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Short communication

First detection of *Leishmania infantum* DNA within the brain of naturally infected dogs

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ABSTRACT

Visceral leishmaniasis is an anthropozoonosis caused by the protozoan *Leishmania infantum* (*L. chagasi*). In dogs, the disease presents with systemic manifestations, including neurological disorders. There are rare reports of the presence of the parasite in the central nervous system of infected dogs, and some evidences of inflammatory lesions and the breakdown of cerebral barriers have been described. The aim of this study was to investigate the presence of *L. infantum* DNA in five specific areas of the brains of 20 naturally infected dogs by real-time PCR. For the first time, the presence of parasite DNA was detected and quantified in the brains of naturally infected dogs, in all evaluated regions. These data provide strong evidence of the presence of the *Leishmania* parasite in the nervous milieu and contribute to a new perspective of the pathogenesis of visceral leishmaniasis.

1. Introduction

Leishmaniasis may develop in two major forms: cutaneous leishmaniasis and visceral leishmaniasis (VL). Visceral leishmaniasis is an anthropozoonosis caused by *Leishmania infantum* (syn. *L. chagasi*) in the Americas and in the Mediterranean basin and by *Leishmania donovani* in Asia and Africa (Mauricio et al., 2000; Alvar et al., 2004; Lukeš et al., 2007; Baneth et al., 2008).

Domestic dogs are the main host of *L. infantum*, and the clinical presentation may range from asymptomatic to systemic manifestations characterized by fever, anemia, progressive weight loss, hepatomegaly, splenomegaly, renal alterations, skin disease, ocular lesions, disorders of the cardiovascular and respiratory system (García-Alonso et al., 1996a; Ciaramella et al., 1997; Blavier et al., 2001; Reis et al., 2009).

Although there are many reports of systemic manifestations, few authors have observed the occurrence of lesions in the central nervous system (CNS) in dogs with VL. There have been reports on parasite migration and the deposition of immunoglobulins and antigens in the CNS of infected dogs (García-Alonso et al., 1996b; Nieto et al., 1996; Viñuelas et al., 2001). More recently, José-López et al. (2012) noticed multiple brain infarcts in two dogs with VL, and Márquez et al. (2013) verified by immunohistochemistry the presence of *L. infantum* amastigotes in the spinal nerves, spinal cord, brain parenchyma and choroid plexus of one chronically infected dog. Furthermore, findings such as glial cell activation, meningitis and choroiditis have also been detected (Nieto et al., 1996; Viñuelas et al., 2001; Melo et al., 2009, 2013; Melo and Machado, 2011; José-López et al., 2012).

The brain is protected from the bloodstream by the blood–brain barrier (BBB) at the level of parenchymal

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blood vessels, and by the blood–cerebrospinal fluid barrier (BCSFB) at the level of the choroid plexus (Dziegielewska et al., 2001; Persidsky et al., 2006). There are evidences of brain barrier breakdown in dogs with VL, mainly via the deposition of *Leishmania* antigen in the walls of blood vessels and in the choroid plexus, along with high antibody titers in the cerebrospinal fluid (CSF), inflammatory cell infiltrates in the choroid plexus, periventricular areas and leptomeninges, and glial cell activation (García-Alonso et al., 1996b; Ikeda et al., 2007; Melo and Machado, 2011; Melo et al., 2009, 2013)

Despite these findings, the pathogenesis of visceral leishmaniasis in the CNS is not fully clarified. Thus, due to the involvement of the barriers and the possibility of parasite migration toward the brain of dogs with VL, the aim of this study was to investigate and quantify the presence of *L. infantum* DNA in specific areas of the encephalon of infected dogs.

2. Material and methods

2.1. Animals

Twenty dogs, twelve male and eight female, ranging in age from 1 to 6 years old that were selected from the Veterinary Teaching Hospital of UNESP, São Paulo State University and from the Zoonosis Control Center in the municipality of Araçatuba, São Paulo State, Brazil, which is an endemic area for VL. Blood and cerebrospinal fluid samples were collected and the dogs were euthanized with the owners' permission, in compliance with state law (São Paulo, 2006). VL diagnosis was achieved using a routine ELISA (enzyme-linked immunosorbent assay) and popliteal lymph node fine-needle aspiration. None of the animals were previously vaccinated against VL. The animals did not present a history of neurological signs and they were also serologically negative for toxoplasmosis and neosporosis, as assessed by indirect immunofluorescence assays.

2.2. Sampling

The dogs were euthanized with an overdose of pentobarbital (Nembutal®) and potassium chloride. Necropsies were performed immediately after euthanasia, when macroscopic lesions were evaluated. The brains were collected and separated into two hemispheres; one of which was placed in 10% buffered-formalin. After fixation, fragments of five areas of the central nervous system were separated and embedded in paraffin (Fig. 1). Tissue sections of 15 μm were used for DNA extraction.

2.3. Serological exam

Routine ELISA tests on serum were performed to diagnose *Leishmania* infections (Lima et al., 2005). ELISA tests were also performed to detect the presence of anti-*Leishmania* antibodies in the CSF, as described by Lima et al. (2003). If any blood contamination was observed, the CSF sample was discarded.

2.4. PCR

Fifteen micrometer sections of formalin-fixed and paraffin-embedded (FFPE) tissue were collected. The paraffin was removed following the protocol of Müller et al. (2003), with some modifications. DNA extraction was performed with the UltraClean® BloodSpin® DNA Isolation kit (MoBio Labs, CA, USA), according to the manufacturer's instructions. DNA was stored at −80 °C until real-time PCR was performed.

PCR analyses were carried out using the Step One Plus® Real-Time PCR System (Applied Biosystems Laboratories, Foster City, CA, USA). The PCR reactions contained 20 μM of each primer (Leish 1: 5′-AACTTTTCTGGTCCTCCGGGTAG-3′ and Leish2: 5′-ACCCCCAGTTTCCCGCC-3′), 10 μM of the TaqMan Probe (FAM-5′-AAAAATGGGTGCAGAAAT-3′-MGB) (Francino et al., 2006) and the iTaq Universal Probes Supermix (BioRad, USA).

In the thermocycler, samples were submitted to one incubation step at 50 °C for 2 min and an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A standard curve was produced using DNA from seven known quantities of *Leishmania* parasites (MCAN/ES/1996/BCN150, zymodeme MON-1) ranging from 250,000 to 0.25 parasites. The standard curve was extracted and analyzed together with the brain samples in triplicate. Samples were considered positive when the Ct was lower than 35 and when amplification was detected in all replicates.

2.5. Statistical analysis

The statistical evaluation of the parasite load in the five areas of the CNS was performed using the Friedman test, followed by Dunn's test. The Spearman test was performed to correlate the data from blood, CSF and brain. Statistical significance was accepted when $p < 0.05$. All statistical analyses were performed using Prism software (Prism 6, GraphPad).

2.6. Ethical issues

This study was approved by the institutional Ethics and Animal Welfare Committee (CEEA—Comissão de Ética e Experimentação Animal, UNESP, process no. 05/06).

3. Results

3.1. Parasite load in the CNS

The parasite load of the CNS samples was evaluated by qPCR using the Leish-1 and Leish-2 primers, which amplify a fragment of the kinetoplast DNA (kDNA) minicircle of the parasite. The presence of the *L. infantum* kDNA was detected in all areas of the CNS of all infected dogs, except two areas of two dogs (dog no. 5, area C; dog no. 9, area E). The standard curve allowed us to quantify the parasite load in the different parts of the brain. Amplification reactions obtained an efficiency value of 92.7% with a determination coefficient of $r^2 = 0.998$ and a slope of −3.509.

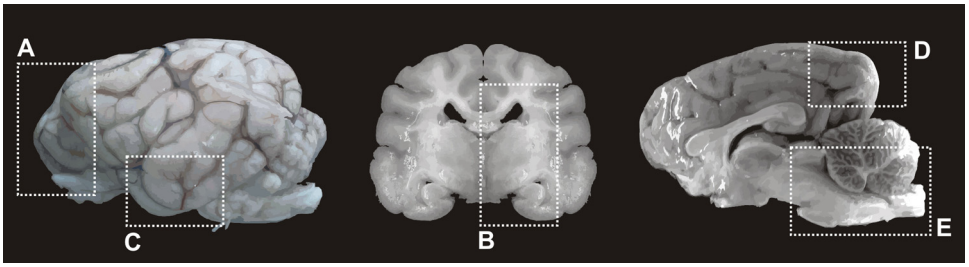

Fig. 1. Graphical representation of the brain areas included in this study. (A) frontal lobe; (B) hippocampus, diencephalon, including the lateral ventricle choroid plexus; (C) temporal lobe; (D) occipital lobe; (E) midbrain, brainstem and cerebellum, including the fourth ventricle choroid plexus.

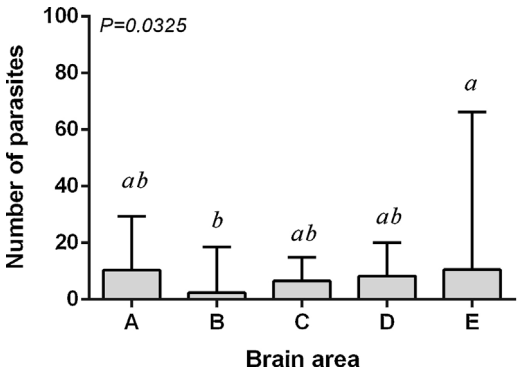

Fig. 2. Parasite load in the central nervous system (CNS) of dogs with VL. Columns represent the median (interquartile range). [ab] Columns with no common superscript letter differ significantly by Dunn's multiple comparison test ($p < 0.05$). Brain areas: (A) frontal lobe; (B) hippocampus, diencephalon, choroid plexus; (C) temporal lobe; (D) occipital lobe; (E) midbrain, brainstem, cerebellum and choroid plexus.

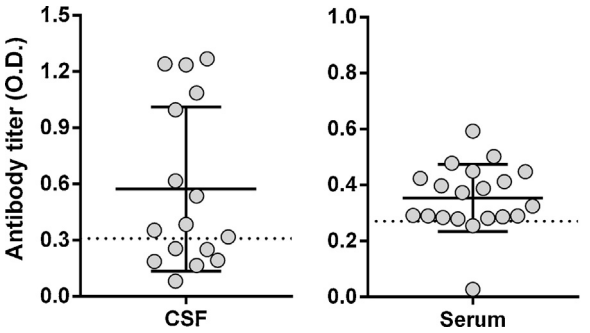

Fig. 3. Scatter plot presenting cerebrospinal fluid (CSF) and serum reactivity (IgG) against *Leishmania* antigens determined by ELISA. Antibody titers were measured by optical density (O.D.; absorbance at 492 nm). Horizontal lines indicate the mean and SD. The dotted lines represent the lower limit of positivity (cut-off): 0.309 for CSF and 0.270 for serum.

The detection range was from 0.84 to 125.22 parasites per section of area A, 0.44 to 144.25 parasites per section of area B, 0.36 to 173.08 parasites per section of area C, 0.96 to 1750.59 parasites per section of area D and 1.92 to 561.78 parasites per section of area E (Fig. 2). A significant difference in the parasite load was detected only between areas B and E ($p = 0.0325$). No amplification was observed in the samples used as the negative control.

3.2. Anti-L. infantum antibody levels in cerebrospinal fluid and sera of infected dogs

Of the infected dogs, 90% (18/20) presented serum antibody titers higher than the cut-off value, ranging from 0.279 to 0.593; 62.5% (10/16) of the infected dogs also presented antibody titers in the CSF higher than the cut-off value, ranging from 0.318 to 1268 (Fig. 3). Four CSF samples that presented blood contamination were discarded. There was no correlation between serum and CSF antibody titers ($p = 0.0715$). Moreover, there was no correlation between the antibody titer in the CSF ($p = 0.6564$) or in the serum ($p = 0.2635$) with the parasite load in the CNS.

3.3. Macroscopic lesions and brain histopathological analysis

The macroscopic alterations observed in the dogs during necroscopic examination and the histopathological findings of the dogs included in this study were previously published by Melo et al. (2009) and Melo and Machado (2011). Immunohistochemistry analysis also was performed, and the authors did not detect the presence of the parasite (Melo and Machado, 2011).

4. Discussion

There is little evidence of the migration of the *Leishmania* parasite toward the CNS of infected dogs. Although some authors have observed the presence of amastigotes of *L. infantum* in the meninges and choroid plexus (Nieto et al., 1996; Viñuelas et al., 2001) and in the peripheral nervous system (Márquez et al., 2013), other studies have failed to detect the parasite in the brains of dogs with VL (Ikeda et al., 2007; Melo and Machado, 2009, 2011).

In the study presented here, for the first time, *L. infantum* kDNA was detected and quantified in all evaluated brain areas of the infected dogs. This is strong evidence of the presence of the *Leishmania* parasite in the nervous system, corroborating the findings of Nieto et al. (1996) and Viñuelas et al. (2001) in natural infection. Our results indicate that there is no brain region of predilection for the detection of parasite kDNA in the brain, especially in the areas evaluated in the present study.

Although a significant statistical difference was found between the parasite load of areas E (brainstem and cerebellum) and B (hippocampus, diencephalon), we do not consider this difference to be pathologically important, considering that there was wide variability in the detection

range of the parasite in area E. Melo et al. (2009) and Melo and Machado (2011) also compared different areas of the brains of dogs with VL, and did not observe any differences in inflammatory infiltrates or glial cell alterations in the brain evaluated areas.

Even though the dogs included in this study did not present any neurological clinical signs, we observed high levels of anti-*Leishmania* antibodies titers in the CSF. There was no correlation between these titers and the brain parasite load. The same was observed for the serum. Other authors have also detected anti-*Leishmania* antibodies in the CSF of dogs with VL, despite the absence of the pathogen in the nervous system (García-Alonso et al., 1996b; Lima et al., 2003; Melo et al., 2009). This provides support to the affirmation that parasite DNA can be detected in the brain, regardless of the presence of antibodies in the serum and CSF.

Dogs with VL and CNS involvement may present neurological clinical signs, such as generalized seizures, tetraparesia, paraplegia and tetraplegia, ocular alterations, depressed mental status, walking in circles, vestibular and cerebellar signs (Font et al., 2004; Ikeda et al., 2007; José-López et al., 2012). In humans, the presence of neurological symptoms has also been observed in patients with VL, mainly, but not only, related to the peripheral nervous system (Chunge et al., 1985; Hashim et al., 1995; Diniz et al., 2010). The development of the disease to a more severe clinical status, including neurological signs, might take longer to occur, maybe by action of immune cells, inflammatory mediators, components of the parasite, and not necessarily the protozoan itself. The dogs in this study presented no history of neurological clinical manifestation, possibly because to the short time of infection, since in Brazil, the infected dogs must be euthanized as soon as the diagnosis is confirmed, due to the current legislation which prevents the treatment of canine VL (São Paulo, 2006). Thus, the detection of the parasite DNA within the brain might represent the early dissemination of *L. infantum* into the CNS, and this fact must be taken into account during the monitoring of both canine and human VL, especially in endemic areas.

In previous studies performed by our research group, *Leishmania* was not detected in the brain of dogs with VL, using HE-stained tissue sections (Melo et al., 2009) or immunohistochemistry (Melo and Machado, 2011). However, the activation of glial cells and leukocyte infiltration in the CNS, mainly by CD3+ T lymphocytes, could indicate breakdown of the brain barriers (Melo et al., 2009, 2013). A hypothesis to explain this may be the release of cytokines and other substances that would result in alterations to brain barrier permeability in response to *Leishmania* infection (Melo et al., 2013). Thus, besides inflammatory cells migration, and pathogens could disseminate from the periphery to the CNS, as has been described in some forms of viral encephalitis (Ryan et al., 2005; Amude et al., 2011), primarily across the choroid plexus, resulting in a localized inflammatory process, or inside infected leukocytes likely via a Trojan horse mechanism (Drevets and Leenen, 2000). This permeability increase could also be permissive to the diffusion of *Leishmania* and/or *Leishmania* DNA into the brain parenchyma, as observed in this study.

The target organs for *Leishmania* are those containing cells of the mononuclear phagocyte system, such as the spleen, lymph nodes, bone marrow, and also the skin (Baneth et al., 2008), but not the CNS, consequently, the dissemination to the CNS is thought to occur to support proliferation and protection against anti-leishmanial drugs (Prasad and Sen, 1996; Ikeda et al., 2007), because of the isolation of the CNS by the BBB. In our study no dogs received any kind of treatment; even so, the presence of *Leishmania* kDNA was verified in the brain.

5. Conclusions

In the data presented here, we describe the first detection of *Leishmania infantum* kDNA in several areas of the brains of infected dogs. We suggest that the inflammatory process observed in the CNS and the neurological clinical signs previously described in dogs with VL may be related to the presence of the parasite or its DNA in the CNS.

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