

## First report of canine visceral leishmaniasis in Bulgaria

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**Abstract:** Seroepidemiological studies on canine leishmaniasis in Bulgaria, even in areas where human leishmaniasis has been detected, are controversial. This report describes 2 clinical cases of canine visceral leishmaniasis. The disease was confirmed by microscopical, serological, and molecular methods. It is, therefore, suggested that infected dogs could constitute the reservoir for human leishmaniasis in the country.

**Key words:** *Leishmania infantum*, canine visceral leishmaniasis, clinical symptoms, IFAT, PCR

### Introduction

Canine visceral leishmaniasis (CVL) is a zoonotic multisystemic disease caused by the protozoan parasite *Leishmania infantum* and transmitted by the bite of an infected phlebotomine sand fly. Clinical manifestations are observed only in a low proportion of infected dogs. A high incidence of infected dogs is considered as one of the risk factors for the emergence of human visceral leishmaniasis (1). CVL is endemic in Mediterranean and Middle Eastern countries (2); however, currently it is spreading in continental climate areas and in previously non-endemic regions such as Germany, Holland, England, Canada, and the USA (3-5). However, there is little information on the epidemiological status of CVL in most Balkan countries (6,7), where human visceral leishmaniasis cases have been reported. In some Balkan countries like Albania, human visceral leishmaniasis cases have shown an increasing trend (8).

In Bulgaria during 1988-2002, 67 human cases were recorded, including a 4-month old baby (9). The cases were geographically situated mainly in south Bulgaria, the Stara Zagora region, with the highest morbidity rate, followed by the Blagoevgrad region.

With regard to CVL in Bulgaria, a study conducted in Petrich (Blagoevgrad region) reported a seroprevalence of 13.8%, but dogs were asymptomatic (10). According to recent studies conducted in south Bulgaria, dogs were found to be seronegative. (11). In another study where 50 canine sera from stray, domestic, and police dogs, were analyzed, only one seropositive dog, which was also asymptomatic, was detected (12). In line with the above were the results of a seroepidemiological survey among 220 healthy dogs from 11 different geographical areas of the country. No antibodies against *Leishmania* were detected (7).

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### Case history

This study reports the diagnosis of CVL in 2 dogs from Petrich which were admitted in a private clinic in October 2006 and were suspected to be infected by leishmania parasites.

Case 1. A 4-year-old female Rottweiler with a history of skin alterations on the head that spread to the limbs for 1 year was admitted to a private clinic (Figure 1). The dog was unsuccessfully treated against dermatophyte and demodicosis. Clinical examination showed alopecia on the ears, exfoliative dermatitis, nasal and auricular hyperkeratosis, conjunctivitis, blepharitis, and pale mucous coats. Furthermore, the dogs showed progressive loss of weight with decreased appetite. The lymph nodes were not enlarged and its body temperature was normal.

Case 2. A 3-year-old female Rottweiler with skin alterations on its head that appeared 6 months before was also admitted to the same private clinic (Figure 2.). The dog received no treatment. The clinical examination showed periocular alopecia, exfoliative dermatitis, blepharitis, pale mucous coats, and nasal and auricular hyperkeratosis. The popliteal lymph nodes were enlarged. The dog had low appetite and it was emaciated as in case 1. Its body temperature was also normal.

### Results and discussion

The owners stated that these dogs had not been imported from another country and they had not travelled outside Bulgaria. To associate the clinical symptoms with CVL, a quick veterinary commercial immunochromatographic test was used (Witness, Synbiotics Corp.). In both cases the results of the test were positive, proving the first serological confirmation of clinical cases for CVL in Bulgaria.

To confirm these results further, 3 different diagnostic methods were applied: parasitological, immunological, and molecular.

Firstly, lymphoid tissue smears of popliteal lymph nodes were stained by Romanovski-Giemsa and examined by direct microscopy. In addition, Giemsa stained spleen touch biopsies were microscopically examined. In both samples, numerous amastigote forms were observed in intracellular, as well as the extracellular areas (data not shown).

Secondly, serum samples were analysed by the indirect fluorescent antibody technique (IFAT) to detect parasite-specific antibodies. IFAT was performed using whole antigen promastigotes of *Leishmania infantum* MON1 (MHOM/GR/78/L4), a strain isolated in Greece (National Reference Centre for Leishmaniasis, Hellenic Pasteur Institute, Athens, Greece). The parasites were cultured in DMEM (Gibco) supplemented with 10% FCS (Seromed) at 25 °C. The IFAT was carried out as



Figure 1. Case 1. Alopecia on the ears, nasal and auricular hyperkeratosis, conjunctivitis, and blepharitis.



Figure 2. Case 2. Periocular alopecia, blepharitis, and nasal hyperkeratosis.

described elsewhere (13). Serial serum dilutions of 1/100 to 1/1600 were performed and a fluorescent anti-dog IgG antibody (BioTech, Paris) was used as a secondary antibody. Antibody titres were considered greater than or equal to 1/400 to be positive for visceral leishmaniasis. In parallel with the results of the immunochromatographic test, samples were found to be positive (titres equal to 1/400).

Thirdly, detection of *Leishmania* DNA by polymerase chain reaction (PCR) in canine blood and spleen samples was performed. The spleen was extracted from case 1 and 2 samples were taken from 2 different sites. DNA was extracted with the Macherey-Nagel Nucleospin Tissue Kit (according to the manufacturer's instructions), final volumes of 100  $\mu$ L and 10  $\mu$ L were used for each PCR reaction (50  $\mu$ L). For each sample 4U of Taq DNA polymerase (Promega in storage buffer A), 1.5 mM of  $MgCl_2$ , and 200  $\mu$ M of each deoxynucleotide were used. RV1 (sense 5'-CTTTTCTGGTCCCGCGGGTAGG-3') and RV2 (antisense 5'-CCACCTGGCCTATTTTACACCA-3') primers (50 pmol/reaction each), giving rise to a 145-bp amplification product of kinetoplast DNA, were used (14). The PCR conditions were: initial denaturation (4 min at 94 °C), 40 cycles of: denaturation (30 s at 94 °C), annealing (30 s at 59 °C),

and polymerization (30 s at 70 °C). PCR was terminated by a final extension at 70 °C for 10 min. The reactions were performed in an Eppendorf Mastercycler Personal. As shown in Figure 3, a specific 145-bp PCR DNA product was detected in both spleen samples and in the blood sample of case 1 (sample 21) but not in that of case 2 (sample 22). Blood samples 23-27 have been obtained from dogs living in great Athens region that were suspected to be infected from *Leishmania* parasites.

A complete blood analysis was also performed using an autoanalyser (Sysmex K4500) (Table). The most characteristic haematological difference between the 2 cases was thrombocyte levels that were  $39 \times 10^9/L$  and  $128 \times 10^9/L$ , respectively. It is known that thrombocytopenia is present in 29.3%-50.0% of CVL cases (15).

Available epidemiological data from Bulgaria indicates a rather sporadic hypoendemic status of human leishmaniasis. However, the incidence of the disease in the town of Petrich in the Blagoevgrad region (11) is indicative of the existence of 'hot spots'. Thus, the identification of the zoonotic reservoir of visceral leishmaniasis in this region is crucial for controlling the disease and understanding its epidemiology.

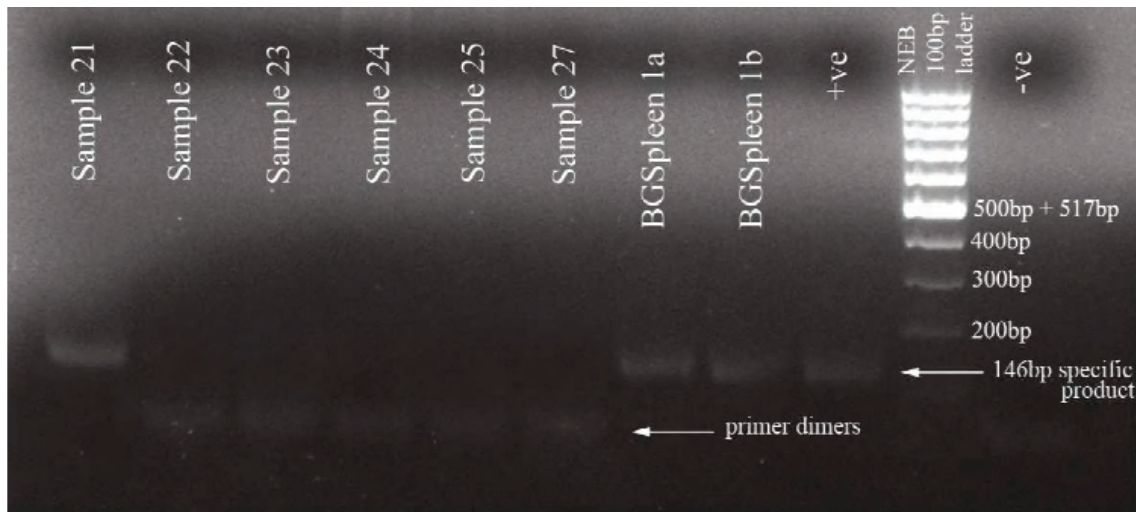


Figure 3. Agarose gel of 1.5% (w/v) electrophoresis showing PCR products using RV1 and RV2 primers of *Leishmania* kinetoplast DNA. PCR DNA product (5  $\mu$ L) was added to each sample tested including negative (-) and positive (+) control.

Table. Haematological parameters obtained from both case 1 and case 2.

Parameter (reference range)	Case 1	Case 2
Total leukocytes ( $6-17 \times 10^9/L$ )	17.0	10.5
Erythrocytes ( $5.5-8.2 \times 10^{12}/L$ )	5.65	5.35
Haematocrit (37%-55%)	56.5	36.4
Haemoglobin (126-194 g/L)	167	122
Mean corpuscular volume (62-70)	88.4	128
Mean corpuscular haemoglobin (22-25 pg)	25.5	22.8
Mean corpuscular haemoglobin concentration (330-360 g/L)	228	335
Thrombocytes ( $150-500 \times 10^9/L$ )	39	128

All together, the clinical manifestations, the positive serology using 2 different tests, the detection of *Leishmania* amastigotes in popliteal lymph nodes smears, the detection of *Leishmania* DNA in clinical samples and the observed thrombocytopenia in both cases confirm the diagnosis of CVL. The negative PCR in the blood sample of case 2 is not surprising since it is known that the sensitivity of the PCR method in blood samples compared to lymph node aspirates is lower (16). Taking into account that in endemic areas, large number of the canine population is exposed to the parasite but only a low proportion of the dogs develop positive CVL PCR, data should be interpreted together with the data obtained from the application of other diagnostic methods.

Overall, this is the first report of canine leishmaniasis in Bulgaria and suggests that since

infected dogs exist they could constitute the reservoir for human leishmaniasis in the country. More studies using molecular epidemiological tools are needed to identify the species of *Leishmania* causing the disease in humans and canines, to characterize the insect vectors and to better understand the epidemiology of leishmaniasis in Bulgaria.

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