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Case Report

Leishmania hide-and-seek: Parasite amastigotes in the choroid plexus of a dog with neurological signs in an endemic municipality in Brazil

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ABSTRACT

A female adult mixed-breed stray dog presented with hind limb paraparesis and clinical signs of visceral leishmaniasis. The cerebrospinal fluid presented signs of blood-brain barrier disruption. Both spleen and brain were positive for *Leishmania* spp. DNA. Besides inflammation, *in situ* hybridization and immunohistochemistry (IHC) revealed the presence of intracellular amastigotes in the choroid plexus (CP). Despite other studies that revealed parasite DNA, the current study describes the presence of *Leishmania* within the brain of a naturally infected dog, specifically in CP, with no previous reports in the Americas, and suggests the CP as a possible pathway to parasite entry into the brain.

1. Introduction

Visceral leishmaniasis (VL) is an important zoonotic disease caused by the parasitic protozoan *Leishmania infantum* (syn. *chagasi*) in the Americas and *Leishmania donovani* in Asia and Africa (Mauricio et al., 2000). Domestic dogs, the main urban reservoir of the disease in South America and in the Mediterranean basin, may develop the clinical form of VL, exhibiting lymphadenopathy, splenomegaly, cachexia and/or skin and renal diseases. Alternatively, dogs may remain asymptomatic for long periods, depending essentially on the immune response that each individual mounts against the infection (WHO, 2010; Solano-Gallego et al., 2011).

Parasites, transmitted through sand fly bites, can virtually spread everywhere, but they are usually located in spleen, bone marrow, liver, lymph nodes and skin, where an immune response is mounted against the protozoan (Solano-Gallego et al., 2011). During the peripheral infection, the brain also presents signs of inflammation and evidences of immune response; nevertheless, the parasite is rarely observed in the central nervous system (CNS) and it has been detected only in isolated cases (Nieto et al., 1996; Viñuelas et al., 2001; Font et al., 2004; Márquez et al., 2013; Gianuzzi et al., 2017; Oliveira et al., 2011). Consequently, the aim of this article is to describe the detection of *Leishmania* amastigotes in the choroid plexus (CP) of a dog with spontaneous VL.

2. Materials and methods

2.1. Animal

A female adult mixed-breed stray dog was captured by the zoonosis control center in an endemic municipality for canine visceral leishmaniasis in Brazil (Araçatuba, Sao Paulo State), as part of the leishmaniasis control program. As required by the Brazilian law, the *Leishmania*-infected dog was euthanized as soon as the diagnosis was achieved (Brasil, 2008) with an overdose of thiopental (Brasil, 2012). No clinical history was available.

2.2. Sampling

Peripheral blood was collected from the jugular vein and cerebrospinal fluid (CSF) samples were collected from the cerebello-medullary cistern. A complete necroscopic examination was performed and samples of the spleen and the brain (section containing cerebral cortex, thalamus, hippocampus, ventricular CP and periventricular white matter) were collected and fixed in formalin or directly frozen.

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2.3. Analysis

Complete blood work was performed, as well as measurements of the level of anti-*Leishmania* antibodies (indirect ELISA; Lima et al., 2005), and paired quantification of albumin and IgG in the serum and in the CSF. The fixed spleen and brain samples were sectioned, paraffin-embedded and submitted for routine histology (hematoxylin and eosin) and for parasite detection by immunohistochemistry (IHC) and *in situ* hybridization, according to Melo et al. (2015). Parasite load in spleen and brain frozen samples was assessed by qPCR (Melo et al., 2015). We excluded other parasitic infections that could culminate in nervous signs, such as toxoplasmosis and neosporosis by immunofluorescence antibody test (IFAT) and ehrlichiosis and babesiosis by enzyme-linked immunosorbent assay (ELISA), using in house routine kits.

3. Results

3.1. Clinical examination

Since no clinical history was available, we could not determine the duration and progression of the clinical signs; however, before euthanasia, the dog presented mild lesions suggestive of VL, such as lymphadenomegaly and hypotrichosis, as well as hind limb paraparesis. During the necroscopic examination, splenomegaly and brain edema were noticed. The dog had normocytic and normochromic anemia, lymphopenia, thrombocytopenia, hypoalbuminemia, hyperglobulinemia and increased urinary protein-creatinine ratio (UPC, 1.16; reference range < 0.5). The diagnosis of VL was positive using serological (ELISA, optical density of 0.837; reference range < 0.270), cytological (popliteal lymph node smear) and molecular (qPCR in spleen) techniques. The cerebrospinal fluid was colorless and had increased levels of albumin (14 mg/dL; reference range < 10 mg/dL) and elevated albumin quota (1.94; reference range < 0.64), indicative of blood-brain barrier disruption, and increased amounts of anti-*Leishmania* antibodies (optical density of 0.751; reference range < 0.309). The parasite load estimated was 2.9×10^4 parasites/mg of tissue in the spleen and 1.3×10^1 parasites/mg of tissue in the brain.

3.2. Brain histopathological analysis

At the histological examination, the brain presented mild mononuclear inflammatory cell infiltration, especially located in the leptomeninges and in the CP. Further, we observed alterations in the CP morphology and thickening of some blood vessel walls; however parasites were not detected using this technique (Fig. 1A). By means of *in situ* hybridization, which detects the parasite DNA in histological sections, we noticed positive signals in round-shaped intracellular structures (Fig. 1B). Finally, with IHC, we confirmed the presence of amastigotes forms of the parasite in the CP, localized in the cytoplasm of mononuclear cells, possibly macrophages (Fig. 1C-D).

4. Discussion

In the municipality of Araçatuba, the presence of *Leishmania infantum* (formerly named *Leishmania chagasi*) was detected for the first time in 1998 (Luvizotto et al., 1999), where the occurrence of neurological signs in naturally infected dogs has already been reported (Ikeda et al., 2007). Inflammation in the brain of dogs with VL has been the subject of our studies since that time. Histological evaluation of hundreds of brains from infected dogs often presented inflammation (Melo et al., 2009; Melo and Machado, 2011; Melo et al., 2013), including in the CP (Melo and Machado, 2009; Grano et al., 2016, 2018). We never found the parasite itself within the CNS, even if the parasite DNA have been detected in different parts of the brain and CSF (Grano et al., 2014; Jose-Lopez et al., 2014; Cardinot et al., 2016; Oliveira et al., 2011).

To the authors' knowledge, the most reports of *Leishmania infantum* detection in the CNS of naturally infected dogs have been from Spain (Nieto et al., 1996; Viñuelas et al., 2001; Font et al., 2004; Márquez et al., 2013) or Italy (Gianuzzi et al., 2017), where the treatment against VL is performed differently from Brazil. In Brazil, there is a recent report performed with 48 infected dogs where L. *infantum* was only detected in one dog by histopathology and IHC, in a general area from brain (Oliveira et al., 2017). In another endemic area for leishmaniasis in Brazil (Maranhão State), *L. infantum* amastigotes were also detected by IHC in the brain from one dog presenting neurological signs (Macau et al., 2017), but the authors did not investigate the CP. In CP, *Leishmania* amastigotes were only reported in one dog from Spain two decades ago (Nieto et al., 1996). Altogether, these data show once more that *Leishmania is* a parasite hide-and-seek.

In humans, the rare reports of neurological symptoms in patients with VL did not include parasite detection in the CNS (Mustafa, 1965; Chunge et al., 1985; Hashim et al., 1995; Diniz et al., 2010; Sedaghattalab and Azizi, 2018), except in one case of a ten year old boy from India, infected by *Leishmania donovani* (Prasad and Sen, 1995). In that case, the authors observed amastigote forms in the cerebrospinal fluid, and they hypothesized that the parasite migration to the CSF could have occurred after splenectomy, forcing the parasites to find a place to proliferate and to keep safe from drugs.

The CP is a well know pathway to inflammatory cell migration into the CNS, and it is considered a mediator between the brain and the periphery (Ransohoff et al., 2003). During infectious diseases, some neurotropic pathogens are able to invade the brain *via* the CP, such as *Neisseria meningitidis, Cryptococcus neoformans* and *Trypanosoma brucei*, and evidence classifies this organ not only as an 'entry door' to pathogens, but as an important actor during the response against the infection (Masocha and Kristensson, 2012; Schwerk et al., 2015; Johanson and Johanson, 2018).

5. Conclusion

In the case presented herein, we describe the detection of *Leishmania* parasites within the brain of a naturally infected dog, specially in CP, with no previous reports in the Americas. It was not possible to establish a concrete link between VL, the parasites in the CP and the nervous signs, since no differential diagnosis of paresis was performed, although the dog presented biochemical changes in the CSF. The pathogenesis of the cerebral form of VL is still to be elucidated since inflammatory lesions occur in the absence of the parasites (or maybe during a rapid and/or intermittent presence of parasites). *Leishmania* detection in the nervous environment of infected dogs tends to be solely a finding, and may not be directly related to the occurrence of brain inflammation. Nevertheless, one possible pathway to *Leishmania* entry into the CNS is *via* the CP, in agreement with previous studies with other neurotropic pathogens.

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Conflict of interest statement

The authors of this work do not have any financial, personal or other relationship with organizations or people that could inadequately influence the content of this paper.

Ethical issues

This study was approved by the institutional Ethics and Animal

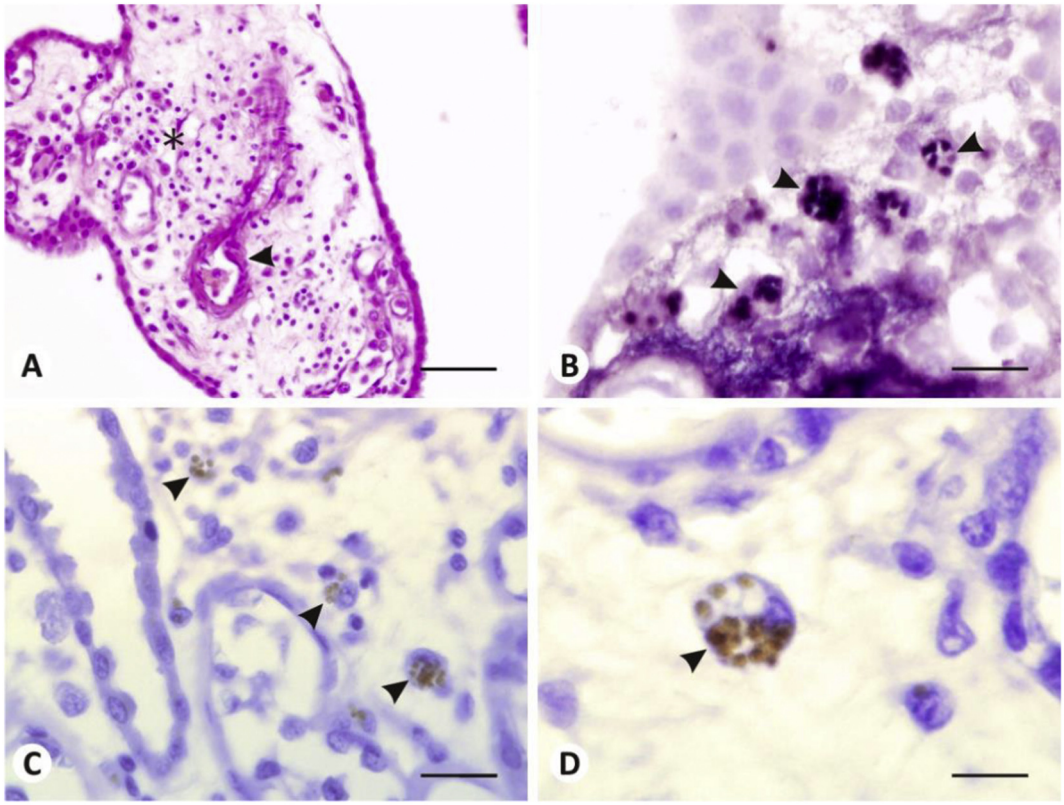

Fig. 1. Representative photomicrography of *Leishmania* detection in the choroid plexus of a naturally infected dog. A. Histopathological section of the choroid plexus exhibiting mononuclear cells infiltration (*) and thickening of a blood vessel wall (arrowhead). With this technique, no parasites were detected. Hematoxylin and eosin, scale bar = 100 µm. B. *In situ* hybridization representative photomicrograph where the dark-purple pigment deposits identify the parasite's DNA (arrowheads). NBT/BCIP, scale bar = 25 µm. CD. Immunohistochemical representative photomicrograph exhibiting round-shaped structures in the cytoplasm of mononuclear cells (arrowheads). Immunoperoxidase, scale bar = 50 µm (C) and 20 µm (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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