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Short communication

First report of vertical transmission of *Leishmania* (*Leishmania*) *infantum* in a naturally infected bitch from Brazil

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ABSTRACT

Dogs are the most important reservoir of *Leishmania* (*L.*) *infantum*, the causal agent of visceral leishmaniasis (VL) in Brazil. Vectorial infection is the main route of transmission of the parasites. This paper reports the first case of vertical transmission of *L. infantum* in Brazil, confirmed by PCR and immunohistochemistry techniques in samples from spleen and liver of two stillborn pups from a bitch naturally infected with *L. infantum* in Belo Horizonte city, endemic area of VL. This result confirms the existence of transplacental transmission of *Leishmania* between dogs, and suggests the need for further studies to determine the rate of occurrence of this fact in endemic areas and what is their role in the epidemiology of the disease.

1. Introduction

Visceral leishmaniasis (VL) is a serious-parasitic-illness which represents a real problem for public health services, with high rates of morbidity and mortality in humans. The disease is widely distributed on all continents, except for Oceania and Antarctica, with >90% of the 500,000 cases per year worldwide affected by VL living in India, Nepal, Sudan, Bangladesh and Brazil (Alvar et al., 2006).

In the New World the disease is a zoonosis, attributed to parasitic protozoa of the complex *Leishmania donovani*, including the species *Leishmania* (*L.*) *infantum* syn. *Leishmania* (*L.*) *chagasi* endemic in Brazil. Transmission of the parasites to man and dogs occurs from bites by infected females of the genus *Lutzomyia* (Diptera: Psychodidae; Phlebotominae), *Lutzomyia longipalpis* being the most important vector of the parasite in Brazil (Laison and Rangel, 2005).

Canine infection is characterized by a marked pleomorphism of clinical signs, ranging from a total absence to various degrees of dermopathy, lymphadenopathy, onycogryphosis, weight loss, abnormalities of the musculoskeletal system and eye lesions (Alvar et al., 2004). In urban areas of Brazil where the VL is highly prevalent, dogs affected by visceral leishmaniasis (CVL) represent an important target for the control of the disease. Among dogs, the high prevalence of infection has suggested other forms of transmission of the parasite, such as from blood transfusion, venereal routes and, more recently, by transplacental transmission (Rosypal et al., 2005; de Freitas et al., 2006; Silva et al., 2008).

The possibility of transmission of parasites between dogs by other forms than sand fly bites requires investigation to elucidate their role in epidemiology of the disease (Mancianti and Sozzi, 1995). This paper reports the first case of vertical transmission of *L. infantum* in Brazil, confirmed by molecular and immunohistochemistry techniques, in two stillborn pups from a bitch with CVL, which was submitted to cesarean section in a veterinary clinic of Belo Horizonte (State of Minas Gerais, Brazil), a region in which VL is endemic.

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2. Materials and methods

An Argentine Dog bitch of 36 months old with a history of infection by *L. infantum* was brought to in a veterinary clinic of Belo Horizonte, where the diagnosis was made by indirect fluorescent antibody test—IFAT (serum titre at 1:640), and by the presence of amastigote forms of *Leishmania* in bone marrow smear. Throughout the period of gestation, which occurred normally, the dog remained without clinical signs of CVL. At 68 days she was examined by ultrasonography, and shown to carry two pups, one without a detectable heartbeat and the other with a much reduced heartbeat. Cesarean section was indicated, and it transpired that both pups were stillborn. The bitch was ovario-hysterectomized. Uterus and placenta samples from the mother and ear skin, spleen and liver samples from the pups were taken, fixed in neutral buffered formalin 10% and processed for the PCR and immunohistochemistry (IHC) tests.

The tissues samples were dehydrated, cleared, embedded in paraffin, cut at 4–5 μm, and stained with hematoxylin and eosin (H and E) before IHC assay by the method described by Tafuri et al. (2004). Sections were deparaffined, incubated with serum of *L. infantum*-infected dog as primary antibody, followed by biotin-conjugated anti-mouse rabbit serum, as secondary antibody (Link-DAKO, LSAB2 kit, Carpinteria, CA, USA). They were incubated with streptavidin-peroxidase (Link-DAKO, LSAB2 kit), shown up by using 0.024% diaminobenzidine solution (DAB, Sigma–Aldrich, St. Louis, MO, USA) and 0.16% hydrogen peroxide 40 (v/v) (Merck, Darmstadt, Germany). Finally, sections were dehydrated, cleared, and counterstained with Harris Hematoxylin and mounted in Canada balsam (Entellan®, Merck, Darmstadt, Germany). The preparations were studied under optical microscopy at 400× magnification, and classified as positive or negative according to the presence or absence of amastigote forms. As controls of the reaction, slides with fragments of each tissue were prepared in the absence of primary antibody, which was replaced by PBS buffer solution.

For PCR analysis, fragments of tissues were extracted according to Xavier et al. (2006), with some modifications. DNA amplification was performed using primers LV_1 (5′ ACGAGGTCAGCTCCACTCC 3′) and LV_2 (5′ CTGCA-ACGCCTGTGTCTACG 3′) obtained from Invitrogen (São Paulo, SP, Brazil), which were specific for the amplification of a repetitive DNA sequence of *L. infantum* (Piarroux et al., 1993). The PCR reaction mixture consisted of 15 pmol of DNA template, 15 pmol of each primer, 7 μL of Go Taq® Green Master Mix (Go Taq® Green Master Mix, Promega, Madison, WI, USA), and 3.5 μL of nuclease-free water (Go Taq® Green Master Mix). Amplification was carried out using an initial denaturation step (5 min, 95 °C) followed by 33 cycles of annealing (30 s, 59 °C), extension (30 s, 72 °C) and denaturation (94 °C, 30 s), and a final extension step (72 °C, 2 min) (Piarroux et al., 1993). Following amplification PCR products (100 bp fragment) were analysed by electrophoresis on a 5% polyacrylamide gel, and the samples were visualised by silver nitrate staining. In all assays, a positive control containing *L. infantum* (MHOM/BR/1972/BH46 strain) genomic DNA and a negative control without DNA were included.

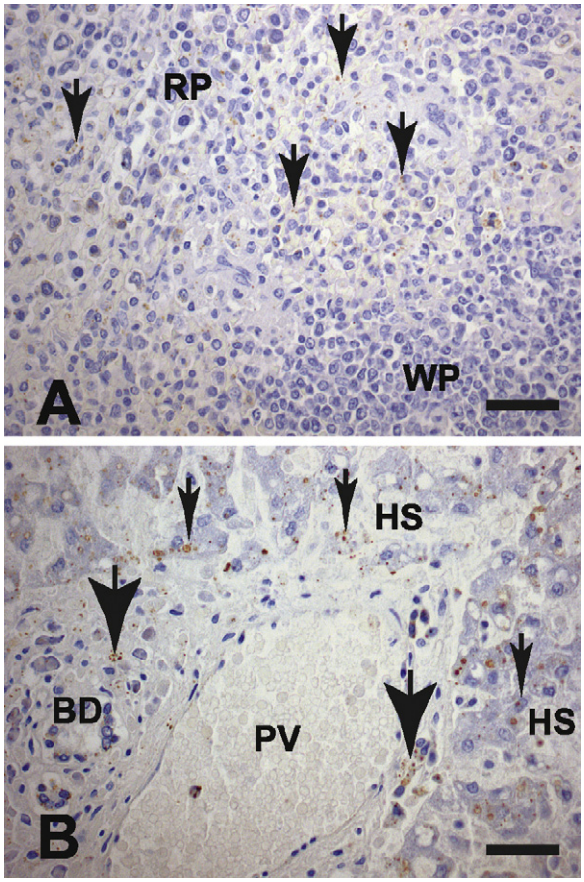

Fig. 1. (A) Paraffined spleen section of a stillborn puppy naturally infected with *L. infantum*. Observe the presence of immunolabeled amastigote forms into macrophages of red pulp (arrows). (RP) Red pulp; (WP) white pulp. Streptoavidin-peroxidase with Harris Hematoxylin counter-staining. Bar = 16 μm. (B) Paraffined liver section of a stillborn puppy naturally infected with *L. infantum*. Observe the presence of immunolabeled amastigote forms into macrophages in the hepatic portal tract (large arrow) and sinus (Kupffer cells) (tiny arrows). (PV) Portal vein; (BD) biliar ducts; (HS) hepatic sinuses. Streptoavidin-peroxidase with Harris Hematoxylin counter-staining. Bar = 16 μm.

3. Results

After cesarean section the bitch recovered well. Analysis of tissues from the pups by IHC assay showed the presence of *Leishmania* immunolabeled amastigotes in the liver and spleen (Fig. 1). This was confirmed by PCR analysis for *Leishmania* DNA in these tissues. In contrast, IHC assay and PCR tests were negative for ear skin from both pups (Table 1). The maternal placenta was positive by PCR, but not by IHC and both techniques showed negative results for fragments of uterus from the bitch.

4. Discussion

Few cases of non-vectorial transmission of *Leishmania* have been reported. The scientific literature contains rare

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Table 1
Results of PCR analysis and IHC assay in tissues taken at necropsy from two stillborn puppies after cesarean section in a Dog Argentine bitch naturally infected by *Leishmania infantum*.

Tissue	IHC		PCR	
	Puppy 1	Puppy 2	Puppy 1	Puppy 2
Liver	+	+	+	+
Spleen	+	+	+	+
Skin	–	–	–	–

reports of cases of vertical transmission among humans. Low et al. (1926) described the first case of VL in a newborn where the mother was ill. Since then, some other cases in humans have been reported in several countries, including Germany, Sudan, India (Eltoun et al., 1992). The possibility of transplacental transmission of *Leishmania* was confirmed by Rosypal and Lindsay (2005), who used a murine model and suggested that the frequency in mice by this route of transmission would be very low.

In humans, other forms of transmission, in addition to the vector and vertical forms have been reported, *e.g.* by iatrogenic route through the sharing of contaminated needles by drug users, especially in cases of co-infection HIV/*Leishmania* and through blood transfusion (Cruz et al., 2006; Dey and Singh, 2006).

In dogs, the occurrence of *Leishmania* parasites transmission by blood transfusion has been reported (Owens et al., 2001; de Freitas et al., 2006). The presence of *Leishmania* in the external genitalia of bitches described by Silva et al. (2008), in association with the report of Diniz et al. (2005) demonstrating the presence of the parasite in lesions of genital tissues and in the sperm of males, suggest that venereal transmission of *Leishmania* could occur.

This is the first report from Brazil of vertical transmission of *L. infantum* from a naturally infected bitch to their offspring. *L. infantum* was found in the spleen and liver of two pups, both by PCR and IHC techniques, and in maternal placenta by PCR. These findings corroborate those of Rosypal et al. (2005), who reproduced the experimental infection by *L. infantum* in a beagle bitch and diagnosed the presence of the parasite in tissue samples from three pups using PCR tests submitted to euthanasia procedure immediately after cesarean section at 60 days of pregnancy.

Cesarean delivery of the pups prevented them coming into contact with the mother's vaginal canal, eliminating the possibility of transmission by direct contact and/or contamination of the specimens we analysed, as might have occurred in the case described by Mancianti and Sozzi (1995).

Gestation in bitches can vary from 57 to 72 days, with average of 63 days (Shimatsu et al., 2007), and its maintenance depends directly on the development of an immunoregulatory process, with amplification of the response mediated by T helper lymphocytes of type 2 (Th2), leading to an increased secretion of growth factor-processing (TGF) β and interleukins (IL6 and 10). In contrast, lymphocyte producers of Th1 response are depleted, with a reduced production of potentially abortive pro-inflammatory cytokines, such as interferon-γ (INF-γ) and tumor necrosis factor-α (TNF-α) (Morein et al., 2007). High levels of TNF-α and INF-γ, one of the main mechanisms that induce death of amastigotes in macrophages in CVL, are present in dogs that are able to muster an effective immune response against the parasite (Barbiéri, 2006). The polarization of the response mediated by Th2 lymphocytes in pregnant dog which occurs during pregnancy, in association with the Th2-like status normally induced in dogs suffering CVL, could lead the increased parasite load, including the number of parasites in the mononuclear cells present in peripheral blood (Francino et al., 2006; Manna et al., 2009). The circulating parasites in the blood of the bitch and the irrigation of placenta and annexes could allow the passage of *Leishmania* through the placenta and into direct contact with the fetal circulation, leading to infection of the organs and tissues of her fetuses.

The effect of the presence of the parasite development in these organs and their possible connection with the death of the pups remains uncertain. The fact that the pups did not survive prevents any inference about the relationship of these animals with the disease, as to whether they would have clinical signs compatible with CVL, and when the skin of these animals would have parasites to infect the sand flies.

In contrast to our results, Andrade et al. (2002) found no evidence of the parasite in tissue samples from pups of bitches naturally infected with *L. infantum*, using cytology, histopathology and/or PCR techniques. These authors identified two positive samples of placenta through PCR. This finding reinforces the possibility of vertical transmission in bitches. The authors suggest that, if the vertical transmission of *Leishmania* occurs in dogs, it only happen in a very small proportion. In our opinion this low rate suggested by these authors could be caused by the parasitism in vital organs of pups during pregnancy, such as liver and spleen, leading to intra-uterine death of pups in most cases of vertical transmission of *Leishmania*.

The case we report is the first demonstration of vertical transmission of *L. infantum* in dogs in Brazil, where the CVL is endemic since the 50s and in a region where the prevalence of canine infection is about 8%, according to data from the Center for Zoonosis Control of Belo Horizonte (Belo Horizonte City Hall, 2008). Because of this, we consider important to determine in which proportion the vertical transmission occurs among pregnant bitches. Another important issue is that maybe this fact could be helpful to guide the veterinary surgeons in these areas with high rates of prevalence and incidence of the disease, to consider the existence of risk, even minimal, of abortions and stillbirths during the gestation of bitches infected with the parasite.

Based on fact that vertical transmission may occurs in bitches suffering CVL, the veterinary surgeons in agreement with the owners could suggest treatment of the bitches before the mount, in order to reduce parasitic load in all tissues, including peripheral blood, which may diminishes the chances of vertical transmission of *Leishmania*, as suggested by Figueiró-Filho et al. (2004) in cases of pregnancy and VL.

Further studies need to be conducted in order to investigate the frequency of vertical transmission of

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Leishmania among dogs and its relevance in the dynamics of the epidemiology of VL and CVL.

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