First report of autochthonous canine leishmaniosis in Hong Kong

Jeanine Rhoda Sandy (j.sandy@cityu.edu.hk)
City University of Hong Kong
Anthony Matthews
Acorn Veterinary Hospital
Yaarit Nachum-Biala
The Hebrew University of Jerusalem
Gad Baneth
The Hebrew University of Jerusalem

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Abstract

Background: Canine leishmaniosis is a zoonotic disease caused by *Leishmania infantum* and transmitted by the bite of phlebotomine sand flies. Infected dogs represent the main domestic reservoir of the parasite for both human and animal transmission.

Methods: *Leishmania infantum* infection was investigated in a kennel of eight dogs in Hong Kong, of which four had cutaneous lesions. All eight dogs were tested serologically by the enzyme-linked immunosorbent assay (ELISA) and by polymerase chain reaction (PCR) targeting the *Leishmania* ribosomal operon internal transcribed spacer 1 and the kinetoplast minicircle on blood and tissues, followed by DNA sequencing.

Results: One dog which never left Hong Kong was seropositive for *Leishmania infantum* and diagnosed with cutaneous and systemic disease with detection of tissue amastigotes by cytology, and positive blood and spleen PCR for *L. infantum*. *Leishmania* amastigotes were seen within inflamed nasal and skin samples from this dog. Another kennel dog imported from the USA which developed cutaneous lesions prior to the local dog, was confirmed as infected by *L. infantum* by PCR and skin histopathology. All other kennel dogs were negative on serology and PCR and no amastigotes were identified in the skin lesions from the additional two dogs with skin disease. Treatment of allopurinol administered to both clinically affected dogs with leishmaniosis resulted in clinical recovery.

Conclusions: A dog from the USA introduced into a Hong Kong kennel was detected as clinically affected by leishmaniosis followed by the development of the disease in a local kennel dog which never left Hong Kong. Both dogs responded well to long term therapy and none of the six in contact dogs showed evidence of being infected. As there are no known suitable sand fly vectors of leishmaniosis reported in Hong Kong, horizontal or vertical transmission is suspected to be responsible for disease transmission. As *L. infantum* causes human and canine disease, and dogs serve as reservoirs for human infection, attention should be paid to the possibility of visceral leishmaniosis emerging in Hong Kong.

Background

Leishmaniosis is an important disease of animals and humans which affects four eco-epidemiological regions of the world, according to the World Health Organisation (WHO), including South East Asia [1]. Canine and human leishmaniosis caused by *Leishmania infantum* are transmitted by the bite of phlebotomine sand flies. Infected dogs are the main domestic reservoir of the parasite for both human and animal transmission. Leishmaniosis is endemic in various parts of China [2], transmitted by four different sand fly species: *Phlebotomus chinensis*, *P. longiductus*, *P. wui*, and *P. alexandri* [1]. According to the Hong Kong Food and Environmental Hygiene department, the government body responsible for monitoring vector-borne diseases of humans, no human leismaniosis cases have been reported in Hong Kong. There are currently no specific sand fly monitoring procedures by the department in Hong Kong and insect monitoring has identified Psychodidae specimens with no *Phlebotomus* spp. found (Personal communication; M. W. LEE, Pest Control Advisory Section, Food and Environmental Hygiene Department). Autochthonous animal leishmaniosis cases have not been reported in Hong Kong, although dogs imported from endemic countries have been diagnosed by the

Hong Kong City University's veterinary pathology department which is part of the Veterinary Diagnostic Laboratory.

Clinical Study

Eight dogs were included in this study (Table 1). All dogs were privately owned adult Belgian Malinois aged between 6 and 10 years, resided in Hong Kong Island, with 4 males and 4 females, housed on the same property as guard dogs. Four dogs were born in Hong Kong (dogs 2,3,5,6), two in the Netherlands (dogs 4, 8), one in the United States of America (dog 1) and one in Sweden (dog 7). Two dogs (dog 2 and 3) were littermates, both born in Hong Kong and dog 5 was the progeny of dog 1.

Table 1
Signalment, origin, main test results and *Leishmania* treatment outcome of eight dogs living in the same kennel investigated for leishmaniosis.

Dog	Dog country of origin	Relationship with other dogs in kennel	Year of birth	Detection and anatomic location of *Leishmania* amastigotes by microscopy	ELISA Serology	PCR result and tissue	*Leishmania* treatment/outcome
1	USA	Sire of dog 5	2011	Yes: Dermis	-	- (blood)*	Disease relapsed but now resolved
2	Hong Kong	Full brother of dog 3	2012	Yes: Spleen and liver	+**	+ (blood, spleen)	Disease relapsed but now resolved
3	Hong Kong	Full sister of dog 2	2019	No	-	- (blood and skin)	NA
4	Netherlands	None	2009	No	-	- (blood and skin)	NA
5	Hong Kong	Son of dog 1	2013	ND	-	- (blood)	NA
6	Hong Kong	None	2011	ND	-	- (blood)	NA
7	Sweden	None	2011	ND	-	- (blood)	NA
8	Hong Kong	None	2012	ND	-	- (blood)	NA

- negative result; + positive result; ND: Not done; NA Not applicable.

* Dog 1 had two rounds of PCR testing on EDTA blood. Quantitative species *Leishmania* PCR performed by IDEXX Reference Laboratory, United Kingdom. Approximately five years later, PCR performed by Koret School of Veterinary Medicine, the Hebrew University, Israel.

** Dog 2 had two rounds of ELISA serology. One performed by Texas A and M veterinary medical diagnostic laboratory. Second test performed 2 months later by Koret School of Veterinary Medicine, the Hebrew University, Israel.

Four dogs exhibited cutaneous lesions (dogs 1, 2, 3, 4). Three dogs had ulcerative dermatitis affecting one toe (dog 1), two toes and pressure points (dog 2) and the left lateral stifle region (dog 4). One dog had chronic alopecia and hyperpigmentation over the caudodorsal skin (dog 3). One of the dogs with skin disease (dog 2) also had respiratory signs with nasal stertor and ulceration and swelling of the nasal vestibule, splenomegaly, lethargy and dullness, and chronic regenerative anaemia indicating systemic illness. Skin biopsies for histopathological assessment were obtained from all four dogs with cutaneous skin disease, as part of the diagnostic process by their attending veterinarians, and a biopsy of nasal mucosa was collected from dog 2 to investigate the concurrent nasal disease. Formalin fixed skin samples were processed routinely into paraffin

blocks and stained with haematoxylin and eosin (H and E) and examined microscopically. Skin biopsies from dogs 2 and 4 with ulcerative dermatitis were also stained by Grocott's methenamine silver (GMS), Periodic acid Schiff (PAS), Ziehl Neelsen (ZN), Gram and Giemsa staining. Cytological assessment of fine needle aspirates were obtained from ulcerative dermatitis lesions of dogs 2 and 4, with additional aspirates collected from the spleen and liver of dog 2 with systemic illness signs. Aspirates were stained with Wright's Giemsa (WG) and examined microscopically.

Blood samples were collected from the cephalic vein of all eight dogs for *Leishmania* serology. Serum collected was stored at -70C before being sent to the Koret School of Veterinary Medicine, Hebrew University, Israel. The systemically ill dog (dog 2) had additional serology performed at the Texas A and M, Veterinary Medical Diagnostic Laboratory, College Station, Texas, USA.

PCR for *Leishmania* detection was performed on EDTA blood from all eight dogs, punch biopsies of skin collected from three of the dogs with skin disease (dogs 2,3,4) placed into sterile saline, paraffin embedded skin from dog number 1 with cutaneous disease, and aspirated splenic tissue, scraped off a stained glass slide from the systemically ill dog (dog 2). *Leishmania* species quantitative PCR on EDTA blood from dog 1 was also performed by IDEXX Reference Laboratories, UK, as part of the initial diagnostic workup of this case.

DNA was extracted from the blood samples of all eight dogs using the Qiagen EZ1 Advanced XL automated system (QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, Germany). DNA was stored at -70C before being sent to the Hebrew University in Israel.

The presence of *Leishmania* DNA in samples was tested using two PCR protocols for amplification of different targets. A 265 bp fragment of the *Leishmania* internal transcribed spacer 1 (ITS1) region of the *L. infantum* rRNA operon was amplified by real-time PCR using primers ITS-219 F (AGCTGGATCATTTTCCGATG) and ITS-219R (ATCGCGACACGTTATGTGAG) and then evaluated by high resolution melt (HRM) analysis as previously described [3]. In addition, a 120 bp of the *Leishmania* kinetoplast DNA (kDNA) minicircle was amplified by real-time PCR using primers JW11 (CCTATTTTACACCAACCCCCAGT) and JW12 (GGGTAGGGGCGTTCTGCGAAA) and then evaluated by melt curve analysis as previously described [3, 4]. All positive PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), at the Center for Genomic Technologies, Hebrew University of Jerusalem, Israel. DNA sequences were evaluated with the ChromasPro software version 2. 1.1 (Technelysium Pty Ltd., Australia) and compared for similarity with sequences available in GenBank®, using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Serology for anti-leishmanial antibodies was performed by the enzyme-linked immunosorbent assay (ELISA) using *L. infantum* antigen, as described previously [5].

Clinical Cases

Dog no. 1, born in the USA, presented with as a referral case, with a 1–2 month history of relapsing ulcerative skin disease affecting a single nailbed in the right foreleg, which partially responded to 30 day treatment with prednisolone (0.25 mg/kg SID, generic, China), cephalexin (16 mg/kg, BID, Stada Pharmaceuticals (Asia)

Limited, Hong Kong) and itraconazole (5 mg/kg, SID, Europharm Lab Co LTD, Hong Kong) (Table 1). The lesion relapsed and punch biopsies of the nail lesion were collected 60 days from initial presentation, and cephalexin (16 mg/kg, BID) was continued for an additional 30 days. The biopsy revealed granulomatous dermatitis with abundant, intracytoplasmic organisms consistent with *Leishmania* amastigotes seen on histology (Fig. 1). Amastigotes were ovoid or round, 2.5–5.0 to 1.5–2.0 um in diameter, with a small nucleus and kinetoplast. *Leishmania* species quantitative PCR on EDTA blood was negative (IDEXX Reference Laboratories, UK) but PCR and DNA sequencing performed at the Hebrew University in Israel on DNA extracted from paraffin embedded tissue from the nail bed stored for six years was positive confirming leishmaniosis caused by *L. infantum*. Allopurinol (generic, China) treatment commenced at the unusually high dose of 30 mg/kg SID for 6 months, and thereafter the dosage was reduced in another unusual protocol to 30 mg/kg SID for 7 days each month, for 7 months, and the dog was placed on a low purine diet (Royal Canin). The dog's skin lesion healed within three months, but against veterinary advice, treatment ceased and 12 months after it was discontinued the dog relapsed with bleeding associated with ulcerative dermatitis at the nail beds of both hind legs. Biopsy was declined by the owner and allopurinol (generic, China) treatment recommenced at 30 mg/kg SID for 6 months. A second ELISA serology and PCR on blood, conducted five years after the initial diagnosis, when the dog was clinically well and had no cutaneous lesions, were negative for leishmaniosis.

Table 1. Signalment, origin, main test results and Leishmania treatment outcome of eight dogs living in the same kennel investigated for leishmaniosis.

(TABLE IS ATTACHED AS AN ADDITIONAL FILE DUE TO LANDSCAPE FORMAT BEING REQUIRED)

Dog 2 was born in and had never travelled outside of Hong Kong (Table 1). Information about the sire was unavailable but the dam, born in America, had died at the time of the dog's presentation and was suspected by the attending veterinarian to have a disease with similar manifestations to leishmaniosis but no laboratory testing, treatment or post mortem were performed. Dog 2 initially presented with ulcerative skin disease affecting a single nailbed in the left forelimb), which failed to respond to cephalexin (13 mg/kg BID for 5 days, Stada Pharmaceuticals (Asia) Limited, Hong Kong) but partially responded to 28 days with enrofloxacin (6mg/kg SID, Dechra Veterinary Products (Australia) Pty Ltd, Australia) and itraconazole (5 mg/kg, Europharm Lab Co LTD, Hong Kong). Six months later, ulcerative dermatitis appeared on two right fore digits and both metacarpal pads. Lesions responded well to the same treatment regimen with enroflaxacin and itraconazole for 21 days, but 4 to 6 months later, ulcerative dermatitis reappeared on the left hind and right forelimb digits, the right hock, accompanied by ulceration and swelling of the nasal vestibule with nasal stertor and lymphadenomegaly of the popliteal, prescapular and submandibular lymph nodes. Incisional biopsies revealed pyogranulomatous inflammation within the nasal mucosa (Fig. 2) and skin lesion, with low numbers of structures suspicious for amastigotes. GMS, PAS, ZN, and Gram and Giemsa staining did not provide additional information. The lesions resolved following treatment with prednisolone (1mg/kg BID for 44 days, tapered to 0.75 mg/kg BID for 2 weeks, Mavlab Pty Ltd, Australia), cephalexin (13 mg/kg, BID, 17 days, Stada Pharmaceuticals (Asia) Limited, Hong Kong), itraconazole (5 mg/kg SID for 35 days, Europharm Lab Co LTD, Hong Kong), but ulceration of the nares recurred four months later along with lethargy, dullness and splenomegaly. Blood count showed a mild regenerative, normocytic, hypochromic anaemia (PCV: 31% reference range 37–54%; reticulocytes 128.1 x 10^9/L reference range 11–92 x 10^9/L; MCHC 321 g/L reference range 330–360 g/L), with 18 nucleated RBC/100 white blood cells and a left shift of neutrophils (9

neutrophilic bands/100 WBC) with a mild increase in plasma proteins (81 g/L reference range 59–78 g/L). Serum biochemistry changes included mild hyperglobulinaemia (43 g/L reference range 19–36 g/L) and hypoalbuminaemia (30 g/L reference range 32–44 g/l). There was mildly increased ALP (104 U/L reference range 17–100 U/L), AST: (95.1 U/L reference range 15–57) and CK (769 U/L reference range 48–261 U/L). Spleen and liver cytology by fine needle aspirates showed granulomatous to pyogranulomatous splenitis and hepatitis with abundant *Leishmania* amastigotes within macrophages in both organs (Fig. 2). Urinalysis was unremarkable.

Indirect fluorescent antibody test (IFAT) performed at the Texas A and M veterinary medical diagnostic laboratory detected a titre > = 2048 for antibodies against *L. infantum* ELISA serology and PCR on blood taken two months later and performed at the Hebrew University, as well as splenic tissue scraped from a cytology slide, were positive for leishmaniosis. The ELISA result had an optical density (OD) of1.436 (cut off 0.4 OD) and PCR which detected the ITS-1 spacer using the ITS219 F/R primers, produced sequences from the blood and spleen which were 100% similar to one another and had 100% identity to *L. infantum* (MN503527.1)_(Table 1).

Dog 2 was treated with allopurinol (16 mg/kg BID for 30 days) reduced to 10 mg/kg, BID, which is ongoing, and subcutaneous meglumine antimoniate (Glucantime, Merial, France) at 80 mg/kg in the morning and 40 mg/kg in the evening for 4 weeks.

The remaining six ‘in contact’ dogs (dogs 3,4,5,6,7,8) were seronegative for *Leishmania* by ELISA and their PCR from blood and skin samples was negative. At the time of sampling, two dogs (dogs 3 and 4) had chronic skin disease and four dogs (dogs 5,6,7,8) were clinically healthy. Dog 3 was born in Hong Kong and was a full sister of the systemically unwell, *Leishmania*-positive dog (dog 2). This dog had a chronic history of alopecia with hyperpigmentation on the dorsum of the thorax and rump region, which clinically resembled flea allergy dermatitis. Skin punch biopsies collected from alopecic regions were PCR negative for *Leishmania* and lacked amastigotes on histology, and the histological diagnosis was chronic hyperplastic dermatitis. Dog 4 was born in the Netherlands, and presented with chronic, pyogranulomatous, ulcerative dermatitis on the left stifle, but histology and cytology failed to demonstrate amastigotes or any infectious agents on hematoxylin and eosin, GMS, PAS, ZN, Giemsa, Gram staining, and PCR from a skin biopsy was negative for *Leishmania*.

Discussion

This report is the first to describe leishmaniosis due to *L. infantum*, in either animals or humans, in Hong Kong. The presence of leishmaniosis in the canine population is of potential public health concern, as dogs are the main reservoir for human infection and canine leishmaniosis has been shown to precede human cases [6]. Although no suitable sand fly vector has been identified in Hong Kong, the emergence of two canine cases in a local kennel is alarming. Canine *Leishmania* infection is often sub-clinical [7–9], however sub-clinically infected dogs are infectious to sand fly vectors that can transmit infection to humans, other dogs [10, 11]. One of the infected dogs showed mainly cutaneous manifestations of the disease whereas the other dog presented both cutaneous and visceral organ involvement with splenitis and hepatitis. Both dogs were clinically stable and received allopurinol treatment at the time of this report. As dog 1 experienced clinical relapse, despite 13 months of allopurinol treatment, and since allopurinol, as well as all other drugs used for the treatment of

canine leishmaniosis, are not known to cure dogs parasitologically and completely eliminate infection, it is likely that these two dogs remained infected [9].

Leishmania infantum is transmitted by female sand fly bites [12] and only specific sand fly species serve as vectors. *Phlebotomus chinensis* Newstead, 1843 has a natural range of distribution geographically close to Hong Kong in the Guangdong and Hainan districts of mainland China, but to date, this species has not been reported in Hong Kong (Pers comm from senior agricultural officer from Agriculture, Fisheries and Conservation department of Hong Kong). Dog 1, imported from the USA, most likely acquired infection via the vertical route [13], but dog 2 had lived its entire life in Hong Kong. Non-vector borne transmission of leishmaniosis in dogs has been associated with *in utero* infection, exposure to parasites within blood products, venereal transmission and potentially by direct contact including dog fights [13–24] with similar incubation periods of 3 months to 7 years [17–19]. The two most likely routes of infection in dog 2 were *in utero* infection and direct contact. The dam of dog 2 died with clinical disease suspicious for leishmaniosis, but unfortunately no investigation into the cause of its death was made. It has been shown that maternal *Leishmania* disease status significantly predicts the likelihood of offspring infection in dogs [13] so it would have been interesting to know whether the dam was sick at the time that dog 2 was born but this information was unavailable. Horizontal transmission may have occurred [20, 21], as multiple episodes of dog fights requiring veterinary treatment occurred between dogs 1 and 2 after leishmaniosis was diagnosed in dog 1 who represented the index case in this kennel, although this mode of transmission has not been proven.

Leishmania infantum infection in dogs has two main patterns; sub-clinical or clinical disease, with about 5%-10% of dogs naturally infected progressing to overt clinical disease [7]. The cutaneous lesions in both dogs due to granulomatous dermatitis were typical but the numbers of amastigotes detected by microscopy varied, with large numbers seen on histology in dog 1, confirmed by PCR, and only few organisms suspicious for *Leishmania* seen within macrophages from cutaneous and nasal mucosal lesions in dog 2. *Leishmania infantum* parasites have been previously reported in the nasal epithelium of dogs and associated in some cases with epistaxis [22]. Dog 2 developed systemic disease with granulomatous splenitis and hepatitis as well as hyperglobulinaemia, similar to previous reports in dogs, with abundant amastigotes seen on histology [9, 16, 19, 23, 24]. Hyperglobulinaemia is associated with anti-*Leishmania* IgG antibodies, detectable via serology which are non-immunoprotective [25], and are responsible for the formation of immune-complex glomerulonephritis, which is a common cause of mortality in infected dogs [9, 26] .

Dog 1 was PCR negative on blood during its initial diagnosis despite the abundant amastigotes seen within macrophages in the toe lesion. This is not surprising as in a previous study, 25% of symptomatic dogs were negative using quantitative real time PCR on peripheral blood, whereas splenic aspirates produced the highest sensitivity of 95.8% [27]. PCR and serology performed on blood 5 years later were negative, despite the dog probably remaining infected, as this dog relapsed 12 months after completing a 13-month treatment regimen with allopurinol [28, 29]. Low parasite burdens due to treatment contribute to negative serological screening tests and overall, serology fails to detect a high portion of sub-clinically infected dogs [30, 31]. Dog 2 which had overt cutaneous and systemic pathology with abundant numbers of amastigotes detected by histopathology in the viscera had positive PCR and serology results, reflecting this high parasite burden.

Conclusion

This report is the first to document autochthonous canine leishmaniosis in Hong Kong. Canine leishmaniosis can precede human disease, therefore continued monitoring to detect phlebotomine vectors in Hong Kong is warranted. Canine *Leishmania* infection is mostly sub-clinical, however sub-clinically infected dogs could be infectious to sand fly vectors that can transmit infection to humans, other dogs [10, 11]and cats [32], and routine diagnostic procedures such as serology and PCR on blood can be negative despite the presence of infection.

Abbreviations

ELISA: Enzyme-linked immunosorbent assay; EDTA:ethylenediaminetetraacetic acid; GMS: Grocott's methenamine silver; H and E: Haematoxylin and Eosin; ITS1: Ribosomal operon internal transcribed spacer 1 region; kDNA: Short fragment of the kinetoplast minicircle; *L. infantum*: *Leishmania infantum*; PAS: Periodic acid Schiff; PCR: Polymerase chain reaction; WBC: white blood cells; WG: Wright's Giemsa; ZN; Ziehl Neelsen

Declarations

Ethics approval and consent to participate not applicable. Dogs in this study were naturally infected, tested for leishmaniosis and evaluated clinically as part of their clinical veterinary management.

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Author's contributions

JS designed the study based on clinical material submitted by AM. BF, YN-B performed serology and analysed the quantitative and molecular data. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

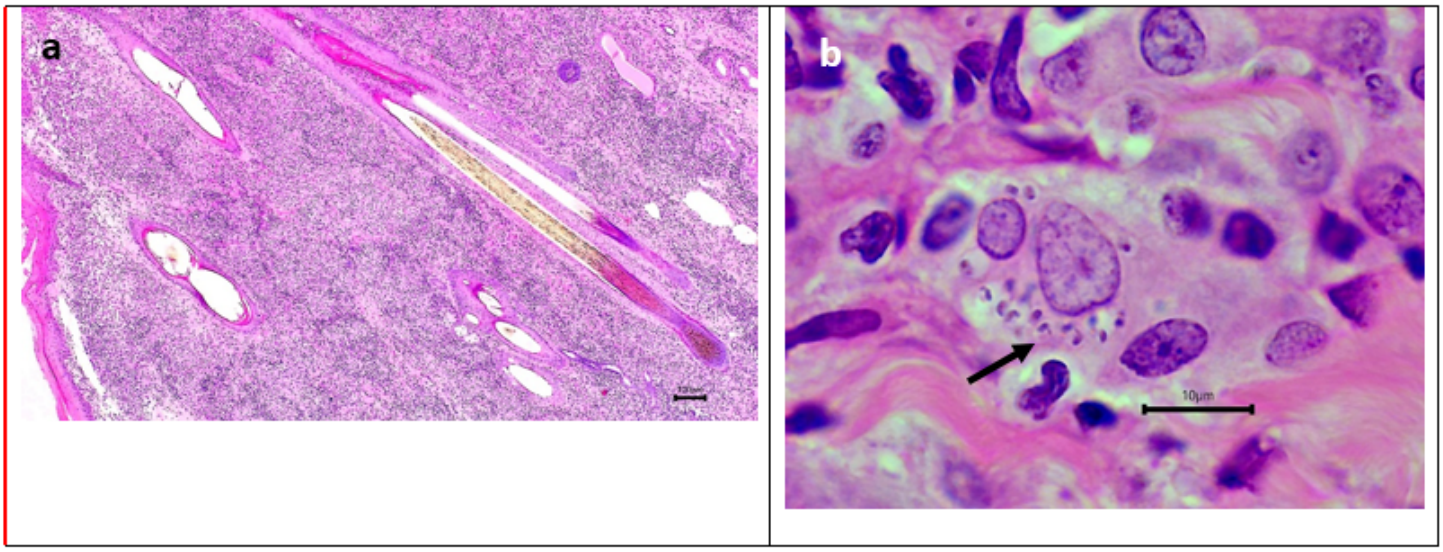

Figure 1

Skin histopathology from dog 1.

a: Skin histopathology from dog 1 showing granulomatous dermatitis. Hematoxylin and eosin staining; Bar = 100 um.

b: Skin histopathology from dog 1 showing multiple *Leishmania* amastigotes within the cytoplasm of a macrophage. Arrow points to a kinetoplast at right angles to the amastigote nucleus. Hematoxylin and eosin staining; Bar = 10 um.

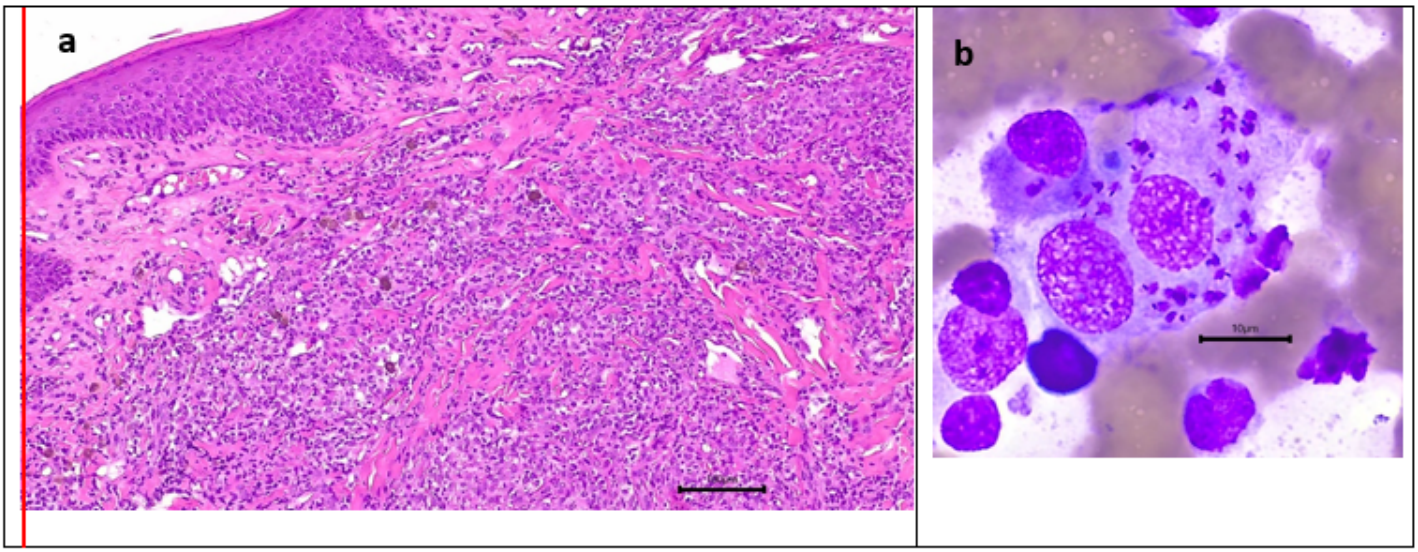

Figure 2

Dog 2. a. Histopathology of nasal mucosa with granulomatous inflammation Hematoxylin eosin staining; Bar = 100 um.

b. Splenic aspirate. Wright's Giemsa stain. *Leishmania* amastigotes within the cytoplasm of macrophages.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- JSandyLeishmaniaAdditionalfile1Table1.docx
- JSandyLeishmaniaAdditionalfile2Figures1and2.docx