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Case Report

Fatal visceral leishmaniosis in a dog caused by *Leishmania infantum* in Bosnia and Herzegovina: A case report

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ABSTRACT

Canine leishmaniosis (CanL) caused by *Leishmania infantum*, is a zoonotic vector-borne disease endemic in the Mediterranean region. Here, we report a molecularly confirmed case of fatal CanL caused by *L. infantum* in the south of Bosnia and Herzegovina where epidemiology data are scarce. A 2.5-year-old, male golden retriever presented with a history of lethargy, prostration, and anorexia. Clinical examination revealed pale mucosae membranes, reduced capillary refill time, anuria, and ulcerated oral mucosae and skin of the legs. Complete blood count discovered severe non-regenerative, normocytic and normochromic anemia. Biochemistry profile showed hyperglycemia, hypoalbuminemia, hypercholesterolemia, increased potassium, and considerably elevated creatinine, urea, and phosphorus. Rapid *Leishmania* SNAP test was negative, as well as the serum neutralization test for leptospirosis. At necropsy, mildly enlarged and firm yellow to tan kidneys were the most prominent lesions. Macrophages laden with amastigotes in bone marrow, liver, spleen, kidneys, lymph nodes and the skin were seen in histopathology. Molecular testing by PCR and sequencing (*cpb* gene) confirmed and identified the pathogen as *L. infantum*. This study highlights the lack of key measures necessary to undertake the proper control of this important zoonosis in the country. Nationwide epidemiologic study on CanL and its vector (s), along with adoption and establishment of proper diagnostic approach with quantitative serologic and molecular methods in place are warranted.

1. Introduction

Leishmaniosis is a zoonotic vector-borne disease caused by different protozoan species of the genus *Leishmania*. The three most important species in Europe are: *Leishmania infantum* reported both in the Old and New world, *L. tropica* present in the Ionian islands and Crete, and *L. major* widespread from West Africa to the Middle East and India (Pennisi, 2015). In Europe, *L. infantum* causes visceral and also cutaneous leishmaniosis in humans, and a mixed or generalized form of disease in dogs (Baneth et al., 2008; Pennisi, 2015). Clinically, canine leishmaniosis (CanL) ranges from subclinical infection to severe lethal disease, with a wide spectrum of clinical signs, which depend on the organ system affected by the parasite (Solano-Gallego et al., 2011; Paltrinieri et al., 2016; Meléndez-Lazo et al., 2018). Based on previous investigations, classification of the clinical manifestations in CanL is heterogeneous, and hinders the comparison of clinical and epidemiological studies (Meléndez-Lazo et al., 2018). However, lesions in dogs caused by *L. infantum* are well characterized (Koutinas and Koutinas, 2014).

Canine leishmaniosis is endemic in many Mediterranean countries (Alvar et al., 2012). In the last decade, a significant increase in the prevalence of infected dogs, and further northwards incursion of the disease into non-endemic regions were observed (Pennisi, 2015; Le Rutte et al., 2018). However, the awareness of the veterinarians and physicians, as well as the general public and animal owners is still low in regards to this threat (Le Rutte et al., 2018).

In Bosnia and Herzegovina, CanL and subclinical canine Leishmania infection are known to occur in the southern and south-eastern regions of Bosnia and Herzegovina, but data on the species involved, its vectors, and clinical and pathology findings are very limited. A recent study (Colella et al., 2018, in press) confirmed the exposure to or infection by *L. infantum* of a large proportion of 408 dogs tested from various regions

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of Bosnia and Herzegovina. Furthermore, the estimated incidence of human leishmaniosis in the country is 2–3/100,000/year, and probably minimally underreported (Alvar et al., 2012). The only existing record of autochthonous human leishmaniosis dates back to the year 1959 (Gvozdenović and Miladinović, 1959). A more recent study on healthy Austrian soldiers returned from the peace-keeping campaigns in Bosnia and Herzegovina revealed five ELISA positive persons and one positive for *Leishmania* DNA (Obwaller et al., 2018). Here we report the first case of fatal visceral leishmaniosis caused by *L. infantum* in a dog from the country.

2. Case report

In December 2017, a 2.5-year-old, male golden retriever was presented to the Small Animal Clinic of the Internal Diseases Department at the Faculty of Veterinary Medicine, University of Sarajevo, with signs of lethargy and in lateral recumbence. The dog originated from Mostar municipality (43°20′35.99″N, 17°48′29.02″E) and had never travelled abroad. The animal was regularly vaccinated (parvovirus, distemper, parainfluenza, canine adenovirus, leptospirosis and rabies), periodically dewormed and treated against ectoparasites. Twenty-five days prior to admission the dog fed on the internal organs of a bovine carcass. Following this were episodes of diarrhea and vomiting which resolved by symptomatic therapy administered by a local veterinarian. However, later on, the animal became severely lethargic, prostrated, and anorexic and had lost > 5 kg of the initial body weight of 25 kg.

At clinical examination, pale mucosal membranes, capillary refill time < 2 s, ulcerations on the mucosa of the lips and oral cavity accompanied by petechial hemorrhages and uremic fetor were recorded. Superficial lymph nodes were unremarkable. Complete blood count revealed hypochromic, normocytic non-regenerative anemia, pronounced monocytosis and mild neutrophilia and basophilia. A routine biochemistry profile showed hyperglycemia, hypoalbuminemia, hypercholesterolemia, increased potassium, enormously elevated creatinine, urea and phosphorus, and decreased level of calcium (Table 1). Furthermore, proteinuria, glycosuria and hematuria were observed by urinalysis (Table 1). Serum neutralization test for leptospirosis as well as *Leishmania* SNAP test (Canine *Leishmania* Antibody test, IDEXX Laboratories, Inc. Westbrook, Maine, USA) were negative.

Upon abdominal palpation the kidneys were firm, but painless. On thoracic auscultation, mild systolic regurgitating sound at the base of the heart was noted. Marked anuria was detected by palpation and ultrasound examination of the urinary bladder upon rehydration. Abdominal ultrasound showed overall kidney enlargement and hyperechogenic thickened kidney cortex. The wall of the bladder was also thickened. The liver and spleen were unremarkable.

The dog was treated for acute kidney failure stage III (IRIS – International Renal Interest Society Kidney Staging). It was rehydrated with 600 mL NaCl (30 mL/kg i.v.) during 5 h. Also, calcium gluconate (1 mL/kg i.v., only at the first day), Ipakitin (1 g/5 kg p.o., 2× per day), Clamoxyl (amoxicillin) (12.5 mg/kg s.c.) and Furosemide (4 mg/kg i.v.) were administered. On the second day, the dog was very lethargic, cachectic, and became oliguric, and the day after he died and complete necropsy was performed.

The animal was in a poor body shape and condition. The tips of the ears were congested and blue with epidermal erosions. Bilaterally, in the region of the elbow joint there were multifocal to coalescing alopecic and ulcerative areas. At cross section, multiple cavities filled with thick yellow material and separated with firm white grey septa (abscesses) were observed. The mucous membranes of the oral cavity were light grey with multifocal irregular ulcerations also present at the apex of the tongue. In the stomach there were multifocal ulcerations on the pyloric mucosa, and multiple areas of serosal mineralization. The serosa of the small intestine was diffusely dry and slightly granular, with prominent peyer's patches in the intestinal wall. The liver and spleen were unremarkable, as well as all the lymph nodes. Popliteal lymph node was collected for molecular investigation of *Leishmania* spp. The kidneys were slightly enlarged, firm, yellow to tan, and had pitted surface. At cross section, there were multifocal grey-white linear stripes in the medulla. Ecchymosed hemorrhages were observed in the subepicardial tissue of the right auricle. Multifocal irregular grey areas were present in the wall of the left ventricular myocardium and in the subepicardial and subendocardial tissue of the left and right ventricles. The bone marrow of the femur was yellow with few light red superficial areas.

Histopathology revealed severely aplastic bone marrow infiltrated with numerous macrophages filled with large numbers of 1–2 µm round light blue bodies with dark nucleus (*Leishmania* amastigotes) (Fig. 1). The kinetoplast was not readily visible in histopathology slides. Macrophages laden with amastigotes were also observed in decreasing number in the liver, the dermis, the spleen, the kidneys, and in the medulla and sinuses of the lymph nodes. Macrophages laden with amastigotes in the liver were mostly aggregated in portal spaces, and often contained dark brown granular material (hemosiderin). Diffuse severe micro- and macro-vesicular degeneration of hepatocytes was

Table 1
Complete blood count and biochemistry profile results.

Parameter	Result	Reference value
Red blood cells	3.38×10^{12}/L	$5.50–8.50 \times 10^{12}$/L
Hematocrit	27.5%	37–55%
Hemoglobin	9.5 g/dL	12–18 g/dL
Reticulocytes	15 K/µL	10–110 K/µL
White blood cells	15.6×10^9/L	$5.50–16.90 \times 10^9$/L
Neutrophils	12.14×10^9/L	$2.00–12.00 \times 10^9$/L
Lymphocytes	0.77×10^9/L	$0.50–4.90 \times 10^9$/L
Monocytes	2.59×10^9/L	$0.30–2.00 \times 10^9$/L
Eosinophils	0.16×10^9/L	$0.10–1.49 \times 10^9$/L
Basophils	0.12×10^9/L	$0.00–0.10 \times 10^9$/L
Platelets	196 K/µL	175–500 K/µL
Glucose	9.92 mmol/L	4.11–7.95 mmol/L
Creatinine	– mmol/L[a]	44–159 mmol/L
Urea	> 46.4 mmol/L	2.5–9.6 mmol/L
Cholesterol	8.36 mmol/L	2.84–8.26 mmol/L
Albumins	18 g/L	23–40 g/L
Globulin	45 g/L	25–45 g/L
Sodium	147 mmol/L	144–160 mmol/L
Potassium	6.5 mmol/L	3.5–5.8 mmol/L
Chloride	111 mmol/L	109–122 mmol/L
Phosphorus	> 5.20 mmol/L	0.81–2.20 mmol/L
Calcium	1.46 mmol/L	1.98–3.00 mmol/L

[a] The level exceeds the measurable level.

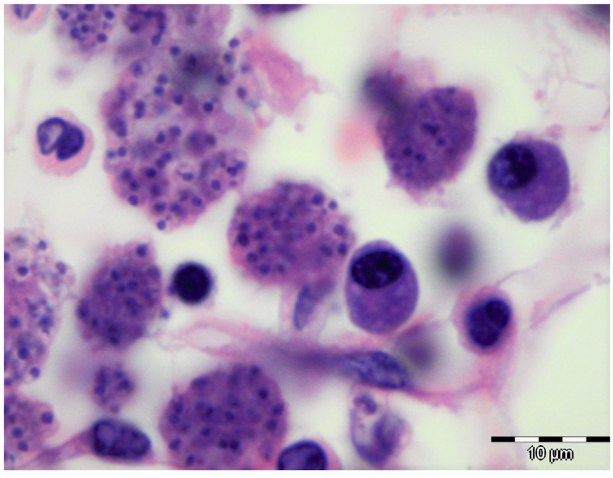

Fig. 1. Photomicrograph of the bone marrow of a dog infected with *Leishmania infantum*. Macrophages laden with numerous 1–2 µm *Leishmania* amastigotes are visible (H&E stain). *Scale-bar* 10 µm.

noted. In the kidneys, there was diffuse interstitial nephritis and membranous glomerulonephritis with fibrosis. Multifocal myocardial degeneration, necrosis, myocarditis and mineralization, multifocal ulcerative gastritis with mineralization, and multifocal to coalescing ulcerative and purulent dermatitis were observed.

To confirm the identity and molecularly characterize *Leishmania* spp., DNA was extracted from the popliteal lymph node and tested by conventional PCR and sequencing of a 702 bp long fragment of the cysteine protease b (*cpb*) gene (Hide and Bañuls, 2006). The obtained sequence showed 100% identity to the sequence of *L. infantum* (GenBank® accession no. JN400124).

3. Discussion

This paper provides the clinical presentation, pathology and molecular confirmation of a fatal *L. infantum* infection in a dog from the southern region of Bosnia and Herzegovina. Our findings contribute to the better understanding of a known endemicity of CanL in this region of the country (Colella et al., 2018, in press).

Prior to this report, the diagnosis of CanL in Bosnia and Herzegovina was merely based on cytology and on qualitative serology with immunochromatographic rapid test (SNAP test). Although characterized by adequate sensitivity and specificity, rapid qualitative serologic tests are still not optimal and may show false negative results (Athanasiou et al., 2014; Mettler et al., 2005), as in our case. The serology is less sensitive in apparently healthy infected dogs compared to clinically affected ones (Porrozzi et al., 2007). However, dogs showing clinical signs consistent with leishmaniosis and positive by quantitative serologic methods should be confirmed by molecular methods as it is the most useful approach for CanL diagnosis (Miro et al., 2008).

Cytological examination of superficial lymph nodes or spleen in dogs with clinical signs of leishmaniosis is rewarding (Paltrinieri et al., 2016). However, the cytology was not performed in our case because the superficial lymph nodes, spleen and liver were not enlarged. Moreover, in the present case, macrophages laden with amastigotes in the lymph nodes and spleen, as seen on histopathology, were the least in numbers and could easily be missed in cytology if performed. Nevertheless, the fact that the enlarged lymph nodes often reduce to the normal size in chronic cases of CanL (Koutinas and Koutinas, 2014) should be considered in suspected cases without obvious organomegaly.

In addition to lymphadenomegaly, spleno- and hepatomegaly, common lesions in CanL (Baneth et al., 2008), were also absent in this case. The absence of these lesions, in conjunction with the negative SNAP test, typically ruled out *Leishmania* spp. as the cause of the illness in this animal. On the other hand, several usually observed clinical features in clinically affected dogs with CanL (Baneth et al., 2008; Paltrinieri et al., 2016) were present in this case (e.g. cachexia, anemia, kidney failure and skin lesions). Histopathology revealed numerous *Leishmania* amastigotes in lesions in parenchymal organs corresponding to these clinical features. As commonly observed, macrophages laden with amastigotes and free amastigotes were most numerous in the bone marrow, almost completely effacing the production of corpuscular elements of the blood and hence leading to non-regenerative, normocytic and normochromic anemia (Koutinas and Koutinas, 2014). Kidney failure could also have contributed to the development of the anemia (Baneth et al., 2008).

The cause of death in the present case was kidney failure caused by *L. infantum* infection. Leptospirosis, one of the most common infectious agents that causes the kidney disease in dogs was ruled out in this case. The progression to clinical leishmaniosis was most probably initiated by the episode of stress caused by the feeding on the rotten meat, contributing to interruption of the immune status of the animal. Glomerulonephritis and interstitial fibrosis observed in this case suggest the progression of the infection into the chronic form. Severity of recorded lesions corresponds with the clinical findings (proteinuria, elevated urea and creatinine) noted in severe damage of the majority of nephrons (Baneth et al., 2008). The short period between appearance of clinical signs and development of severe chronic lesions with lethal outcome suggests, however, that this dog had probably been infected long before the apparent clinical picture. Signs of kidney failure could go undetected for long periods before the clinical presentation of CanL, late in the progression of the disease (Baneth et al., 2008). Also, it is known that clinical leishmaniosis may take a long time to develop and depends on the immune status of the host (Cardoso et al., 2007). The presented dog probably became infected in the area he was living through sand fly bites. However, other routes of direct transmission could not be excluded.

4. Conclusion

The case presented here highlights the lack of several key aspects in the control of CanL in the country. Despite recent confirmation of its endemicity (Colella et al., 2018, in press) the status of the infection is still unknown in the majority of the canine and feline population, and data on the existence and distribution of vectors are also missing. Further epidemiologic studies are necessary to broaden the basic knowledge on the status of CanL and presence of vectors in order to plan future control measures. Most importantly, diagnostic methods in place (cytology and qualitative serology) are insufficient for proper diagnosis of CanL, especially in asymptomatic cases. The spread of CanL from the south to northern and inland areas, as well as the possibility of direct transmission routes from infected to uninfected dogs, and/or to humans are of particular concern. Establishment of a proper clinical protocol with more advanced serologic and confirmatory molecular methods is warranted to diagnose and control this important parasitic zoonosis.

Conflict of interest

The authors declare that they have no competing interests.

Ethical statement

The authors declare that no experimentation on animal has been conducted to obtain data presented in this paper. The study was performed in accordance with the Veterinary law in Bosnia and Herzegovina, and the Animal Protection Law in Bosnia and Herzegovina.

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