Tongue nodules in an atypical canine leishmaniasis in Brazil

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ABSTRACT. We aimed to report a case of canine leishmaniasis with the only visible clinical sign being the presence of nodules in the lateral region of the tongue. The bitch was treated for a mandibular fracture, when multiple small nodules were observed on the tongue. We identify nodular glossitis with the presence of structures compatible with amastigote forms of *Leishmania*. The bitch was positive by ELISA, RIFI and PCR assays. Clinical re-evaluation after one year of treatment for leishmaniasis showed clinical improvement, but there was maintenance of antibody titers and infectivity. Lingual nodules as the only clinical sign of the disease is rare, especially in endemic areas, but should be included as differential diagnosis for leishmaniasis in the country.

KEY WORDS: diagnosis, endemic area, *Leishmania,* PCR, tongue nodule

In August of 2018, a 7-year-old female canine Shih Tzu, duly vaccinated (parvovirus, distemper, parainfluenza, canine adenovirus, leptospirosis, and rabies), with no history of previous treatment, was treated at the Veterinary Hospital of the Federal University of Uberlândia (VH-FUU), presenting as main complaint a painful tenderness in the jaw and dysphagia after a supposed fall. The animal originated from the municipality of Uberlândia and had never traveled to another state of the country. There were no reports of alteration of the animal's physical condition on previous occasions.

During the physical examination it was observed that the patient presented multiple rounded, firm, reddish nodules with whitish, non-ulcerated parts, measuring around 0.5 cm located on the right lateral face of the tongue (Fig. 1). In addition, mobility was observed between the right and left sides of the mandible, in the mandibular symphysis region, as a result of the fall.

Laboratory tests included blood count, creatinine, alanine aminotransferase (ALT) and total protein levels; The observed changes were: mild anemia (35.3% hematocrit; red cells $5.13 \times 10^6/mm^3$), and increase in total protein (9.8 g/dl; Table 1). Radiographic examination confirmed fracture of the mandibular symphysis and ultrasound examination of the abdomen revealed no changes.

The treatment of choice for the main complaint was surgical reduction of the fracture through the use of cerclage suture, followed by excisional biopsy of the nodules of the tongue, which were immediately referred for histopathological examination. Before the surgery, an intramuscular injection of midazolam 0.4 mg/kg and methadone 0.3 mg/kg was given as pre-anesthetic medication. Ten min later, propofol 4 mg/kg was given intravenously, followed by continuous inhaled isoflurane. During the postoperative period, it was observed that healing of the fracture and tongue occurred within the expected time and without complications.

Histopathological examination of the nodules (Fig. 2a and 2b) identified the presence of severe inflammatory nodular to diffuse infiltrate affecting various tissues of the tongue epithelium, including the superficial and deep lamina propria and predominantly composed of histiocytes and plasmocytes, with formation of rare granulomas, and a lower proportion of neutrophils and lymphocytes; however, no evidence of malignancy was observed in the evaluated tissues. Structures compatible with amastigote forms of *Leishmania* were also detected in the histiocyte cytoplasm. In view of these observations, nodular glossitis was diagnosed as severe diffuse lymphoplasmic cells, with the presence of amastigote forms of *Leishmania* spp. To confirm the presence of amastigotes in the lesion, immunohistochemistry was used, in general terms, according to Tafuri *et al.* [30] (Fig. 2c and 2b). Endogenous peroxidase blocking

was performed with 2 ml of 30 volume hydrogen peroxide, 50 ml of methanol and 50 ml of distilled water, non-specific blocking was performed with 3% powdered milk (Molico®) in 0.01 M PBS pH 7.2. As primary antibody, heterologous hyperimmune serum from a dog naturally infected with *L. infantum* was used (ELISA titer 0,989; RT-PCR 1,109.5 ng/µl of DNA), diluted 1:50 in 0.01 M PBS. And as secondary antibody EnVisionTM FLEX / HRP (DAKO) for 40 min at room temperature. 3,3′-diaminobenzidine (DAB) (Dako Corp., Carpinteria, CA, USA) was used as a chromogen.

For the indirect diagnosis of the suggestive result obtained in the histopathological examination, enzyme immunoabsorption serological examinations (Enzyme-Linked Immunosorbent Assay−ELISA S7® kit) (Biogene Indústria e Comércio, Recife, Brazil) were performed using the amastigote S07 protein. The indirect immunofluorescence reaction (RIFI) method was also performed for the detection of anti-*Leishmania* IgG antibodies (Immunodot, São Paulo, Brazil) [1, 7, 9]. Both tests are validated by the country's official agency: MAPA–7,434/2,000 and MAPA–9,347/2,007, respectively, and were positive for leishmaniasis (Table 1).

As a direct diagnostic method, real-time qPCR was performed to confirm the molecular identity of the *Leishmania* spp. The DNA was extracted (Maxwell® RSC 48 Instrument) (Promega, São Paulo, Brazil) from a sample collected from the animal's bone marrow and tested for conserved cysteine protease b (cpb) fragment gene (StepOne Real-Time PCR System) (Thermo Fisher Scientific, Jaguaré, Brazil) [11, 15, 32]. The result indicated positivity for the

Fig. 1. Dorsal surface of the tongue moments before excisional biopsy (before treatment initiation). Round lesions, measuring about 0.5 cm, ranging from red to off-white in color, present along the right lateral border of the tongue (arrow).

Table 1. Results of blood count, biochemical, serological and molecular profile of the animal treated at the Veterinary Hospital of the Federal University of Uberlândia in 2018

Exams	Results	Reference values
Red blood cells (million/mm^3)	5.13	5.5–8.5
Hemoglobin (g/dl)	12.1	12–18
Hematocrit (%)	35.3	37–55
Total leukocytes	5,300	6,000–17,000
Neutrophils	2,915	3,000–11,500
Eosinophils	954	150–1,250
Basophils	0	Rare
Monocytes	0	150–1,350
Lymphocytes	1,431	1,000–4,800
Platelets	338,000	200,000–500,000
Creatinine (mg/dl)	1.16	0.8–1.8
ALT (UI/l)	48	Up to 50
Serology (ELISA)	Reactive–0.463	Negative–below the cut-off: 0.221
Serology (RIFI)	Reactive–1:160	Positive–Reactive Negative–Nonreactive
qPCR *L. infantum* (bone marrow)	Positive–59,069.63 DNA copies of the pathogen/µl	Positive–Presence of one copy of DNA/µl

agent with 59,069.63 DNA copies of *L. infantum* per µl of the sample (Table 1).

Mandatory notification procedures to the authorities were made in accordance with local legislation [8], as well as the tutor's awareness of the animal condition and the risks to public health.

Therefore, the tutor suggested to treat the leishmaniasis using Milteforan 2 mg/kg SID for 28 days; Allopurinol 10 mg/kg BID for 90 days; Domperidone 0.4 mg/kg BID for 30 days; Prednisolone 0.4 mg/kg BID for 7 days, in addition to measures to prevent sandfly bites, such as the use of repellent collars, the use of repellents by the tutors and cleaning the environment to avoid creating a favorable habitat for sandfly presence and multiplication. Periodic returns were scheduled to monitor the parasite load behavior and maintenance of treatment by observing the antibody titer and the DNA copy number of the pathogen found by qualitative PCR examination of the collected bone marrow material.

The animal responded satisfactorily to the treatment, and after one year after treatment, the patient showed no signs of relapse and had complete regression of the tongue nodules (Fig. 3).

Post one year, serological analyzis revealed a reduction in the blockade titer to 0.346 in the ELISA and maintenance of the value according to the IFAT (1:160). Quantitative PCR, on the other hand, detected a significant increase in the number of DNA copies, about 10-fold compared to when it was found at the time of the previous treatment (698,576.88 DNA/µl copies of the sample), euthanasia being indicated for the infected animal according to Brazilian legislation due to the maintenance of infectivity and the tutor's negligence in maintaining the periodic and constant treatment recommended [7].

The study provided the clinical presentation, pathology and molecular confirmation of an atypical *L. infantum* infection in a bitch from the state of Minas Gerais, Brazil. These findings contribute to the inclusion of nodular glossitis in the differential diagnosis of
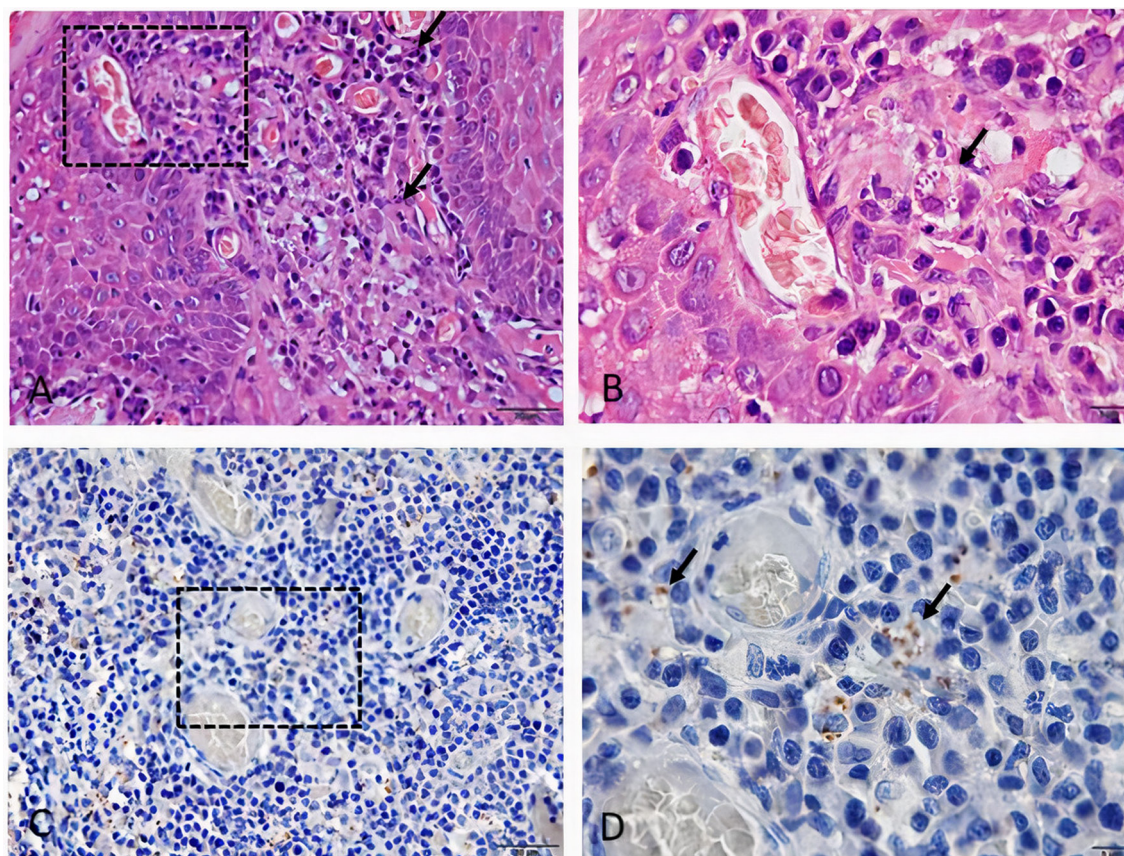
Fig. 2. Photomicrograph of the granulomatous lesions in the tongue of a dog infected with *Leishmania* spp. **A**: Severe inflammatory diffuse infiltrate in the lamina propria predominantly composed by histiocytes and plasma cells. Also a lower proportion of neutrophils and lymphocytes are observed (arrows); Structures compatible with amastigote forms of *Leishmania* spp. were observed inside and outside the cytoplasm of histiocytes. (H-E; Bar=20 μm). **B**: Histiocytes loaded with *Leishmania* spp. (arrows), (H-E; Bar=5 μm). **C**: Amastigote forms immunostained in brown (dotted frame), inside histiocytes and free (arrows). (DAB; Bar=20 μm). **D**: Higher magnification showing amastigotes inside histiocytes (arrow) (DAB; Bar=5 μm).

Fig. 3. Dorsal surface of the tongue one year after anti-leishmania treatment, demonstrating complete regression of nodular lesions.

canine leishmaniasis (CanL), allied to the worrying difficulty in the maintenance of leishmaniosis treatment in this endemic region.

Despite the common occurrence of dogs asymptomatic for CanL, the manifestation of nodular glossitis is quite rare. In Brazil, there are few studies reporting this occurrence [33], and none specifying the occurrence of this evident clinical sign in Minas Gerais, Brazil.

An unidentified sign in our case was the increased volume of superficial lymph nodes, spleen and liver, widely found in CanL and relevant in clinical diagnosis [20]. However, regression to normal size is possible in chronic cases of the disease, which is consistent with our suspicion [16].

In addition to lymphadenum, splenic and hepatomegaly, common changes in CanL such as cachexia, alopecia, anorexia, fever, lesions in the nasal and ear planes, onychogryphosis, anemia, renal failure and skin lesions were also absent or unclear (mild anemia) in this case. Identifying only nodular glossitis would generally exclude *Leishmania* spp. as a cause of disease in this animal [20, 26].

It is known that the most common signs of CanL may not be detected for long periods before its clinical presentation [4] due to the host's immune status [10]. It is possible that progression to a more obvious clinical stage may occur after some event in which the animal is exposed to a disturbance in immune status.

Histopathology revealed numerous amastigote forms of *Leishmania* spp. in various tissues of the tongue epithelium, including the superficial and deep lamina propria. The suspicion of leishmaniasis was initiated only after this identification. This represents an important finding for the diagnosis, as some authors report that for cases of canine leishmaniasis-associated lingual lesions there is always cytological and histopathological evidence, ruling out other possible differential diagnoses [13, 21, 33].

Involvement of the oral mucosa occurs and may manifest as nodular, as in our case, or ulcerative lesions [5, 6, 31]. Other cases were identified by Lamouthe and Poujade [17] and Tangalidi [31], who reported reddish ulcerative lesions with raised edges and a central depression; skin lesions are frequent in the cases of *L. infantum* in humans.

In most cases, lingual lesions are found in the context of a multisystem infection with a clinical manifestation of the disease. On the other hand, the restriction of clinical signs may represent a rare form of localized leishmaniasis, possibly due to accidental ingestion of infected sandflies bites in the region of the tongue that promote local infection in the region, or a systemic spread of *Leishmania* that, for some indefinite reason, induces multifocal lesions exclusively on the tongue [13, 27]. The latter hypothesis seems plausible in our case, based on the positive result found in molecular analysis of a bone marrow sample indicative of systemic dissemination. The occurrence of this type of lesion in a restricted region has also been described in the intestinal mucosa, a place where direct inoculation by the sandfly would not be possible, in which amastigote forms of *Leishmania* were identified in hyperemic areas, with erosion, nodules, diffuse thickening or even with no changes [22].

The final diagnosis of nodular granulomatous glossitis associated with canine leishmaniasis was established based on cytology and histopathology associated with serological and molecular diagnosis in our study. Serological tests are known to be less sensitive in infected but apparently healthy dogs compared to affected dogs [2], different from our case (Table 1). However, diagnosis in dogs that show few consistent signs, even if they are positive for leishmaniasis by serological methods, must be confirmed by molecular techniques as they are methods of higher sensitivity and specificity for CanL [19].

Considering the low possibility of spontaneous injury resolution, complete recovery of lingual lesions was possible after specific medical treatment was instituted. Overall, the response of CanL-associated tongue lesions to *Leishmania*-specific medical treatment appears to be quite effective, as several studies using various anti-leishmaniasis protocols have demonstrated complete regression of lesions, as well as the results of the present report [13, 21, 31, 33].

The maintenance or reduction of the antibody titer after treatment was not unexpected discovered in this case. It has been described previously that post-treatment kinetics of antibodies are unpredictable in canine leishmaniasis, with several permanently seropositive dogs and others with significant reductions [3].

The alarming result was the increase in post-treatment infection, as evidenced by increased DNA copies. The high number of identified DNA copies, both in the initial phase of the study and after one year by qPCR may be associated with the technique used which produces absolute quantification from synthetic targets that can repeating sequence in *L. infantum* and the type of sample selected for testing. Solcá *et al.* [29], Solano-Gallego *et al.* [28], and Ramos *et al.* [25] also detected high values in bone marrow samples collected from symptomatic dogs and equivalent to 186,000,000/100 mg of host tissue DNA; 468,000,000 and 34,940,375 DNA particles/ml, respectively. Although the vast majority of symptomatic cases have a higher parasitic DNA burden, equivalent values can be identified in bone marrow samples from asymptomatic animals, as detected by Quaresma *et al.* [24], but represent exceptions. The high sensitivity (92.9%) and specificity (99.64%) provided by the qPCR technique used combined with the collection of bone marrow samples to detect the number of DNA copies guarantees the reliability of the results of the present study [11], but it is not possible to establish a correlation for the quantitative parasites/μl or parasites/ml in our study, but the quantitative results are comparative parameters that must be correlated with the treatment and with the clinical presentation of the animal.

This result suggests the effect of the likely negligence by the animal's guardian in the treatment maintenance. It is possible that the performance of the treatment in an adequate manner initially was sufficient to promote the regression of nodular lesions on the tongue, but the subsequent negligence ensured the maintenance and increase of the parasitic load on the bone marrow, considered one of the main sites of protozoan location [25, 28, 29]. It is also necessary to consider the risks of parasitic tolerance due to treatment interruption. According to Deep *et al.* [12] *Leishmania* resistance levels have increased by about 10% over the past five years in African countries and in India. The authors attributed the increasing presence of tolerant parasites to inadequated drug exposure. Especially for *L. infantum*, the possibility of resistance to miltefosine has already been reported in the literature, which demonstrates that despite efficiency in controlling lesions, the parasite may not be eliminated after treatment [18, 23]. According to Gómez Pérez *et al.* [14] incorrect therapeutic intervention can promote a significant decrease in the susceptibility of *L. infantum*, as identified in an isolated strain in an infected dog in Spain.

Our case highlights the need to include nodular glossitis in the differential diagnosis of CanL, even when this is the only evident sign presented by the animal and especially when it resides in endemic areas. In addition, it is important to highlight the effectiveness of treatment in lesion resolution and, paradoxically, its inefficiency related to reducing infectivity, when there is tutor negligence. Finally, it is important to emphasize the importance of establishing adequate clinical protocols, combining advanced histological, serological and molecular methods for safe diagnosis with monitoring and verification of treatment efficacy to help control this important zoonotic parasite.

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