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INFECTIOUS DISEASE

Cutaneous Leishmaniosis caused by *Leishmania martiniquensis* in a Horse in Florida

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Summary

We report a new case of cutaneous leishmaniosis caused by *Leishmania* (*Mundinia*) *martiniquensis* in a horse in Florida, USA. A 10-year-old neutered male Quarter horse was presented with multifocal to coalescing, raised, ulcerated and oozing, non-healing wounds on both pinnae of several weeks' duration. After a few months, the lesions regressed spontaneously. Biopsies of the lesions were performed with microscopical findings of epidermal hyperplasia with multifocal ulceration and focally extensive, dermal pyogranulomatous inflammation with numerous intact and degenerate neutrophils being surrounded by epithelioid macrophages, lymphocytes and plasma cells, as well as rare eosinophils. Within the macrophages, and freely within the inflammatory infiltrate, were small (2–4 μm) round, basophilic protozoal organisms. Immunohistochemistry and colourimetric in-situ hybridization were positive for amastigote forms of *Leishmania* spp. The species *L. martiniquensis* was identified by polymerase chain reaction targeting the *ITS-1* gene performed with extracts from formalin-fixed and paraffin wax-embedded samples of skin lesions. *L. martiniquensis* causes an ulcerative pyogranulomatous dermatitis in horses with spontaneous healing. This second autochthonous case in Florida, 5 years after the first case, suggests that this parasite may have become endemic in this state.

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Leishmanioses are diseases caused by the protozoan *Leishmania* spp., which infects man, mammals and reptiles and is endemic in nearly 100 countries (Burza *et al*., 2018). The transmission of *Leishmania* spp. to man and animals mainly occurs by the bites of female phlebotomine sandflies. There are three main forms of leishmaniosis: cutaneous leishmaniosis (CL), visceral leishmaniosis (VL) and mucocutaneous leishmaniosis (MCL) (WHO, 2019). CL (annual incidence of 0.6–1 million human cases worldwide) is characterized by skin lesions, mainly ulcers (Burza *et al*., 2018; WHO, 2019). MCL causes ulcerative lesions of the mucous membranes of the nose, mouth and throat (WHO, 2019). Both CL and MCL can evolve to scarring, disfiguring and disabling lesions (Gontijo and Carvalho, 2003). VL (annual incidence of 50,000 to 90,000 human cases worldwide) is characterized by inflammatory lesions in viscera, especially the liver, spleen and bone marrow, and causes high mortality if left untreated (Burza *et al*., 2018; WHO, 2019).

In the USA, VL caused by *Leishmania infantum* is enzootic in the hunting dog population in many states (Toepp *et al*., 2017). Recently, the first autochthonous human case of CL, caused by *L. donovani* species complex, was reported in North Dakota (Douvoyiannis *et al*., 2017). CL caused by *L. mexicana* is endemic in Texas and affects people, dogs and cats (McIlwee *et al*., 2018). In addition, an autochthonous case of

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CL was reported in a horse in Florida (Reuss *et al.*, 2012). The species in this case, and of other emergent cases of CL in nine horses in Germany and Switzerland (Müller *et al.*, 2009), in a cow in Switzerland (Lobsiger *et al.*, 2010) and also of CL and VL of people in Thailand and Myanmar (Leelayoova *et al.*, 2017; Jariyapan *et al.*, 2018) were formerly identified as '*Leishmania siamensis*'. This species name is now taxonomically invalid (Sereno, 2019). Based on taxonomic studies using molecular methods, the *Leishmania* species of these cases in animals and in the majority of human cases in Thailand and Myanmar were identified as *Leishmania* (*Mundinia*) *martiniquensis* (Leelayoova *et al.*, 2017; Jariyapan *et al.*, 2018; Sereno, 2019).

The species *L. martiniquensis* was first isolated in human patients with CL in the Martinique Island in 1995, but named in 2014 (Desbois *et al.*, 2014). Later, this species was assigned to the newly created subgenus *Mundinia* (Espinosa *et al.*, 2018). This subgenus also includes *L. enrietti* of guinea pigs in Brazil, *L. macropodum* of macropods in Australia, *L. orientalis* of people in Thailand and *Leishmania* species of people in Ghana (Jariyapan *et al.*, 2018; Sereno, 2019). However, the prevalence, life cycle, vectors and zoonotic potential of the species in this worldwide emergent subgenus are still unknown.

In this context, the aim of this study was to describe the occurrence, clinical signs, histological alterations and molecular diagnosis of the second autochthonous case of CL caused by *L. martiniquensis* in Florida, USA.

In August 2016, a 10-year-old neutered male Quarter horse from Lake Wales (27°54′17″ N and 81°35′3″ W), Florida, USA, was presented with a several week history of bilateral, non-healing wounds on the ear pinnae. Lesions were raised, ulcerated, oozing and multifocal to coalescing. There were three horses on the farm and only this horse was affected. Two 3–4 mm punch biopsy samples were obtained from the wounds on the left pinna after local disinfection with 70% alcohol and anaesthesia with 2% lidocaine. The biopsy samples were fixed for 48 h in 10% neutral buffered formalin for histopathological examination. After several months, the lesions resolved and the skin of the formerly affected ears appeared unremarkable.

The skin biopsy samples were processed routinely and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin (HE), Grocott's methenamine silver stain (GMS), periodic acid–Schiff (PAS) and Giemsa (Carson and Cappellano, 2015). Microscopically, the haired skin had mild hyperplasia of the epidermis and epidermal adnexa, but in most areas, the epidermis was extensively ulcerated (Supplementary Fig. 1). There was a diffuse pyogranulomatous inflammatory infiltrate composed of large numbers of neutrophils, macrophages, lymphocytes and plasma cells, as well as rare eosinophils, which obscured and sometimes effaced the normal dermal architecture (Fig. 1, Supplementary Fig. 2). Within parasitophorous vacuoles in the cytoplasm of macrophages and, rarely, neutrophils were small (2–4 μm) round, basophilic protozoal organisms (Fig. 1). In some organisms, a basophilic nucleus was identified at one end of the protozoal organism. Giemsa staining highlighted and accentuated the organisms, although a kinetoplast was not obvious. GMS and PAS stains were negative. Based on the morphological characteristics of the observed protozoan organisms, the suspected diagnosis was leishmaniosis.

For the confirmation of the amastigote form of *Leishmania* spp., serial sections were processed for colourimetric in situ hybridization (CISH) and immunohistochemistry (IHC). IHC was performed using an in-house rabbit polyclonal anti-*Leishmania* serum (Oliveira *et al.*, 2017). For CISH, we used a digoxigenin-labelled oligonucleotide probe that detects a 5.8S rRNA sequence specific to all relevant *Leishmania* species (Dinhopl *et al.*, 2011) in an automated protocol (Menezes *et al.*, 2013). Amastigote forms of *Leishmania* spp. were detected by both IHC (Fig. 2) and CISH (Fig. 3).

For the identification of *Leishmania* spp. at species level, a conventional polymerase chain reaction (PCR) followed by sequencing of PCR products was performed. For PCR, eight 5 μm serial sections were cut from the paraffin wax block containing the

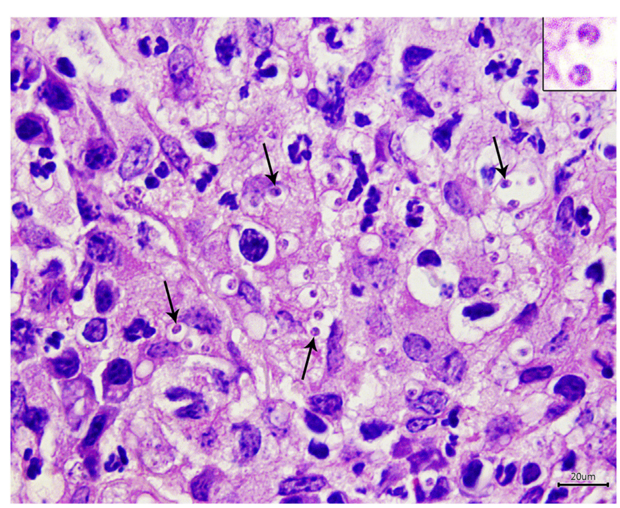

Fig. 1. Diffuse pyogranulomatous dermatitis composed of large numbers of neutrophils, macrophages, lymphocytes and plasma cells. Amastigote forms of *Leishmania* (arrows and inset) are observed within macrophages. HE.

samples of skin lesions and subjected to DNA extraction. DNA extraction was done using the QIAamp® DNA FFPE tissue commercial kit (Qiagen, Valencia, California, USA) on the semi-automated Qiacube (Qiagen) nucleic acid extraction platform, following the manufacturer's recommendations. The extracted DNA was amplified using a pair of primers for the region of the ribosomal internal transcribed spacer 1 (*ITS-1*) gene, according to previously described protocols (Schönian *et al.*, 2003; Graça *et al.*, 2012). As a positive control for the reaction, 10 ng/µl of the *L. infantum* reference strain (MHOM/BR/74/PP75) was used. Bands of expected size for PCR products (300 base pairs) were visualized in 2% agarose gel and purified using the PCR Clean-up System™ kit (Promega, Madison, Wisconsin, USA). Sequencing was performed with the PCR product at a concentration of 3 ng DNA and primers *ITS-1* at 3.2 pmol in the sequencer ABI3730xl (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The sequences were multiple aligned with a set of *Leishmania* strains retrieved from GenBank using Program MEGA (Molecular Evolutionary Genetics Analysis), version 4.

After alignment of the sequences, an identity of 100% was observed for the PCR target with the strain GQ281278.1, which reportedly caused CL in a horse in Germany (Müller *et al.*, 2009) and was identified by molecular analyses as *L. martiniquensis* (Leelayoova *et al.*, 2017) (Fig. 4).

This was the second autochthonous case in Florida of *L. martiniquensis* in a horse and it also occurred in summer. The first case was reported in a 10-year-old mare in August 2011 (Reuss *et al.*, 2012). Another case of CL in a horse was also reported during the same time period in Florida (Reuss, 2013), but the species was identified as '*L. siamensis*', with no further taxonomic reclassification. *L. martiniquensis* has also been reported in animals from Central Europe, but no new cases in horses and cattle have been reported there since 2010 (Müller *et al.*, 2009; Lobsiger *et al.*, 2010). The reappearance of CL caused by *L. martiniquensis* in horses in Florida indicates that this parasite is cycling in this region and may have become endemic in horses. A hypothesis for the

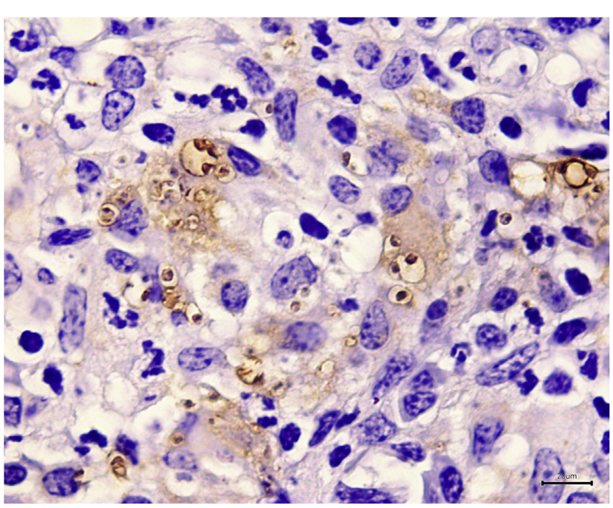

Fig. 2. Amastigote forms of *Leishmania* within macrophages in the dermis. IHC.

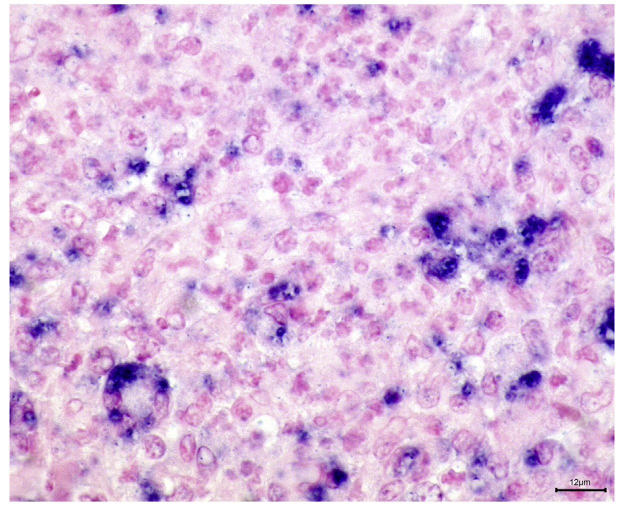

Fig. 3. Amastigote forms of *Leishmania* within macrophages in the dermis. CISH.

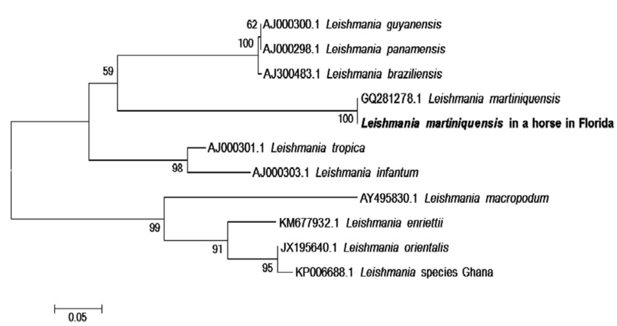

Fig. 4. Phylogenetic analysis of DNA sequences of the main species of *Leishmania* using *ITS-1* sequences and evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.25315367 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated. There were a total of 172 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

emergence of *L. martiniquensis* in Florida, Central Europe, Thailand and Myanmar is the possible increase in abundance of potential insect vectors due to global warming, which should be investigated. In Florida, such potential vectors include the phlebotomine sandflies *Lutzomyia shannoni*, *Lutzomyia cubensis*, *Lutzomyia vexator* and *Lutzomyia cruciata* (Reuss *et al*., 2012). In addition, there is strong evidence that biting midges (Diptera: Ceratopogonidae) of the genera *Culicoides* and *Forcipomyia* can be the vectors of *Leishmania* of subgenus *Mundinia*, because promastigote forms of this parasite can survive in the gut of these insects (Dougall *et al*., 2011; Šeblová *et al*., 2015). In Florida, the nine species of biting midges that infest horses are *Culicoides insignis*, *Culicoides stellifer*, *Culicoides niger*, *Culicoides alachua*, *Culicoides venustus*, *Culicoides scanloni*, *Culicoides debilipalpis*, *Culicoides pusillus* and *Culicoides edeni*, which are not anthropophilic (Greiner *et al*., 1990). However, further investigation of *L. martiniquensis* infections in biting midges and phlebotomine sandflies in Florida is needed to confirm their role in the transmission of this protozoan.

The multiple ulcerated lesions in the skin of the pinnae observed in this horse are similar to the lesions described by other authors in horses and a cow infected by *L. martiniquensis* (Müller *et al*., 2009; Lobsiger *et al*., 2010). These animals also presented with multiple nodules, masses and plaque-like lesions in the skin of the head, axilla, neck, shoulder, withers, thorax, flank, legs, muzzle and udder. Regardless, lesions are non-specific and similar to those lesions described in horses infected by *Leishmania braziliensis* (Barbosa-Santos *et al*., 1994) or *L. infantum* (Koehler *et al*., 2002; Solano-Gallego *et al*., 2003). Clinical differential diagnoses also include cutaneous neoplasms, mycoses, cutaneous habronemiasis or hypersensitivity reactions (Solano-Gallego *et al*., 2003). Although only CL with frequent spontaneous healing and rare recurrence has been observed in animals infected by *L. martiniquensis* (Müller *et al*., 2009; Lobsiger *et al*., 2010; Reuss *et al*., 2012), this parasite can cause lethal VL in immunocompetent people or people immunocompromised by human immunodeficiency virus infection/acquired immunodeficiency syndrome (Pothirat *et al*., 2014; Liautaud *et al*., 2015).

Microscopically, ulcers and hyperplasia of the epidermis as well as pyogranulomatous or granulomatous dermatitis associated with amastigote forms of *L. martiniquensis* within macrophages were observed in previous cases in horses and in a cow (Müller *et al*., 2009; Lobsiger *et al*., 2010; Reuss *et al*., 2012). These lesions were similar to the histological findings in the present case, but are also similar to lesions reported in horses and dogs infected with *L. braziliensis* or *L. infantum* (Barbosa-Santos *et al*., 1994; Koehler *et al*., 2002; Solano-Gallego *et al*., 2003; Miranda *et al*., 2010). However, different from previous cases of *L. martiniquensis* dermatitis in horses (Müller *et al*., 2009; Reuss *et al*., 2012), eosinophils were present in the lesion in this horse, while multinucleated giant cells were absent. Eosinophils were also present in the dermatitis associated with *L. martiniquensis* in a cow (Lobsiger *et al*., 2010), but in larger numbers compared with this equine case. Eosinophils appear to be an important defence mechanism for the control of *Leishmania* spp. infections, since their presence in cutaneous lesions or affected lymph nodes is associated with smaller numbers of amastigote forms of this parasite (Gutierrez *et al*., 1991; Costa *et al*., 2018). Another possible reason for the presence of eosinophils in the present case is a hypersensitivity reaction to the bites of insects, mainly biting midges of the genus *Culicoides* (Oliveira-Filho *et al*., 2012), which is a common disease in horses in Florida (Greiner, 1995). These small variations in the composition of the inflammatory infiltrate in infections by *L. martiniquensis* in animals may also reflect differences in the immune response of individual hosts. Amastigote forms of *L. infantum* are frequently detected in the intact skin of dogs, which have become an important reservoir of this parasite in some parts of the world (Madeira *et al*., 2009). Therefore, investigation of whether amastigote forms of *L. martiniquensis* may be found in the intact skin of horses may provide important information about the potential of horses as reservoirs of this parasite.

In conclusion, *L. martiniquensis* can cause an ulcerative pyogranulomatous dermatitis in horses with spontaneous healing. The second autochthonous case in Florida, 5 years after the first case being recognized, suggests that this parasite may have become endemic in this state.

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Conflict of Interest Statement

The authors declare no conflict of interest regarding the publication of this manuscript.

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jcpa.2019.09.011.

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