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RESEARCH

An experimental challenge model for *Leishmania donovani* in beagle dogs, showing a similar pattern of parasite burden in the peripheral blood and liver

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Abstract

Leishmania donovani and *Leishmania infantum* are closely related species. However, the former is considered the causative agent for anthroponotic visceral leishmaniasis (AVL), while the latter is known to be responsible for zoonotic visceral leishmaniasis (ZVL) with dogs as the main reservoir host. Although molecular detection of *L. donovani* from naturally infected dogs has been reported in AVL endemic areas, the experimental infection of dogs with this species is very limited. Here, we constructed an experimental canine visceral leishmaniasis (CVL) model with *L. donovani* infection using beagle dogs. During an observation period of 8 months after parasite inoculation, few clinical symptoms were observed in the three inoculated dogs. The overall hematological and biochemical data of the dogs showed normal levels, and there were no remarkable changes in the peripheral $CD4^+$, $CD8^+$, $CD25^+$, or $FoxP3^+$ T cell populations. Liver biopsy sampling was conducted to monitor the parasite burden in the liver. A similar pattern of the amount of mitochondrial kinetoplast DNA was observed in the peripheral blood and liver by real-time PCR analysis. In addition, parasite antigens were detected from the liver biopsy sections by immunohistochemical analysis, further supporting the existence of parasites in the liver. These results showed a subclinical CVL model for *L. donovani* in beagle dogs with a similar kinetics of parasite burden in the peripheral blood and liver.

Keywords Animal experimentation · Dogs · *Leishmania donovani* · Liver · Real-time polymerase chain reaction

Introduction

Visceral leishmaniasis (VL) in humans is caused by mainly two closely related species, *Leishmania donovani* and *Leishmania infantum* (syn. *Leishmania chagasi*). The former species is considered responsible for anthroponotic VL (AVL) in the Indian subcontinent (India, Bangladesh, and Nepal) and East Africa. On the contrary, *L. infantum* is responsible for zoonotic VL (ZVL) because dogs act as the main reservoir of the parasite in the Mediterranean, North Africa, Middle East, West Asia, China, and Latin America (Chappuis et al. 2007; Baneth et al. 2008; World Health Organization, 2010; Aslan et al. 2016). While *L. infantum* causes canine visceral leishmaniasis (CVL) with a broad spectrum of clinical manifestations, from subclinical to fatal symptoms (Ciaramella et al. 1997; Alvar et al. 2004; Baneth et al. 2008; Dantas-Torres et al. 2012), the investigation of pathogenesis of *L. donovani* in natural infected dogs has been inconsiderable and neglected.

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To assess the clinical, parasitological, and immunological responses in dogs against *L. infantum*, the construction of experimental canine models for *L. infantum* was carried out by consideration of many factors, including the breed of dogs, stage of the parasite, number of parasites, route of inoculation, and use of vectors (Campino et al. 2000; Leandro et al. 2001; Moreno and Alvar 2002; Paranhos-Silva et al. 2003; Aslan et al. 2016; Abbehusen et al. 2017). However, studies on experimental infection of dogs with *L. donovani* have never been reported except one report in two separate studies in which hematological, biochemical, and pathological changes were assessed on German shepherd dogs (Keenan et al. 1984a, b). The lack of experimental studies of CVL by *L. donovani* may be attributed to the concept of anthroponotic transmission of *L. donovani* in humans (World Health Organization 2010).

It is worth to note that the detection of *L. donovani* DNA by PCR assay or anti-*Leishmania* antibodies by rk39 immunochromatographic test in peripheral blood collected from stray dogs have been reported in India (Sharma et al. 2009), Bangladesh (Alam et al. 2013; Akter et al. 2016), Sudan (Dereure et al. 2003; Hassan et al. 2009; Shamboul et al. 2009), and Ethiopia (Kalayou et al. 2011). Furthermore, *Phlebotomus argentipes* and *Phlebotomus orientalis*, the known vectors for *L. donovani* in the Indian subcontinent and Ethiopia, respectively, have been reported to take blood meals from domestic animals, including dogs (Burniston et al. 2010; Aklilu et al. 2018). The presence of *Leishmania* DNA in the canine blood suggests that dogs play a role in the reservoir of the *L. donovani* transmission cycle in VL endemic areas although parasite isolation was rarely successful. Thus, experimental assessment is needed to understand the parasitological and immunological aspects in dogs with *L. donovani* infection.

In the present study, beagle dogs were inoculated with *L. donovani* promastigotes to construct an experimental challenge model for *L. donovani* in dogs. Challenged dogs were examined for clinical signs and hematological and biochemical parameters for up to 8 months after inoculation. T lymphocyte subsets, including $CD4^+$, $CD8^+$, and $CD4^+CD25^+FoxP3^+$ T cells in the peripheral blood, were examined by flow cytometry as the role of regulatory T cells has been reported in persistence of the same *L. donovani* parasites in the liver of immunodeficient murine VL model (Tiwananthagorn et al. 2012). The detection of parasite DNA from the peripheral blood, liver biopsy materials, and spleen autopsy materials was conducted by real-time PCR. Furthermore, the detection of parasite antigens in the liver was performed by immunohistochemistry.

Materials and methods

Animals and ethics statement

A total of three 1-year-old male beagle dogs (approximately 10 kg) were purchased from Hokudo Co., Ltd. (Sapporo, Japan). The dogs were born of different parents in a breeding company. None of the negative control dogs were used due to the 3R principle in animal experiments, in which the number of animals per experiment is reduced to the absolute minimum. All dogs received physical examination and vaccines against leptospirosis, rabies, hepatitis, adenovirus, coronaviruses, distemper, parainfluenza, and parvovirus before the initiation of the experiment. Dogs (A, B, and C) were housed in an animal biosafety level 2 (ABSL2) facility in strict accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of Graduate School of Veterinary Medicine, Hokkaido University. Dogs were kept in separate cages and given a standard dog diet and tap water. This study was conducted following the guidance of the Institute for Laboratory Animal Research, which was based on Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. This experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Hokkaido University (Permit Number: 13–0149), following the guideline of the American Association for Accreditation of Laboratory Animal Care International.

Parasites and experimental infection

Because of the unavailability of *L. donovani* strains isolated from dogs, we used the *L. donovani* Sudan strain (HOM/SU/62/2S-25M-C2), which was originally isolated from humans and has been characterized by in vitro experiments as well as mouse models in previous studies (Katakura and Kobayashi 1988; Tiwananthagorn et al. 2012; Chiba et al. 2014; Katakura 2016). Promastigotes of *L. donovani* of the frozen stabilized parasites were cultured at 25 °C in M199 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, and 50 µg/ml gentamycin. Dogs were inoculated with 5×10^8 stationary-phase promastigotes/kg body weight in 2 ml saline administered intravenously via the jugular vein. Dogs were observed for a period of up to 8 months after parasite inoculation. At the end of this study, dogs were euthanized by intravenous injection of 50 mg/kg sodium pentobarbital (Kyoritsu Seiyaku Co., Tokyo, Japan) and

1.0 g/kg potassium chloride. The liver and spleen tissues of euthanized dogs were subjected to DNA extraction and paraffin sections after fixation in a 10% neutral-buffered formalin solution.

Clinical assessment

Clinical manifestations of challenged dogs were checked by a facility staff veterinarian every day. At the time of blood and liver biopsy sampling, dogs were extensively examined for symptoms of visceral leishmaniasis, such as skin lesions, weight loss, enlarged lymph nodes, and hepatosplenomegaly. Rectal temperature was not monitored because of avoidance of direct contact with challenged dogs without anesthesia as much as possible.

Hematological and biochemical analysis of peripheral blood

Dogs were sedated by intramuscular injection with 0.1 mg/kg midazolam (midazolam; Teva Pharma Japan Inc., Nagoya, Japan) and 0.2 mg/kg medetomidine hydrochloride (Domitor; Nippon Zenyaku Kogyo (ZENOAQ), Fukushima, Japan). Anesthesia was reversed by intramuscular injection with atipamezole hydrochloride (Antisedan; ZENOAQ). A total of 10 ml of peripheral blood samples was collected from the jugular vein into an EDTA-coated tube before parasite inoculation and after parasite inoculation with a monthly interval. Approximately 2 ml, 3 ml, and 5 ml of the samples were used for hematological examination, biochemical examination, and flow cytometry, respectively. In addition, plasma samples were collected after centrifugation of EDTA-blood samples at $1100 \times g$ at 25 °C for 10 min.

Clinical laboratory tests were outsourced to a reference laboratory (Hokudo Co., Ltd.). Hematological parameters included the number of erythrocytes, white blood cells, and platelets, and the value of hematocrit, hemoglobin level, mean cell hemoglobin level, mean cell volume, and mean cell hemoglobin concentration. Differential white blood cells were also determined using an automated counter and hemoglobinometer. In addition, biochemical parameters, such as albumin, albumin/globulin ratio, creatinine, urea, alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, and γ-glutamyl transpeptidase were determined using an autoanalyzer in the company (The Daiichi Kishimoto Clinical Laboratories, Sapporo, Japan).

rk39 immunochromatographic test

A rapid immunochromatographic strip test, using the rk39 dipstick (Kalazar Detect™; InBios International, Inc., Seattle, WA, USA), was conducted on each canine plasma sample (50 µl) for the qualitative detection of antibodies to visceralizing *Leishmania* species. According to the manufacturer's guideline, when two pink lines (control and test) appeared, even in faint, the test was determined as positive.

Liver biopsy

Dogs were sedated as described above, and liver biopsy was performed under ultrasound guidance using a portable ultrasound imaging system (SeeMore™; Medico's Hirata, Inc., Tokyo, Japan). A 14-gauge biopsy needle (Fine-Core; Toray Medical Co., Ltd., Tokyo, Japan) was percutaneously inserted and maintained in the left and lateral lobes to avoid the gallbladder during penetration of the liver. Two liver tissue samples (20–30 mg each) were taken from each dog for real-time PCR and histopathological examination. The liver biopsy samples were collected before and at 1 month, 3 months, 5 months, and 7 months after parasite inoculation.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were separated using a Percoll reagent (Percoll™ Plus; GE Healthcare Japan, Tokyo, Japan) adjusted to a density of 1.077 g/ml in phosphate-buffered saline (PBS). Buffy coat samples were collected after centrifugation at $400 \times g$ at 25 °C for 30 min and treated with NH_4Cl solution (HLB; Immuno-Biological Laboratories Co., Ltd., Takasaki, Japan) to lyse red blood cells. PBMCs were adjusted to 1×10^7 cells/ml in PBS containing 1.7 mM EDTA (PBS-E) supplemented with 10% heat-inactivated FBS. To evaluate the number of canine $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, and $FoxP3^+$ cells by flow cytometry, the following antibodies were used: fluorescein isothiocyanate (FITC)-labeled mouse anti-dog CD3, FITC-labeled rat anti-dog CD4, phycoerythrin (PE)-labeled rat anti-dog CD4, Alexa Fluor® 647–labeled rat anti-dog CD8 (AbD Serotec®; Bio-Rad, Kidlington, UK), PE-labeled mouse anti-dog CD25 (clone P4A10; eBioscience, San Diego, CA, USA), and allophycocyanin (APC)-labeled rat anti-mouse FoxP3 (clone FJK-16s, eBioscience). The clone FJK-16s is cross-reactive with canine FoxP3 (Biller et al. 2007), and clone P4A10 can be used to select canine regulatory T cells (Tregs) (Abrams et al. 2010). Isotype-matched control antibodies, including PE-mouse IgG1κ, FITC-rat IgG2aκ, and APC-rat IgG2aκ (eBioscience), were also used.

$CD4^+$ and $CD8^+$ T cell subsets were identified by a combination of canine anti-CD3 with anti-CD4 or anti-CD8 antibodies. FoxP3 staining was carried out using a fixation/permeabilization solution following the manufacturer's instructions. A number of 1×10^5 cells were analyzed for each sample by a BD FACSVerse™ flow

cytometer and BD FACSuite™ Software (Becton, Dickinson and Company, USA).

Parasite culture

A small piece of liver and spleen sample was aseptically taken from challenged dogs at necropsy and homogenized in PBS. The homogenate was tenfold serially diluted with Medium 199 (5 µg to 50 mg tissue samples/ml) and cultured in duplicate on 24-well plates in Medium 199 supplemented with 15% heat-inactivated FBS, 25 mM HEPES, and 50 µg/ml gentamycin at 25 °C for 4 weeks (Katakura 2016).

DNA extraction and real-time PCR

Total DNA was extracted from 100 µl of whole blood as well as from 20−30 mg of liver biopsy, liver necropsy, and spleen necropsy samples using a DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). Genomic DNA was also extracted from cultured *L. donovani* promastigotes using a DNeasy Blood & Tissue kit. DNA concentration was measured by a NanoDrop 2000 Spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA).

Real-time PCR was performed on the LightCycler® Nano System (Roche Diagnostic Japan, Tokyo, Japan) using primers specific for *Leishmania* mitochondrial minicircle kinetoplast DNA (kDNA), RV1 (5′-CTTTTC TGGTCCTCCGGGTAGG-3′), and RV2 (5′-CCACCCGGC CCTATTTTACACCAA-3′) (Mary et al. 2004). A typical 20-µl reaction mixture contained 1 × FastStart Essential DNA Green Master (Roche), 0.25 µM of each primer, and 100 ng DNA templates from the blood, liver, and spleen. The reaction was carried out with the initial denaturation at 95 °C for 5 min, followed by 40 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A standard curve was created using *L. donovani* DNA ranging from 0.005 to 500 pg. Amplification plot, melting temperature (T_m), and threshold cycle of amplification (Ct) values were generated according to the software provided with the instrument. The amount of parasite kDNA (ng/100 ng DNA of blood and tissue) was determined from three different assays in duplicate or triplicate and is expressed as the average ± SD. Based on the concentration of parasite DNA (0.4 pg/cell) determined in this study, the number of parasites per ml blood was estimated by the following formula:

$$\text{No. parasites/ml blood} = A/B \times 1/0.4 \times 10^{-3}$$

where *A* is the amount of kDNA in 100 ng blood DNA (pg) and *B* is the blood volume equivalent to 100 ng blood DNA (µl).

Histopathology and immunohistochemistry

Liver biopsy, liver necropsy, and spleen necropsy specimens were prepared to 2−4-µm-thick serial paraffin sections, and at least one section was stained with hematoxylin and eosin (HE). As a positive control, paraffin-embedded liver sections prepared from a mouse inoculated with the same *L. donovani* strain in this study (Tiwananthagorn et al. 2012) were included. For immunohistochemistry, paraffin sections were deparaffinized using xylene and rehydrated with 70−100% ethanol. Sections were then boiled in 0.1 M citrate buffer (pH 6.0) for 20 min to retrieve the epitopes and incubated in 0.3% hydrogen peroxide in methanol at 25 °C for 15 min to block endogenous peroxidase activity. After blocking nonspecific immunoglobulin binding by incubation in 10% nonfat milk at 25 °C for 10 min, sections were reacted with a mouse monoclonal antibody against *Leishmania* peroxiredoxin (kindly supplied by Dr. Y. Matsumoto, University of Tokyo) diluted 1:500 in PBS-Tween 20 containing 10% nonfat milk at 25 °C for 1 h. Sections were washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (*N*-Histofine® Simple Stain MAX-PO (M); Nichirei Biosciences Inc., Tokyo, Japan) at 25 °C for 1 h. After washing sections with PBS, immunoreactivity was visualized by using a 3,3′-diaminobenzidine (DAB) (Dojindo, Tokyo, Japan) solution. Hematoxylin was used for a nuclear counterstain.

Results

Clinical assessment and blood analysis

All three dogs inoculated with *L. donovani* promastigotes showed no obvious clinical signs, skin abnormality, nail growth, bleeding, or weight loss throughout the experimental period (8 months) (data not shown). No remarkable changes in hematological (Table S1) and biochemical (Table S2) parameters were detected, revealing none of the indications of anemia or liver dysfunction in these dogs.

Detection of anti-Leishmania antibodies by rk39 test

Plasma samples collected before inoculation were negative, while very faint positive bands were detected in all three canine samples at 1 month and 3 months after parasite inoculation. As the test lines were very faint and difficult in appearance in a photograph (Fig. 1a), the pictures of the test bands were enhanced for visualization using photo editing software (Fig. 1b). However, plasma samples

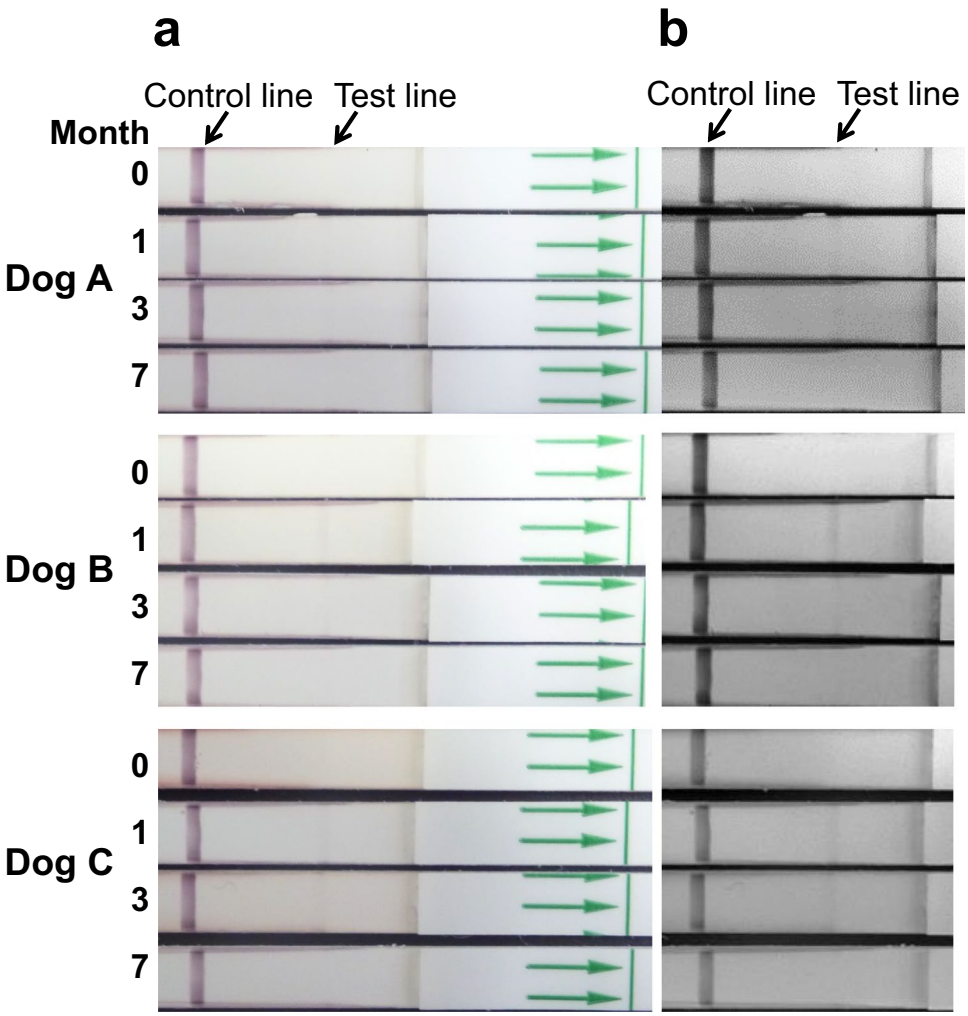

Fig. 1 Detection of antibodies against *Leishmania* parasites in dogs inoculated with *Leishmania donovani* promastigotes. The reactions in rk39 dipsticks (**a**) were digitally enhanced using photo editing software (**b**)

obtained at 7 months of inoculation showed negative reactions in all dogs (Fig. 1b).

Flow cytometry

In an immunodeficient mouse model, FoxP3$^+$ Tregs are involved in the persistence of *L. donovani* infection in the liver (Tiwananthagorn et al. 2012). To address whether Tregs are also involved in the parasite load in the present canine model of *L. donovani* infection, the kinetics of CD4$^+$FoxP3$^+$ and CD4$^+$CD25$^+$FoxP3$^+$ cells in the peripheral blood were examined. The light-scatter characteristics of lymphocytes were used to gate CD3$^+$ and CD4$^+$ T cells (Fig. S1a, b) for further analysis of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cell subpopulations (Fig. S1c). CD25$^+$FoxP3$^+$ T cells were gated from CD4$^+$ T cells (Fig. S1d). The ratio of CD25$^+$FoxP3$^+$ to CD4$^+$ T cells varied in three dogs and slightly increased at 6 months of parasite inoculation, but the percentages appeared to be consistent and no conclusive evidence was obtained (Fig. 2).

Detection of the parasite by culture

Necropsy liver and spleen samples from the three challenged dogs were cultured in the medium for up to 4 weeks. However, no parasites were detected in the culture medium.

Real-time PCR

To assess the presence of *Leishmania* kDNA in the canine peripheral blood, real-time PCR was performed on blood samples at 1 month, 3 months, 4 months, 5 months, 6 months, and 7 months after inoculation. In a standardization for real-time PCR assay, an amplification (Fig. S2a) and a standard curve ($r^2 = 0.9936$, $E = 1.813$, error = 0.521)

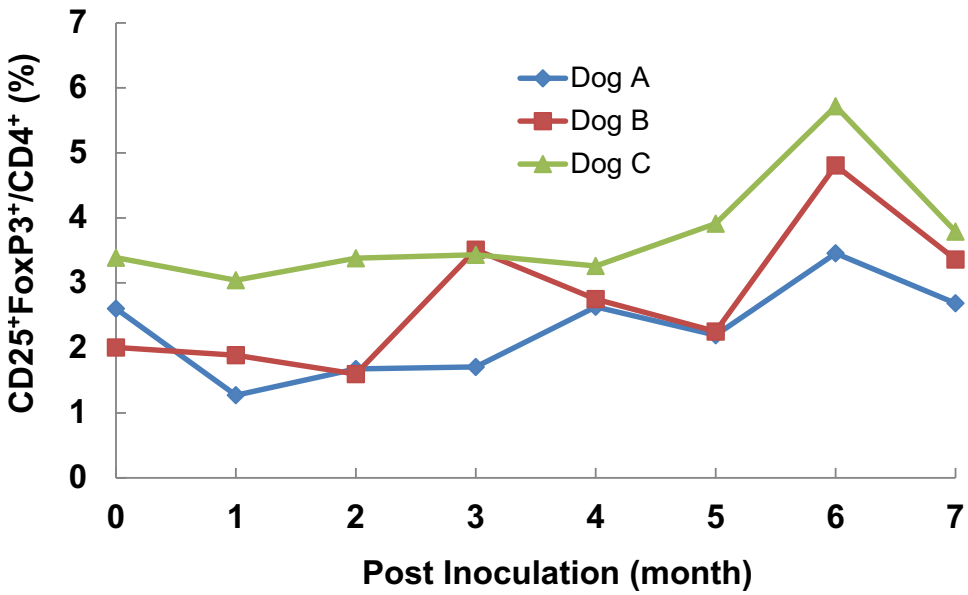

Fig. 2 Kinetics of $CD25^+FoxP3^+/CD4^+$ T cell subpopulations in the peripheral mononuclear cells from dogs inoculated with *Leishmania donovani*

(Fig. S2b) were clearly created in the range of 0.005 pg (5 fg) and 500 pg of *Leishmania* DNA. The T_m ranged from 83.19 to 83.36 °C, indicating the specific amplification (Fig. S2c). As the DNA concentration of the parasites used in this study was 0.4 pg/cell, the detection limit in the real-time PCR assay using SYBR Green reagents was 5 fg, equivalent to a 1.25×10^{-5} parasite. A considerable amount of *Leishmania* kDNA was detected from all three canine blood DNA samples collected at 4 months, 5 months, 6 months, and 7 months after inoculation (Fig. 3a). The highest amount of kDNA was calculated as 0.81 ± 0.11 pg, 2.35 ± 2.22 pg, and 1.10 ± 1.31 pg/100 ng of blood DNA at 5 months after inoculation for dogs A, B, and C, respectively (Fig. 3a). The DNA amount was equivalent to $0.77-2.27 \times 10^{-4}$ parasites per ml of the original peripheral blood.

Similarly, a considerable amount of parasite kDNA was also detected from the liver biopsy DNA samples in all three dogs at 5 months and 7 months after inoculation (Fig. 3b). A similar pattern of kDNA amount was observed in the peripheral blood and liver, in which the peak parasite load was detected at 5 months of parasite inoculation (Fig. 3a, b). Further, parasite kDNA was detected from the spleen necropsy samples at 8 months after inoculation, although the amount was less compared to the level in the liver (Fig. 4).

Histopathology and immunohistochemistry

Although careful microscopic examination of HE-stained sections was conducted, there were no remarkable inflammatory responses or granuloma formation in the canine liver and spleen. Based on the results of parasite burden by real-time PCR assay, the liver biopsy sections at 5 months and 7 months after parasite inoculation were further examined by immunohistochemical staining using a monoclonal antibody against *Leishmania* peroxiredoxin. The immunohistochemistry showed many reacted areas in a positive control murine liver section (Fig. 5a). Similar staining portions were rarely observed in the liver biopsy sections, but a typical immunochemical reaction was detected in the liver biopsy section at 5 months in dog B (Fig. 5b). However, HE counter-staining revealed no remarkable inflammatory response around the parasite-antigen positive area (Fig. 5c).

Discussion

In the present study, an experimental canine challenge model for *L. donovani* promastigotes was constructed using beagle dogs. Although this model showed an asymptomatic CVL with very low parasite burden, a considerable amount of parasite kinetoplast DNA was detected from the peripheral blood, liver, and spleen of challenged dogs. Liver biopsy sampling enabled us for the first time to compare the amount of parasite DNA in the peripheral blood and liver of the same animal. It is worth to note that the amount of parasite DNA during the experimental period showed a similar pattern with a peak at 5 months in the circulating blood and liver tissue. The blood parasite DNA may be attributed to the proliferation and dissemination of parasites or destruction of parasites in the liver at the late phase of this experimental infection.

The parasite isolation from the necropsy samples was unsuccessful. This may be due to the reduction of the number of parasites after the peak parasite burden at 5 months in the liver and to the lower parasite burden in the spleen, even though parasite DNAs remained in the organs. Nevertheless,

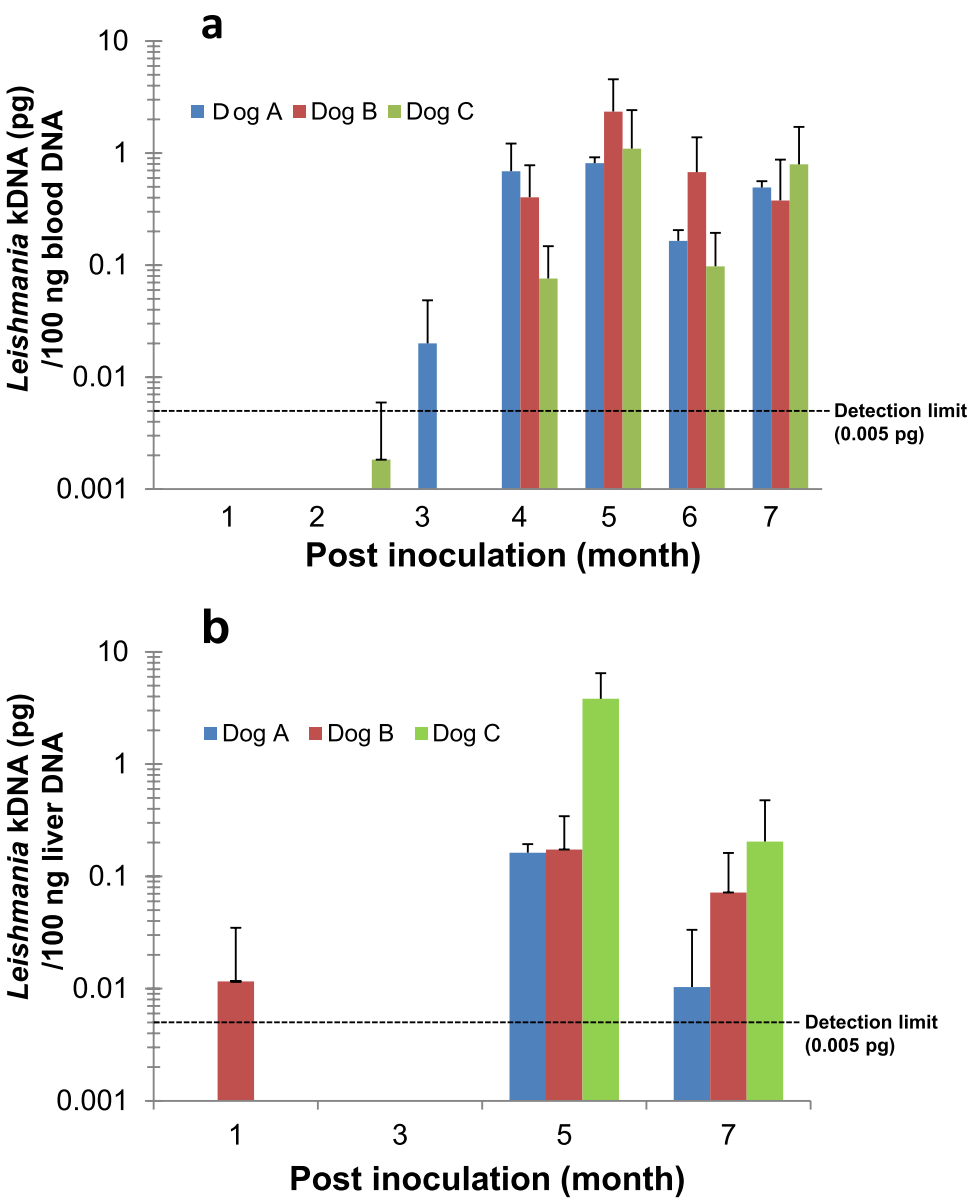

Fig. 3 Detection of *Leishmania* kDNA using real-time PCR from the peripheral blood (**a**) and liver biopsy (**b**) samples of dogs after inoculation with *Leishmania donovani*

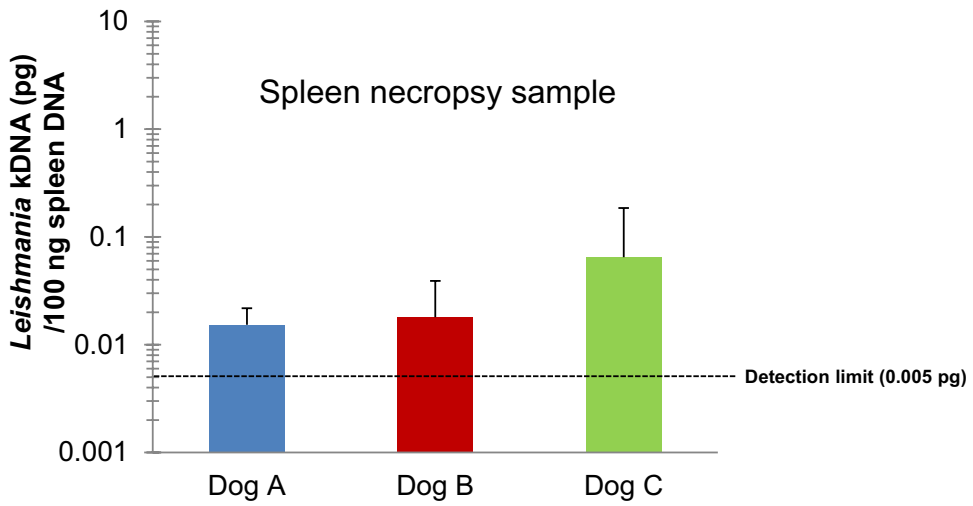

Fig. 4 Detection of *Leishmania* kDNA using real-time PCR from the spleen necropsy samples of dogs at 8 months after inoculation with *Leishmania donovani*

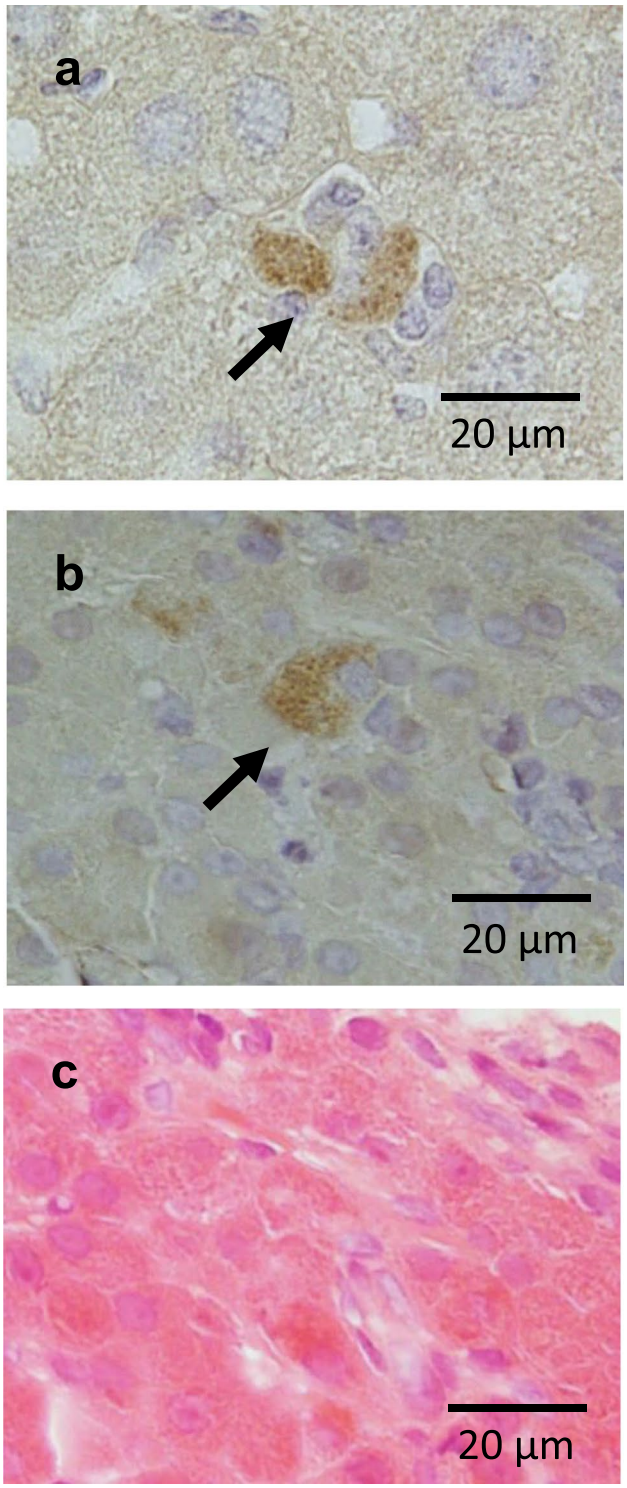

Fig. 5 Immunohistochemistry for detection of parasite antigens. Monoclonal antibody against *Leishmania* peroxiredoxin was reacted with a murine liver section prepared from a mouse infected with *Leishmania donovani* in the previous study as a positive control (**a**). A canine liver biopsy section from dog B after 5 months of inoculation with the same *Leishmania donovani* strain in this study was reacted with the monoclonal antibody (**b**). One of the canine liver consequent sections was stained with hematoxylin and eosin (**c**). Arrows in **a** and **b** indicate cells expressing *Leishmania* peroxiredoxin

detection of parasite DNA in the blood in this study may support the concept that dogs play a role in maintaining *L. donovani* parasites, as suggested by several reports on VL endemic areas (Dereure et al. 2003; Hassan et al. 2009; Shamboul et al. 2009; Sharma et al. 2009; Kalayou et al. 2011; Akter et al. 2016).

Only one experimental infection of dogs (German shepherd) with *L. donovani* (Khartoum strain, probably human isolate) was reported in two separated papers (Keenan et al. 1984a, b). The dogs received intravenous inoculation with the amastigotes. The dogs developed CVL symptoms, including moderate splenomegaly and lymph adenomegaly. Parasites were detected in cultures from the spleen, lymph node, liver, and bone marrow. In the present study, the limitation of CVL model included the small sample size of experimental animals, the use of murine-adapted *L. donovani* strain, intravenous inoculation of a large number of cultured promastigotes, unavailability of timely biopsy for the spleen and other organs except for the liver, and uncertainty of the effect of reagents for sedation and euthanasia on the growth of parasites. Thus, the use of *L. donovani* canine strains isolated from naturally infected dogs will be required for further immunopathological studies of CVL by *L. donovani*.

Visceralizing species of *Leishmania*, *L. donovani* and *L. infantum,* establish long-term infection within different organs, including the liver, spleen, and bone marrow. Organ-specific immune responses have been associated with VL in murine models infected with *L. donovani*, revealing self-limitation in hepatic infection and persistent infection in the spleen, with profound structure alterations (Engwerda and Kaye 2000; Engwerda et al. 2004). The present CVL beagle model suggested the self-limiting hepatic proliferation of the parasites in the early phase of infection with the peak of parasite burden at 5 months. However, the persistent infection in the spleen was unclear because the experiment was terminated after 8 months.

Clinical manifestations and hematological abnormalities are not clearly observed in asymptomatic CVL cases and cytological or histopathological detection of parasites appear to have low sensitivity (Chappuis et al. 2007; Saridomichelakis 2009; World Health Organization 2010). The diagnosis can be confirmed using PCR-based techniques (Solano-Gallego et al. 2011). Mitochondrial kDNA is the most sensitive target for PCR amplification because of the presence of approximately 10^4 copies in a single protozoan cell (Lambson et al. 2000). Among various primers for the amplification of kDNA, a primer set of RV1 and RV2 showed rare artefactual PCR products (Lachaud et al. 2002; Maia and Campino 2008) and detected 1×10^{-3} and 1×10^{-4} parasites per ml of the blood by conventional PCR (Lachaud et al. 2002) and real-time PCR (Mary et al. 2004; Mohammadiha et al. 2013), respectively. In the present real-time PCR assay using RV1 and RV2 primers, 0.81−2.35 pg kDNA

equivalent to $0.77-2.27\times10^{-4}$ parasites per ml of blood was detected, indicating a similar sensitivity to that in the previous reports (Mary et al. 2004; Mohammadiha et al. 2013). Thus, this study verified the usefulness of real-time PCR assay using RV1 and RV2 primers for the diagnosis of even asymptomatic CVL.

Detection of specific antibodies in combination with PCR detection is useful for the diagnosis of VL. In this study, all three challenged dogs showed a weak positive reaction to the rk39 dipstick strip at as early as after 1 month of parasite inoculation. In general, asymptomatic dogs infected with *L. infantum* showed negative or low positive antibody levels (Solano-Gallego et al. 2011). A follow-up study of experimental infection of beagle dogs with *L. infantum* amastigotes showed that the rk39 test became positive in four of 12 infected dogs at 60 days or 90 days after infection (Maia et al. 2010). However, it is possible that a faint band on the rk39 dipstick might not be counted as positive, although an ELISA test became positive at as early as after 1 month of *L. infantum* infection (Maia et al. 2010).

From the CVL model in this study, no conclusive evidence showed an increase or decrease in the percentage of $CD4^+$, $CD8^+$, $CD4^+FoxP3^+$, or $CD4^+CD25^+FoxP3^+$ T cell populations in the blood, although $FoxP3^+$ Treg subset was involved in hepatic parasite persistence in an immunodeficient mouse model (Tiwananthagorn et al. 2012). A significant increase of $CD3^+CD8^+$ T cells and a reduced percentage of $CD3^+CD4^+FoxP3^+$ T cells in the peripheral blood have been reported in dogs naturally infected with *L. infantum* (Cortese et al. 2013). A decrease of $CD4^+FoxP3^+$ T cells in the peripheral blood and spleen was reported in dogs naturally infected with *L. chagasi* (Silva et al. 2014). Thus, cellular immune responses might not be strongly induced in the present asymptomatic CVL model by *L. donovani* inoculation.

Finally, comparative whole-genome analysis of four major *Leishmania* species revealed that 55 out of 8032 protein-coding genes were identified as specific to *L. donovani*, and 60 out of 8241 were specific to *L. infantum* (Kumar et al. 2014). More recently, a comprehensive genome-wide global study, with 151 cultured field isolates of *L. donovani* and *L. infantum*, revealed that *L. infantum* strains from across the sampling range fall mainly into a single clade, while *L. donovani* are separated into five major groups with great diversity (Franssen et al. 2020). Furthermore, the identified genetic groups varied in heterozygosity and levels of linkage, suggesting different recombination histories. In that study by Franssen et al. (2020), only one *L. donovani* canine isolate from Iraq was included, and this strain was classified into *L. donovani* group 4, which contained one of each human isolate from Iraq, Saudi Arabia, and Ethiopia. Group 4 showed the most cross-relationship to the group 3, which contained human isolates from Sudan and Ethiopia (Franssen et al. 2020). Further isolation of canine *L. donovani* strains and comparative genetic analysis of *L. donovani* and *L. infantum* isolates could determine factors involving host preferences and elucidate why *L. donovani* is more infective to humans than dogs and vice versa and why *L. infantum* is more virulent to dogs than humans.

Conclusions

An experimental challenge model for *L. donovani* promastigotes was constructed in beagle dogs. Although the challenged dogs showed an asymptomatic infection, *Leishmania* DNA was detected in the peripheral blood, liver, and spleen. This model may contribute to the development of diagnostic, prognostic, and chemotherapeutic tools for canine leishmaniasis with *L. donovani* infection.

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Author contribution Hiroya Konno: writing of the original draft, formal analysis, investigation, and methodology.

Nozomu Yokoyama, Yu Tamura, and Keisuke Aoshima: investigation, validation, and methodology.

Mitsuyoshi Takiguchi: supervision and resources.

Ryo Nakao: methodology and data curation.

Ken Katakura: project administration, conceptualization, and writing including review and editing.

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Data availability All data presented here are available upon request.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval and consent to participate This study was carried out under the guidance of the Institute for Laboratory Animal Research (ILAR) under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. This experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Hokkaido University (Permit Number: 13–0149). Consent to participate is not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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