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CASE REPORT

Companion or pet animals

Proliferative papulo-nodular glossitis due to *Leishmania infantum* in a dog

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Abstract

Canine leishmaniosis, caused by the protozoan *Leishmania infantum*, is an endemic vector-borne disease in Europe. From a diagnostic point of view, the disease can sometimes be a real challenge for the clinician, due to the variability of clinical presentations in a sick dog. These clinical signs can be classified, based on the frequency of presentation, in typical and atypical signs. In dogs is common to find different clinical presentations of cutaneous disease and systemic involvement, while clinical presentations in mucosa are not so common. A 6-year-old, entire, male German shepherd crossbred dog was examined for evident nodular lesions on the tongue. The most important laboratory alteration detected was hyperglobulinemia with an increase in the gamma fraction classified as polyclonal gammopathy. High anti-*Leishmania* antibodies were detected by ELISA. Tongue biopsy samples were obtained from the lesions with the presence of a diffuse inflammatory infiltrate characterised by macrophages and neutrophils with no compatible forms of *Leishmania* parasites. However, the presence of *Leishmania* amastigotes was confirmed by specific immunohistochemistry. A good clinical response to the anti-*Leishmania* based on meglumine antimoniate and allopurinol was observed after meglumine antimoniate administration. This clinical case describes the presentation of proliferative papulo-nodular glossitis in an *L. infantum*-positive dog as the most evident clinical finding reported.

BACKGROUND

Canine leishmaniosis is a zoonotic vector-borne disease caused by *Leishmania infantum*. The main route of transmission is through the bite of infected phlebotomine sand flies.[1] Other transmission routes described in dogs include transplacental,[2] blood transfusion[3] and venereal transmission.[4]

The clinical and laboratory alterations detected are the result of the immune response of the dog against the parasite.[5] The most common clinical signs include weight loss, anorexia, skin lesions, lymphadenomegaly, eye injuries, epistaxis, among others.[6,7] By contrast, atypical clinical signs are also described with the presence of lesions in other tissues and organs such as striated musculature, central nervous system, gonads, endocrine glands, as well as mucous areas such as oral cavity.[8,9]

Oral cavity lesions caused by the parasite have been considered as an infrequent form of presentation, and sometimes reported as the only clinical sign of the disease.[10] There is not much information available about oral leishmaniosis, a term named by some authors.[10] In animals with this presentation, other clinical signs associated with the location of the lesions may be observed, such as hypersalivation, halitosis, anorexia, lymphadenopathy and weight loss as a consequence of possible difficulty in ingesting food.[10]

This article reports a case of proliferative papulo-nodular glossitis in a dog associated with *L. infantum* infection. Confirmatory tests used for the diagnosis were quantitative serology, cytological examination, histopathology and immunohistochemistry specifically for *L. infantum*.

CASE PRESENTATION

A 6-year-old, entire, male, German shepherd crossbred dog was presented to the dermatology service of the Veterinary Hospital, University of Veterinary Medicine, Zaragoza (Spain) for evaluation of two nodular lesions in the dorsal surface of the tongue detected by the tutor, showing these lesions for several months (Figure 1). The dog lived outdoors as a guard dog, and no information was available on the progression of the

FIGURE 1 Presence of multiple oral lesions in the dorsal area of the tongue.

lesions. There was no evidence of difficulty eating, swallowing or pain. The patient had no travel history, and received routine heartworm prophylactic treatment and long-acting topical insecticide during the entire season of risk of exposure to activity of vectors. The patient was dewormed twice a year with a combination of febantel, pyrantel pamoate and praziquantel tablet, and the dog was also properly vaccinated. Finally, no previous treatments were administered to treat nodular lesions.

At the initial physical examination, the dog was aggressive, alert, with a bodyweight of 25 kg and body condition score of 3/5, normothermic, properly hydrated with pink mucous membranes. The dog´s abdomen was distended without painful palpation, with absence of organomegaly or the presence of palpable masses. Cardiac auscultation was within normal limits. Respiratory sounds were also normal, and there was no evidence of lymph node enlargement and other lesions in mucosal surface like preputial, ocular, nasal or rectal. Apart from lesions, the general examination was unremarkable. An oral examination under anaesthesia was also performed, revealing the presence of bilateral proliferative lesions along the lateral border and ventral surface of the tongue (Figures 2 and 3). After the American Society of Anesthesiologists (ASA) protocol, the patient was assigned with ASA 2 classification (slight risk of a slight to mild systemic disease). The anaesthetic protocol of the patient started with a combination of dexmedetomidine (3 μg/kg), butorphanol (0.4 mg/kg) and ketamine (3 mg/kg), combined in a single use syringe for intramuscular administration, with an adequate effect, allowing the clinician to manipulate the patient and placing of an intravenous catheter. The anaesthetic induction was carried by a dose effect of intravenous propofol (2 mg/kg) in combination with midazolam (0.3 mg/kg). These lesions detected under anaesthesia were not initially observed by the tutor before the physical examination.

LEARNING POINTS/TAKE-HOME MESSAGES

- Quantitative serological tests are suitable for diagnosing oral nodular forms, although in these types of lesions, it is necessary to perform a biopsy to rule out other nodular conditions.
- Canine leishmaniosis should be considered in the differential diagnosis of nodular and ulcerative lesions in the oral cavity.
- It is possible to detect animals with generalised oral lesions caused by leishmaniosis.

Cytological evaluation was done from a sample of the dorsal surface taken by needle aspiration and stained with Diff-Quick. A full-thickness biopsy from the proliferative lesions located in the lateral border and ventral surface of the tongue was obtained to characterise the type of infiltrate.

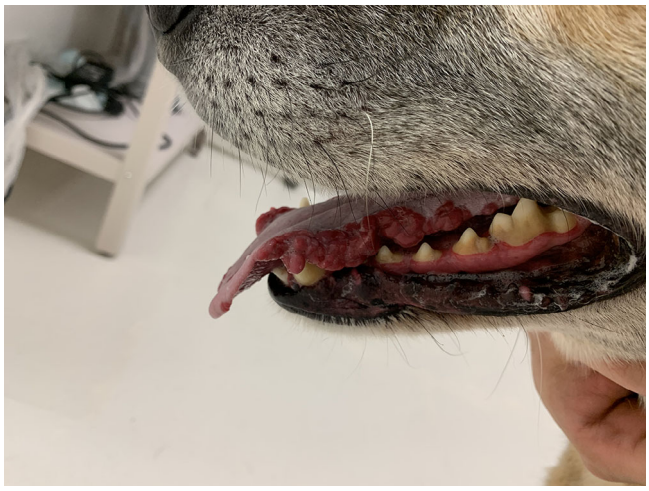

FIGURE 2 Bilateral proliferative lesions through the lateral plane of the tongue.

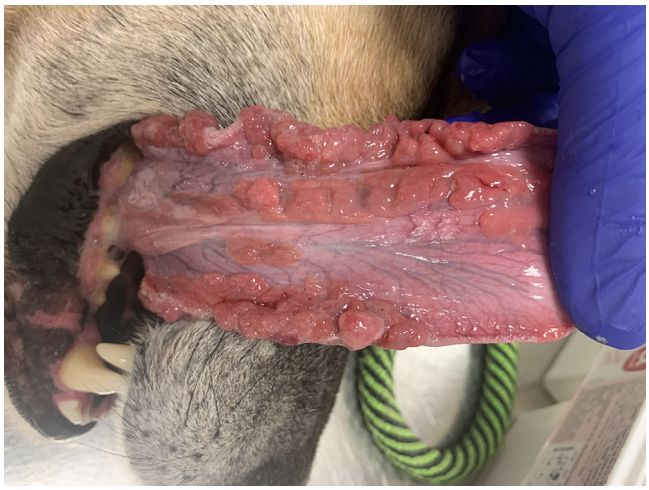

FIGURE 3 Bilateral proliferative lesions in the ventral plane of the tongue.

INVESTIGATIONS

In this patient, different clinical pathology tests were performed including haematology (LaserCyte analyser, Idexx); serum biochemical profile (AmiShield, Protect Life International Biomedical) to determine the following parameters: glucose, total protein concentrations, albumin, blood urea nitrogen, creatinine, calcium, inorganic phosphorus, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transferase, total bilirubin, amylase and globulins. Urine analysis was performed including urine specific gravity and urine sediment analysis. The only main clinicopathological finding was the presence of a very mild hyperglobulinemia (4.6 [1.9–3.7 g/dL]).

Serum protein electrophoresis was also performed by agarose gel electrophoresis system with HYDRAGEL Kit (Sebia). Serum protein electrophoresis was run manually with agarose gels (Sebia), and densitometer (Sebia) was used for scanning the electrophoretograms. The electrophoresis detected an increase in the gamma fraction classified as polyclonal gammopathy.

A quantitative serology based on in-house ELISA technique was performed to detect the presence of anti-*Leishmania* antibodies.[11] Briefly, each plate was coated lightly with 100 μL/well of the 20 μg/mL antigen solution (strain MHOM/FR/78/LEM 75 belonging to *L. infantum* zimodeme MON-1) in 0.1 M carbonate/bicarbonate buffer (pH 9.6), and incubated overnight at 4°C. Plates were then frozen and stored at −20°C. One hundred microlitres of dog serum, diluted 1:800 in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) and 1% dry skimmed milk (PBST-M) were added to each well. The plates were incubated for 1 hour at 37°C in a moist chamber. After washing the plates three times with PBST for 3 minutes, followed by one wash with PBS for 1 minute, 100 μL of Protein A conjugated to horseradish peroxidase (Thermo Fisher Scientific) diluted 1:20,000 in PBST-M was added to each well. The plates were incubated for 1 hour at 37°C in a moist chamber, followed by washes with PBST and PBS as described above. The substrate solution (ortho-phenylene-diamine) and stable peroxide substrate buffer (Thermo Fisher Scientific) were added (100 μL per well) and developed for 20 ± 5 minutes at room temperature (RT) in the dark. The reaction was terminated by adding 100 μL of 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm (reference wavelength) in an automatic microELISA reader (ELISA Reader Labsystems Multiskan). Each plate included serum samples from a dog infected with *L. infantum* as confirmed by cytological examination as a positive control (calibrator) and serum samples from a healthy, non-infected dog from the blood donor programme as a negative control. The same calibrator serum sample was used for all assays, and the plates with an interassay variation greater than 10% were tested again. All samples and controls were analysed in duplicate. The results were quantified as ELISA Unit (EU) compared to a positive control serum sample used as a calibrator that was arbitrarily set to 100 EU. The cut-off value was set to 30 EU (mean + 4 standard deviations of values from 70 apparently healthy dogs from a non-endemic area and that were not included in this study). Sera with an EU ≥ 200 were classified as high positive, with an EU ≥ 100 and less than 200 as moderate positive, and with an EU greater than 30 and less than 100 as low positive. A high positive result was detected in this patient (250 EU).

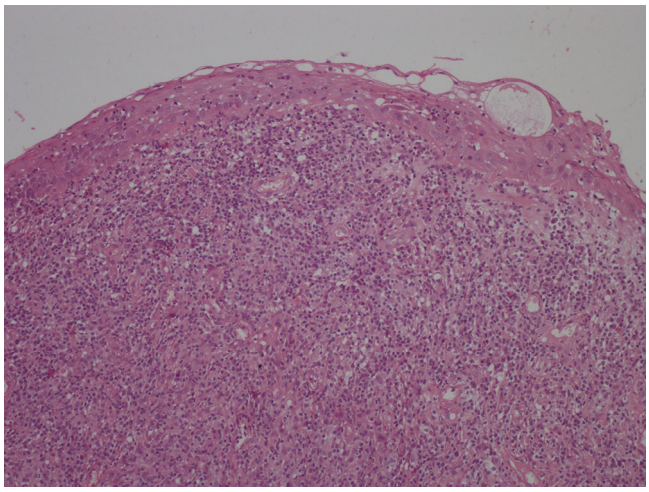

FIGURE 4 Histopathological examination. A vesicle is observed in the epithelium at the intraepidermal area with the presence of an intense inflammation (×4). Haematoxylin and eosin.

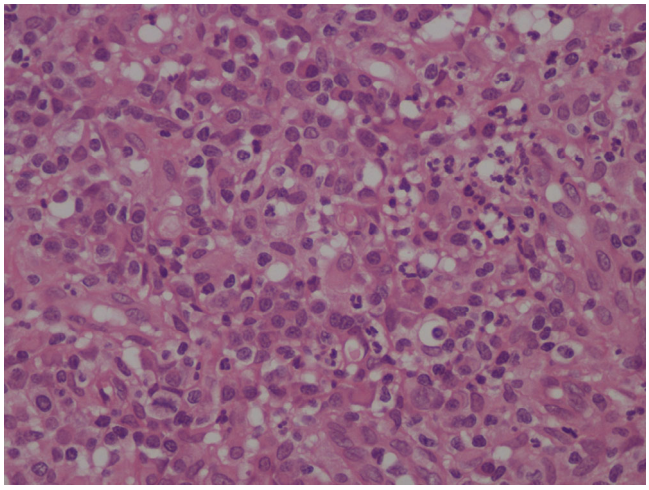

FIGURE 5 The type of infiltrate is composed of neutrophils and cells compatible with macrophages (pyogranulomatous inflammation) (×40). Haematoxylin and eosin.

Other additional tests were performed, including a rapid immunochromatographic test for the qualitative detection of *Dirofilaria immitis*-specific antigens (Uranovet), and commercial immunofluorescence antibody test for *Anaplasma phagocytophilum* (Megacor Diagnostik) and *Ehrlichia canis* (Megacor Diagnostik). All tests showed negative results for the investigated pathogens.

Cytological examination of the dorsal surface of the tongue revealed pyogranulomatous dermatitis; no infectious agents were visualised. The tongue mucosa biopsy sample was fixed in 10% formalin and embedded in paraffin. Four micrometre-thick sections were stained with haematoxylin and eosin. Histologically, nodules at the ventral surface of the tongue were composed of a diffuse inflammatory infiltrate characterised by numerous foamy macrophages, a fewer number of neutrophils and generalised oedema that expanded the submucosa and elevated the overlying epithelium (Figures 4 and 5). There was also moderate fibrosis within the affected

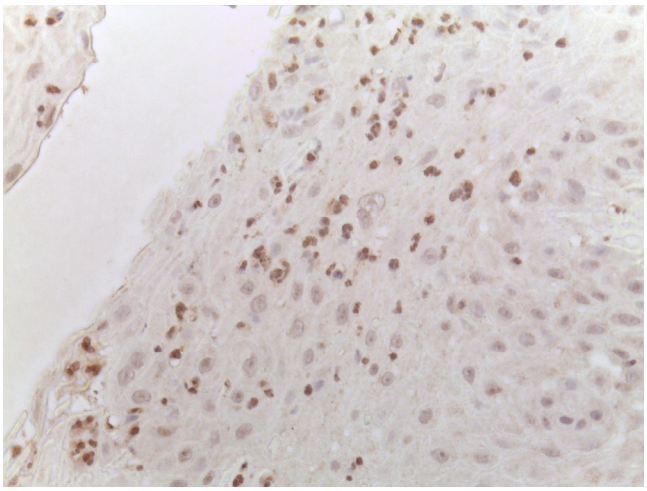

FIGURE 6 Immunohistochemical staining-enabled labelling of *Leishmania* species amastigotes. Several *Leishmania* amastigotes are detected. Note the amastigote forms labelled in brown (×40).

submucosa. Multifocally, adjacent epithelium was eroded or ulcerated, and occasionally it showed hyperplastic changes (acanthosis) with increased intercellular space with conspicuous intercellular bridging (spongiosis) and intracellular oedema.

To determine the presence of *Leishmania* parasites in tissue section, immunohistochemistry was performed using a standard protocol with an Autostainer Link48 (Dako) and an in-house rabbit polyclonal antibody specific for *L. infantum*.[12] Blocking of endogenous peroxidase activity (Dako REAL Peroxidase-Blocking Solution, Dako, Glostrup, Denmark) was performed before sections were incubated for 30 minutes with the primary antiserum at RT (1:500 dilution in EnVision Flex antibody diluent, Dako). Thereafter, sections were incubated for 30 minutes at RT with Dako EnVision+System-HRP,Rb. The substrate used for detection was 3,3′-diaminobenzidine incubated for 10 minutes. Sections were then counterstained with haematoxylin for 8 minutes (EnVision FLEX Haematoxylin, Dako) and covered in slides. For the negative control, the primary antibody was replaced with non-immune rabbit serum. A biopsy sample of the lymph node from other dog with clinical leishmaniosis was included as a positive control. Immunohistochemistry revealed positive signal within macrophages (Figure 6).

DIFFERENTIAL DIAGNOSIS

Differential diagnosis for oral lesions in dogs includes neoplasia (histiocytoma, cutaneous lymphoma, mast cell tumour, carcinoma/adenocarcinoma), fungal granuloma, bacterial furunculosis, trauma, autoimmune diseases, uraemic lesions, eosinophilic granuloma, mucocutaneous amyloidosis, periodontal disease and oral leishmaniosis.

TREATMENT

Anti-*Leishmania* treatment protocol was administered based on meglumine antimoniate (at 40 mg/kg twice daily [BID]) subcutaneously for 5 weeks and allopurinol (at 10 mg/kg BID orally) sine die. In the case of antimoniate meglumine was initially administered from 25 mg/kg BID the first week to 40 mg/kg BID the third, fourth and fifth weeks BID subcutaneously.

FIGURE 7 Physical examination 3 months later. A significant reduction in the size of all the lesions was observed.

OUTCOME AND FOLLOW-UP

A good clinical response was observed after finishing meglumine antimoniate (5 weeks later) administration. No abnormalities were detected by serum protein electrophoresis, and a reduction of the anti-*Leishmania* antibody level (185%) was observed. Three months later, a complete physical and oral examination and laboratory tests were performed, including haematology, serum biochemistry, quantitative serology and serum protein electrophoresis. No laboratory findings were detected and a decrease of *Leishmania* seropositivity (97%) was observed. A significant reduction in the size of all lesions was also detected (Figure 7).

DISCUSSION

Oral leishmaniosis in dogs represents an uncommon and rare dermatological clinical presentation of the disease due to *L. infantum*, with the presence of single or multiple, ulcerated or non-ulcerated oral lesions have been described in dogs and no predisposition based on gender or breed has been found. The first report of the presence of multiple nodular lesions on the ventral surface of the tongue due to *L. infantum* in dogs was described several years ago in 1996.[13] Nevertheless, oral lesions have been described in various areas, including the tongue,[14,15] gingiva, soft palate and lips.[10] Lesions can be

circumscribed as a sole clinical manifestation of the disease, and they in combination with other clinical signs are associated with the systemic disease such anorexia, lymphadenomegaly and weight loss.[14,16–19] Moreover, granulomatous laryngitis problem associated with *L. infantum* has also been described in a dog treated with glucocorticoids, and the authors of this case report suggested that immunosuppressive treatment may have led to the onset of the lesion.[20] In the case of cats, the first report of oral lesions in feline leishmaniosis has been recently described in 2022.[21] In general, skin lesions are the most common findings on physical examination in cats with leishmaniosis.[22]

In humans, leishmaniosis is caused by *L. infantum* and other *Leishmania* species with mucocutaneous and visceral tropism. The parasite is able to cause oral lesions, which can be often detected in immunosuppressed patients.[23–27] Different forms have been described in *L. infantum* clinical cases in humans, such as the presence of tumour-like presentation, granulomatous glossitis, nodular form from a single nodule in the tongue to multiples nodules located in the palate, tumour-like lesions with increased lip size, granulomatous plaques on the tongue and lips, ulcers at the base of the mouth or in the buccal mucosa of the cheek and in more severe cases fistulous processes in the hard palate.[25,27–31]

The pathogenesis of oral leishmaniosis in dogs is not clearly described, and two different mechanisms have been proposed. *Leishmania* parasites have been reported to directly invade the tongue mucosa through the bites of infected sandfly vectors, similar to the immunopathogenic mechanism that occurs with the clinical form of papular dermatitis associated with the bite sites of the transmitting sandfly vectors in the skin.[15] By contrast, indirectly *Leishmania* amastigotes migrate from the skin or visceral organs to oral and tongue lesions is the other hypothesis.[14]

In our case, the presence of hyperglobulinemia together with the presence of a polyclonal gammopathy suggests that the lingual lesions observed are a consequence of parasite migration through infected macrophages from other infected organs to the tongue, being a non-conventional location of the *Leishmania* amastigotes. However, oral leishmaniosis in infected dogs is poorly understood, and there is no information available to explain the reason of the *Leishmania* migration to the tongue or other oral locations.

Generally, serological techniques can detect the presence of anti-*Leishmania* antibodies from moderate to high levels with different techniques such as immunofluorescence antibody test and ELISA technique.[32] In this sense, a difference between oral leishmaniosis and papular dermatitis is observed considering anti-*Leishmania* antibody levels where the quantitative serological results are negative or weakly positive in papular dermatitis.[33] In the present case report, a reduction of anti-*Leishmania* antibodies was detected by ELISA from high positive result to low positive result. At the time of diagnosis, the presence of a polyclonal gammopathy was detected; however, in clinical cases of papular dermatitis due to *L. infantum*, laboratory abnormalities are not usually detected.[33] This serological result confirms that this clinical picture is different from *Leishmania* papular dermatitis.

The diagnosis of leishmaniosis in this clinical presentation was possible using different confirmatory diagnostic techniques such as ELISA and immunohistochemistry with the visualisation of the *Leishmania* amastigotes. In this type of mucocutaneous clinical presentation, the definitive diagnosis is obtained through the histopathological examination of biopsies, where it is sometimes possible to detect in haematoxylin–eosin preparations, the presence of forms compatible with *Leishmania*. In oral canine leishmaniosis, different histopathological patterns could be detected with the presence of a lymphoplasmacytic and macrophage inflammatory infiltrate.[10] Nevertheless, the infiltrate detected in the present case was composed of neutrophils and cells compatible with macrophages. At other times, the observed histological pattern is compatible with *Leishmania*, but forms of the parasite are not observed in the preparations; additional tests, such as the parasite-specific immunohistochemical technique can help in the diagnosis and the amastigote forms are labelled in brown.[34,35] No intracytoplasmic forms compatible with *Leishmania* amastigotes were observed, while the specific immunohistochemistry was useful to detect the presence of *Leishmania* amastigotes in the present case report.

In conclusion, in the presence of oral proliferative papulonodular lesions in endemic areas of leishmaniosis, the parasite infection should be considered. Moreover, canine leishmaniosis should be ruled out in dogs with nodular and/or ulcerative glossitis; it is necessary to perform histopathological studies to characterise the type of inflammatory infiltrate and specifically immunohistochemistry to detect the presence of *Leishmania* amastigotes. Systemic anti-*Leishmania* treatment is necessary to achieve the clinical resolution of the oral lesions.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions. Sergio Villanueva-Saz and Maite Verde managed the case and performed the clinical laboratory analyses. Estela Pérez and Alex Gomez performed histopathological study. Sergio Villanueva-Saz and Andrés Yzuel drafted the manuscript. All authors participated in critically appraising the manuscript and revising it for intellectual content. All authors gave final approval of the completed manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflicts of interest.

FUNDING INFORMATION

The authors declare that no funds, grants or other support were received during the preparation of this manuscript.

ETHICS STATEMENT

The dog was sampled with the owner´s consent and for clinical reason. No additional ethical approval was required.

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