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Immunologic progression of canine leishmaniosis following vertical transmission in United States dogs

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ABSTRACT

Canine leishmaniosis (CanL) is caused by *Leishmania infantum*, an obligate intracellular protozoan parasite, endemic in U.S. hunting dog populations. CanL has been found in dogs in 28 states and two Canadian provinces. Previous studies by our group, (Boggiatto et al., 2011), demonstrated that vertical transmission of *Leishmania* was the predominant means of transmission within U.S. dogs. Very little is known regarding how this alternative means of transmission, alters the long-term immunity and clinical presentation of leishmaniosis in dogs born to a positive bitch. This study follows the immunological progression of CanL in three pups after birth to an infected bitch. During the course of the study, these dogs were tested every six months over the course of six years. Both immunologic (IFN-γ, T cell proliferation, antibody production) and parasitological parameters (qPCR) of vertically-infected dogs were measured. Within the six years after birth to an *L. infantum*-infected, oligosymptomatic bitch, all dogs had at least one *L. infantum* PCR-positive test. Interestingly, despite living in the same location for their entire lives and being full siblings, these pups demonstrate three different disease progression patterns of *L. infantum* infection. One dog progressed to oligosymptomatic disease, maintaining a positive titer and had intermittent positive PCR results. One asymptomatic dog had positive serological titers and demonstrated a robust $CD4^+$ immune response to infection. The third dog had a negligible response to *L. infantum* antigen and was healthy. This work demonstrates the biologic variability associated with vertically-transmitted infection similar to the variety of presentations observed during vector-borne leishmaniosis.

1. Introduction

Leishmaniasis is a spectral vector-borne disease caused by intracellular protozoan parasites of the genus *Leishmania*. Natural hosts include dogs and humans (Dantas-Torres et al., 2012; Millan et al., 2014; Ravendra and Anuradha, 2006). *Leishmania* is endemic in human and canine populations in more than 98 countries affecting more than 1 million people per year and countless dogs (Alvar et al., 2013). Global canine mortality due to leishmaniosis is unknown. Data generated based on CanL seropositive dog culling in multiple countries including Brazil, suggests that canine mortality associated with CanL is 20-30 times greater than that of humans (Alvar et al., 2013; Costa et al., 2007; Esch et al., 2012; Palatnik-de-Sousa et al., 2001). Visceralizing leishmaniasis, due to its systemic presentation, is commonly termed leishmaniosis. Signs of CanL include: weight loss, lethargy, decreased appetites, enlarged lymph nodes, kidney failure and immune compromise (Oliveira et al., 2010; Solano-Gallego et al., 2011).

Leishmaniosis is not commonly found in people within the U.S. but has become endemic within the hunting dog population. The first documented autochthonous hunting dog infection was reported in 1980 in Oklahoma (Anderson et al., 1980). The first large outbreak of CanL occurred in 1999 in the Northeastern U.S. Follow-up studies demonstrated that more than 18 U.S. states and 2 Canadian provinces had serologically positive hunting dogs (Duprey et al., 2006; Gaskin et al., 2002). Since then, CanL has spread further within these hunting dog populations to 35 states and 2 Canadian provinces (Petersen, 2009; Schantz et al., 2005). The current prevalence of leishmaniosis in a specific breed of U.S. hunting dogs is greater than 3 per 1000 dogs (Toepp et al in preparation).

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Multiple diagnostic techniques can be used to assess presence or absence of *Leishmania* infection. For the purposes of this study, real time quantitative polymerase chain reaction (qPCR) was used to identify and quantitate parasitic infection. Immune response to infection was evaluated by antibody titer, T cell proliferation and IFN-γ production. Measurement of canine IFN-γ-producing CD4+ T cells was shown previously to predict T helper 1-based immune control of infection, or loss thereof (Boggiatto et al., 2010).

To date, no naturally *L. infantum*-infected sand fly within the U.S. has been found and therefore no hunting dog leishmaniosis case has been determined to be secondary to sand fly transmission (Duprey et al., 2006). Instead vertical transmission was shown to be the predominant means of transmission in U.S. hunting dogs (Boggiatto et al., 2011). This study found that 10 of 12 naturally infected puppies had *L. infantum* parasites in multiple organs, including bone marrow, liver, lymph node, lung, spleen, thymus, blood, and umbilicus (Boggiatto et al., 2011). Multiple other studies have demonstrated that vertical transmission is not unique to these dogs or the U.S. (da Silva et al., 2009; Masucci et al., 2003; Naucke and Lorentz, 2012; Pangrazio et al., 2009; Rosypal et al., 2005).

Although there have been cross-sectional and case studies of vertically transmitted *L. infantum* infection in dogs, CanL progression from birth after vertical transmission has not been evaluated (Avila-Garcia et al., 2013; da Silva et al., 2009; Esch and Petersen, 2013; Gibson-Corley et al., 2008; Osorio et al., 2012; Pangrazio et al., 2009; Tanczos et al., 2012). This study follows three hunting dogs from birth as part of the litter described in (Boggiatto et al., 2011). These pups were born to a naturally infected, oligosymptomatic, dam within a controlled laboratory facility then returned to their kennel of origin and followed over six years. We hypothesized that by observing this cohort over time we would see similar immune progression and development of clinical leishmaniosis as previously described after classical, vector-borne transmission.

2. Materials and methods

2.1. Animals

Three pups born to an *L. infantum* infected, oligosymptomatic dam from a litter of 15 (12 euthanized as part of Boggiatto et al., 2011) were followed from birth until age 6. These dogs were sampled every 6 months to evaluate *L. infantum* infection status (Fig. 1). Animals were enrolled with informed consent of the owner and followed with approval by the Iowa State University and University of Iowa Animal Control and Use Committees (IACUC). Animals were housed according to AAALAC accreditation standards.

2.2. Serology (IFA)

Canine serum samples were stored at −20 °C and sent to the Centers for Disease Control and Prevention (CDC) for IFAT and in vitro promastigote culture as previous described (Badaro et al., 1983; Duprey et al., 2006).

2.3. PCR

Quantitative real-time qPCR was used to assess parasite ribosomal and/or kinetoplast DNA isolated from whole blood as previous (Boggiatto et al., 2011; Esch et al., 2013). *L. infantum* kinetoplastid specific primer sets and probe were used for qPCR diagnostic tests run from 2009-2011 F,5′-CCGCCCGCCTCAAGAC and R, 5′-TGCTGAATATTGGTGGTTTTGG (Integrated DNA Technologies, Coralville, IA), probe [6-FAM]-AGCCGCGAGGACC-3′ MGB (Applied Biosystems, Foster City, CA). Ribosomal specific *Leishmania spp.* primer and probe were used in 2010 through present F 5′-AAGCCACCCCAGAGGTAAAAA, R 5′ GACGGGTCTGACCCTTGGTT, (Integrated DNA Technologies, Coralville, IA), probe 5′ 6FAM-CGGTTCGGTGTGTGGCGCC-MGBNFQ (Applied Biosystems, Foster City, CA). Results were analyzed using ABI 7000 System SDS Software (Applied Biosystems, Life Technologies, Grand Island, NY).

2.4. CD4± T cell proliferation and IFN-γ production via FACS analysis

Canine CD4+ T cell IFN-γ production and proliferation were assessed by intracellular flow cytometry labeling and BrdU or EdU incorporation, respectively, as previously described by (Esch et al., 2013). Briefly, peripheral blood mononuclear cells were isolated plated in 96-well tissue culture, round bottom plates at 5×10^5 cells per well. Cells were stimulated with 1 μg/mL of freeze-thawed *L. infantum* antigen for 7 days. Brefeldin-A (Sigma–Aldrich, St. Louis, MO) was added to each well at a final 1X concentration per manufacturer's recommendation 6 hours prior to intracellular labeling. Interferon-γ was labeled with anti-Bovine-RPE clone CC302 (AbD Serotec, Raleigh, NC). Cells were fixed with BD™ Stabilizing Fixative (BD Biosciences, Franklin Lakes, NJ). Cells were collected for analysis utilizing an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo vX (Treestar, Ashland, OR).

2.5. Statistical analysis

Statistical analyzes were completed using Excel (Microsoft, Redmond, WA) and Graph Pad Prism version 6.05 (Graph Pad Software Inc., La Jolla, CA).

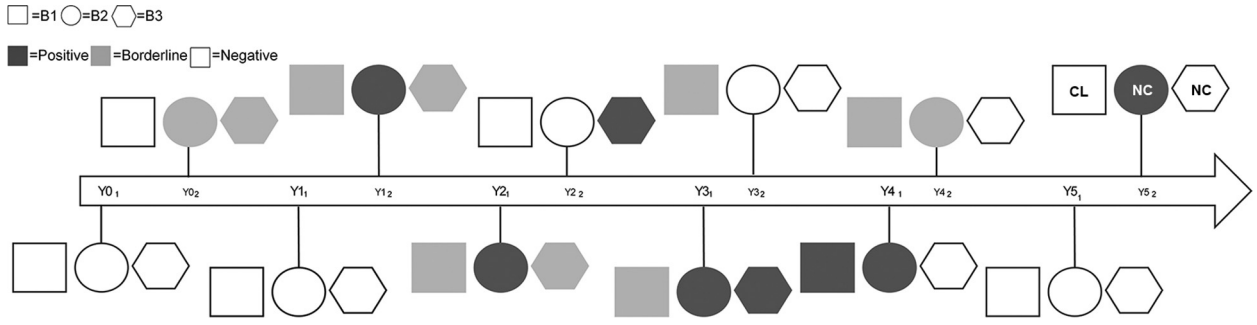

Fig. 1. Timeline of diagnostic progression of *Leishmania*-infected dogs. Dog B1 = square, B2 = circle, and B3 = hexagon. Positive qPCR or serology = dark grey. Borderline qPCR or a CDC IFAT titer of 1:16 or 1:32 = light grey. Negative for all diagnostic tests = white. Clinical status provided at last time point; CL indicates dog had multiple signs of clinical CanL, NC indicates one or no signs of disease based on physical exam.