ORIGINAL ARTICLE

Equine infection with *Leishmania spp.* in Costa Rica: Study of five cases

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Abstract

Background: Cutaneous forms of leishmaniosis due to *Leishmania braziliensis* have been reported in horses in the New World. Domestic animals play a role in the transmission of the disease. In Costa Rica, human cases of *L. braziliensis*, *L. panamensis* and *L. infantum* have been reported.

Objectives: The present report describes five cases of equine cutaneous leishmaniosis in Costa Rica. The aetiological diagnosis was based on the presence of the parasite within the lesions.

Methods: Skin biopsies were used to perform histopathological analyses of the lesions. Immunohistochemistry was used to detect the presence of the *Leishmania spp.* antigens in tissue sections. Laser-capture micro-dissection and quantitative real-time PCR techniques were carried out to detect the pathogen nucleic acid within the microscopic lesions.

Results: Histopathological analyses showed a granulomatous inflammation within the dermis, with multi-nucleated giant cells, macrophages, lymphocytes and few neutrophils and eosinophils. We detected the parasite by immunohistochemistry, using a rabbit polyclonal antibody raised against *Leishmania spp.* However, we could not identify *Leishmania spp.* by quantitative real-time PCR in formalin-fixed paraffin-embedded tissues, using specific primers for the conserved region in the minicircle of the *Leishmania* DNA kinetoplast.

Conclusions: Our results emphasise the importance of *Leishmania spp.* not only as a causative agent of equine cutaneous disease in the New World, but also as a possible emerging pathogen. Leishmaniosis is one of the most prevalent parasitic public health problems worldwide, and equines may have a role in the epidemiology of the disease.

KEYWORDS
cutaneous leishmaniosis, histopathology, horse, immunohistochemistry, *Leishmania spp*, leishmaniosis

1 | INTRODUCTION

In the New World, cutaneous forms of leishmaniosis are caused by *Leishmania braziliensis*, *L. guyanensis*, *L. panamensis*, *L. shawi*, *L. naïffi*, *L. lainsoni*, *L. lindenbergi*, *L. peruviana*, *L. mexicana*, *L. venezuelensis* and *L. amazonensis*. In Europe, however, *L. infantum* causes the visceral forms and, more rarely, the cutaneous forms (OIE, 2012). Domestic animals play a role in the transmission since they are in close contact with humans and are also present in agricultural and pastoral areas that are generated by deforestation. *Leishmania spp.* infection in horses in the New World has been reported in Brazil, Venezuela, Puerto Rico and the United States. In all these cases, *L. braziliensis* was isolated (Truppel et al., 2014). *Equus caballus* has been reported to be naturally infected by *L. infantum* in the Old World since 2002 (Koehler et al., 2002) and in the New World since 2013 (Soares et al., 2013).

In Costa Rica, only human cases of infection have been reported. The most frequent aetiologic agent reported in these human cutaneous leishmaniosis cases has been *L. panamensis*, although some cases of *L. braziliensis* have also been described (Jaramillo-Antillón et al., 2009; Jaramillo-Antillón et al., 2018). Additionally, some cases of atypical cutaneous leishmaniosis caused by *L. infantum* have also been reported (Jaramillo-Antillón et al., 2009).

In the present study, five endemic cases of equine cutaneous leishmaniosis due to *Leishmania spp.* in Costa Rica are described.

2 | MATERIALS AND METHODS

Five horses (#1–#5) were taken to local veterinary practitioners in Costa Rica due to the presence of stubborn ulcerated or nodular cutaneous lesions affecting different parts of the body. Most lesions were located in the pinna and neck areas (Figure 1).

Skin biopsy samples ranging from 0.40 to 1.50 cm in diameter were surgically excised. Samples were fixed in 10% neutral-buffered formalin and embedded into paraffin wax. Sections (4 μm thick) were cut and processed for histopathological examination using haematoxylin and eosin staining, as well as immunohistochemical detection of *Leishmania spp.* antigens. Briefly, tissue sections were dewaxed and rehydrated at 19°C and then placed in a fresh solution of 3% hydrogen peroxide in methanol for 15 min in order to block endogenous peroxidase activity. Samples were then washed with tap water, antigen was retrieved by enzymatic digestion using a solution of 2% proteinase K (Dako, Glostrup, Denmark) in tris-buffered saline (TBS; 0.05 M Tris–HCl pH 7.5). Slides containing the tissue sections were then washed with TBS. To prevent nonspecific binding of antibodies, slides were blocked for 20 min with 190 μl of Universal Blocker™ Blocking Buffer in TBS (Thermo Fisher Scientific). Then, slides were incubated with 190 μl of a rabbit polyclonal antibody (diluted 1:10 in TBS) to detect *Leishmania spp.*, counterstained with haematoxylin and mounted for light microscopy examination as previously described (Salguero et al., 2018).
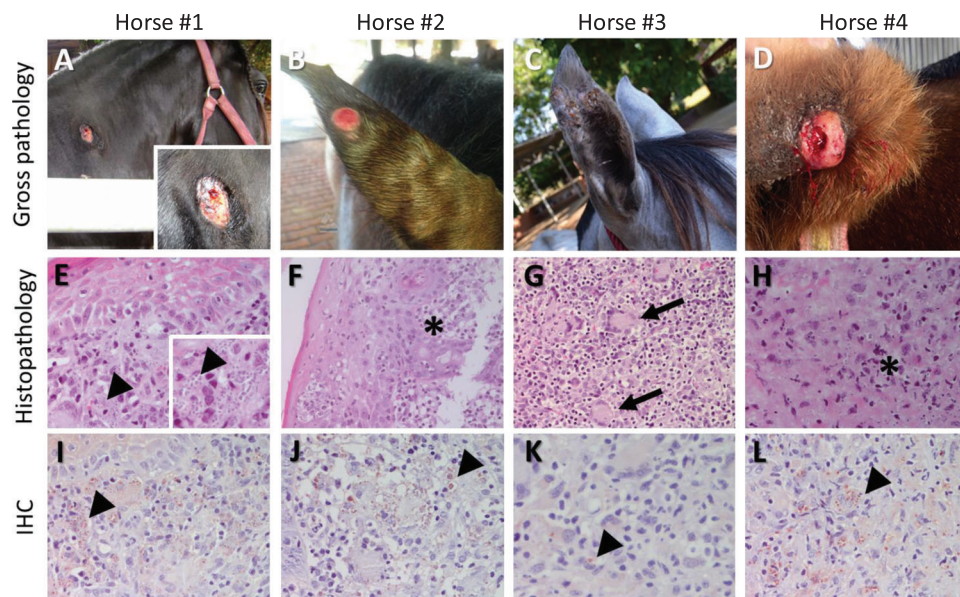

FIGURE 1 Gross pathology, histopathology and immunohistochemical detection of *Leishmania spp.* antigen in horses #1, #2, #3 and #4. Gross pathology (a, b, c, d), histopathology (e, f, g, h) and immunohistochemical detection of *Leishmania spp.* antigens (IHC; i, j, k, l) in horses #1, #2, #3 and #4. Gross lesions were non-healing skin wounds in the neck (a) neck skin or pinna (b, c, d). At histopathology, various stages of necrosis (*) and infiltration of dermis by mixed inflammatory cells, including multi-nucleated giant cells (arrowheads) are observed. Multiple ovoid amastigotes within histiocytes and in between collagen fibres can be identified (arrows), ranging from 2 to 4 μm in diameter. Amastigotes show a small round nucleus surrounded by a clear halo.
Immunoreactivity (IHC) to *Leishmania spp.* amastigotes within the inflammatory cell infiltrates, including macrophages and multi-nucleated giant cells within granulomas as well as of extracellular stages (arrowheads). The distribution of immunostaining is variable and is correlated with the degree of inflammatory response.
Magnification: 200× (f, g); 400× (e, h, i, j, l) and 600 (k)

Appropriate positive and negative controls were used in each immuno-histochemistry run.

Laser capture microdissection using polymer membrane slides (PEN-Membrane 2.0 lm Leica Microsystems, Wetzlar, Germany) was performed on skin sections (10 μm thick) from two horses (#2 and #3) that immunostained strongly against *Leishmania spp.* The sections were stained with haematoxylin and eosin, and tissues were microscopically identified, then dissected using a laser capture micro-dissection microscope (Leica LMD6500) and collected in RNase-free and DNAse-free PCR tubes (Greiner bio-one, Stroudwater, UK). Tissues showing no anti-*Leishmania spp.* immunostain were also dissected and analysed as control tissue.

Total genomic DNA was extracted from the laser-micro-dissected tissue sections using the QIAamp DNA FFPE Tissue kit (QIAGEN, Hilden, Germany), according to the manufacturert's instructions with some modifications. These modifications were the following: the xylene was preheated at 60°C for 10 min in advance, samples were heated at 37°C for 30 min to eliminate residual ethanol, samples were kept at 56°C overnight after the addition of the ATL buffer and the proteinase K, and samples were maintained at −20°C overnight after the addition of the AL buffer and ethanol. DNA obtained from biopsy specimens obtained by laser micro-dissection was suspended in 60 μl of elution buffer and frozen at −40°C until use. The concentration and quality of DNA obtained from tissues was determined using a spectrophotometer (Nanodrop 2000; Thermo Scientific). Good laboratory practices were used to avoid DNA cross-contamination. During all DNA extraction procedures, positive controls were processed using DNA from promastigotes of *L. infantum* [MCAN/ES/97/10,445] zymodema MON-1, *L. chagasi*, *L. amazonensis* and *L. braziliensis*, as were negative controls including only kit reagents without DNA.

Extracted total genomic DNA was analysed using SYBR Green-based real-time PCR and high-resolution melting as described Ceccarelli et al. (2014), with slight modifications. The primers MLF (5′-CGTTCTGCGAAAACCGAAA-3′) and MLR (5′-CGGCCCTATTTTACACCAACC-3′) were used to amplify a fragment of 111 base pairs from the conserved region in the minicircle of the *Leishmania* DNA kinetoplast. All reactions were performed using 4 μl of the PyroTaq master mix solution EvaGreen qPCR Mix Plus (Cultek Molecular Bioline, Madrid, Spain), 0.5 μl of each primer (20 μM), and 5 μl of DNA in a total reaction volume of 20 μl. The following thermal protocol was used: one cycle at 95°C for 15 min, followed by 40 cycles at 95°C for 15 s, 60–65°C for 20 s and 72°C for 20 s. All reactions were carried out in a LightCycler 480 thermal cycler (Roche, Basel, Switzerland). After PCR amplification, high-resolution melting curves were acquired from 75°C to 92°C, with a slope of 0.1°C/s and 2 s for each temperature. The curves were analysed using the Roche LightCycler 480 Basic v1.2 software.

3 | RESULTS

This retrospective study covers five horses that were brought to local veterinarians in Costa Rica between April 2009 and April 2016. Horse

#1, a 4-year-old Pinto Americano male, had an alopecic ulcerative lesion of 2.0 cm of diameter on the right side of the neck. Horse #2, an adult Andalusian female, had several cutaneous masses in the external left pinna, some of them ulcerated. Horse #3, a 2.5-year-old Andalusian female, also showed cutaneous nodular, crusting and ulcerated lesions. Horse #4, a 2.5-year-old Andalusian female, displayed an ulcerated cutaneous lesion in the internal right pinna. Horse #5, another 2-year-old Andalusian female, exhibited some cutaneous ulcerated and crusting masses in the neck and inner left pinna.

In all horses, histopathological evaluation based on tissue sections stained with haematoxylin and eosin showed various stages of necrosis in the dermis, together with a diffuse infiltration of mixed inflammatory cells, containing neutrophils, lymphocytes, plasma cells, macrophages and multi-nucleated giant cells. Multiple ovoid amastigotes of 2–4 μm in diameter were observed within macrophages and among collagen fibres. Amastigotes had a small round nucleus surrounded by a clear halo. These morphological findings suggested the inclusion of cutaneous leishmaniosis in the differential diagnosis (Figure 1).

In all horses, positive immunostaining against *Leishmania spp.* was observed in multiple macrophages within the inflammatory cell infiltrates, in macrophages within granulomas, and in extracellular stages. Immunostaining was less strong in horse #4 than in the other horses, and its intensity correlated with the extent of inflammatory cell infiltration described before (Figure 1).

No positive reaction was observed after real-time PCR and high-resolution melting of genomic DNA isolated from laser-micro-dissected paraffin-embedded tissues. In contrast, the expected positive reactions were observed in control samples of promastigote DNA from *L. infantum*, *L. chagasi*, *L. amazonensis* and *L. braziliensis* (Table 1 and Figure 2).
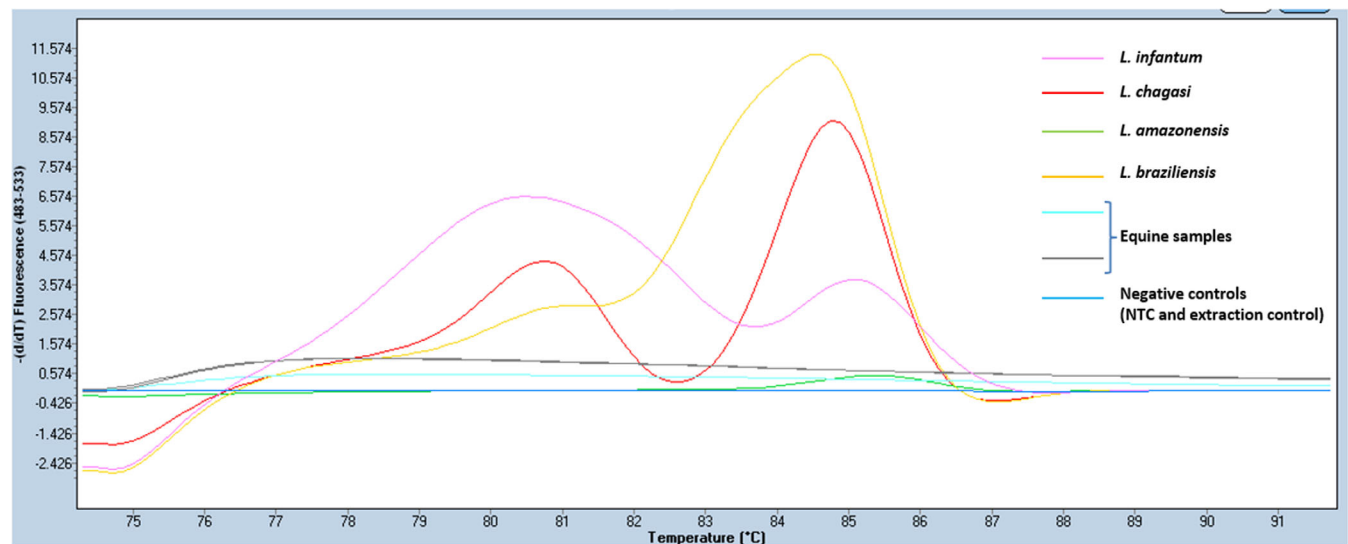
4 | DISCUSSION

In Europe, equine leishmaniosis caused by *L. infantum* and *L. siamensis* (the name *siamensis* is a nomen nudum as described by Espinosa et al., 2018) has been described in Germany (Koehler et al., 2002; Müller et al., 2009), Spain (Solano-Gallego et al., 2003), Portugal (Gama et al., 2014; Rolão et al., 2005), Switzerland and Germany (Müller et al., 2009). Cutaneous leishmaniosis in *Equidae* has been diagnosed in South America (Aguilar et al., 1986), North America (Reuss et al., 2012) and the Caribbean (Ramos-Vara et al, 1996). In the Americas, the first report of *E. caballus* naturally infected with an aetiological agent of visceral leishmaniosis (*L. donovani* complex) occurred in Brazil, and *L. infantum* was the infective species (Soares et al., 2013). So far, there are no reported cases of cutaneous leishmaniosis in horses caused by any species from the *L. donovani* complex in Costa Rica. The present study describes for the first time, five cases of equine leishmaniosis that occurred during a 7 years' period.

In Costa Rica, cases of New World cutaneous leishmaniosis in humans are common in rural areas and can also occur in semi-urban and urban areas, as well as rainforests and arid areas; cases have also been reported in Texas and Oklahoma in the southern US, in Mexico, and in

TABLE 1 High-resolution melting curve analysis

Sample	Cq	Tm peak 1 (°C)	Tm peak 2 (°C)
L. infantum 10445 (positive control)	18.44	80.25–80.51	85.11–84.89
L. chagasi (positive control)	24.10	80.51–80.86	84.77–84.82
L. amazonensis (positive control)	35.00	NA	85.33–85.40
L. braziliensis (positive control)	20.66	NA	84.38–84.53
Animal #2	35.00	81.00	
Animal #3	Negative		
NTC	Negative		

Cq: cycle of quantification; Tm: temperature of melting; NTC: no template control; NA: not applicable.

FIGURE 2 High-resolution melting curve analysis of qPCR products. No positive results were obtained for horses #2 and #3 (equine samples) after laser-capture micro-dissection and qPCR

Central and South America, mostly Brazil and Peru (iamat Costa Rica, 2016) . In the Costa Rica humans cases, the most frequently agent isolated is *L. (Viannia) panamensis*, although some cases of *L. (V.) braziliensis* have been reported. The known parasite wildlife reservoir hosts in Costa Rica are the sloths (*Bradypus griseus* and *Cholepus hoffmani*). *B. griseus* shows an infection rate of 3.5% and occupies low-lying, humid areas, while *C. hoffmani* shows an infection rate of 3.1% and occupies highlands. Neither species is found in northwestern Costa Rica, including the Nicoya Peninsula. Other *Leishmania* species and hosts have also been reported in Costa Rica: *L. panamensis* has been isolated from the wild mouse *Heteromys desmarestianus*, and dogs and rodents as hosts of atypical cutaneous leishmaniosis caused by *L. infantum* (*L. chagasi*) has been described (Jaramillo-Antillón et al., 2009; Jaramillo-Antillón et al, 2018).

In the five cases described here, we were able to demonstrate that the infectious agent belonged to *Leishmania spp.* by using immunohistochemistry with an in-house developed primary antibody, which may become a useful reagent for the detection and characterization of *Leishmania spp.* infections (Salguero et al., 2018). In con-trast to the clear signals observed with immunohistochemistry, we failed to detect *Leishmania* spp. DNA using qPCR after laser-capture micro-dissection. We attribute this failure to the small amount and poor quality of DNA extracted from formalin-fixed, paraffin-embedded samples.

The five horses described here presented cutaneous lesions located in at least one of the most frequent sites of presentation of the disease in horses, including the ears, the legs, the neck, and the axillary and inguinal regions, in agreement with other studies (Aguilar et al., 1984; Barbosa-Santos et al., 1991; Bonfante-Garrido et al., 1981; Falqueto et al., 1987; Gama et al., 2014; Koehler et al., 2002; Müller et al., 2009; Oliveira-Neto et al., 1988).

Since the horses described in this study were born in Costa Rica (three of them are from the dry Pacific cost while the other two are from the central valley) and never left the country, endemic infection cannot be excluded and suggests the adaptation of *Leishmania spp.* to this new mammalian host, as already reported in Germany (Koehler et al., 2002) and more recently in Brazil (Soares et al., 2013).

This appears to be the first report of cutaneous leishmaniosis by *Leishmania spp.* in horses in Costa Rica. The disease was diagnosed in the five horses in this study using anti-*Leishmania spp.* immunohistochemical detection. Additional studies with fresh and snap-frozen samples are needed to determine the *Leishmania* species involved. Furthermore, the prevalence of cutaneous leishmaniosis in horses from these areas of Costa Rica and their role in the parasite life cycle should be analysed.

AUTHOR CONTRIBUTIONS

Conceptualization, data curation, formal analysis, writing-review and editing: Maria Victoria Ortega-Garcia, Francisco Javier Salguero and Alexis Berrocal. *Data curation, formal analysis, writing-review and editing*: Inmaculada Moreno, Mercedes Dominguez and Nerea Garcia.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.587.

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