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Short Communication

Cutaneous leishmaniosis in a horse from northern Portugal

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ABSTRACT

The first case of cutaneous leishmaniosis in a horse from the north of Portugal, with a 1.5 cm in diameter ulcerated nodular lesion on the left face, is reported. The skin nodule was surgically excised and assessed by histopathology, including an immunohistochemistry method applied for the first time to equine tissues which clearly demonstrated leishmanial amastigote forms. Two serological determinations with the direct agglutination test performed 13 months apart showed seroconversion specific for *Leishmania* from a <25 to a 200 antibody titre. Polymerase chain reaction followed by kinetoplast DNA sequencing provided a 116-bp sequence with 98% identity to *Leishmania infantum* closest sequence deposited in GenBank. No recurrence was observed after complete surgical excision. Leishmaniosis should be included in the differential diagnosis of cutaneous nodular or papular lesions in the equine species in Portugal.

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1. Introduction

Leishmaniosis due to *Leishmania infantum* (syn. *Leishmania chagasi*) is an endemic zoonotic disease in countries of the Mediterranean basin, America and Asia. Phlebotomine sand flies are the vectors and dogs the reservoirs of *L. infantum* transmission to humans (Palatnik-de-Sousa and Day, 2011), but infection has also been described in other hosts, including horses (Lopes et al., 2013). In particular, cutaneous leishmaniosis caused by *L. infantum* has been sporadically found in horses from Central (Koehler et al., 2002) and southern Europe (Solano-Gallego et al., 2003; Rolão et al., 2005) and most recently from Brazil (Soares et al., 2013). Clinical manifestations are usually characterized by single or multiple nodular or papular lesions on the head, limbs or axillary and inguinal regions. *Leishmania siamensis*, a distinct species originally described from Thailand (Sukmee et al., 2008), was also detected in some horses from Switzerland and Germany (Müller et al., 2009). Here a case of equine cutaneous leishmaniosis in northern Portugal, where zoonotic leishmaniosis is endemic, is presented.

2. Materials and methods

The horse, a two-year-old male Belgisch Warmbloed Paard, originated from the municipality of Vila Real (41°20′34″ N, 7°44′54″ W), in the North region of Portugal. The animal was born in Portugal and had never travelled abroad. A thorough physical examination revealed an ulcerated nodular lesion in the left face with 1.5 cm in

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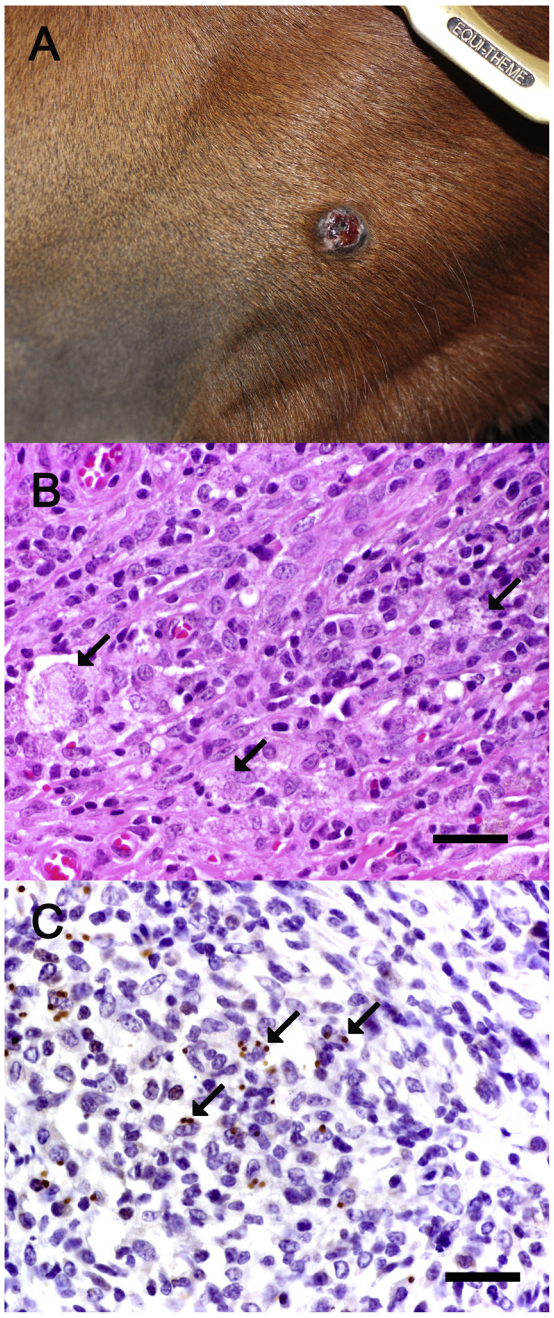

Fig. 1. (A) Horse presenting an ulcerated nodular lesion (1.5 cm in diameter) on the left face. (B) Equine dermis: diffuse periadnexal inflammatory reaction comprising macrophages and multinucleate giant cells, with *Leishmania* amastigotes in the cytoplasm (arrows); H&E staining, bar = 30 μm. (C) Equine dermis: immunohistochemical demonstration of *Leishmania* with labelled amastigote forms (arrows) within macrophages; streptavidin-biotin peroxidase complex method, bar = 30 μm.

diameter (Fig. 1A), but no other lesions. The skin nodule was surgically excised and fixed in 10% neutral buffered formalin.

The sample was embedded in paraffin, sectioned at 3 μm-thick pieces, and stained with haematoxylin and eosin (H&E) and Giemsa methods. Additional sections were used for immunohistochemistry (IHC), using an alternative method, according to Tafuri et al. (2004). Briefly, the deparaffinised slides were hydrated and antigen retrieval was carried out by microwave treatment in 10 mM citrate buffer, pH 6.0. After cooling (20 min at room temperature), sections were immersed in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Sections were then incubated with a blocking serum (Ultra V Block, UltraVision; Labvision, Freemont, CA, USA) for 10 min, to block nonspecific immunoglobulin (IgG) absorption to tissues, and then incubated overnight at 4 °C in a humid chamber with the primary antibody (a canine hyperimmune serum, obtained from one dog naturally infected with *L. infantum*), diluted 1:100 in PBS. After washing in PBS, the slides were incubated with a secondary antibody (Biotinylated Goat Anti-Polyvalent, UltraVision) for 10 min, washed in PBS, and then incubated with a streptavidin-peroxidase complex (Streptavidin Peroxidase, UltraVision) for 10 min at room temperature. Reaction was developed with a 3,3 diaminobenzidine tetrahydrochloride (DAB) solution. Finally, slides were counter-stained with Gill's haematoxylin, dehydrated, cleared, and mounted for evaluation by light microscopy. Skin from a naturally infected dog with numerous *Leishmania* amastigotes was used as positive control.

The direct agglutination test (DAT), with a standard freeze-dried antigen at a concentration of 5×10^7 promastigotes/ml (KIT Biomedical Research, Amsterdam, The Netherlands), was used to determine the antibody titre to *Leishmania* in a plasma sample, as previously described (Schallig et al., 2002; Lopes et al., 2013). Plasma from a horse with a DAT titre of 800 was used as positive control; plasma from another horse that lived in a non-endemic region was used as negative control (DAT titre <25).

DNA was isolated from a small section of tissue (25 mg). Paraffin and fixative were removed by extraction with xylene and washing with PBS, respectively. DNA was isolated with a commercial purification kit (QIAamp® DNA Mini; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Primers 5′-CCTATTTTACACCAACCCCCAGT-3′ and 5′-GGGTAGGGGCGTTCTGCGAAA-3′ were used to amplify a 116-bp fragment of the kinetoplast minicircle DNA (kDNA) (Nicolas et al., 2000). The *Leishmania*-specific amplified products were directly ligated in the PGEM-T plasmid vector (Promega, Madison, WI, USA) and cloned into *Escherichia coli* DH5α and placed on LB agar plates with ampicillin X-gal, and isopropylthiogalactoside for recombinant selection. Three white recombinant colonies were individually used for plasmid preparation, and the inserts were sequenced (Eurofins MWG Operon, Ebersberg, Germany) with the M13 rev (−29) and M13 uni (−21) primers. The obtained sequences were analyzed with the BLAST web server, hosted by NCBI using the BLASTn utility (Zhang et al., 2000).

3. Results and discussion

Histological examination of the cutaneous lesions revealed a nodular to diffuse periadnexal inflammatory

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