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Original article

Leishmania incidence and prevalence in U.S. hunting hounds maintained via vertical transmission

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ABSTRACT

Leishmania is the causative agent of leishmaniasis, a deadly protozoan disease which affects over 1 million people each year. Autochthonous cases of canine leishmaniasis are generally associated with tropical and subtropical climatic zones. However, in 1999, U.S. hunting dogs were found to have leishmaniasis with no history of travel outside the country. Transmission of this disease was found to be primarily vertical. In endemic areas, dogs are a dominant domestic reservoir host for *Leishmania infantum*. This study evaluated *L. infantum* infection prevalence and incidence within US dogs tested over a nine-year span (2007–2015). This investigation used both passive and active surveillance, following an initial outbreak investigation by the Centers for Disease Control. *L. infantum* infection incidence and prevalence over time and across regions were examined to evaluate whether transmission was sufficient to maintain ongoing infection within this population. These studies also established whether this disease is becoming more or less prominent within this reservoir host, dogs. There was no significant difference between prevalence and incidence rates via as measured by passive vs. active surveillance. Although due to fluctuations in sample submission there were significant changes in both incidence and prevalence of *L. infantum* in US hunting dogs over this nine year span, these differences were not outside of the interquartile range and therefore there is likely to be a steady-state of transmission within U.S. dogs. Based on these findings, if vertical transmission is the primary means of *L. infantum* spread in U.S. dogs, with appropriate husbandry and infection control procedures, elimination of *L. infantum* from US dogs could be possible.

1. Introduction

Leishmaniasis is a zoonotic disease predominantly transmitted by the bite of an infected sand fly. In non-endemic areas vertical transmission may be a predominant means of transmission. *Leishmania* infects and causes combined disease in approximately 1 million people per year with visceral disease leading to > 20,000 deaths annually (Alvar et al., 2012; Ready, 2014). Visceral leishmaniasis (VL), is characterized by lymphadenopathy, splenomegaly, hepatomegaly and renal failure. VL is fatal if not treated once symptoms develop (Murray et al., 2005). *Leishmania* spp. infection causes the second largest number of parasite-induced human mortalities globally (den Boer et al., 2011). *Leishmania* also infects and causes diseases within millions of dogs around the globe (Colwell et al., 2011; Dantas-Torres, 2009; Reguera et al., 2016). While many dogs are asymptomatic for disease they can progress to clinical stages of disease with both cutaneous and visceral signs. The clinical signs are similar to humans including lymphadenopathy, renal failure, and severe weight loss (Otranto and Dantas-Torres, 2013; Palatnik-de-Sousa, 2012).

For thousands of years, dogs have been domesticated human companions, working or guard dogs (Killian, 2007), with full integration into human life. The dog is a natural host for *Leishmania* in endemic countries. Dogs live with or near humans, propagating a domestic transmission cycle in areas with sufficient sand fly vector populations (Costa et al., 2013). In the United States, the hunting hound population is endemic for canine leishmaniasis (CanL). The first recognized case of CanL in the U.S. was reported in 1980 in Oklahoma (Anderson et al., 1980) however it was not until 1999 that an outbreak of disease in New York prompted broader disease surveillance and awareness (Gaskin et al., 2002). Subsequent testing demonstrated that several thousand dogs were infected with *L. infantum* in the U.S. (Petersen, 2009; Petersen and Barr, 2009). Although epidemiological studies have demonstrated incidence and frequencies of CanL in Asia (Rosypal et al., 2010), Europe (Franco et al., 2011; Mattin et al., 2014; Sifaki-Pistola

Abbreviations: VL, visceral leishmaniasis; CanL, canine leishmaniasis; PCR, polymerase chain reaction

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et al., 2014) and Latin/South America (Acosta et al., 2015; Campos et al., 2017; D'Andrea et al., 2015; Franco et al., 2011; Leca Junior et al., 2015) studies in the United States, where vector transmission has not been found, have been limited (Duprey et al., 2006). To understand the impact of the U.S. CanL outbreak in 1999 and subsequent stability of transmission within the United States, we initiated a prospective study of *L. infantum* infection within the affected hunting hound population while maintaining our laboratory's ongoing passive surveillance of the disease.

Active surveillance in United States dogs performed by our group and others, reported in reviews since the 1999 outbreak, indicated that dogs throughout the Eastern and Midwest regions were infected with *L. infantum* (Duprey et al., 2006; Petersen, 2009; Petersen and Barr, 2009). The primary transmission route of *Leishmania* in these infected dogs was transplacental, supported by significant evidence from our group and others (Boggiatto et al., 2011; Gibson-Corley et al., 2008; Rosypal et al., 2005). Vertical transmission has also been identified in dogs residing in endemic areas (Caldas et al., 2003; da Silva et al., 2009; Figueiro-Filho et al., 2004). Despite multiple studies, and proof that vector transmission using *Lu. longipalpis* is experimentally possible from these hounds to other mammalian hosts (Schaut et al., 2015), there is no to date evidence of vector involvement in the transmission of *Leishmania* through hunting hounds in North America (Duprey et al., 2006; Schantz et al., 2005; Schaut et al., 2015; Weng et al., 2012). The possibility of vector transmission still remains, but in the meanwhile very little is known about how vertical transmission propagates this disease within a population.

The present study used qPCR analysis to establish trends in active infection, which has a 55% sensitivity and 100% sensitivity in our hands to detect disease (Larson et al., 2017). This is in comparison to previous studies, which examined *Leishmania* seroprevalence. This study used both active and passive surveillance techniques and thus evaluated whether there are significant differences in the demographics, prevalence and incidence rates reported, and overall trends identified via these two surveillance techniques. More awareness of the continued risk of this disease within U.S. hunting hounds and prevention or control options are necessary as there is ongoing transmission of this disease. Furthermore, more targeted risk management methods, including not breeding infected dogs and canine vaccination, will be necessary if this disease is to be controlled or eliminated from U.S. dogs.

2. Materials and methods

2.1. Active surveillance cohort

Through collaborations with kennels in the Midwest an active surveillance cohort of 4 large (> 50 dogs each) kennels was established over this 9 year period. Animals were enrolled with informed consent of the caretakers and followed protocols as approved by the Iowa State University and University of Iowa Institutional Animal Care and Use Committees (IACUC). The Petersen laboratory visited each of these kennels annually for at least three years, at which point two of the kennels elected to control leishmaniasis in their kennel via euthanasia. Licensed veterinarians collected 1–5 cm^3 whole blood and serum from all dogs present at these kennels. Demographic information regarding sex and age were also collected. The active surveillance cohort testing period extended from 2007 to 2015. This surveillance effort started eight years, or at least one hunting-dog life-span, after the major reported *L. infantum* outbreak in 1999.

2.2. Passive surveillance cohort

The Petersen laboratory has developed an ongoing collaboration with a hunting hound organization in the US and is a laboratory for *Leishmania* diagnostic testing for this group and others. Due to this relationship, the Petersen laboratory maintains passive surveillance for *Leishmania* based on samples sent to us from this hunting hound group for voluntary diagnostic testing.

2.3. Blood collection/diagnostic status

Across both active and passive groups, over 9 years, 21 kennels participated in the study. This was a total of 1699 samples (~188 per year). Peripheral blood was collected from dogs in heparinized or EDTA-treated tubes, aliquoted and stored at − 80 °C until used for DNA isolation and analysis. Animals were divided into 2 diagnostic groups (positive and negative). All qPCR was completed in duplicate with two full strength and two 1:10 dilutions. Dogs with 1 or more positive wells were considered positive. Negative samples had no amplification in any of the 6 wells/dog. These diagnostic cut-offs were based on qPCR standards and controls as previously described (Boggiatto et al., 2011, 2010; Schaut et al., 2016; Vida et al., 2016).

2.4. DNA isolation/qPCR

DNA was isolated from 1 mL canine blood using QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) per manufacturer's specifications. Isolated DNA was checked for quantity and quality using a NanoDrop 2000 (Thermo Scientific, Waltham, MA). RT-qPCR was performed as previously described (Boggiatto et al., 2010; Esch et al., 2013; Vida et al., 2016). Briefly, isolated DNA was tested for RT-qPCR in duplicate at full strength and in 1:10 dilutions in a 96 well plate format. All RT-qPCR plates included positive and negative controls. Primer sets for kinetoplastid DNA optimized to *L. infantum*: F 5′-CCGCCCGCCTCAAGAC, R 5′-TGCTGAATATTGGTGGTTTTGG, (Integrated DNA Technologies, Coralville, IA) and TaqMan probe, 5′-6FAM-AGCCGCGAGGACCMGBNFQ, were used (Applied Biosystems, Foster City, CA). Kinetoplastid primer and probe targets were used from 2007 to 2011. Ribosomal primer sequences were as follows: F 5′-AAGCCACCCCAGAGGTAAAAA, R 5′-GACGGGTCTGACCCTTGGTT (Integrated DNA Technologies). TaqMan probe was 5′-6FAM-CGGTTCGGTGTGTGGCGCC-MGBNFQ (Applied Biosystems). Ribosomal primer and probe targets were used from 2012 to 2015. All assays were performed using Super MasterMix (Rox) (Quanta Biosciences, Gaithersburg, MD) on an ABI 7000 system (Applied Biosystems). ABI 7000 System SDS Software (Applied Biosystems) was used for RT-qPCR analysis.

2.5. Statistical analyses

Prevalence rates were determined as the number of qPCR positive dogs in that year/total number of dogs tested in that year. Incidence rates were determined as the number of new qPCR positive or borderline dogs in that year/total number of dogs tested in that year. All prevalence and incidence rates were reported as a rate per 1000 dogs so that the two surveillance techniques, active and passive, could be appropriately compared. Student's *t*-tests were used to determine whether there were statistically significant differences in the ages of the two surveillance cohorts. D'Agostino & Pearson normality test was performed to determine normality in both the passive and active cohorts. Both active and passive prevalence and incidence patterns were assessed via Pearson's correlation to determine whether the patterns were significantly similar. A paired *t*-test was used to determine whether overall there was a statistically significant difference rates between the two surveillance techniques. A chi-squared test for trend was completed to determine whether the prevalence and incidence changes over time had a linear trend. The mean prevalence and incidence were calculated for both active and passive surveillance across the study. To determine if there were significant fluctuations/changes in prevalence and incidence rates compared to the mean biologically relevant outliers were identified as single year rates outside of the interquartile range (IQR:25–75%). Graphical analyzes and calculations of incidence and prevalence were completed using Excel (Microsoft, Redmond, WA), SAS