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Case Report

Adrenalitis associated with *Leishmania infantum* infection in a maned wolf (*Chrysocyon brachyurus*)

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Abstract

This report describes a case of visceral leishmaniasis characterized by adrenalitis with intralesional *Leishmania* sp. amastigotes in a 16 year-old maned wolf (*Chrysocyon brachyurus*). The animal had been previously diagnosed as infected with *Leishmania infantum* by serology and xenodiagnosis. The only organ in which amastigotes were detected by histopathology and immunohistochemistry was the adrenal gland, which presented multifocal infiltration of lymphocytes, plasma cells and macrophages containing intracytoplasmic amastigotes. The animal had no other lesions of visceral leishmaniasis, except for renal and splenic amyloidosis and pancreatitis that may be associated with the disease. Importantly, the maned wolf had an intratubular seminoma in the testis, which to the best of our knowledge is the first reported case of testicular tumor in this species.

Key words: *Leishmania infantum*, maned wolf, *Chrysocyon brachyurus*, adrenalitis, amyloidosis, seminoma.

Introduction

Visceral leishmaniasis (VL) is caused by a protozoan of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). In Brazil, VL is caused by *L. infantum* (synonym of *L. chagasi*), which is an obligate intracellular parasite of monocytes and macrophages of the vertebrate host (15, 41, 45). *L. infantum* is usually transmitted by the bite of female sand flies of the genus *Phlebotomus* in the Old World (18) or *Lutzomyia* in the New World, while in Brazil *Lutzomyia longipalpis* is the most important invertebrate vector species (20). VL is a zoonotic disease and the dog is the most important reservoir of the disease (1, 9), particularly in urban areas (10), although several species of wild mammals have been incriminated as reservoirs (47). Canine leishmaniasis is a severe zoonotic disease that affects millions of dogs with a marked impact on public health (29). Moreover, the World Health Organization (WHO) included VL among the six most relevant endemic diseases in the world (54). In several regions of the world, there is an increase in the number of human and canine cases (9), and Belo Horizonte is currently considered an endemic area (45), with a similar situation in other Brazilian metropolitan areas (10).

The adrenal gland can be directly infected by pathogens, including viruses, fungi, and bacteria (2). In dogs, there are reports of adrenal infection with canine herpesvirus (11), *Listeria monocytogenes* (44), *Aspergillus deflectus* (39), *Neospora caninum* (34), *Trypanosoma cruzi* (4), and *Leishmania* sp. (27, 51).

The maned wolf (*Chrysocyon brachyurus*) is the largest wild canid in South America, weighing in average

25 kg (53). A population of 21,746 animals has been estimated in Brazil. The maned wolf is considered an endangered species (3, 16, 40) since its population will likely reach 10% of the original numbers over the next decade, as a result continued loss of habitat, and other threats (16, 33). There are a few previous reports of serological and molecular evidences of *Leishmania* sp. infection in maned wolves in Brazil. These reports included two out of seven free-living maned wolves (8), one out of two maned wolf living close to urban areas (17), and one out of seven maned wolves kept in captivity in Belo Horizonte, Brazil (23). However, to the best of our knowledge there are no previous reports of adrenal lesions associated with *Leishmania* sp. in maned wolves. Therefore, considering the unfavorable perspective to this species in the wild, maintenance of maned wolves in captivity is a relevant approach for conservation. However, captivity near or within urban areas that are endemic for leishmaniasis, increases exposure and the risk of infection.

The goal of this report was to describe for the first time the pathological, parasitological and immunohistochemical findings in a case of adrenalitis in a captive senile maned wolf associated with natural infection with *Leishmania infantum*.

Case report

This report describes the pathological findings in a male maned wolf (*Chrysocyon brachyurus*) with approximately 16 years of age. The animal was kept at the Zoo-Botanical Foundation of Belo Horizonte and was diagnosed with leishmaniasis by ELISA, and confirmed by xenodiagnosis. ELISA was performed using the rK39 antigen and a commercially available kit (Biomanguinhos/FIOCRUZ, Rio de Janeiro, Brazil) according to the manufacturer's instructions. Wells (in 96-well microplates) were coated with rK39 in carbonate buffer (0.015 M of sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6), and then rinsed four times with 0.05% Tween 20 in PBS followed by incubation with 2% casein in PBS. Serum samples (1:80 dilution) were added and incubated at 4°C for 12 hours. Plates were then washed and incubated with the secondary antibody anti-canine IgG (1:5,000 dilution; Sigma-Aldrich) and incubated for 40 minutes. Plates were then washed and incubated with o- phenylenediamine (OPD) and H_2O_2 for 10 minutes. Optical density (OD) was measured at 492 nm, and the cut-off was set at two standard deviations above the average OD from negative controls.

The xenodiagnosis was performed as previously described (46) with minor modifications. This procedure has been approved by the Institutional Ethics Committee for Animal Experiments Experimentation (CEUA/UFMG, protocol number 94/2013). The maned wolf was physically contained, and 40 to 50 4-day-old *Leishmania*-free female sand flies (*L. longipalpis*) from the Department of Parasitology (Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG) were placed into a FleboContainer (6), and fed directly on the right ear of the wolf for 30 minutes. The sand flies were kept in the laboratory for 5 days, and then their medium intestine was dissected. DNA was extracted from the sand fly intestine by using guanidine as previously described (36). Ten female sand flies were analyzed by quantitative PCR as described below. Male and non engorged female sand flies were used as negative controls, and they all tested negative for *Leishmania* DNA. Quantitative real time PCR was performed using primers for *L. infantum* DNA polymerase and β-actin (Table 1). PCR was performed in a final volume of 25 μL with 200 nM of each primer, 1X SYBR Green PCR mastermix (Applied Biosystems, USA) and 5 μL of template DNA, under the following parameters: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were established by sequential dilutions of target sequence cloned into pGEM-T Easy Vector System Plasmid (Promega, USA). A ABI Prism 7500 (Applied Biosystems, USA) was used for PCR.

A serum sample from the maned wolf was positive by both serologic methods. Xenodiagnosis resulted in detection of two out of ten engorged female sand flies that contained DNA sequences of *L. infantum*, and therefore the xenodiagnosis was considered positive.

The clinical condition of the animal worsened. The wolf had chronic otitis, which became associated with hypothermia, hypotension, and anemia. Due to the lack of response to intensive care, and senility, euthanasia was elected.

A necropsy was performed and samples were taken for histopathology. Samples of kidney, pancreas, brain, stomach, trachea, testes, lungs lymph nodes, intestine, omentum were processed for histopathology and immunohistochemistry. The animal had marked cachexia, moderately icterus mucosa, and severe dehydration. There was partial edentulism, characterized by the absence of right ventral canine tooth. Large amount of foamy fluid was observed in the trachea and in the lungs, and small whitish firm small nodules diffusely distributed in the parenchyma. Histologically, these nodules corresponded to multifocal mineralization, which was associated with multifocal areas of interstitial fibrosis, and anthracosis. The liver was enlarged and histologically there was diffuse moderate to severe hepatic lipidosis, portal fibrosis, proliferation of bile ducts, and multifocal accumulation of proteinaceous material compatible with amyloidosis, which was confirmed by Congo Red staining under polarized light.

Kidneys and adrenal glands had a yellowish discoloration (icterus). Microscopically, there was accumulation of proteinaceous amorphous material in the glomeruli, which was confirmed to be amyloid by Congo Red staining under polarized light (Fig. 1). Renal tubules contained multiple hyaline cylinders with mild tubular ectasia. There was also a mild multifocal

Table 1. Primers used in this study.

Target gene	Primers (5' – 3')	Product size (base pairs)	Reference
DNA polymerase	TGTCGCTTGCAGACCAGATG GCATCGCAGGTGTGAGCAC	90	26
β-actin	CTTCTACAACGAGCTGCGCG TCATGAGGTAGTCGGTCAGG	307	26

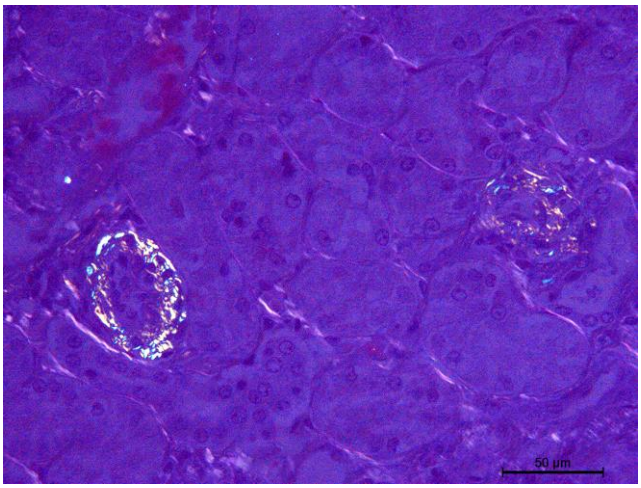

Figure 1. Maned wolf (*Chrysocyon brachyurus*). Kidney; birefringent amyloid in a glomerulus as demonstrated by Congo Red staining under polarized light; bar = 50 µm.

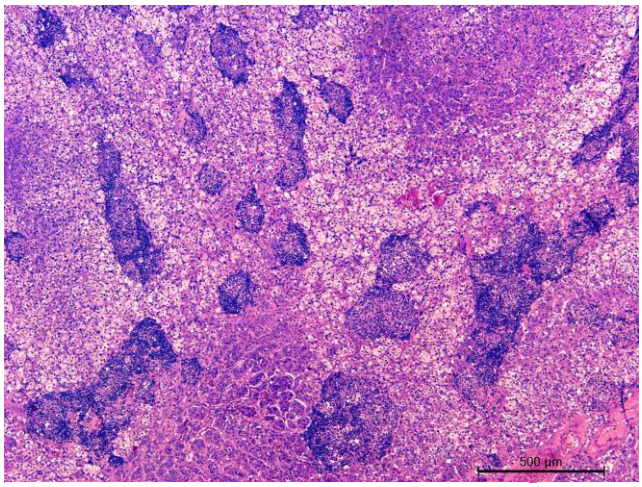

Figure 2. Maned wolf (*Chrysocyon brachyurus*). Adrenal gland; adrenalitis characterized by multifocal to coalescing inflammatory infiltrate. Hematoxylin and eosin; bar = 500 µm.

lymphoplasmacytic interstitial nephritis, and mild multifocal mineralization.

The adrenal glands had chronic severe multifocal lymphohistiocytic infiltrate, predominantly in the *zona reticularis*, *zona fasciculata*, and medulla, with macrophages containing numerous rounded 3-4 µm protozoa with basophilic nucleus and distinct kinetoplast, compatible with amastigotes of *Leishmania* sp. (Fig. 2 and 3). Immunohistochemistry was performed using serum from dogs naturally infected with *Leishmania infantum* (1:100) as primary antibody, as previously described (48). Immunohistochemistry confirmed amastigotes of *Leishmania* sp. within macrophages in the adrenal glands (Fig. 4). All other organs were negative by immunohistochemistry.

Testes were significantly reduced in volume, and histologically there was absence of spermatogenesis and marked atrophy of seminiferous tubules with loss of germ cells. There was also a neoplastic proliferation of germ cells, obliterating the lumen of some atrophic seminiferous tubules. Neoplastic cells were polygonal, with a high nucleus:cytoplasm ratio, prominent nucleolus, anisocariosis and moderate anisocytosis, with rare binucleated cells, supporting the diagnosis of intratubular seminoma (Fig. 5).

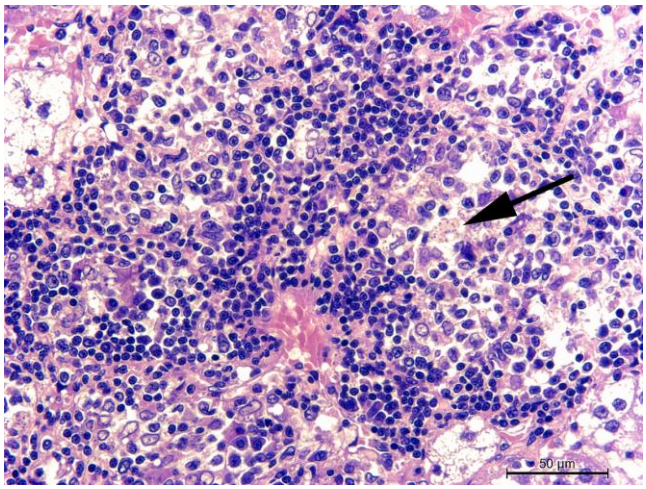

Figure 3. Maned wolf (*Chrysocyon brachyurus*). Adrenal gland; adrenalitis characterized by infiltration of lymphocytes, plasma cells, and macrophages containing intracytoplasmic amastigotes (arrow). Hematoxylin and eosin; bar = 50 µm.

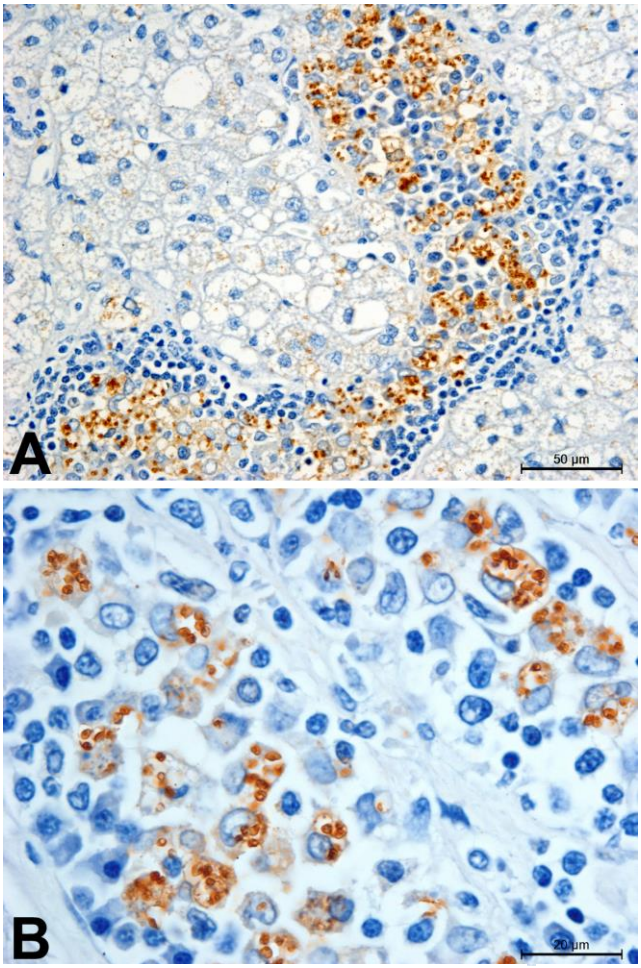

Figure 4. Maned wolf (*Chrysocyon brachyurus*). Adrenal gland, immunohistochemical detection of amastigotes of *Leishmania* sp. intracytoplasmic in macrophages. Streptavidin-biotin-peroxidase. (A) bar = 50 µm; (B) bar = 20 µm.

The pancreas was grossly pale, enlarged, and firm, while histologically there was an interstitial lymphoplasmacytic and histiocytic infiltrate, with multifocal areas of fibrosis (Fig. 6), which were consistent with chronic interstitial pancreatitis. Fragments of the mesentery had a severe and diffuse neutrophilic infiltrate associated with large numbers of lymphocytes and histiocytes, and moderate hyperemia. Microscopically, there was a marked lymphoid depletion in the spleen, associated with severe and diffuse amyloidosis, which was confirmed by Congo Red staining under polarized light.

Together, our clinical pathological and laboratorial findings support the diagnosis of VL in a senile maned wolf, with an atypical manifestation of VL characterized primarily by an adrenalitis associated with myriad of intralesional *Leishmania* amastigotes. In addition, the animal had a marked splenic and renal amyloidosis, which was probably secondary to *Leishmania* infection as well as other significant lesions, including intratubular seminoma, hepatic lipidosis, and pancreatitis.

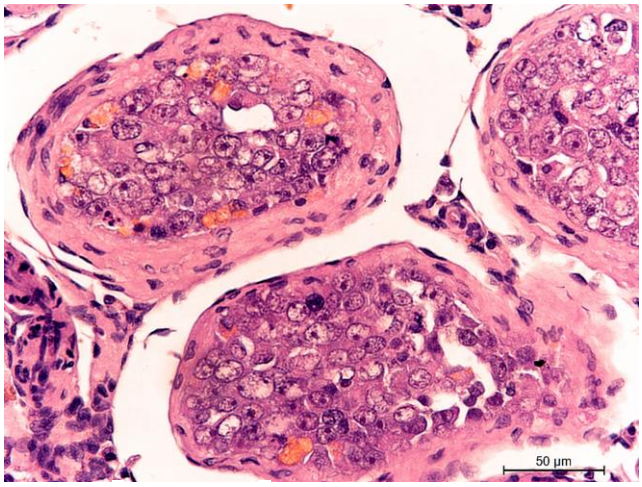

Figure 5. Maned wolf (*Chrysocyon brachyurus*). Testis; intratubular seminoma, characterized by the proliferation of neoplastic germ cells obliterating the lumen of atrophic seminiferous tubules. Hematoxylin and eosin; bar = 50 µm.

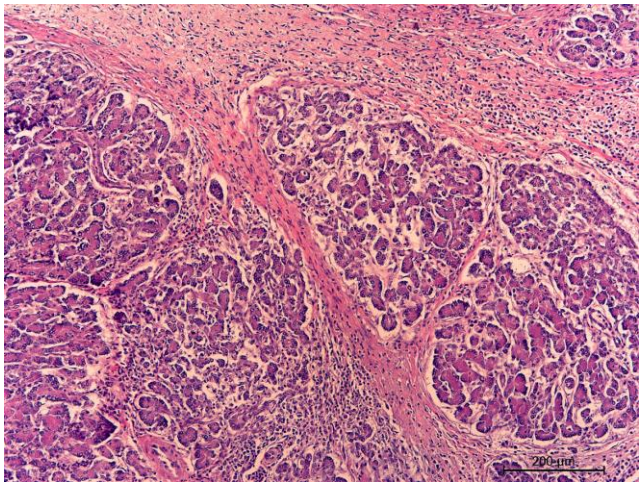

Figure 6. Maned wolf (*Chrysocyon brachyurus*). Pancreas; interstitial pancreatitis characterized by interstitial infiltration of inflammatory cells and fibrosis replacing some acini. Hematoxylin and eosin; bar = 200 µm.

Discussion

Although there are previously reported serologic and molecular evidences of *Leishmania* sp. infection in maned wolves (8, 17, 23), in this case we were able to achieve confirmation of infection by xenodiagnosis. Furthermore, for the first time we were able to describe histopathological and immunohistochemical findings associated with VL in a maned wolf. Interestingly, the clinical and pathological manifestation of the disease in this case could be considered atypical based on the usual manifestation of the disease in domestic dogs (52), although clinical and pathological characterization of VL in wild canids are very scarce (23). This notion is supported by the finding that the adrenal glands were the only site where amastigotes were identified by

histopathology and immunohistochemistry. In contrast, several lesions are often associated with VL in domestic dogs (*Canis familiaris*), including chronic dermatitis, granulomatous or histiocytic hepatitis, lymphadenopathy associated with lymphoid hyperplasia and accumulation of plasma cells and macrophages, bone marrow hypoplasia, and membrano-proliferative glomerulonephritis. Importantly, in all these lesions there are often macrophages containing intracellular amastigotes (13, 21, 28, 48). Interestingly, a study that included 45 domestic dogs serologically positive for canine leishmaniasis, demonstrated that eight dogs had positive immunostaining for *Leishmania* sp. in the adrenal glands (27). In those cases, the inflammatory infiltrate was composed of plasma cells and macrophages that infiltrated mostly medulla and *zona reticularis* of the cortex, and only occasionally the *zona fasciculata* and *zona glomerulosa* were involved (27). In this case, the inflammatory infiltrate involved primarily the *zona reticularis*, *zona fasciculata* and medulla. Another study demonstrated amastigotes in the adrenal medulla, but only rarely in the adrenal cortex of dogs (51).

Incubation of VL in domestic dogs varies from a few months to several years (31). Similarly, clinical features of VL are highly variable in domestic dogs, ranging from a subclinical or asymptomatic infection to a fatal disease (19, 31).

Glomerular amyloidosis has been described in dogs with VL as well as amyloid deposit in the basal membranes of renal tubules (51). In this case, amyloidosis was interpreted as secondary to chronic inflammation since macrophages release cytokines that stimulate hepatic synthesis of serum amyloid associated protein (SAA) and the P component of amyloid, which accumulate in tissues such as the mesangium, basal membrane of renal tubules, and vascular walls (42).

VL has become an urban disease (10), which poses a risk to endangered susceptible species kept in captivity in endemic areas. Previous studies conducted at the Zoo-Botanical Foundation of Belo Horizonte, located in an endemic area for VL, demonstrated serological and molecular evidences of *Leishmania* infection in several wild canids including two hoary fox (*Lycalopex vetulus*), one crab-eating fox (*Cerdocyon thous*), and one maned wolf (*Chrysocyon brachyurus*) (23). The report by LUPPI et al. (23) also described a clinical case of VL in a bush dog (*Speothos venaticus*). Non-human primates belonging to the same zoological also had molecular evidences of *Leishmania* infection (24). Finally, a comprehensive molecular survey performed in the same zoological garden, including several wild species potentially susceptible to *Leishmania*, resulted in evidences of infection restricted to canids and non-human primates (22).

Interestingly, to the best of our knowledge, this is the first reported case of seminoma, or any testicular tumor, in a maned wolf (*Chrysocyon brachyurus*). Seminoma is a common testicular tumor in domestic dogs (35, 37, 50). Compared to other testicular neoplasms of the dog, seminomas have a frequency ranging from 29.85 to 32% (32, 43) in dogs with 11.46 ± 3.47 years of age, while the frequency of canine testicular tumors increases very significantly with age (43). Although apparently this is the first report of testicular tumor in a maned wolf, other tumors have been reported in this species, including extraosseous osteosarcoma (38), lung adenocarcinoma (14), fibrosarcoma (25), synovial cell sarcoma (7), mammary tubule-papillary carcinoma (12), mammary ductal papilloma (5), and ovarian tumors (30).

In conclusion, we reported a case of VL in a maned wolf with an atypical manifestation that was primarily characterized by an adrenalitis associated with large numbers of intralesional and intramacrophagic amastigotes of *Leishmania* sp. Furthermore, this animal had the first reported case of seminoma in this species.

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