Case Report Rapport de cas

Leishmania infantum infection in a dog imported from Morocco

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Abstract — A mixed breed dog rescued from Morocco was presented at a Quebec veterinary practice for facial lesions. Leishmaniosis, an exotic disease caused by the zoonotic protozoan *Leishmania infantum,* was suspected. Genomic DNA extraction from blood samples and polymerase chain reaction (PCR) were used to confirm *L. infantum* parasitemia. Parasites were successfully cultured from lesion biopsies, and dose-response assays demonstrated susceptibility to miltefosine, a drug that requires importation from Europe. Twenty-eight days of treatment led to the disappearance of lesions, but relapse occurred several months later (consistent with persistent parasitemia on post-treatment analysis). Further treatment would require importation of drugs and significant delays, offering a poor prognosis.

Key clinical message:
Diagnosis of tropical diseases in Canada will likely become more common in the near future. Having proper diagnostic tools, effective drugs, and stricter control of animal importation are essential to preventing the spread of these dangerous and frequently zoonotic diseases.

Résumé — Infection par *Leishmania infantum* chez un chien importé du Maroc. Un chien de race croisée réchappé du Maroc fut présenté dans une pratique vétérinaire du Québec pour des lésions faciales. La leishmaniose, une maladie exotique causée par le protozoaire zoonotique *Leishmania infantum*, fut suspectée. L'extraction d'ADN génomique d'échantillons sanguins et la réaction d'amplification en chaine par la polymérase (PCR) furent utilisées pour confirmer la parasitémie à *L. infantum*. Les parasites furent cultivés avec succès à partir de biopsies des lésions et des essais dose-réponse ont démontré une sensibilité au miltefosine, un médicament devant être importé d'Europe. Vingt-huit jours de traitement ont mené à la disparition des lésions, mais une rechute se produisit plusieurs mois plus tard (compatible avec une parasitémie persistante lors d'analyses post-traitement). Des traitements supplémentaires nécessiteraient l'importation de médicaments et des délais significatifs, offrant ainsi un pronostic peu optimiste.

Message clinique clé :
Le diagnostic de maladie tropicale au Canada devrait devenir plus fréquent dans un avenir rapproché. Il est essentiel d'avoir les outils diagnostiques appropriés, des médicaments efficaces et un contrôle plus sévère des importations d'animaux afin de prévenir la propagation de ces dangereuses et fréquentes maladies zoonotiques.

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Leishmaniosis is a serious disease transmitted by sandflies (1). The etiologic agent of canine leishmaniosis is the zoonotic protozoan species *Leishmania infantum,* and dogs serve as a reservoir for this infectious agent (2,3). Leishmaniosis in humans is a neglected tropical disease, affecting the eastern Mediterranean region, southeast Asia, and parts of Africa, Europe, and Central and South America, with an estimated 1.3 million new cases annually (4,5). Conservative estimates based on serological data indicate that approximately 2.5 million dogs are affected in endemic western Mediterranean countries alone (6). Leishmaniosis in humans can manifest in several ways, including a disfiguring cutaneous form, mucocutaneous form, and fatal visceral form (1). In dogs, *L. infantum* infections typically lead to cutaneous lesions with lymphadenomegaly, lethargy, and weight loss, sometimes progressing to deposition of immune complexes and corresponding immune-mediated signs such as glomerulonephritis, keratoconjunctivitis sicca, and vasculitis (3,7,8). Diagnosis of canine leishmaniosis can be challenging due to a number of factors, including the high percentage of apparently healthy carriers, nonspecific nature of clinical

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signs, and lack of accessible diagnostic tools in non-endemic areas (1,3). This disease poses a serious public health risk, and prevention is based on vector control, reducing sandfly/human contact, and control of the animal reservoir (dogs), among others (4). While the phlebotomine vector of this protozoan parasite is not present in Canada, certain indigenous species may be competent vectors of *Leishmania* (9). Moreover, recent studies have shown the potential role of ticks in the infectious cycle of leishmaniosis (10,11). Given predictions that climate change may lead to expansion of *Leishmania* to North America, surveillance for this important zoonosis (among other exotic diseases) should be implemented and enforced (12). Current rules for importing animals into Canada require only that the animal be rabies-free (vaccinated or accompanied by a certificate of health from a licensed veterinarian) and appear healthy at inspection (13). Here we describe the case of a dog imported from Morocco to Quebec, with lesions on the face and muzzle that were later diagnosed to be infection with *Leishmania infantum.*

Case description

A mixed breed dog was presented to a private clinic in Quebec for lesions on the muzzle and medial canthus of the left eye. The dog had recently arrived in Quebec from a shelter in Morocco and had an unknown history. The lesions were granulomatous and weeping and were originally thought to be wounds from a fight. The dog was treated with Cefaseptin (Vétoquinol, Lavaltrie, Quebec), 12.5 mg/kg body weight (BW), PO, q12h for 15 d. Three months later, the dog was again presented because the lesions had not disappeared; however, they were stable (granulomatous without purulent secretions) and no new lesions had appeared. The veterinarian suspected possible autoimmune disease and implemented treatment with Prednisolone (Rafter 8; Calgary, Alberta) at an anti-inflammatory dose (0.5 mg/kg BW, PO, q12h for 7 d, q24h for 10 d, then q48h). Inflammation receded, but the lesions persisted. Approximately 1 mo later, the lesions began to spread to the limbs of the dog. Leishmaniosis was suspected.

The veterinarian consulted with the Faculté de médecine vétérinaire — Université de Montréal (FMV). Biopsies (both fresh and in formalin) of lesions on the lateral face of the right tarsus and medial face of the left carpus (firm, alopecic masses with dry, red skin), and muzzle (where lesions had progressed to become ulcerated and crusty), as well as a biopsy of the right submandibular lymph node were sent to the FMV Pathology and Microbiology department. Blood and serum samples were sent to the FMV Molecular Parasitology department, where they were processed by our team. DNA was extracted from whole blood using the QIAmp DNA Blood Mini Kit (Qiagen, Germantown, Maryland, USA). The pteridine reductase 1 gene *(PTR1),* which allows for the identification of *Leishmania* species, was amplified using compatible primers (14). Two additional *Leishmania* genes, LINF_230008200 (Fw: 5′-ATGCGCTTCAAGGCG-3′; Rv: 5′-CTACACCCTTGCGGGG-3′) and LINF_230007700 (Fw: 5′-ATGCTCAACGAGGTGC-3′; Rv: 5′-CTAAATAC CAACCAGA-3′), were amplified and sequenced to further corroborate *L. infantum* as causative agent. Polymerase chain reactions (PCRs) were performed in 50 μL volumes using the compatible primers and contained 100 ng of total gDNA, 0.5 μM of each primer, 0.2 mM of dNTPs, and 1 U of Phusion DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA). Amplification was performed in 25 cycles: denaturation at 98°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 1 to 2 min (depending on the size of the PCR amplicon). A final extension was performed at 72°C for 5 min. PCR products were electrophoresed on 0.7% agarose gels, purified with the Monarch DNA Gel Extraction Kit (New England Biolabs, USA) and sequenced. Cell culture, considered the gold standard method for *Leishmania* diagnosis (15), was also carried out with samples from the affected dog's lesions in 5 mL of M199 medium (Gibco, ThermoFisher, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum (Wisent, Saint-Jean-Baptiste, Quebec), 5 μg/mL of hemin at pH 7.0 (Sigma, St. Louis, Missouri, USA), 100 IU of penicillin/mL (Sigma, USA), and 100 μg of streptomycin/mL (Sigma). Preliminary pathological testing of biopsies did not allow visualization of *Leishmania,* which can often be found inside macrophages. However, Sanger sequencing of DNA extracted from the blood sample (performed at the FMV sequencing service) detected the presence of *Leishmania infantum.* The veterinarian, therefore, instituted therapy with allopurinol (Jamp Pharma Boucherville, Quebec), 100 mg, PO, q24h, a leishmaniosis treatment available in Canada, until the lengthy process of importing miltefosine (Virbac, Carros, France), a more effective drug, could be completed. Miltefosine was received 1 mo later and therapy was initiated at a dose of 2 mg/kg BW, PO, q24h for 28 d while awaiting final parasitological results. Parasites were successfully cultivated from skin samples after approximately 3 wk of growth. The culture was then separated into 2 aliquots; the first was subjected to genomic DNA extraction using *DNAzol* (Invitrogen, ThermoFisher, USA) as described by the manufacturer. Polymerase chain reactions were performed as described. The resulting sequences were aligned against *Leishmania* reference genomes using TriTryp.DB (16). Results once again confirmed *L. infantum.* The second aliquot was used for dose-response tests (in comparison with standard *L. infantum* wild type strain MHOM/MA/67/ITMAP-263) to determine the drug susceptibility profile of the isolate by monitoring the growth of parasites after 72 h of incubation in the presence of increasing drug concentrations by measuring absorbance at 600 nm (17) (Cytation 5 multimode reader; BioTek, Winooski, Vermont, USA). EC_{50} values were calculated based on dose-response curves analyzed by non-linear regression with Prism 8.2. software (GraphPad, San Diego, California, USA). Final results of dose-response assays indicated that the dog was infected by *L. infantum* susceptible to antimonial drugs and miltefosine, confirming the relevance of the already instituted drug therapy. After approximately 1 mo of miltefosine treatment, lesions had completely disappeared, and new blood samples were sent to the FMV for analysis. DNA extraction followed by standard PCR still allowed detection of *Leishmania infantum* in the blood. Consistent with these results, the dog relapsed 6 mo later.

Discussion

Leishmania infantum is a zoonotic parasite of worldwide importance, and is responsible for severe illness in humans and dogs (18). Canada is not currently a *Leishmania*-endemic

country. That said, importation of animals into Canada requires no quarantine and no serological testing; all that is required is proof that an animal is rabies-free (13). With countless owners traveling with their pets and numerous organizations rescuing dogs from other countries, leishmaniosis, along with other exotic diseases, presents a risk to public health in Canada. Despite the fact that the phlebotomine vector of *L. infantum* is not present in this country, similar indigenous insects may well prove to be competent vectors of the disease (9). Ticks have recently been demonstrated to have a possible role in the infectious cycle of *Leishmania;* this is noteworthy considering the rapidly spreading tick populations in Canada (10,11,19). Certain articles have also documented transmission of leishmaniosis through dog bite wounds or transplacentally; these types of dog-to-dog transmission may contribute to maintaining disease in North America (20,21). Furthermore, Canadian veterinarians are not equipped to diagnose and treat many of the diseases of concern in imported animals due to a lack of available tests and appropriate therapeutic drugs. For example, the recommended first step in diagnosing a dog with clinical leishmaniosis is a quantitative serology enzyme-linked immunosorbent assay (ELISA) that is unavailable in Canada (18). It is not always possible to identify *Leishmania* through pathological analysis of lesion biopsies; there may be low parasite burden or lack of migration to the lymph nodes. In such cases, without available serological tests, specialized PCRs and/or lengthy parasite cultures are necessary. An additional consideration is that many of the drugs necessary to treat *Leishmania,* such as miltefosine, require an onerous importation process to obtain the medication from *L. infantum*-endemic countries. Due to the complexity of *Leishmania* diagnosis, the difficulties in obtaining appropriate treatments, and the vast number of infected but clinically healthy dogs in endemic areas, managing this neglected infectious disease presents a substantial challenge for doctors, veterinarians, and public health officials. It is worth noting that even with appropriate treatment, it can be difficult to obtain complete clearance of *Leishmania* because the parasite is capable of harboring within macrophages and "hiding" in different organs and tissues such as the spleen, liver, and bone marrow. Dogs serve as a reservoir for this disease, and therefore control of canine infection is a fundamental pillar of disease prevention and needs to be prioritized (3,18).

In conclusion, veterinarians should be vigilant when examining animals with a travel history. Given the significant public health risk posed by many exotic diseases, relevant diagnostic tests and therapeutic molecules should be made available in Canada, and stricter animal importation control measures are imperative. Delays in diagnosis as well as the lengthy process of drug importation are detrimental to affected patients' prognoses. As demonstrated by the current case, exotic diseases may not be so exotic anymore.

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