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Case report

Clinical manifestations and diagnostic approaches in cases of canine leishmaniasis in Bulgaria

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Abstract

Leishmaniasis, a parasitic disease found in many parts of southern Europe, is transmitted in both humans and canines through the bite of phlebotomine sandflies, and can present in a variety of ways, such as cutaneous, mucocutaneous, diffuse, and visceral. In Bulgaria there are endemic areas of canine leishmaniasis, with sporadic cases in humans. However, no detailed studies of the animal population and vectors have been performed. Here we describe a few clinical cases of canine visceral leishmaniasis in two districts in western Bulgaria: one endemic and one without previously detected cases in humans or dogs. Diagnosis was confirmed serologically and molecularly using both real time and conventional PCR. Specific anti-leishmanial antibodies were confirmed in three of the cases via ELISA, with 50% of them returning extremely high values. In the majority of the cases DNA fragments were detected in the skin or lymph node aspirate but not in the blood. This paper highlights the need for further studies updating the current knowledge on the epidemiology, diagnosis, and control of visceral leishmaniasis in the reservoir host population.

Keywords

Canine leishmaniasis, clinical case, molecular methods, serology

Introduction

Canine leishmaniasis (CanL) is a parasitic zoonotic infection of dogs caused by *Leishmania* spp. parasites and is transmitted by the bite of infected female *Phlebotominae* (*Diptera, Psychodidae*) sandflies (Akhoundi et al., 2016). Although cases of CanL due to *Leishmania major* and *Leishmania tropica* infection are documented (Baneth et al., 2017), CanL caused by *Leishmania infantum* is considered the most important vector-borne parasitic diseases of dogs in Europe (Alvar et al., 2004).

In humans, more than 20 *Leishmania* species have been described as agents of leishmaniasis. All of them are morphologically indistinguishable, but they can be differentiated through molecular methods or isoenzyme analysis. Even though leishmaniasis is classified as a neglected tropical disease (NTD) by the World Health Organization (WHO), between 1,500,000 and 2,000,000 new cases occur each year, causing more than 70,000 human deaths worldwide (Torres-Guerrero et al., 2017).

The first field study on CanL in Bulgaria was performed by Drenovski in 1941 on 100 dogs from Petrich municipality. Thereafter, Matov and Filipov conducted two serological studies in 1958 and 1997, without observing clinical presentation of the disease (Filipov et al., 1997; Matov, 1958). The first autochthonous cases of CanL in Bulgaria were reported in 2006 in two dogs from Petrich showing typical cutaneous lesions. After this report 6 additional dogs from the same municipality tested positive for antibodies presence (Tsachev, 2009; Tsachev et al., 2010). Petrich is situated in south-west Bulgaria close to the borders with North Macedonia and Greece, both of which are known to be leishmaniasis endemic countries. A recent sandfly field investigation performed in this region of Bulgaria, confirmed the presence of competent vectors of CanL (Dvorak et al., 2020). Up to date, *Leishmania* parasites have not been isolated from sandflies collected in the country. Although sporadic cases in humans are reported annually in southern Bulgaria, canine leishmaniasis in this endemic part of the country remains unmonitored.

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The aim of this article is to report the ongoing presence of *Leishmania infantum* in Bulgaria and to describe the performance of a laboratory algorithm including multiple tests for detection of CanL cases among reservoir hosts, highlighting the importance of this approach for successful detection, control, and prevention of the infection.

Materials and Methods

The study group comprised of four animals: two 6- and 7-year-old mixed breed dogs (cases 1 and 2) living together in Mendovo village, Petrich municipality; a 10-year-old husky (case 3) from Samuilovo village, Petrich municipality; and a 2-year-old mixed breed dog (case 4) located in a shelter in Kostinbrod municipality.

Clinical examination

Dogs were examined for presentation of clinical signs associated with canine visceral leishmaniasis such as enlarged lymph nodes, alopecia, keratoconjunctivitis, blepharitis, onychogryphosis, lesions of the skin, ears, or muzzle, bristle condition, depigmentation, weight loss, decreased appetite, fever, vomiting and diarrhea.

Samples

Blood samples were collected in vacutainer tubes with spray-coated silica and with EDTA for whole blood (BD Vacutainer® Plus Plastic Serum Tubes, Becton Dickinson, Franklin Lakes), from the cephalic vein with a 21G needle. Blood designated for serology was allowed to clot for 30 min at room temperature before centrifugation at 2000 *g* for 10 min to separate the serum, after which it was stored at -20°C until analyzed. EDTA blood samples, lymph node aspirate (case 4) and skin lesions (cases 1 and 2) were kept at +4°C. The skin samples were homogenised with antibiotic media containing Eagle's Minimum Essential Medium (EMEM), 10 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 mg/ml gentamicin and 2.5 µg/ml amphotericin B. After 24 h incubation at +4°C, the suspensions were centrifugated at 2000 *g* for 15 min and the supernatants were used for extraction. EDTA blood samples and lymph node aspirate were directly subjected to analysis.

Hematology and blood biochemical analysis

All samples were tested for complete blood count (CBC) and blood biochemical parameters using the hematology analyzer BC-2800 and the bio-chemistry analyzer BA-88A (Mindray, Shenzen, China).

Serology

For antibodies detection two methods were used: An in clinic rapid Leishmania Ab Test kit (BioNote, Big Lake, USA) and a commercially available enzyme-linked immunosorbent assay (ELISA) (ID Screen® Leishmaniasis Indirect, IDVet, Grabels, France) following the manufacturer's protocols.

Molecular methods

Extraction was performed with IndiSpin® Pathogen Kit (Indical Bioscience, Leipzig, Germany) and specific DNA fragments were detected with real time and conventional polymerase chain reaction (PCR). For the real time PCR, a multiplex assay including primers and probe targeting the parasite specific kinetoplast DNA (kDNA) minicircle and primers and probe detecting an exogenous internal DNA control was applied (Applied Biosystems, Waltham, USA) (Lombardo et al., 2012). Conventional PCR was carried out by using 18 µl of Virotype Mix +IC-DNA (Indical Bioscience), 1 µM of each primer, amplifying a 145 base-pair (bp) size fragment from the conserved region of the kDNA minicircles, and 5 µl of DNA (le Fichoux et al., 1999). The sequences of the specific primers/probe and the temperature regimes of the reactions are shown in Table I.

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	Real time PCR		Conventional PCR	
Primers (Forward)	5′-GGCGTTCTGCGAAAACCG -3′		5′-CTTTTCTGGTCCCGCGGGTAGG-3′	
Primers (Reverse)	5′- AAAATGGCATTTTCGGGCC -3′		5′- CCACCTGGCCTATTTTACACCA-3′	
Probes	5′FAM-TGGGTGCAGAAATCCCGTTCA-3′- BHQ1		-	
Temperature regime	50 °C – 150 s	1 cycle	95 °C – 120 s	1 cycle
	95 °C – 10 min	1 cycle	95 °C – 5 s	1 cycle
	95 °C – 15 s	45 cycles	62 °C – 150 s	45 cycles
	60 °C – 35 s		70 °C – 30 s	
	-	-	70 °C – 10 min	1 cycle

Table I. Sequences of the specific primers, probe and temperature regime of real time and conventional PCR protocols.

Differential diagnosis

SNAP 4Dx Plus Test (IDEXX Laboratories Inc., Westbrook, USA) rapid test was used for additional testing for *Dirofilaria immitis*, *Ehrlichia canis*, *Ehrlichia ewingii*, *Borrelia burgdorferi*, *Anaplasma platys* and *Aanaplasma p hagocytophilum*.

Results

Clinical examination revealed alopecia and necrosis on the ears, exfoliative dermatitis, nasal and auricular hyperkeratosis, onychogryphosis (cases 1 and 2), conjunctivitis and keratitis (case 1) (Figure 1). Furthermore, all dogs showed progressive weight loss and decreased appetite. One of the animals (case 3) exhibited nasal lesions, chronic diarrhea, and epistaxis. Clinical manifestation in case 4 included periorbital skin lesions. In none of the animals fever and solitary or generalized lymphadenomegaly was observed.

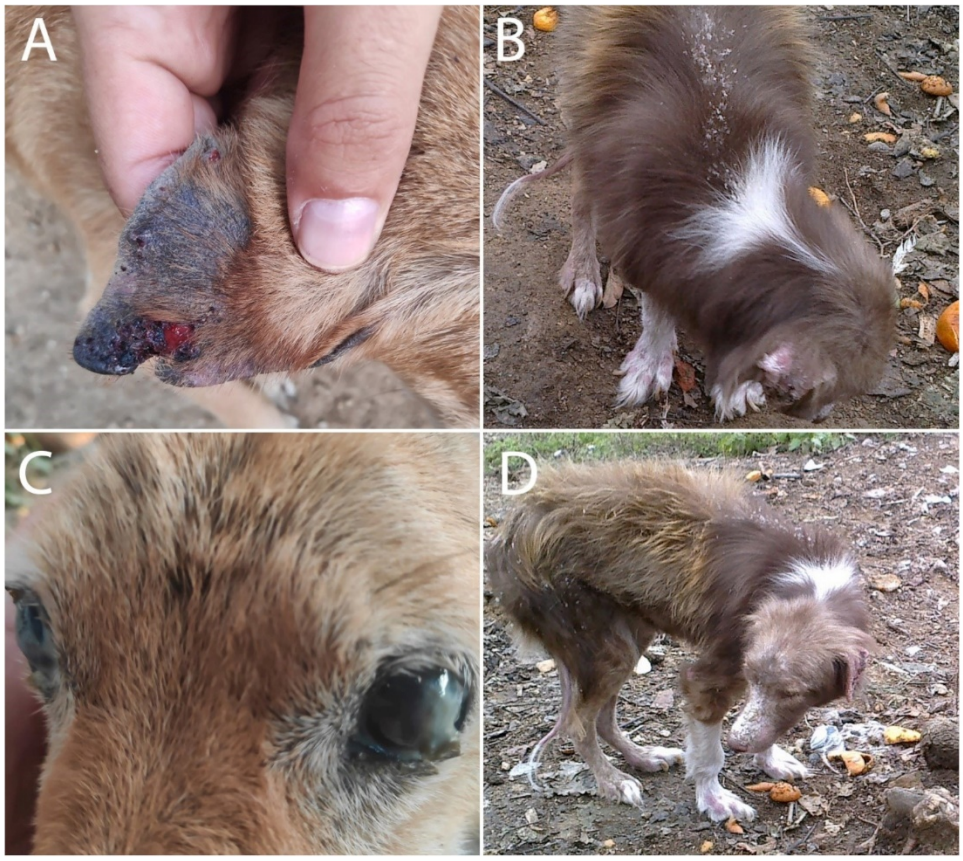

Figure 1 Clinical signs of canine visceral leishmaniosis. A: alopecia and necrosis on the ears; B: exfoliative dermatitis; C: conjunctivitis, keratitis; D: nasal hyperkeratosis, auricular alopecia, and necrosis.

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Blood biochemical profiling (Table II) and CBC (Table III) were performed on all dogs. The most important biochemical changes found in the plasma were mild to severe hyperproteinemia, hypoalbuminemia with a decrease in the albumin/globulin (A/G) ratio and in cases 2 and 3, increased plasma creatinine and urea. In all cases, leukocytosis and mild to moderate anemia were detected.

Blood biochemical profile of leishmania infected dogs					
PARAMETER	REFERENCES	CASE 1	CASE 2	CASE 3	CASE 4
ALB	22-44 g/L	12.6	18.6	12.2	21.8
TP	52-82 g/L	**91.8**	**139**	**113**	**139**
GLO	23-52 g/L	**79.2**	**89.4**	**100**	**74**
A/G	–	0.2	0.2	0.1	0.3
Ca	1.98-2.95 nmol/L	2.12	2.02	2.32	2.42
GLU	3.89-7.95 nmol/L	5.23	5.88	4.13	3.47
BUN	2.5-9.6 nmol/L	5.65	**15.25**	**78**	6.29
P	0.81-2.2 nmol/L	1.37	2.06	4.4	1.36
AMY	400-2500 U/L	2140	1590	2150	2345
CHOL	2.84-8.26 nmol/L	5.49	5.86	3.89	4.25
ALT	10-118 U/L	34	42	58	19
TBIL	2-15 nmol/L	**20.23**	12.37	10.36	6.37
ALP	40-300 U/L	57	72	152	51
CRE	27-124 nmol/L	53	**150**	**300.63**	72

Table II Blood biochemical profile of dogs with confirmed leishmania infection. Parameters exceeding reference values are indicated with bold, while parameters below reference values are underlined. ALB: albumin; TP: total protein; GLO: globulin; A/G: albumin/globulin ratio; Ca: Calcium; GLU: blood sugar; BUN: blood urea nitrogen; P: phosphate; AMY: Amylase; CHOL: total cholesterol; ALT: alanine aminotransferase; TBIL: total bilirubin; ALP: alkaline phosphatase; CRE: creatinine; – : no reference value.

Hematological findings in leismania infected dogs					
PARAMETER	REFERENCES	CASE 1	CASE 2	CASE 3	CASE 4
WBC	6.0-17.00 × 10^9/L	**22.5**	**21.6**	**20.5**	**17.7**
Lymph	0.8-5.1 × 10^9/L	6.7	**5.8**	3.8	4.7
Mid	0.0-1.8 × 10^9/L	**3.7**	**2.6**	1.6	1.2
Gran	4.0-12.6 × 10^9/L	12.1	12.1	**17.1**	**14.1**
Lymph	12.0-30.0%	29.7	28.2	20.2	14.8
Mid	2.0-9.0%	**16.4**	**15.3**	6.4	6.4
Gran	60.0-83.0%	53.9	58.3	82.3	81.3
RBC	5.50-8.50 × 10^{12}/L	3.58	4.88	3.49	4.31
HGB	110-190 g/L	68	95	70	90
HCT	39.0-56.0%	20.2	30.2	25.0	27.0
MCV	62.0-72.0 fL	56.7	65.0	61.0	64.0
MCH	20.0-25.0 pg	18.9	22.1	19.1	19.6
MCHC	300-380 g/L	336	300	280	220
RDW	11.0-15.5%	**16.0**	12.5	15.5	**16.0**
PLT	117-460 × 10^9/L	154	316	286	245
MPV	7.0-12.9 fL	6.3	8.7	7.2	8.1
PDW	–	14.8	18.5	18.8	19.7
PCT	– %	0.097	3.026	2.136	3.247

Table III Hematological findings in dogs with confirmed leishmania infection. Parameters exceeding reference values are indicated with bold, while parameters below reference values are underlined. WBC: white blood cell; Lymph: Lymphocytes; Mid: other white blood cells; Gran: Granulocytes; RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean red blood cell volume; MCH: mean corpuscular hemoglobin; MCHC: mean red blood cell hemoglobin concentration; RDW: red blood cell distribution width; PLT: platelets; MPV: mean platelet volume; PDW: platelet distribution width; PCT: plateletcrit; – : no reference value.

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Due to the present clinical symptoms consistent with CanL, dogs were screened with a rapid test for antibody detection to confirm the clinical observations. All four dogs yielded positive results. High amounts of specific anti-leishmanial antibodies were confirmed in cases 3 and 4 when serum samples were subsequentially tested with ELISA, while IgG levels were marginally above the assay's positive cut-off limit (S/P% ≥50%) in case 2, and in the gray zone in case 1 (S/P%: >40% – <50%). The results obtained from the laboratory investigations are presented in Table IV.

	Serology		PCR	
	Rapid test	ELISA (S/P%)	Real time PCR (Ct)	Conventional PCR
CASE 1				
Serum	+	49.7	NA	NA
EDTA	NA	NA	-	-
Skin	NA	NA	39.6	+
CASE 2				
Serum	+	52.0	NA	NA
EDTA	NA	NA	-	-
Skin	NA	NA	27.6	+
CASE 3				
Serum	+	260.0	NA	NA
EDTA	NA	NA	31.9	+
CASE 4				
Serum	+	474.0	NA	NA
EDTA	NA	NA	-	-
Lymph node aspirate	NA	NA	28.6	+

Table IV Serological and molecular results in dogs with confirmed leishmania infection. S/P%: sample positivity expressed in % (S/P% ≤ 40%-negative; 40%< S/P%

CanL infection was confirmed in all animals through the real time PCR method. *Leishmania infantum* specific DNA fragments were detected only in one of the examined EDTA samples (case 3), while the presence of the parasite in the rest of the affected animals was discovered in the skin (cases 1 and 2) and the lymph node aspirate (case 4). Afterwards, results were verified with the conventional PCR and a 145 bp fragment was detected in all samples which were positive on the real time PCR (Figure 2). No co-infection with heartworm or antibodies against Lyme disease and anaplasmosis were identified in any of the four dogs.

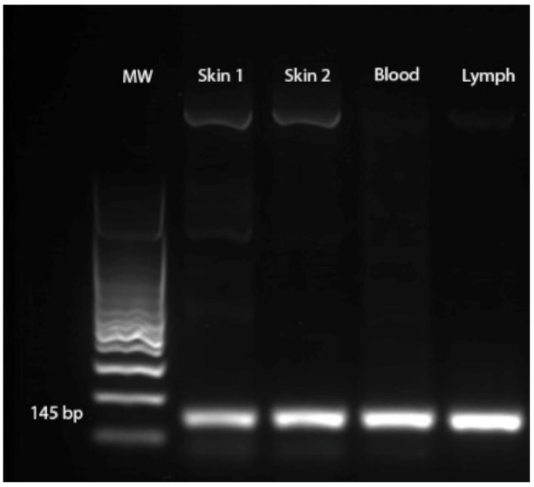

Figure 2 Electrophoresis of conventional PCR amplification for the 145 bp sequence of Leishmania infantum kinetoplast DNA. Positive bands corresponding to the 145 bp kDNA sequence were observed in all 4 samples tested (Skin sample 1, Skin sample 2, Blood, and Ly mph node aspirate). MW = 100 bp molecular weight marker.

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Discussion

Clinical manifestation of CanL is often variable, from subclinical to severe progressive disease, depending on the immune response of the host (Solano-Gallego et al., 2009). All four dogs in the present study showed clinical signs associated with leishmaniasis. During physical examination exfoliative dermatitis, nasal and auricular hyperkeratosis, conjunctivitis, keratitis, weight loss and chronic diarrhea were observed. The main blood biochemical abnormalities detected in all animals were related to changes in the protein levels, which are common findings, especially in the acute phase of the disease (Paltrinieri et al., 2010). Renal disease, due to deposition of immune complexes at the glomerular level (Grauer, 2005) can be present in approximately 50% of the cases (Cortadellas et al., 2006). Kidney function loss, associated with proteinuria (Almeida et al., 2005) can cause further decrease in the A/G ratio. Deviations in the creatinine level were present in two of the examined cases.

The main abnormalities detected in the CBC were leukocytosis and anemia. The observed leukocytosis may be a consequence of a systemic inflammatory response and often is prominent when ulcerative cutaneous lesions, with secondary bacterial infection are present. The most consistent hematological change in dogs naturally infected with *Leishmania infantum* is anemia (Kiral et al., 2004). The pathogenesis of the anemia in affected dogs has different mechanisms (Day, 2010; Smith et al., 2004; Tvedten, 2010; Weiss, 2010) and may appear six months after exposure to infection (Foglia Manzillo et al., 2013). Based on the blood biochemical results obtained in our study, pathogenesis can be attributed to depression of the metabolic activity of the bone marrow and the renal failure in cases 2 and 3, leading to decreased iron availability for erythropoiesis and reduced erythropoietin synthesis.

Immunochromatographic tests (ICT) and ELISA methods are extensively used for serological analysis of CanL. Although ICTs have been established as a screening assay performed routinely in the clinics, they have several limitations, including low diagnostic sensitivity (30-70%) and qualitative presentation of the results (EFSA Panel on Animal Health and Welfare, 2015; Maia and Campino, 2008). Due to these limitations, it is advisable to confirm the presence of antibodies with methods with better diagnostic performance, such as immunofluorescent antibody test (IFAT) or ELISA, which are designed to measure their level (Solano-Gallego et al., 2011).

Laboratory algorithms with high diagnostic sensitivity are essential for the detection and management of CanL. The most successful approach to identify*Leishmania* infected dogs is the combination of serology and molecular methods (Miró et al., 2008). This methodology could be further improved by testing various types of samples for DNA detection. In the present study, three of the animals were confirmed as clinical cases based on the positive results from both ELISA and real time PCR tests. In these patients, parasite DNA fragments were detected in the skin or lymph node aspirate but not in the EDTA blood samples. Skin and lymph node aspirate/tissue are among the recommended types of samples to be tested in order to identify symptomatic and asymptomatic dogs with CanL (Morales-Yuste et al., 2022; Reis et al., 2006). Although PCR on whole blood is considered as less sensitive method, which was confirmed in this investigation also, PCR testing on EDTA samples is accepted as eligible approach in certain scenarios. In addition, all results obtained through the real time PCR were confirmed with a highly sensitive conventional PCR targeting kDNA, which enhanced the reliability of the performedlaboratory diagnosis.

Conclusion

In Bulgaria CanL remains unreported and infected dogs are usually diagnosed at the late stages of the disease, when the cost of the treatment is high and the prognosis for the outcome is poor. Delayed diagnosis affects not only the quality of life of the CanL cases but also favors the spread of the infection among other susceptible animals. Reliable diagnostic tests are crucial for the successful management of this infection and multiple analytical modalities can overcome limitations of singular analytical techniques for confirming the presence or absence of the disease. The combination of different methods increases the effectiveness of the laboratory algorithm and should be implemented in field investigations, particularly in endemic regions, where the transmission of the infection is high. The detection of subclinical cases is an additional challenge and should be considered when the strategy for disease control is framed.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement

None required.

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