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ORIGINAL ARTICLE

Pathogenic effects of *Hepatozoon canis* (Apicomplexa: Hepatozoidae) on pet dogs (*Canis familiaris*) with amplification of immunogenetic biomarkers

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Abstract
Hepatozoon species (phylum Apicomplexa: Adeleorina, Hepatozooidae) are the cause of canine hepatozoonosis, a vector-borne disease (VBD) spread by ticks (Ixodidae). The aim of the present study was to determine the prevalence of *Hepatozoon canis* (*H. canis*) infections in different pet dogs admitted to different clinics in Egypt as well as determine the effect of *H. canis* on dogs through analysis of the immunogenic genes and the oxidative stress markers. One hundred dogs with different clinical signs, fever, fatigue, tick infestation around the ears and neck region, were examined. Thin blood films were performed and stained with Giemsa. Thirty examined dogs (30%) were positive for *H. canis* by direct observation of the gamonts in circulating leucocytes on blood smears stained with Giemsa. Two hundred ticks were identified morphologically and all ticks were classified as *R. sanguineus.* Hematological and biochemical results of sampled dogs were recorded. The AST and ALT levels were higher than control-negative healthy dogs. MDA levels in *H. canis* infected dogs were higher than that of control negative dogs. The transcript levels of the different targeted genes (IL-1β; IL6; TNF-α and IFN-γ) were upregulated in infected dogs with *H. canis* significantly than control healthy dogs. Canine hepatozoonosis induced tissue reaction evaluated by different immunological genes and oxidative stress.

Keywords *Hepatozoon canis* · *R. sanguineus* · MDA · Blood parasites · Gene expression analysis

Introduction

Hepatozoon species (phylum Apicomplexa: Adeleorina, Hepatozooidae) are the cause of canine hepatozoonosis, a vector-borne disease (VBD) spread by ticks (Ixodidae). According to Attipa et al. (2018), only two *Hepatozoon* species, *H. canis and H. americanum*, have been linked to canine infections. While *H. americanum* has only been documented from the North American continent, *H. canis* is widely spread over several nations in Europe, Asia, Africa and America (Giannelli et al. 2013; Léveillé et al. 2019).

H. canis is believed to be mostly transmitted by the brown dog tick, *Rhipicephalus sanguineus* sensu lato, although more recently, an experimental investigation also supported the role of *Rhipicephalus turanicus* as a vector (Giannelli et al. 2017); *Amblyomma ovale*, *Haemaphysalis longicornis*, *Haemaphysalis flava* and *Rhipicephalus* are among the other tick species. *H. canis* transplacental infections have also been reported (Murata et al. 1993). *Hepatozoon* spp. infection occurs when an intermediate vertebrate host (such as a mammal, amphibian, reptile or bird) eats the infected definitive host (Kwon et al. 2017). Sporozoites enter the body through the mouth and then pass through the intestinal wall to reach the hemolymphatic target organs (liver, spleen and bone marrow). These organs undergo merogony, and the ensuing meronts contain merozoites, which enter neutrophils and grow into gamonts, which thereafter manifest in the peripheral circulation.

H. canis infection in dogs causing anaemia, fever, lethargy, cachexia, and weight loss are some of the clinical symptoms, which range from asymptomatic to severe and lethal disease. Severe clinical symptoms characterize the majority of *H. americanum* infection cases (Baneth et al. 2003).

Giemsa-stained blood smears (peripheral blood or buffy coat) are typically examined under a microscope to diagnose *Hepatozoon* spp. infections by looking for gamonts in the

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cytoplasm of neutrophils rarely in the cytoplasm of monocytes (Kwon et al. 2017). *Hepatozoon* spp. infection can also be found using more contemporary diagnostic methods such as the polymerase chain reaction (PCR), indirect fluorescent antibody testing and enzyme-linked immunosorbent assays (Li et al. 2008). Additionally, the detection of hemoprotozoan DNA in the peripheral blood of infected dogs and in infected ticks using PCR assays targeting the nuclear18S ribosomal (r) RNA gene has shown to be effective, providing trustworthy information about the prevalence of the parasites globally (Daz-Sánchez et al. 2021). The aim of the present study was to determine the prevalence of *Hepatozoon canis* (*H. canis*) infections and to confirm the species identity and phylogenetic relationships of the causative agent using the 18S-rRNA gene as a genetic marker.

Materials and methods

One hundred dogs (admitted to clinic with different clinical signs, fever, fatigue, tick infestation around the ears and neck region) were examined for blood parasites, from the period of May to August 2022. Blood and sera were collected from each examined dogs; whole blood on EDTA was used for haematological parameters, and sera were used for biochemical parameters. Faecal samples were collected from each examined dogs for parasitological examination. *This study was approved by the ethical committee of the Faculty of Veterinary Medicine; Cairo University with number Vet CU 01122022624*.

Parasitological examination

Each anti-coagulated blood sample was processed for thin blood film and Giemsa staining to check for the presence of any blood parasites (Soulsby 1986); faecal samples were tested for the presence of any other concurrent parasitic infection. Five canines were about 3 months old and served as the control negative group (Attia and Salem 2022).

Haematological and biochemical analysis of blood and sera from the infected dogs with *H. canis*

Blood was drawn into two tubes: one EDTA for whole blood and one plain for serum samples collection (both tubes were centrifuged for 10 min at 3000 rpm). Blood that had been altered by *H. canis* infection in dogs was used in gene expression analysis.

Using a Fujifilm dri-chem (NX500V, Japan), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and urea were measured for analysis of liver and kidney enzymes.

Measurement of oxidative stress markers

Using particular kits made by the manufacturer, the sera samples from the diseased dog were examined for stress markers as malondialdehyde (MDA; catalog no: MBS2540407; MYBiosource; AbdElKader et al. 2020).

Transcript levels of the interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) through the use of quantitative real-time PCR from the infected blood.

Infected blood was aseptically preserved at − 20 °C, which was taken from sick dogs that had ticks and tested positive on blood smear examination, along with the negative controls (Salem et al. 2021), and was used for transcript levels of different genes.

RNA isolation

Following the manufacturer's instructions, RNA was isolated from 10 mg of blood samples using an RNA extraction kit (Ambion, Applied Biosystems; USA). The RNA purity was measured by Thermo Scientific NanoDrop to measure the RNA quantity. Then, 500 ng of RNA was made with DNase-I amplification grade (Invitrogen; USA) (Attia et al. 2020, 2021a). The reverse transcription of treated RNA was performed by a High-Capacity cDNA Archive Kit (Applied Biosystems; USA).

Quantitative real-time PCR protocol

Dog-specific PCR primers for interleukin-1 (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were created based on sequences deposited in the GenBank (Table 1; Maissen-Villiger et al. 2016 and Attia et al. 2021b). For sample normalization and as a reference gene, β-actin was utilized. A different pool of cDNA made from 10 negative control, non-infected dogs that had previously been checked for the presence of any parasites was used to evaluate the expression of the genes used in this investigation.

The DNA undergoes initial denaturation for 10 min at 95 °C, then 40 cycles of denaturation at 95 °C for 30 s (annealing at 60 °C; extension at 72 °C for 45 s) and final extension at 72 °C for 10 min (Attia et al. 2020).

Statistical analysis

For group comparison, a two-way analysis of variance was used, and a *P* value of 0.05 or less was considered statistically significant. The PASW Statistics, version 18.0, software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Table 1 Sequence base of the genes used in this study

Genes	Sequence (5′ > 3′)	Sequence number	References
Interleukin 1 beta (IL-1β)	F-AGTTGCAAGTCTCCCACCAG R-GTAGGGTGGGCTTTCCATCC	NM_001037971	Jiménez (2023)
Tumor necrosis factor (TNF-α)	F-GTGCCGTCAGATGGGTTGTA R-TCGGTTTGGGCAAGAATGGA	NM_001003244.4	Mankowska et al. (2016)
Interferon gamma (IFN-γ)	F-CAGATGTATCGGACGGTGGG R-CTGAAGCACCAGGCATGAGA	NM_001003174	Dąbrowski et al. (2020)
Interleukin-6 (IL-6)	F-TCCAGGACCCCAGCTATGAA R-GTCTGTGGTTGGGTCAGGAG	CFU12234	Kukielka et al. (1995)

Results

Two hundred ticks were identified morphologically, and all ticks were classified as *R. sanguineus* (Figs. 1 and 2).Thirty examined dogs (30%) were positive for *H. canis* by direct examination of blood smears which reveals presence of the gamonts in circulating leucocytes (Fig. 3). Table 2 lists the haematological and biochemical data of the sampled dogs, including information on the dogs' health status during infection with *H. canis*.

The total blood indices were lower in infected dogs than in healthy dogs. The majority of the dogs with low haematological levels also showed signs of anaemia clinically. Notably, dogs infected with *H. canis* showed leucocytosis and a low platelet count. *H. canis*–infected dogs had elevated AST and ALT levels. Edoema and jaundice were the clinical manifestations of liver injury. Infected dogs with *H. canis* had elevated levels of creatinine and blood urea nitrogen, which suggested renal failure. Emaciation and bloody urine are clinical signs of renal failure.

Oxidative stress markers in infected dogs

MDA levels in *H. canis*–infected dogs were, on average, higher (8.65 ± 1.41 nmol/ml) than in control-negative dogs (2.55 ± 0.50 nmol/ml) (Fig. 4).

Transcript levels of different evaluated genes in infected dogs with *H. canis*

The targeted genes (IL-1β, IL6, TNF-α and IFN-γ) had considerably higher transcript levels in *H. canis*–infected dogs than in healthy control-negative dogs (Fig. 5).

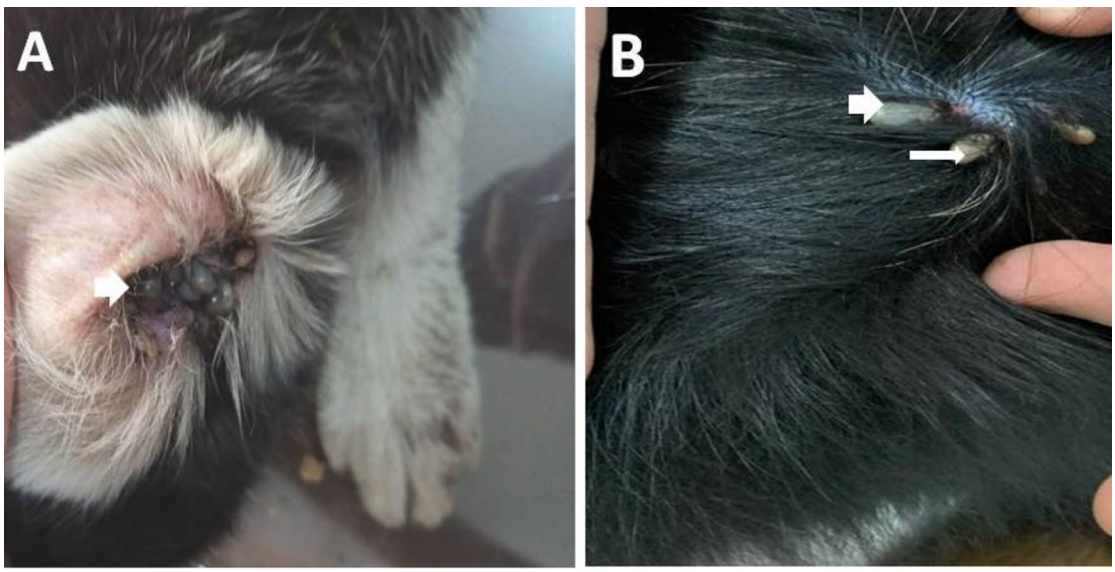

Fig. 1 Examined dogs infested with *Rhipicephalus sanguineus* ticks

Fig. 2 Collected stages of *Rhipicephalus sanguineus* ticks from infected dogs

Discussion

In the present study, *Hepatozoon* infections were detected by microscopy in 30% of dogs. By microscopic examination, in Egypt, Hegab et al. (2022) did not detect *H. canis* in 208 blood smears. In Italy, Otranto et al. (2011) recorded high incidence of *H. canis* infection (43.9%) in dogs. While in Japan, El-Dakhly et al. (2013) revealed *H. canis* gametocytes in the peripheral blood of 45 (23.6%) dogs. Barati and Razmi (2018) in Iran observed *Hepatozoon* spp. gamonts were 5/150 (3.3%) in blood smears. Orkun et al. (2018) in Turkey reported that the infection rate was 3.8% (4/103). Lakshmanan et al. (2018) and Kaura et al. (2020) in India revealed that 4% (6/150) and 1.86% (6/322) were positive for *H. canis* respectively. Díaz-Sánchez et al. (2021) in Cuba revealed that the infection rate was 10% (8/80).

Differences in *H. canis* prevalence may vary as a consequence of several factors, such as the geographical distribution and population density of the vector, climatic conditions, sampling methodology used, the host immune status and the targeted dog population (Aktas et al. 2015). Moreover, it had also been described that stray and shelter dogs showed a significantly higher prevalence of *H. canis* infection in comparison with pet dogs (Otranto et al. 2011).

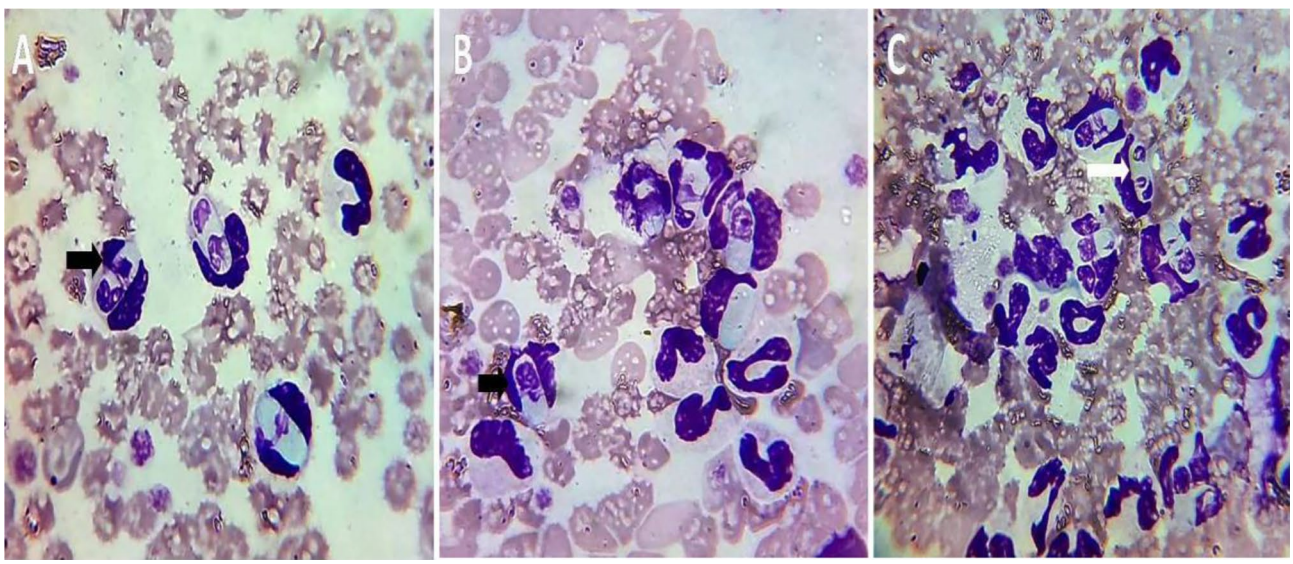

Fig. 3 Blood smears from infected dogs with *H. canis*; the gamonts appeared in leucocytes

Table 2 Haematological and biochemical parameters in healthy and infected dogs

Parameters (normal/reference range)	Mean ± SE		Reference range
	Healthy dogs	*H. canis*-infected dogs	
Haematology			
Total erythrocyte count	8.50 ± 0.05^{a}	4.50 ± 0.25^{b}	($5.5–8.5 \times 10^{6}$ µl)
Haemoglobin concentration	14.33 ± 0.13^{a}	7.95 ± 0.50^{b}	(12–18 g%)
Platelet count	357.32 ± 5.52^{a}	255.59 ± 10.49^{a}	($200–400 \times 10^{9}$/l)
Total leukocyte count	9.55 ± 0.15^{a}	20.48 ± 0.68^{a}	($6–17 \times 10^{6}$ cell/µl)
Neutrophil	7.86 ± 1.55	15.59 ± 5.57a	3–10.5 × 103 cell/µl
Lymphocyte	2.69 ± 2.55	4.35 ± 1.57	1–4.7X 103 cell/µl
Monocyte	0.65 ± 0.55	1.50 ± 1.50^{a}	0.15–1.45 × 103 cell/µl
Eosinophil	0.55 ± 0.57	0.65 ± 0.45	0.1–1.45 × 103 cell/µl
Biochemical			
AST (IU/l)	38.58 ± 2.59^{b}	100.29 ± 5.50^{a}	
ALT (IU/l)	59.46 ± 2.54^{b}	136.55 ± 4.50^{a}	
Creatinine	0.56 ± 0.24^{c}	1.95 ± 0.50^{b}	(0.5–1.6 mg%)
Blood urea nitrogen	24.15 ± 0.59^{c}	45.49 ± 2.49^{b}	(8.8–26 mg%)

SE standard error, *AST* aspartate amino transferase, *ALT* alanine amino transferase

[a,b,c]Different superscripts within the same row indicate significant difference at $P < 0.05$

When compared to healthy animals, WBC levels were elevated in 44% of dogs infected with *H. canis*, a sign of leukocytosis. The high WBC counts here correlated with higher neutrophil (48%) and monocyte (37%) numbers, which is consistent with the earlier findings even if a single study had different results (Salakij et al., (1999); Zaki et al. 2021). These increased cell counts were greater than those found in other blood parasite infections in dogs, which could be because of the inflammatory reaction brought on by the invasion and proliferation of *Hepatozoon* organisms in tissue (Thongsahuan et al. 2020).

According to Kiral et al. (2005), dogs infected with *Hepatozoon* spp. had significantly higher serum levels of GSH, MDA and NO than control samples. Ceruloplasmin concentration, on the other hand, was unaffected in animals with illness. MDA, GSH, NO and ceruloplasmin concentrations

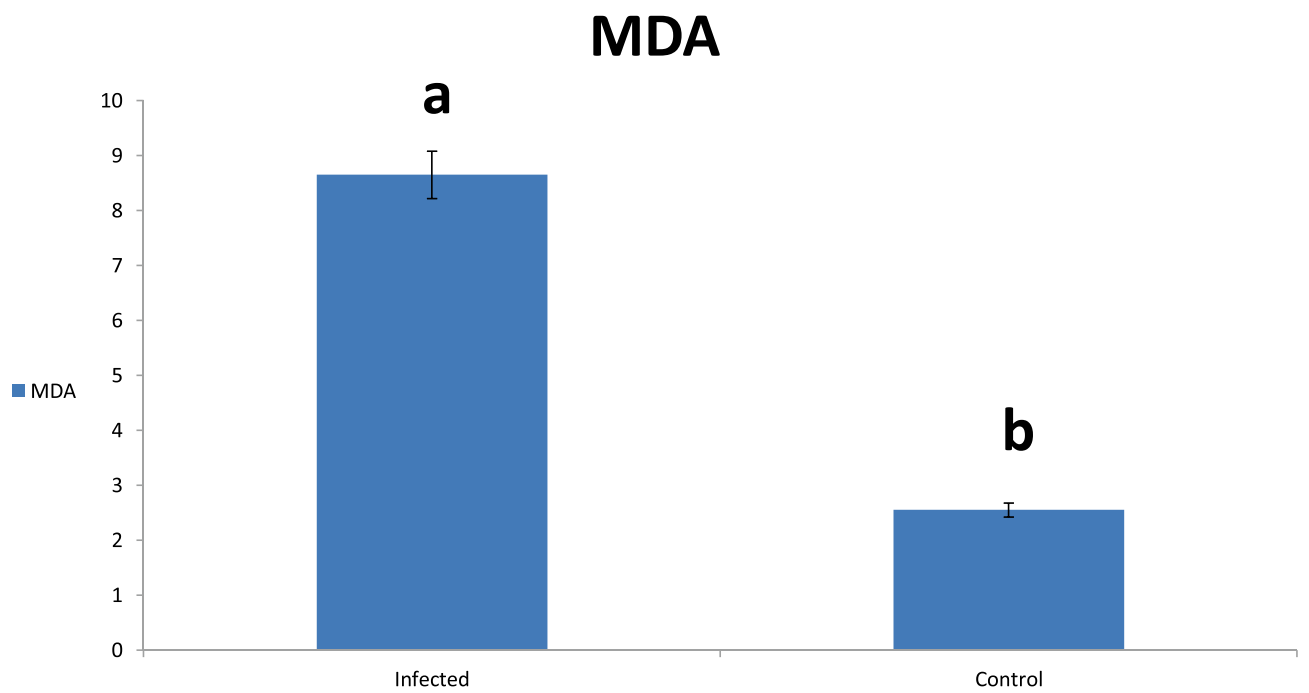

Fig. 4 Malondialdehyde (MDA) in infected *H. canis* in comparison to control healthy one

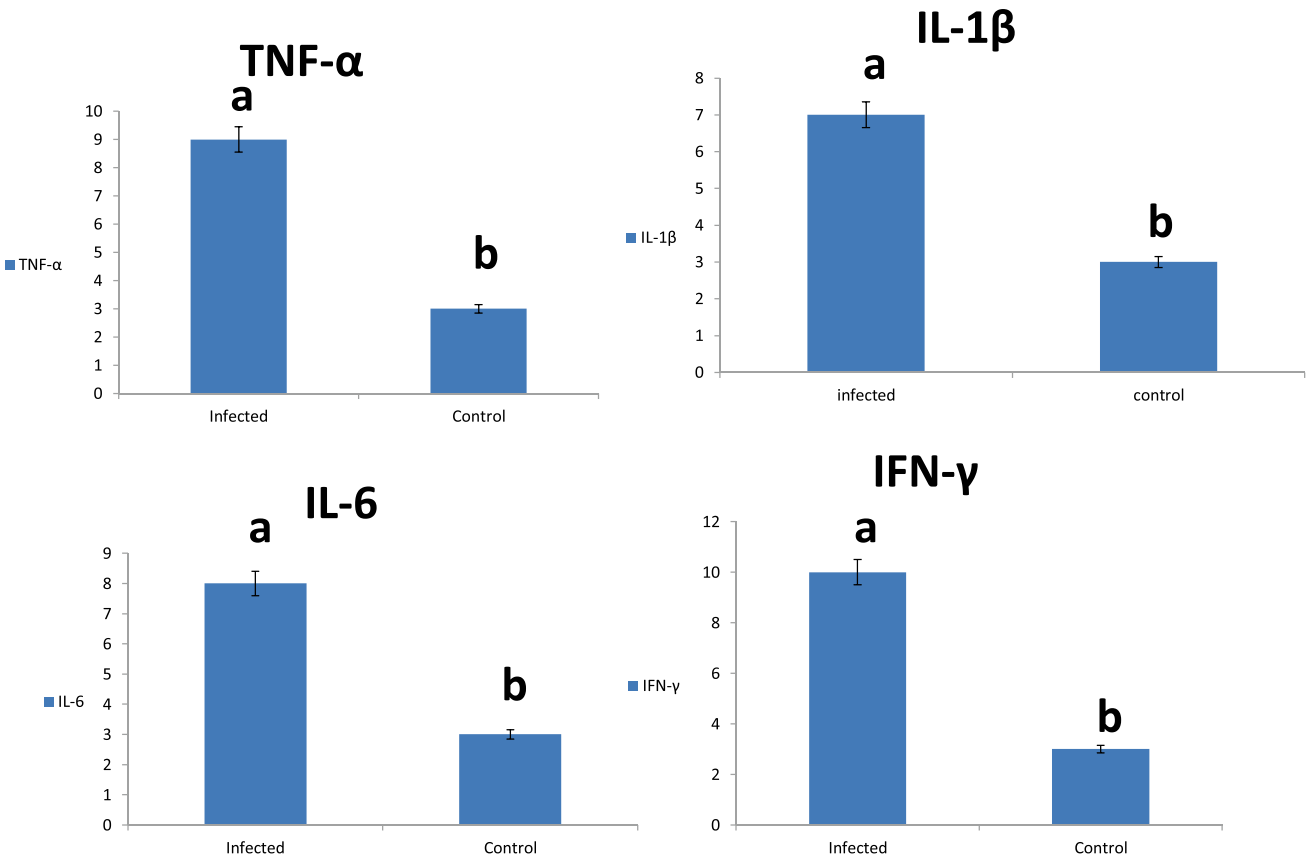

Fig. 5 Transcript levels of the interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) in infected *H. canis* in comparison to control healthy one

could not be linked to the rate of parasitemia in specific animals. According to Sarma et al. (2012), SOD levels decreased but lipid peroxidation (LPO) levels increased, so in an infection with *Hepatozoon*, the oxidative stress was elevated as recorded in this study.

Interleukin-1, along with TNF-α and IFN-γ, is essential for both acute and chronic inflammation, both locally and systemically in infection. The two subtypes are known as IL-1α and IL-1β. Both kinds are primarily produced by fibroblasts, endothelial cells, monocytes and macrophages. In the body, IL-1 has a pyretic effect. This increases liver production of acute phase proteins, lymphocyte activity and the release of collagenases and prostaglandins (Dinarello 1996).

Our results revealed that high transcript levels of the four genes in the infected dogs are due to the inflammation in animals which elevates IL-1β, IL-6, TNF-α and IFN-γ as a subsequent increase in IL-1β as recorded by Dinarello (2011) and Prachar et al. (2013). In IL-12, IL-1β causes natural killer cells to secrete IFN-γ, which causes macrophages to become activated. Only monocytes and macrophages can produce IL-1α, which has a role in several immunological processes, most notably the preservation of the dermal immunological barrier. In addition, oligodendroglia, platelets, osteoblasts, astrocytes, adrenal cortical cells and many T-cell subtypes produce IL-1β. Like many other cytokines, IL-1β is not retained in cells or tissue since it resembles a hormone.

While the other pro-inflammatory cytokine is inhibited by IL-6, IL-1β increases the synthesis of IL-6. One of the first pro-inflammatory cytokines to increase in inflammation, aside from TNF-α, is IL-1β, which is significant since each cytokine has a specific role in regulating the expression of other cytokines (Dinarello 2011; Prachar et al. 2013).

Data availability All data presented in the manuscript; no additional data were used for the research described in this article.

Compliance with ethical standards

Funding This study was not supported by any funding.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors declare that the sampling from animals was conducted under local Ethical Committee laws and regulations as regard to the care and use of laboratory animals *Vet CU 01122022624.*

Informed consent For this type of study, informed consent is not required.

Consent for publication For this type of study, informed consent is not required.

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