT = > 50			
Week	**Kappa**	**Sensitivity %**	**Specificity %**
7	0.17	83	0
12	0.67	100	75
18	0.5	0	67
24	0.5	0	67
34	0.65	80	86
47	0.4	0	88
61	-0.02	0	0
77	0.28	100	0
All Weeks	0.2	92	27
IFAT = or > 100			
Week	**Kappa**	**Sensitivity %**	**Specificity %**
7	0.4	88	0
12	0.5	80	71
18	0.83	86	100
24	0.33	67	67
34	0.52	63	0
47	0.5	50	100
61	0.11	63	0
77	0.28	100	0
All Weeks	0.2	89	29
rK39 Test			
Week	**Kappa**	**Sensitivity %**	**Specificity %**
7	0.56	89	0
12	0.83	86	100
18	1	100	100
24	0.67	100	75
34	0.65	80	86
47	0.63	60	100
61	-0.167	0	0
All Weeks	0.16	90	27
PCR			
Week	**Kappa**	**Sensitivity %**	**Specificity %**
7	0.56	67	89
12	0.83	100	89
18	0.83	100	89
24	0.67	83	83
34	0.5	83	67
47	0.4	88	0
61	0.33	0	100
All Weeks	0.51	78	74

CHAPTER 6

FLOW CYTOMETRIC ANALYSIS OF CELLULAR IMMUNE RESPONSES IN DOGS EXPERIMENTALLY INFECTED WITH A NORTH AMERICAN ISOLATE OF *LEISHMANIA INFANTUM*

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Key words: Leishmaniasis, cellular immunity, CD4, CD8, *Leishmania infantum*, dog

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ABSTRACT

Canine leishmaniasis caused by *Leishmania infantum* is endemic in the foxhound population in the United States and parts of Canada. Studies of Mediterranean leishmaniasis indicate a role for both CD4+ and CD8+ lymphocytes in disease resistance. Limited information is available on the strain of *L. infantum* infecting foxhounds in North America. The present study investigated changes in cellular immune responses in dogs experimentally infected with 1 x 10^7 (low dose, LD) or 2 x 10^8 (high dose, HD) promastigotes of a United States isolate of *L. infantum* for 83 weeks. Density gradient separation was used to enrich for peripheral blood lymphocytes from canine blood. Lymphocyte subsets (CD4+ and CD8+) were quantified by flow cytometric analysis. Lymphocyte population expression levels were compared to clinical status of the dog and antibody responses in infected and control dogs. No significant differences ($P > 0.05$) were observed in either CD4+ or CD8+ lymphocyte expression in any of the groups over the experimental period and they did not reveal changes in clinical status or serology. This study suggests that the cellular immune responses to North American *L. infantum* in experimentally infected dogs may differ from other strains of *L. infantum*.

1. INTRODUCTION

Zoonotic visceral leishmaniasis is caused by *Leishmania infantum* and the genetically similar or identical parasite *L. chagasi* (Mauricio et. al., 1999). Dogs are recognized as the major reservoir hosts for human infections (Slappendel and Ferrer, 1998). Flagellated *Leishmania* promastigotes are transmitted by sand flies and multiply as amastigotes within mononuclear phagocytes in the mammalian host. Zoonotic visceral leishmaniasis is endemic in Latin America, Africa, Asia, and the Mediterranean region of Europe (Enserink, 2000).

Leishmaniasis caused by *L. infantum* has emerged in the foxhound population in many parts of North America (Rosypal et. al., 2003). Isoenzyme electrophoresis revealed the parasites belonged to *L. infantum* MON-1 zymodeme (Gaskin et. al., 2002), the most common type causing both human and canine leishmaniasis in the Mediterranean basin (Alvar, 1999). An extensive serological survey of over 12,000 dogs conducted by the U.S. Centers for Disease Control and Prevention (CDC) in the United States and Canada has detected *Leishmania* infections in 2% of foxhounds, but not in other dog breeds (Schantz, 2003). Although sand flies are widespread in much of the United States (Young and Perkins, 1984), *L. infantum*-infected sand flies have not been documented (Monti, 2000). The means by which *L. infantum* infections are transmitted in North America remains unclear, although one study using experimentally infected laboratory beagles suggests vertical transmission can occur (Rosypal et. al., 2005a). The variant of the parasite infecting foxhounds in North America may have been selected in the absence of sand fly transmission, making it biologically different from other strains of *L. infantum* (Rosypal et. al., 2005b).

Canine visceral leishmaniasis (CVL) is a chronic wasting disease. Clinical signs of CVL are variable and many dogs infected with *Leishmania* parasites do not develop clinically evident disease (Berrehal et. al., 1996). Disease outcome in CVL is largely determined by T-cell-mediated immune response (CMI) to *Leishmania* infection. T cells play a pivotal role in immunity to leishmaniasis by influencing cytokine production and interacting with infected macrophages. Previous work demonstrated that chronically infected dogs have lower numbers of T cells with reduced function compared to non-infected dogs (Brandonisio et. al., 1988). Studies of Mediterranean canine leishmaniasis have demonstrated that the lack of appropriate CMI is related to decreased levels of CD4+ T cells (Bourdoiseau et. al., 1997a; Moreno et. al., 1999). Additionally, infected macrophages are deficient in co-stimulatory molecules that are required

for T cell activation (Pinelli et. al., 1999). Thus, infected macrophages have a reduced ability to interact with T cells, which subsequently interferes with initiation of IFN-γ production and parasite destruction. *In vitro* analysis of cytotoxic T cells from asymptomatic dogs demonstrated a role for CD8+ T cells in resistance to CVL by increased IFN-γ production and lysis of infected macrophages compared to asymptomatic dogs (Pinelli et. al., 1995). In symptomatic dogs, strong humoral responses (Nieto, et. al, 1999) and simultaneous depressed cellular immunity result in disease progression (Pinelli et. al., 1994). Cytokine analysis from peripheral blood mononuclear cells revealed that asymptomatic dogs develop a Th1 type response marked by increased secretion of interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin 2 compared to symptomatic dogs (Pinelli et. al., 1994; Santos-Gomes et. al., 2002).

The strain of *L. infantum* infecting foxhounds in North America appears to have undergone selection that reduces its dependence on insect-borne transmission. Other aspects of the biology of North American *L. infantum* may differ from those of other strains. Much of what is known about the canine immune response to *Leishmania* infection comes from work on Mediterranean isolates or naturally infected dogs from southern Europe (Abranches et. al., 1991; Pinelli et. al., 1995; Bourdoiseau et. al., 1997a; Pinelli et. al., 1999; Moreno et. al., 1999). The present study was conducted to examine changes in CMI parameters in dogs experimentally infected with *L. infantum* isolated from a naturally infected foxhound from Virginia. Canine lymphocyte populations (CD4+ and CD8+ T-cells) from peripheral blood were assessed and compared to antibody responses and clinical disease.

2. MATERIALS AND METHODS

2.1. Dogs and inoculations

Isolation of infective *L. infantum* promastigotes from a naturally infected Virginia foxhound (LIVT-1 isolate) used in this study has been previously described (Rosypal et. al., 2003). Female laboratory beagles were obtained from Covance Research Products, Inc. (Cumberland, VA). Eight 6-month old female beagles were injected with 6-day-old LIVT-1 promastigotes. Dog inoculations have been described elsewhere (Rosypal et. al., 2005b). Infection parameters and clinical status are summarized in Table 1. Briefly, promastigotes were suspended in Hank's balanced salt solution (HBSS) for infection. Dogs were intravenously injected with 2×10^8 (N=4; high dose, HD group) or 1×10^7 promastigotes (N=4, low dose, LD group) in 1 ml of HBSS. Two female beagles were inoculated with 1 ml HBSS (control group) and served as negative controls.

2.2. Clinical findings

The Virginia Tech Animal Care and Use Committee approved all procedures performed on dogs. Dogs in this study were followed for a period of 61-79 weeks. Control dogs did not exhibit clinical manifestations of disease that could be attributed to *Leishmania* during the study period (Table 1). Body weight and activity levels control dogs remained normal. Both control dogs had normal estrous cycles during the course of study. All four dogs in the HD group exhibited clinical signs compatible with leishmaniasis by 6 months post-infection. Clinical signs included ocular discharges, facial, periocular, and aural dermatitis, alopecia, seborrhea, weight loss, draining skin lesions, lameness, polyarthritis, and generalized lymphadenopathy. Alopecia was generalized and involved the face, trunk and extremities. One HD dog died approximately 61 weeks post-infection following complications from cesarean section surgery. Two dogs in the HD group developed hypoalbuminemia and hyperproteinemia approximately 10 months post-infection. One HD dog developed proteinuria. All four dogs in the LD group remained clinically normal. No significant laboratory abnormalities were present in the four LD dogs during the course of study (83 weeks).

2.3. Blood collection

Blood was collected by jugular venipuncture into 7 ml heparinized tubes. Blood samples were obtained at 2-4 week intervals after inoculation. Baseline data were determined from blood

collected 3 days prior to infection. An aliquot of whole blood was used to separate plasma and plasma were kept at -20°C until used for serology.

2.4. Serology

Plasma from infected and control dogs were tested for anti-LIVT-1 IgG antibodies by the indirect fluorescent antibody test (IFAT) as previously described (Rosypal et. al., 2005b). The threshold for the IFAT was 1:100 for the dog to be considered positive (Table 1). The Centers for Disease Control indicate that dogs with reciprocal titers > 64 are suggestive of active infection (Schantz, 2004). IFAT titers of inoculated dogs ranged from 0 to 1:400.

2.5. Lymphocyte isolation

Density gradient separation was used to isolate peripheral blood lymphocytes from canine blood. Blood was diluted 1:4 using room temperature Dulbecco's phosphate buffered saline (DPBS). Diluted blood was layered over room temperature Lymphoprep™ (Greiner, Longwood, FL) at a 2:1 ratio and centrifuged at 1000 x g for 25 min at 25ºC, brake off. The layer of cells at the interface was removed and transferred to a 15 ml conical tube containing 5 ml RPMI 1640 media. Tubes containing cells and media were filled completely with RPMI 1640 and centrifuged at 400 x g for 10 min at 8ºC. Supernatant was discarded and cells were resuspended in 5 ml RPMI 1640 media. Cell suspension was centrifuged at 400 x g for 7 minutes at 8ºC. Supernatant was discarded and cells were resuspended in 3 ml cold DPBS for cell counting.

2.6. Cell enumeration

Lymphocytes were enumerated by CASY® 1 automated cell counter. Suspended cells were centrifuged at 400 x g for 7 min at 8ºC. Supernatant was discarded and cells were adjusted to a final concentration of 2 x 10^6 cells/ml in complete media (RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin, streptomycin, and non-essential amino acids).

2.7. Antibodies and flow cytometry

Monoclonal antibodies (mAB) used in the present study are listed in Table 2. All mAB were obtained from Serotec, Raleigh, NC.

Lymphocyte subsets (CD4+ and CD8+) were quantified by flow cytometric analysis. One hundred µl of cell suspension was placed in appropriate wells in a 96-well round-bottom culture plate. Briefly, 100 µl of of rat anti-canine fluorescein isothiocyanate (FITC)-conjugated CD4 or rat anti-canine phycoerythrin (PE)-conjugated CD8 monoclonal antibody was added to

appropriate wells. Cells were incubated on ice for 30 minutes on a shaker. Following incubation, 100 μl of DPBS was added to all wells. The culture plate was centrifuged at 1200 rpm for 10 minutes at 8ºC. Supernatant was discarded and 200 μl DPBS was added to all wells and the plate was refrigerated overnight. Lymphocyte subset expression was analyzed on an Epics XL flow cytometer (Coulter, Hilaleah, FL). Lymphocyte CD4+ and CD8+ expression was standardized according to the total cell recovery and the original volume of blood from which the cells were obtained.

2.8. Statistical analysis

Tests of group effect and group by time interaction effects were performed with a mixed effects repeated measures analysis of variance (ANOVA). Differences were considered significant at the 5% significance level ($P<0.05$). Data were analyzed using the MIXED procedure of the SAS System (Ver. 9.12, SAS Institute Inc., Cary, NC 27513). Model adequacy was assessed using plots of standardized residuals.

3. RESULTS

No significant differences ($P > 0.05$) in group effect or group by time interaction effect were seen in CD4+ or CD8+ expression levels over the course of the study. The means of interactions for the present study are shown in Figure 1a and b.

4. DISCUSSION

Dogs naturally and experimentally infected with *L. infantum* present a range of clinical manifestations (Rosypal et. al., 2003). A review of experimental models of canine leishmaniasis indicates the irregular response to experimental infection is consistent with variable disease manifestations observed in naturally infected dogs (Moreno and Alvar, 2002). Dogs are often infected with *Leishmania* parasites but never develop clinical disease (Lindsay et. al., 2002). Resistance to CVL is associated with a strong Th1 type cellular immune response and the production of associated cytokines (Rosypal et. al., 2003). The pathogenic potential of the particular *Leishmania* isolate may also be related to individual dog susceptibility to disease (Moreno and Alvar, 2002). Susceptible dogs with Mediterranean CVL lack protective CMI that is associated with significantly decreased levels of CD4+ T cells (Bourdoiseau et. al., 1997a; Moreno et. al., 1999). A role for CD8+ T cells has also been reported in resistance to CVL (Pinelli et. al., 1995).

No significant differences ($P > 0.05$) were observed in CD4+ and CD8+ expression in dogs during this study. An overall decrease in CD4+ and CD8+ expression was observed in dogs from all groups between 9 and 11 months post-infection although the difference was not significant. This effect may be attributable to management techniques during this time period. Some dogs were in estrus and were attempting to be bred in close proximity to the other dogs. Poor cell recovery was noted in some samples during these times due to clotted blood in original peripheral blood samples.

Previous studies of naturally infected dogs from the Mediterranean region demonstrated reduced levels of CD4+ expression (Bourdoiseau et. al., 1997a; Bourdoiseau et. al., 1997b; Moreno et. al., 1999) that correlates with decreased CMI and disease progression. In addition, Guarga et. al. (2000) found that dogs with lower numbers of CD4+ T cells were more infective to sand flies thereby increasing the epidemiological risk of *L. infantum* transmission compared to dogs with high CD4+ cell counts.

A role for CD8+ T cells in control of visceralizing species of *Leishmania* has been demonstrated in humans and mice (Mary et. al., 1999; Gomes-Periera at. Al., 2004; Tsagozis et. a., 2003; Murray and Hariprashad, 1995). Generation of CD8+ T cell lines from asymptomatic dogs revealed a direct cytolytic effect on *L. infantum*-infected macrophages that may be an effector mechanism in resistance to CVL (Pinelli et. al., 1995).

The strain of *L. infantum* endemic in North America apparently infects foxhounds in the absence of an identifiable vector at this time. This observation and results from this work suggest the biological behavior, including immune responses directed against the parasite, may differ from other strains of *L. infantum*. We did not observe trends in CD4+ or CD8+ expression in either control or LIVT-1 inoculated dogs over the course of the study. Lymphocyte subset expression did not reveal changes in clinical status or presence of antibody response in the dogs during the experimental period. Although there were no trends observed in cellular immune responses from these dogs, the cytokines produced by the T cells may have contributed to the clinical status. Further studies using higher numbers of dogs are needed to further investigate the complete immune response to North American canine leishmaniasis. This work should include studies of CD4+ T helper subsets and cytokine profiles.

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Table 1

Definition of experimental groups of dogs

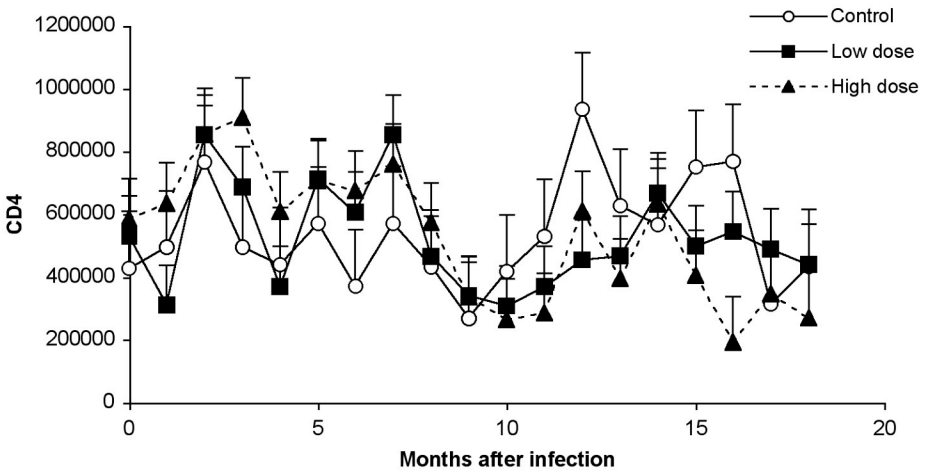
Group	Inoculation	Clinical status	Serology*
Control group (*n*=2)	1 ml HBSS	Healthy dogs	negative
LD group (*n*=4)	10^7 LIVT-1 promastigotes	Good physical condition, mild generalized lymphadenopathy	positive
HD group (*n*=4)	$2x10^8$ LIVT-1 promastigotes	Symptomatic dogs with skin lesions, ocular discharge, polyarthritis, lymphadenopathy	positive

*IFAT titer of ≥ 1:100 at least once over study period

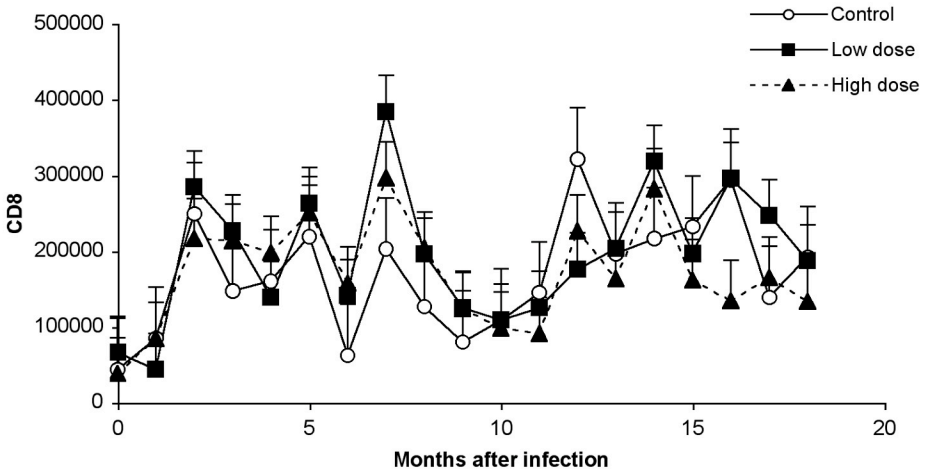
Table 2

Monoclonal antibodies used for characterization of canine lymphocytes

CD	Antibody name	Dilution	Clone	Isotype
CD4 Control	Mouse IgG2a negative control: FITC	1/40	MRC OX-34	Mouse IgG2a
CD8 Control	Mouse IgG1 negative negative control: RPE	1/40	W3/25	Mouse IgG1
CD4	Rat anti-canine CD4: FITC	1/40	YKIX 302.9	Rat IgG2a
CD8	Rat anti-canine CD8: RPE	1/40	YCATE 55.9	Rat IgG1



(a)



(b)

Figure 1. Isolated peripheral blood lymphocytes collected monthly from LIVT-1-inoculated dogs (High dose and low dose groups) and control dogs were labeled for CD4+ (a) and CD8+ (b) markers. Percent expression of CD4+ and CD8+ cells were multiplied by the total lymphocyte recoveries to express absolute numbers of CD4+ and CD8+ cell expression. Each point is representative of the group by time interaction mean and the standard error.

CHAPTER 7

TRANSPLACENTAL TRANSMISSION OF A NORTH AMERICAN ISOLATE OF *LEISHMANIA INFANTUM* IN AN EXPERIMENTALLY INFECTED BEAGLE.

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Key words: transmission, vertical, dog, infection, *Leishmania infantum*

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ABSTRACT

Leishmania infantum, an etiologic agent of zoonotic visceral leishmaniasis, is widespread among foxhounds in the United States. Although sand flies are widely distributed throughout the U.S., epidemiological data do not support a major role for sand flies in the transmission of *L. infantum* in foxhounds in this country. Congenital transmission of visceral leishmaniasis is reported in humans and may also occur in dogs. We have previously isolated *L. infantum* from Virginia foxhounds and used this isolate (LIVT-1) to experimentally infect beagles. Four female beagles, chronically infected with LIVT-1, were bred to a male beagle chronically infected with *L. infantum chagasi*. One beagle was able to maintain her pregnancy and four puppies were delivered by cesarean section. One puppy was dead and autolyzed at delivery and tissues were not collected or analyzed. The remaining puppies were killed at the time of cesarean section and selected tissues were collected for parasite culture and PCR. Promastigotes were not cultured from tissues in any of the puppies. *Leishmania* sp. DNA was detectable by PCR in liver, bone marrow, and heart from all 3 puppies and in the spleen, lymph node, kidney, and placenta in 2 puppies. Placental tissue from the dam was PCR negative. This is the first report of maternal transmission of a North American isolate of *L. infantum* from an experimentally infected dog.

INTRODUCTION

Leishmaniasis is a vector-borne disease caused by infection with parasites in the genus *Leishmania*. These parasites are usually transmitted by phlebotomine sand flies. Studies have suggested that transmission may also occur infrequently by sexual contact (Catone et. al., 2003), blood transfusions (Owens et. al., 2001; Giger et. al., 2002), and direct contact (Lainson and Bray, 1964; Nuwayri-Salti and Khansa, 1985). Rare cases of congenital transmission have been documented in humans (Low and Cooke, 1926; Nyakundi et. al., 1988; Yadav et. al., 1989; Eltoum et. al., 1992; Meinecke et. al., 1999). Similarly, it has been suggested that maternal transmission can occasionally occur in dogs (Mancianti and Sozzi, 1995; Masucci et. al., 2003).

Leishmania infantum, an etiologic agent of human visceral leishmaniasis, is widespread among foxhounds in the U.S. (Rosypal, 2003). Sand flies are present throughout much of the country, particularly in the southeast (Young and Perkins, 1984). However, epidemiological data do not support a role for sand flies in the transmission of *L. infantum*. This suggests that an alternate mode of transmission may maintain infections in U.S. foxhounds. The risk of zoonotic spread from infected dogs to humans is currently unknown for the U.S. The present study was conducted to determine if vertical transmission can occur in dogs infected with a North American isolate of *L. infantum*.

MATERIALS AND METHODS

Intact female beagles (n=4) were experimentally infected with promastigotes of the LIVT-1 strain of *L. infantum* originally isolated from a naturally infected foxhound from Virginia (Rosypal et. al., 2003). All of the dogs had clinical signs compatible with canine leishmaniasis. The parasite had been re-isolated from the beagles by culture of lymph node and bone marrow aspirates and it was detectable by polymerase chain reaction (PCR) conducted on bone marrow (Rosypal et. al., unpublished). The female dogs were bred to a male beagle chronically infected with *L. infantum chagasi* both by natural service and by artificial insemination. Prior to breeding, semen was collected from the male dog and placed in *Leishmania* sp. culture media (30% v/v fetal bovine serum, 1% penicillin/streptomycin, 2% human urine, in Grace's Insect Media) at 25 C.

One of the 4 dogs became pregnant and was able to sustain the pregnancy, but the other 3 dogs either did not conceive or did not maintain their pregnancies. For this reason, the following data were collected from a single female dog that sustained her pregnancy. Four puppies were delivered by cesarean section on day 60 of gestation (normal gestation period=63 days) to preclude the possibility of transvaginal transmission during natural birth. Puppies were either unable to breathe at birth or were killed immediately following delivery. One puppy was non-viable and deformed at the time of delivery and tissues were not collected. Portions of liver, femoral bone marrow, spleen, heart, lymph node, kidney, and placental tissues were collected from the remaining 3 puppies for parasite culture and PCR.

DNA was extracted from tissue samples using a commercial kit (DNA Mini Kit, Quiagen®, Valencia, California). For each 50 µl reaction, 1 µl of DNA was added to 45 µl of Platinum® PCR Supermix (Invitrogen™ Life Technologies, Carlsbad, California) in a 0.5 ml thin-walled microcentrifuge tube. To the reaction tube, 2 µl of primers 13A and 13B were added; these primers amplify a conserved minicircle region of kinetoplast DNA from all species of *Leishmania* (Rodgers et. al., 1990).

Optimal PCR amplification conditions consisted of initial denaturation at 95 C for 2 min, 38 cycles consisting of denaturation at 94 C for 30 sec, annealing at 62 C for 30 sec, and extension at 68 C for 30 sec, and a final extension at 72 C for 10 min. One µl of PCR product was used as template DNA in a second 50 µl reaction and was subjected to the same cycling conditions described above. PCR products were electrophoresed on a 2% agarose gel and with

size markers to detect the 116 bp PCR product. DNA extracted from LIVT-1 promastigotes was used a positive control and a negative control without DNA was included. To determine the sensitivity of the PCR assay, DNA was extracted from a known number of LIVT-1 promastigotes. Ten-fold serial dilutions of DNA were made and subjected to PCR. DNA detection limit was <1 organism.

RESULTS AND DISCUSSION

Promastigotes were not cultured from any puppy tissues. Parasites were not isolated from sire's semen culture. The expected 116 bp product was amplified by PCR in liver, bone marrow, and heart tissue from all 3 puppies (Table I). *Leishmania* sp. DNA was also detectable in spleen, lymph node, kidney, and placental tissues from 2 puppies. No PCR product was detectable in testes collected at necropsy from the sire (data not shown). *Leishmania* sp. DNA was demonstrable by PCR in uterine tissue collected at necropsy from the dam (data not shown). Placental tissue from the dam was PCR negative.

This is the first report of maternal transmission of a strain of *L. infantum* from North America. The most likely route of transmission was across the placenta. Puppies were delivered by cesarean section, which eliminated the possibility of transmission through microscopic lesions in the birth canal and *Leishmania* sp. was detected by PCR in placenta and uterine tissue from the dam.

Leishmania sp. parasites circulate in blood. The placental blood supply is in close proximity to the maternal circulation and parasites may pass into the fetal circulation. This mode of transmission has been previously reported in humans (Low and Cooke, 1926) and in experimentally infected mice (Nuwayri-Salti and Khansa, 1985). Paternal transmission was excluded because no parasites were cultured from semen and there was no detectable *Leishmania* sp. DNA in testes tissue tested by PCR.

Pregnancy has a systemic effect that biases the immune system towards a Th2 immune response that protects the feto-placental unit while simultaneously increasing susceptibility to intracellular pathogens (Wegmann et. al., 1993). It has been previously shown that Th1 cytokines can be harmful to the placenta and may compromise fetal survival (Wegmann et. al., 1993; Raghupathay, 1997). Resistance to leishmaniasis is associated with a strong Th1 type response marked by increased secretion of interferon-γ (IFN-γ), tumor necrosis factor (TNF), and interleukin 2 compared to symptomatic dogs (Pinelli et. al., 1994; Santos-Gomes et. al., 2002). Moreover, dogs with clinical disease develop a strong humoral response (Nieto et. al, 1999) and a simultaneous lack of an appropriate cell mediated immune response resulting in disease progression (Pinelli et. al., 1994). Humoral immunity is driven by a Th2 immune response and the production of cytokines by type 2 helper T cells.

The dogs used in this study were clinically symptomatic with high anti-*Leishmania* sp. antibody responses (data not shown), so they were already biased towards a Th2 immune response. In addition to generalized immunosuppression required during pregnancy to prevent immune reactions directed against foreign antigens on the fetus, a shift to a Th2 from a Th1-type response during clinical disease may increase both the severity of disease and the chance of congenital transmission of canine leishmaniasis. Previous work in experimentally infected mice demonstrated increased susceptibility to leishmaniasis during pregnancy (Krishnan et. al., 1996).

In the present study, 3 of 4 beagles that were bred were unable to conceive or maintain their pregnancies. Similarly, in a murine model, Krishnan et. al. (1996) found that *L. major* infection was associated with increased frequencies of fetal resorption and implantation failure in pregnant mice. Although mice and dogs have different types of chorio-allantoic placentas (Loke, 1982), the various numbers of placental layers and types of placental attachments found between species may play a role in the probability of congenital transmission of leishmaniasis.

Zoonotic visceral leishmaniasis is caused by both *L. infantum* and *L. chagasi* (Mauricio et. al., 1999). The finding that a North American isolate of *L. infantum* is transmitted vertically is in contrast to previous work that inferred maternal transmission of *L. chagasi* does not occur in dogs, even though some placental tissue was PCR positive (Andrade et. al., 2002). Rare cases of vertical transmission of *L. infantum* from the Mediterranean Basin have been previously described in humans and in dogs (Mancianti et. al., 1995; Meinecke et. al., 1999; Masucci et. al., 2003). *Leishmania infantum* MON-1 is the most common zymodeme causing canine leishmaniasis in southern Europe and is the same type identified in infected foxhounds in the U.S. (Gaskin et. al., 2002). It is possible that the strain of *L. infantum* infecting U.S. foxhounds has the unique ability to routinely cross the placenta. This mode of transmission may be responsible for maintaining the infections in this group of dogs. Additional research using larger numbers of dogs experimentally or naturally infected with the North American strain of *L. infantum* need to be conducted to determine the efficiency of maternal transmission and of parasite survival in transplacentally infected puppies.

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TABLE I. PCR results from selected tissues taken at necropsy from puppies delivered from a beagle experimentally infected with a North American isolate of *Leishmania infantum*.

Tissue	Puppy 1	Puppy 2	Puppy 3
Liver	Pos	Pos	Pos
Bone marrow	Pos	Pos	Pos
Spleen	Pos	Pos	Neg
Heart	Pos	Pos	Pos
Lymph node	Pos	Neg	Pos
Kidney	Neg	Pos	Pos
Placenta	Pos	Pos	Neg

CHAPTER 8

NON-SAND FLY TRANSMISSION OF A NORTH AMERICAN ISOLATE OF *LEISHMANIA INFANTUM* IN EXPERIMENTALLY INFECTED BALB/C MICE

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Key words: transmission, sexual, direct, maternal, mouse, *Leishmania infantum*

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ABSTRACT

Leishmania infantum, an etiologic agent of zoonotic visceral leishmaniasis is endemic in the foxhound population in United States and Canada. Leishmaniasis is usually transmitted by blood feeding sand flies, however epidemiological data do not support a significant role for sand flies in the maintenance of foxhound infections in North America and an alternate mode of transmission may exist. The present study was conducted to determine if transplacental or direct transmission occurs in pregnant BALB/c mice experimentally infected with *L. infantum* isolated from a naturally infected foxhound from Virginia and in males used to breed the mice. Female BALB/c mice were intravenously inoculated with1 x 10^6 promastigotes of the LIVT-1 strain of *L. infantum*. Mice were bred to uninfected male BALB/c mice 2 mo post inoculation. Pregnant mice were killed between days 13-18 of gestation. Pups and placentas were collected at necropsy, divided, and used for parasite culture and polymerase chain reaction (PCR). Culture and PCR was performed on spleens from the male mice to determine the possibility of sexual transmission. *Leishmania* DNA was detected in a 4/88 pups and 3/16 placentas from LIVT-1 inoculated mice. One male mouse used to breed infected females was PCR positive. This work provides evidence for a low level of non-vector transmission of North American *L. infantum* in a mouse model.

INTRODUCTION

Leishmania parasites are flagellated protozoans that are normally transmitted by blood sucking phlebotomine sand flies. In North America, *L. infantum*, a member of the *L. donovani* complex and a causative agent of zoonotic visceral leismaniasis, is endemic in the foxhound population (Gaskin et. al., 2002, Rosypal et al., 2003). Sand flies are distributed throughout many parts of North America (Young and Perkins, 1984), however *L. donovani*-infected sand flies have not been described from the United States (Monti, 2000). Infections acquired by blood transfusions from infected U.S. foxhounds have been described infrequently (Owens et. al., 2001; Giger et. al., 2002). The transmission mode by which *Leishmania* infections are maintained in foxhounds in North America is currently unknown.

Rare cases or non-sand fly-vectored leishmaniasis by direct contact (Nuwayri-Salti and Khansa, 1985; Lainson and Bray, 1964) and sexual contact (Catone et. al., 2003; Symmers, 1960) have been described. Additionally, occasional reports of congenital transmission of leishmaniasis have been reported in humans (Low and Cooke, 1926; Nyakundi et. al., 1988; Yadav et. al., 1989; Eltoum et. al., 1992; Meinecke et. al., 1999) and in dogs (Mancianti and Sozzi, 1995; Masucci et. al., 2003). It has been suggested that maternal transmission may play a role in the spread of *L. infantum* among foxhounds in North America (Gaskin et. al., 2002; Rosypal et. al., 2003). We have previously shown that a North American isolate of *L. infantum* was transplacentally transmitted to puppies from an experimentally infected dog (Rosypal et. al., 2005a). In the present work, we report results of a study of direct and maternal transmission of North American *L. infantum* isolated from a naturally infected foxhound using experimentally infected BALB/c mice.

MATERIALS AND METHODS

Parasites and mouse infections

Leishmania infantum promastigotes were isolated from popliteal lymph node and bone marrow tissues from a naturally infected foxhound from Virginia as previously described (Rosypal et. al., 2003) (LIVT-1 strain, ATTC # pending, Manassas, VA). Promastigotes were subcutaneously inoculated into interferon-γ-gene knockout (IFN-γ-KO) (BALB/c genetic background) mice. Chronically infected mice were killed and their spleens were harvested, homogenized and cultured in vitro at 25 C in *Leishmania* culture media (30% v/v fetal bovine serum, 1% penicillin/streptomycin, 2% human urine, in Grace's Insect Media).

Six-day-old LIVT-1 promastigotes cultured from murine spleen tissue were used for inoculum in the present study. Female 12-week-old BALB/c mice (*N*=20, Jackson Laboratories, Bar Harbor, ME) were intravenously (IV) inoculated with 1 x 10^6 LIVT-1 promastigotes suspended in 0.05 ml Hank's balanced salt solution (HBSS). Female BALB/c mice (*N*=4) IV inoculated with 0.05 ml HBSS served as controls and were housed separately. Prior to injection, mice were anesthetized by intraperitoneal injection of 60 mg/kg bodyweight (BW) ketamine and 5 mg/kg BW xylazine. Mice were warmed on a heating pad to induce vasodilation prior to IV injection into the lateral tail vein. *Leishmania*-infected female mice were housed together for 8 wk to allow the mice to establish a chronic infection before breeding.

Breeding

Beginning 8 wks post inoculation female mice were bred to uninfected male BALB/c mice. Female mice were checked daily for the presence of sperm plugs and they were separated from the males after breeding. Pregnant mice were euthanized between days 13-18 of gestation to preclude potential transvaginal transmission of the parasite during natural birth. In order to verify infections in the mothers, spleens were harvested and divided at necropsy. Half of the spleen tissue was homogenized and cultured in vitro at 25 C in *Leishmania* culture media. The remaining spleen halves were used for polymerase chain reaction (PCR).

Mouse pups and *Leishmania* PCR

At necropsy mouse pups were removed from the mother and placentas, divided, and homogenized. Half of each pup was cultured *in vitro* in *Leishmania* culture media at 25 C. Cultures were examined for parasite growth with an inverted microscope every day for 4 wk.

The remaining halves of the mouse pups were used for PCR. Placental tissue was divided and used for culture and PCR.

DNA was extracted from mouse pups, placentas, and maternal spleen tissue using a commercial kit (DNA Maxi Kit, Quiagen®, Valencia, CA). For each 50 µl reaction, 1 µl of DNA was added to 45 µl of Platinum® PCR Supermix (Invitrogen™ Life Technologies, Carlsbad, CA) in a 0.5 ml thin-walled microcentrifuge tube. To the reaction tube, 2 µl of primers 13A (5'-GTGGGGGAGGGGCGTTCT-3') and 13B (5'-ATTTTACACCAACCCCCAGTT-3') were added, which amplify a conserved minicircle region of kinetoplast DNA from all species of *Leishmania* (Rodgers et. al., 1990).

Optimal PCR amplification conditions consisted of initial denaturation at 95 C for 2 min, 38 cycles consisting of denaturation at 94 C for 30 sec, annealing at 62 C for 30 sec, and extension at 68°C for 30 sec, and a final extension at 72°C for 10 min. PCR products were electrophoresed on a 2% agarose gel and with size markers to detect the 116 bp PCR product. DNA extracted from LIVT-1 promastigotes was used a positive control and a negative control without DNA was included. Sensitivity of the PCR assay was < 1 organism, as previously determined (Rosypal et. al., 2005b).

Direct transmission studies

To determine if the LIVT-1 strain of *L infantum* was transmitted by direct contact, male mice were maintained to allow the mice to establish infections following sexual contact. After mating, male mice that bred LIVT-1-infected female mice were housed together for 12 wks. Male mice that bred control mice were housed together after breeding. Mice were bled from the retro orbital plexus and killed 12 wks post breeding. At necropsy spleens were harvested and divided. Half of the spleen was used for parasite culture in *Leishmania* culture media. The remaining spleen halves were subjected to PCR as described above. Mouse sera were tested for anti-*Leishmania* antibodies by the indirect fluorescent antibody test (IFAT) as previously described (Rosypal et. al., 2005c).

RESULTS

The results of culture and PCR from the dams and offspring are summarized in Table 1. None of the LIVT-1 or HBSS inoculated mice developed clinical signs of leishmaniasis. Of the 16 female mice inoculated with LIVT-1 promastigotes, infections were confirmed by culture and/or PCR of splenic tissue in 15 mice. *Leishmania* DNA was detected in placental tissue from 3 LIVT-1 inoculated mice. Promastigotes were not cultured from placentas from any of the mice. Culture of mouse pups yielded all negative results, and 4 pups from infected mice were PCR positive. Spleens, placentas, and mouse pups from HBSS inoculated mice were all culture and PCR negative.

Promastigotes were not cultured from spleen tissue from male mice. *Leishmania* DNA was demonstrable by PCR in one of 8 male mice bred to LIVT-1 inoculated female mice. Spleens from males that mated HBSS-inoculated mice were PCR negative. The male mouse with PCR positive spleen tissue had an IFAT titer of 1:50. All other mice that were bred to either LIVT-1- or HBSS-inoculated female mice did not develop antibody titers to *L. infantum*.

DISCUSSION

The results from this study suggest that BALB/c mice experimentally infected with a United States isolate of *L. infantum* are able to transmit the organisms by vertical and sexual transmission at a low level. *Leishmania* PCR products were present in 4/88 (5%) mouse pups from LIVT-1-inoculated mice. The pups were most likely infected *in utero*. This is supported by the finding of *Leishmania* DNA in 3/16 (19%) placentas from *L. infantum* inoculated mice. In addition, the pups were removed from the dam prior to natural delivery thereby eliminating the chance of infection during parturition. *Leishmania* DNA was detected in 1/8 (13%) male mice used to breed the infected mice. This evidence supports the possibility of direct or sexual transmission of *Leishmania* parasites.

There are 3 basic diagnostic methods used to detect *Leishmania* infections that include parasitological diagnosis by microscopy or parasite culture, anti-*Leishmania* serological tests, and molecular assays to detect *Leishmania* DNA (Ferrer, 1999). Although *in vitro* cultivation provides definitive proof of parasite presence, this method suffers from low sensitivity (Piarroux et. al., 1994), but the diagnostic dilemma can be overcome by the use of PCR (Smyth et. al., 1992; Ashford et. al., 1995). Additionally, *Leishmania* may not grow in culture if few parasites are present (Reale et. al., 1999). No parasites were cultured from mouse pups, placentas or spleens from male mice. This may be due to a low parasite load transmitted by the transplacental or sexual routes or to inherent low sensitivity of the diagnostic test.

It is important for both human and veterinary medicine to understand transmission dynamics of *L. infantum* in North America in order to implement strategic control measures. This work provides evidence for non-vector transmission of a North American isolate of *L. infantum* in experimentally infected BALB/c mice. It is possible that a higher rate of parasite transmission occurs late in gestation, during natural delivery, or by nursing. Future studies should examine potential transmission during the perinatal period.

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Table 1. Results of North American *L. infantum* inoculation in groups of BALB/c mice used for breeding.

	LIVT-1 group (n=16)	Control Group (n=4)
Treatment	1 x 10^6 LIVT-1 promastigotes	HBSS
Total number of pups from all females in group	88	30
Average litter size	5.5 pups	7.5 pups
Total number of culture positive spleens from dams	5	0
Total number of PCR positive spleens from dams	15	0
Total number of culture positive placentas from dams	0	0
Total number of PCR positive placentas from dams	3	0
Total number of positive cultures of pups	0	0
Total number of PCR positive pups	4	0

CHAPTER 9

GENERAL CONCLUSIONS

This dissertation describes the first isolation of *Leishmania infantum* from naturally infected foxhounds from Virginia with no history of foreign travel. Genetic typing of the parasites indicated that they are similar to Mediterranean *L. infantum* previously found in foxhounds elsewhere in North America, as described in Chapter 3. The strain of *L. infantum* infecting North American foxhounds is considered to be of the zymodeme MON-1 genotype. The isoenzyme analysis used to determine zymodeme is not sensitive enough to determine subtle differences in parasite DNA that may confer variability in transmission mechanisms or infectivity. For this reason, biological differences may exist in parasites with identical isoenzyme profiles. The variant of *L. infantum* present in North America appears to have been selected for non-sand fly transmission and consequently, other biological variation may exist. Characterization of canine leishmaniasis was explored in order to investigate North American *L. infantum*.

Chapter 4 of this dissertation described the development of a mouse model of North American leishmaniasis. Results from this study demonstrated that the IV inoculation route was superior to the SC route to induce consistent experimental infections in mice. Promastigotes were cultured from all IV infected mice by 3 days post culture, while spleens from SC inoculated mice became culture positive 3-24 days post culture. Interestingly, the genetically immunodeficient mice inoculated by either IV or SC route did not develop overt disease. Resistance to leishmaniasis is associated with a Th1 type immune response and IFN-γ-induced nitric oxide production to kill intracellular amastigotes. The lack of clinical signs in the mice may have been due to the short study period. The murine model of North American leishmaniasis could be used to test chemotherapeutic agents. The immunocompetent mouse model could also be used to evaluate immune responses in vaccination trials.

The first study of experimental canine leishmaniasis using a North American isolate of *L. infantum* is described in Chapters 5. Beginning approximately 6 months after IV infection, dogs that received 2 x 10^8 promastigotes developed clinical signs of leishmaniasis including skin

lesions, arthritis, ocular discharge, weight loss, and generalized lymphadenopathy. Dogs that were infected with 10^7 parasites did not develop clinical disease other than mild lymphadenopathy. This is consistent with the range of disease manifestations observed in natural infections and in dogs experimentally infected with Mediterranean isolates of *L. infantum*. Evaluation of techniques used to diagnose canine leishmaniasis was performed. Results indicated that parasite culture of lymph node aspirates was more useful for identifying infected dogs than bone marrow culture of cytology. Most veterinarians diagnose *Leishmania* infections in dogs by demonstration of amastigotes in stained preparations of skin, lymph node or bone marrow aspirates. It is possible that veterinarians may miss diagnoses of leishmaniasis in North America because parasite culture is not routinely used in veterinary practices. Serological testing by IFA revealed erratic antibody responses in LIVT-1 inoculated dogs. This finding is in agreement with variable antibody levels observed in naturally and experimentally infected dogs in the Mediterranean basin. Kappa statistics indicated PCR had the highest level of agreement with parasitological diagnosis, although the rK39 dipstick assay consistently identified dogs with active disease.

Cellular immune responses in dogs experimentally infected with LIVT-1 are presented in Chapter 6. Flow cytometric analysis revealed no significant differences ($P > 0.05$) in CD4+ or CD8+ lymphoctye expression in the groups over the experimental period and their expression did not reveal changes in clinical status or serology. Resistance to Mediterranean canine leishmaniasis is associated with CD4+ and CD8+ T cells. Results from this work indicated that the cellular immune response directed against North American *L. infantum* in dogs may differ from Mediterranean canine leishmaniasis and further research should be conducted in this area.

Evidence for non-sand fly transmission was described in Chapters 7 and 8. As previously stated, epidemiological data do not support a role for sand flies in the transmission of *L. infantum* in the North American foxhound population. A low level of parasite transmission was detectable in mouse pups from LIVT-1 inoculated BALB/c mice and in male mice used breed infected females. *Leishmania* DNA was detectable in placenta and in neonatal tissues from a LIVT-1 infected beagle. The strain of *L. infantum* infecting foxhounds in North America may be transmitted predominantly by a transplacental route. Transplacental transmission may be responsible for maintaining infections in this group of dogs.

Canine leishmaniasis caused by *L. infantum* is widespread in foxhounds in North America. This dissertation demonstrated that leishmaniasis developed by dogs experimentally infected with a Virginia isolate of *L. infantum* is similar to natural disease and experimentally induced disease using Mediterranean isolates. Cellular immune responses in dogs may differ from those of Mediterranean leishmaniasis. Evidence for non-insect transmission was described. This work will help veterinarians in the United States and Canada in the diagnosis and management of canine leishmaniasis.

The strain of *L. infantum* endemic in North America apparently infects foxhounds in the absence of an identifiable vector at this time. Current evidence does not implicate infected foxhounds as reservoirs for human infections in the United States, however, it is important to understand the transmission dynamics of *L. infantum* in North America in order to implement strategic control measures. Future work should assess the extent to which *L. infantum* is an insect vectored zoonotic disease in North America.

CHAPTER 10

APPENDIX

MICE LACKING THE GENE FOR INDUCIBLE OR ENDOTHELIAL NITRIC OXIDE ARE RESISTANT TO SPOROCYST INDUCED *SARCOCYSTIS NEURONA* INFECTIONS

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ABSTRACT

Equine protozoal myeloencephalitis (EPM) is a neurologic syndrome in horses from the Americas and is usually caused by infection with the apicomplexan parasite, *Sarcocystis neurona*. Little is known about the role of immunobiological mediators to this parasite. Nitric oxide (NO) is important in resistance to many intracellular parasites. We, therefore, investigated the role of inducible and endothelial NO in resistance to clinical disease caused by *S. neurona* in mice. Groups of interferon-γ-gene knockout (IFN-γ-KO) mice, inducible nitric oxide synthase gene knockout (iNOS-KO) mice, endothelial nitic oxide synthase gene knockout (eNOS-KO) and appropriate genetic background mice (BALB/c or C57BL/c) were orally fed sporocysts or Hanks balanced salt solution. Mice were observed for signs of clinical disease and examined at necropsy. Clinical disease and deaths occurred only in the IFN-γ-KO mice. Microscopic lesions were seen only in the brains of IFN-γ-KO mice. Results of this study indicate that iNOS and eNOS are not major mediators of resistance to *S. neurona* infections. Results of this study suggest that IFN-γ mediated immunity to *S. neurona* may be mediated by non-NO-dependent mechanisms.

1. INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is a neurologic syndrome in horses from the Americas and is usually caused by infection with the apicomplexan parasite, *S. neurona* (Dubey et. al., 1991). The condition has been recognized for more than 20 years in horses from the Americas (reviewed by Dubey et. al. (2001b)) and EPM is considered the most important protozoal disease of horses in the Americas (MacKay et al., 1992). Serological surveys using the Western Blot test indicate that 33-60% of horses have antibodies to *S. neurona* (see Dubey et al., 2001b) indicating that exposure to the parasite is high. Sporocysts excreted by opossums (*Didelphis virginiana* or *Didelphis albiventris*) are the source of *S. neurona* infection (Dubey and Lindsay, 1998; Tanhauser et al., 1999; Dubey et al., 2001a) and this explains why this disease is only observed in the Americas in the range of the opossum. The nine-banded armadillo, *Dasypus novemcinctus*, is a natural intermediate host (Cheadle et al., 2001a; Tanhauser et al., 2001). Domestic cats (*Felis domesticus*) and striped skunks (*Mephitis mephitus*) are experimental intermediate hosts (Dubey et al., 2000; Cheadle et al., 2001c).

Outbred mice are resistant to infection with *S. neurona* infection whereas certain immunodeficient mice are susceptible to *S. neurona* infection. For example, nude mice may suffer disease and death when inoculated parenterally with merozoites (Marsh et al., 1997) and interferon-γ-knockout (IFN-γ-KO) mice fed sporocysts of *S. neurona* will develop clinical encephalitis (Dubey and Lindsay, 1998; Cheadle et al., 2001b). These studies indicate that T cell mediated immunity and IFN-γ are important in resistance to *S. neurona* infection.

Nitric oxide (NO) is derived from L-arginine oxidation by nitric oxide synthase (NOS). NO is a free radical with multiple functions. It has been implicated in the functioning of diverse biological systems including the immune and cardiovascular systems. NO is important in immunity to some protozoan parasites, such as *Leishmania major* (Li et al., 1999), *Toxoplasma gondii* (Shluter et al., 1999), and malarial parasites (Taylor-Robinson and Smith, 1999). Recent molecular genetic studies have shown that NOS exists in three isoforms that are encoded by separate genes and are regulated by diverse signaling pathways (Huang and Fishman, 1996; Ma et al., 1996). These include neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). nNOS and eNOS are constitutively expressed in low concentrations, while iNOS is induced in response to cytokines such as IFN-γ, TNF and MIF, and LPS. The present study

was done to determine whether or not mice deficient in NO production of two isoforms were susceptible to *S. neurona* infection.

2. MATERIALS AND METHODS

2.1. S. neurona sporocysts

Sporocysts of *S. neurona* were collected from the intestine of a naturally infected opossum (SN15-OP of Dubey, 2000). Bioassay in birds and mice indicated that some sporocysts of *S. falcatula* were also present (Dubey, 2000). Sporocysts were counted in a hemacytometer and approximately 4 x 10^4 were orally fed to each mouse. The identity of merozoites isolated from the brains of inoculated mice was examined by PCR using primers JNB33 (5'-CGAACAGAGATGAGGAAAAT-3') and JNB54 (5'-GTTGTGGTGTTGCGTGAGTC-3') described by Tanhauser et al. (1999) and the methods of Lindsay et al. (2000a). The PCR products were digested separately with the restriction enzymes *Hin*f I or *Dra* I (Promega, Madison, WI) and were analyzed by electrophoresis on 1% agarose gel with appropriate size markers. Merozoite DNA from merozoites of the SN7 strain of *S. neurona* was used as a positive *S. neurona* control and DNA from merozoites of the Cornell strain of *S. falcatula* wre used as a control for *S. falcatula*.

2.2. Mouse strains and examinations

Two strains of NOS knockout (iNOS-KO and eNOS-KO) mice and one strain of IFN-γ gene knockout (IFN-γ-KO) (BALB/c-Ifng^{tm1ts}) mice were used to examine their susceptibility to infection with *S. neurona* sporocysts. The eNOS-KO mice were obtained from the laboratories of Drs. P.L. Huang and M.C. Fishman, Massachusetts General Hospital, Harvard Medical School, Boston, MA. They consisted of three males and three females. These mice were of mixed background, F2 background cross of SV 129 and C57BL/c strain. The iNOS (C57BL/6 genetic background, *N* = 9) and IFN-γ-KO mice (BALB/c genetic background, *N* = 5) were obtained from Jackson Laboratories, Bar Harbor, ME, and were females. The IFN-γ-KO mice served as positive controls for susceptibility (Dubey and Lindsay, 1998; Cheadle et al., 2001b). Control mice consisted of C57BL/6 (*N* = 9) and BALB/c mice (*N* = 6) (Jackson Laboratories) either fed sporocysts or not fed sporocysts (Table 1).

In unrelated studies, we have found that supernatants of Con-A stimulated splenic lymphocytes from the same source IFN-γ-KO mice do not have IFN-γ as determined by ELISA (Kapuzoglu-Sahin et al., 2001a,b). The lack of IFN-γ in these IFN-γ-KO mice confirms that the IFN-γ gene is deleted. Similar cultures of iNOS-KO mice from the same source had relatively reduced levels of IFN-γ (~2000 pg/ml) compared to wildtype C57BL/c (10,000 pg/ml). Neither

IFN-γ-KO nor iNOS-KO mice have detectable levels of NO as measured by the Griess reaction assay (Sigma Chemical, St. Louis, MO) (Karpuzoglu-Sahin and Ansar Ahmed, unpublished).

At necropsy, the brain was removed and fixed in 10% neutral buffered formalin solution and processed routinely for histological examination after hematoxylin and eosin staining. Histological sections were coded with a number and examined for lesions by one of us (RD) without knowledge of mouse strain or inoculation status.

3. RESULTS

A 1100 bp PCR product was observed for reactions using DNA from merozoites isolated from the brains of infected IFN-γ-KO mice and the SN7 strain of *S. neurona*. The reaction products were cut into 884 and 216 bp segments by *Dra* I but were not digested by *Hin*f I (data not shown) indicating that the isolated merozoites were those of *S. neurona*.

All five IFN-γ-KO mice developed clinical signs of encephalitis and four were euthanized when clinically ill on days 28, 29, 30, and 31 after infection. One mouse was found dead 30 days after infection (Table 1). Microscopic lesions were present in the brains of these mice and were associated with *S. neurona* schizonts and merozoites. Lesions were similar in all IFN-γ-KO mice. There was a moderate multifocal meningoencephalitis characterized by infiltrates of lymphocytes, neutrophils and eosinophils in the meninges and the Virchow-Robins spaces of vessels that penetrated the neutrophil. There was exudation of neutrophils and eosinophils into the neutrophil with obscuration of the preexisting architecture. Lesions were most severe in the cerebellum and tended to center on blood vessels penetrating into the molecular layer from the meninges. There was congestion of adjacent vessels with endothelial cell hypertrophy and exudation of eosinophils and neutrophils.

One inoculated female eNOS-KO mouse was observed to have clinical signs consisting of body flipping 13 days after inoculation. It was euthanized and its condition was not attributed to *S. neurona* infection. None of the other inoculated eNOS-KO, iNOS-KO, C57BL/c or BALB/c mice developed clinical signs. Microscopic lesions were not observed in the brains of these mice. None of the noninoculated iNOS-KO, C57BL.c or BALB/c mcie developed clinical signs. Microscopic lesions were not observed in the brains of these mice.

4. DISCUSSION

The susceptibility of IFN-γ-KO mice, but not the wild type BALB/c or C57BL/6 mice to *S. neurona* sporocyst infection strongly implies that IFN-γ may be a central cytokine conferring immunity against *S. neurona*. Further, it is of interest that iNOS-KO mice secrete IFN-γ albeit at reduced levels. It is tempting to speculate that reduced amounts of IFN-γ may be sufficient to induce protection. It is well known that IFN-γ activates iNOS gene through inducing IRF-1 transcriptional factor that binds to the promotor of the iNOS gene. NO has been thought to be involved in the physiological functioning of the central nervous, peripheral nervous, immune and the cardiovascular systems (reviewed by Huang and Fishman, 1996). We, therefore, examined whether iNOST-KO are as susceptible as IFN-γ-KO mice to *S. neurona* infection. This suggests that protection conferred by IFN-γ is not mediated through IFN-γ induced NO. Rather, this study suggest that IFN-γ mediated immunity to *S. neurona* may be mediated by non-NO-dependent mechanisms. Unfortunately, nNOS mice are not commercially available and we were not able to examine the role of nNOS in resistance to sporocyst induced *S. neurona* infections. In a pilot study (Lindsay et al., 2000b), we did find that nNOS and eNOS mice were resistant to merozoite induced infections with *S. neurona*.

It is interesting to note that our results with iNOS-KO and eNOS-KO mice and *S. neurona* are similar to those of others with iNOS-γ-KO mice and *T. gondii* infections. It has been shown that iNOS is not essential for protection or for long-term immunity to toxoplasmosis (Khan et al., 1996-1998).

Presently, there is no immunocompetent rodent model for EPM. It is well known that the strains of mouse and *T. gondii* can influence the outcome of experimental infections with this parasite. It is also apparent that mouse strain can influence the susceptibility to *S. neurona*. For example, ICR SCID mice which are defective in both T and B cell immune responses were resistant to parenteral inoculation of *S. neurona* merozoites, while nude C57BL/6 mice defective in T cell immune responses developed infections (Marsh et a., 1997). These findings suggest that host genetic background may also influence host susceptibility to *S. neurona*. Marsh et al. (1997) demonstrated that C57BL/6 mice were resistant to merozoite induced infections in the present study. This is important because merozoites of *S. neurona* may lose their pathogenicity when passed in cell cultures (Dubey et al., 2001c). We also have demonstrated that BALB/c

mice are resistant to sporocyst induced infections. Further studies into immunocompetent mouse strain susceptibility to *S. neurona* are warranted because of the lack of a rodent model.

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Table 1. Results of feeding *S. neurona* sporocysts to control and immunodeficient mice.

Mouse strain	Inoculated	Day killed (k) or died (d)	Lesions
IFN-γ-KO	Yes	28 k	Yes
IFN-γ-KO	Yes	29 k	Yes
IFN-γ-KO	Yes	30 k	Yes
IFN-γ-KO	Yes	30 d	Yes
IFN-γ-KO	Yes	31 k	Yes
BALB/c	Yes	50 k	No
BALB/c	Yes	50 k	No
BALB/c	Yes	64 k	No
BALB/c	No	36 k	No
BALB/c	No	50 k	No
BALB/c	No	64 k	No
iNOS	Yes	36 k	No
iNOS	Yes	36 k	No
iNOS	Yes	50 k	No
iNOS	Yes	50 k	No
iNOS	Yes	64 k	No
iNOS	Yes	64 k	No
iNOS	No	36 k	No
iNOS	No	50 k	No
iNOS	No	64 k	No
eNOS	Yes	36 k	No
eNOS	Yes	50 k	No
eNOS	Yes	50 k	No
eNOS	Yes	64 k	No
eNOS	Yes	64 k	No
C57BL/6	Yes	36 k	No
C57BL/6	Yes	50 k	No
C57BL/6	Yes	50 k	No
C57BL/6	Yes	64 k	No
C57BL/6	Yes	64 k	No
C57BL/6	No	36 k	No
C57BL/6	No	36 k	No
C57BL/6	No	50 k	No
C57BL/6	No	64 k	No

CHAPTER 11

VITA

Alexa Chamonix Rosypal was born January 18, 1978 in Richmond, Virginia. She received her high school diploma from the Collegiate School in Richmond in 1996. She graduated with her Bachelors of Science degree in Animal and Poultry Sciences from Virginia Tech in May 2000. In July 2000 she entered graduate school at the Virginia-Maryland Regional College of Veterinary Medicine at Virginia Tech to pursue her doctoral degree in Parasitology. While at VMRCVM, she became a Morris Animal Foundation Fellow (2001-2005). She is an active member of several professional societies including, American Association of Veterinary Parasitologists (AAVP), American Society of Parasitologists (ASP), American Society of Tropical Medicine and Hygiene (ASTMH), and Southeastern Society of Parasitologists (SSP).