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High-Sensitivity Cardiac Troponin (hs-cTnI and hs-cTnT) Levels in Dogs with *Hepatozoon Canis*

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Abstract

The most common etiologic agent that causes canine hepatozoonosis is *Hepatozoon canis (H. canis).* High parasitemia is associated with symptoms of fever, anorexia, weight loss, anaemia, ocular discharge, and paralysis of the hind legs. The agent has also been reported to cause myocardial lesions. Troponins are considered the gold standard as they have high sensitivity and specificity for myocardial injury. The study consisted of a total of 27 dogs brought to Iğdır University Animal Hospital, 15 dogs diagnosed with *H. canis* infection by polymerase chain reaction analysis, and 12 healthy dogs. Of the examined dogs, 59.3 % were female, and 40.7 % were male. Of the dogs, 55.6 % were 4 years old and under, and 44.4 % were 5 years old and over. This study determined high-sensitivity cardiac troponin I (hs-cTnI) and T (hs-cTnT) levels in healthy dogs as 0.1262 and 0.1054 ng mL^{-1}, respectively. In dogs infected with *H. canis*, hs-cTnI and hs-cTnT levels were 0.4706 and 0.3056 ng mL^{-1}, respectively. In addition, there was a significant difference in serum Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and albumin levels in dogs infected with *H.canis* compared to the control. There was no significant increase in cholesterol and calcium levels. In conclusion, there was a significantly higher increase in hs-cTnT and hs-cTnI levels in dogs infected with *H. canis*. Considering that even the slightest change in troponin levels is important for prognosis, *H. canis* may have caused myocardial injury in dogs in our study. Besides, the relative increase in hs-cTnT and hs-cTnI detected in the healthy animal group may indicate various possible causes, such as low clearance and distant myocardial ischaemic events.

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1. Introduction

H. canis is an apicomplexan parasite from the family Hepatozoidae (Guo et al., 2020), first detected in India (James, 1905), and is transmitted by *Rhipicephalus sanguineus*, known as the brown dog tick (Aktas et al., 2015; Mohanapriya et al., 2021). This tick is frequently observed in dogs in the Balkan peninsula and the Mediterranean region (Aktas et al., 2015). Although studies have revealed more than 340 species in the Hepatozoon genus, canine hepatozoonosis is known to be caused by *H. canis* and *H.americanum* (Baneth and Allen, 2022). *H. canis* has been reported as the most common species causing canine hepatozoonosis (Aktas et al., 2015; Aydin et al., 2015).

H. canis has a prevalence rate of 2.5-58.7 % (Rojas et al., 2014; Harris et al., 2015; Hamel et al., 2016; Pacifico et al., 2020) and generally progresses in a subclinical form. Clinically, the agent causes long-term parasitemia (Baneth and Allen, 2022). High parasitemia is associated with symptoms of fever, anorexia, weight loss, anemia, ocular discharge, and paralysis of the hind legs (Vincent et al., 2021). It is possible to encounter *H. canis* in the lungs, heart, skeletal muscles, liver, spleen, and lymph nodes during schizogony (Ivanov and Tsachev, 2008). Another study reported that the agent causes myocardial lesions (Kegler et al., 2018). However, the most sensitive diagnostic method for canine hepatozoonosis is real-time polymerase chain reaction (PCR)(Tołkacz et al., 2023).

Troponins are globular proteins that play a role in the contraction and relaxation of myofibrils (Basbugan et al., 2010). Cardiac troponin I (cTnI) and T (cTnT) have a high sensitivity to myocardial necrosis (Kırbaş et al., 2021; Ayvazoğlu et al., 2023). These troponins and their isoforms are essential markers of cardiac tissue-specific cellular damage (Mahendran et al., 2022). High-sensitivity cTnI (hs-cTnI) and T (hs-cTnT) can be detected at lower levels than troponin (I and T), thus contributing to the detection of mild myocardial injury that may occur in the early stages of diseases (Ayvazoğlu et al., 2022).

In the literature, while some studies observed no clinical symptoms in dogs with *H. canis* who have reached high parasitemia, some research reported clinical symptoms with co-infections accompanying the infection (Schäfer et al., 2022; Tołkacz et al., 2023) Scarce studies have investigated this parasite's clinical and immunopathological co-infection mechanisms (Ortuño et al., 2022). Furthermore, the number of literature studies reporting the association between *H. canis* infection and myocarditis is scant. Although these studies have emphasized myocardial effects, there is also a potential for vertical transmission of the disease (Schäfer et al., 2022). It is essential to prevent severe myocardial damage in animals and detect damage at the prognosis stage. This situation will enable the determination of accurate, rapid, and sensitive testing strategies for disease diagnosis that will allow early intervention. In order to evaluate myocardial necrosis, this research analyzed serum enzyme levels (ALT, AST, LDH, ALP) and albumin, cholesterol, and calcium values together (De Bonis et al., 2021; Hassanein et al., 2023). The current study aimed to compare high-sensitive hs-cTnI and hs-cTnT levels in "dogs with *H. canis*" with healthy animals, to fill out the gap in the literature on clinical diagnosis of canine *H. canis* infection and myocarditis (Hamacher et al., 2015; Kegler et al., 2018). Our results were aimed to fill the gap in the relationship between *H. canis* infection and serum troponin levels, which is missing in the literature.

2. Materials and methods

This study was conducted by obtaining approval from the Animal Experiments Local Ethics Committee of Kafkas University (Kars, Turkey, KAÜ-HADYEK\2022-056).

2.1. Animal material

The study consisted of 27 dogs brought to Iğdır University Tuzluca Animal Hospital in 2022, 15 dogs diagnosed with *H. canis* infection by polymerase chain reaction (PCR) analysis, and 12 healthy dogs (Figure 1). Of the examined dogs, 59.3 % (16/27) were female (The oestrous cycle was not taken into account), and 40.7 % (11/27) were male. Of the

dogs, 55.6 % (15/27) were 4 years old and under, and 44.4 % (12/27) were 5 years old and over. A blood sample of 5 mL was collected from the Vena cephalica of sick and healthy animals into serum tubes. These blood samples were centrifuged at 3000 rpm for 10 minutes, and their serums were separated. The serums were stored at -20ºC until measuring the hs-cTnI and hs-cTnT levels.

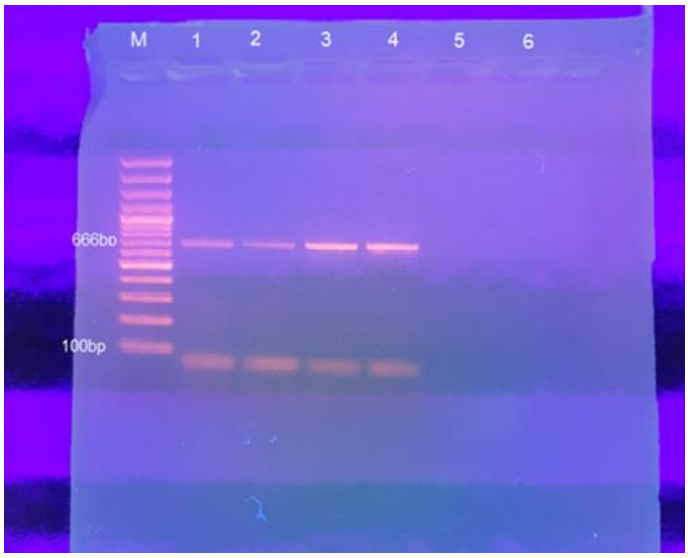

Figure 1. Gel image of samples positive and negative for *H. canis*.
M: marker; 1: *H. canis* positive control; 2,3,4: positive site sample; 5: negative field example; 6: negative control (distilled water)

2.2. PCR analysis

DNA extraction of collected dog blood was performed using the blood DNA extraction kit (Quick-DNATM Miniprep Kit, Zymo Research, Irvine, USA) in accordance with the manufacturer's instructions. DNA samples obtained from *Hepatozoon spp*. PCR was performed using Hep F (5'-ATACATGAGCAAAATCTCAAC-3') and Hep R (5'-CTTATTATTCCATGCTGCAG-3') primers specific to *H. canis*, which amplify the 666 bp fragment of the 18S rRNA gene (Inokuma et al., 2002).

2.3. Measurement of cardiac markers

Levels of serum hs-cTnI and hs-cTnT, were determined by ELISA equipment (Thermo Scientific Multiscan GO, TYPE: 1510) and calculated with commercial test kit (ng mL^{-1}) as instructed by the manufacturer (Canine High Sensitivity Cardiac Troponin I and T Kit, BT Lab, Shanghai). Serum enzymes, albumin, cholesterol, and calcium analysis were performed in an auto-analyzer at Iğdır University Animal Hospital Laboratories (*Randox Monaco*)(Gultekin et al., 2023).

2.4. Statistical analysis

SPSS 20 software package was used for statistical analysis of the obtained data. To reveal the differences in hs-cTnI and hs-cTnT levels of healthy and *H. canis* infected dogs according to age and gender, first of all, conformity of data to normal distribution was determined using a normality test, and then an independent t-test was performed. All results are presented as Mean±SE. Results with a P-value less than 0.05 were considered statistically significant.

3. Results and Discussion

Of the 27 Akbash crossbreed dogs used in this study, 44.4 % (12/27) were healthy, and 55.6 % (15/27) had *H. canis* infection confirmed by PCR analysis. The hs-cTnI and T levels were 0.4706±0.073, and 0.3056±0.052 ng mL^{-1}, respectively, in dogs infected with *H. canis*, and 0.1262±0.008, and 0.1054±0.009 ng mL-1 in healthy dogs (Table 1). Statistical analysis revealed higher hs-cTnI and hs-cTnT levels in dogs with *H. canis* ($P<0.01$).

Table 1. Changes in hs-cTnI and T levels in healthy and *H. canis* infected dogs

Parameters	Group	N	Mean±SE (Min-Max)	T/P
hs-cTnI (ng mL^{-1})	Healthy	12	0.1262±0.008 (0.0720-0.1652)	T=4.196
	H. canis	15	0.4706±0.073 (0.1284-0.9396)	P<0.01
hs-cTnT (ng mL^{-1})	Healthy	12	0.1054±0.009 (0.0460-0.1431)	T=3.392
	H. canis	15	0.3056±0.052 (0.1201-0.7836)	P<0.01

Table 2. Changes in Hs-cTnT levels in healthy and *H. canis* infected dogs by sex

Parameter	Group	Gender	N	Mean±SE	T/P
hs-cTnT (ng mL^{-1})	Healthy	Female	7	0.1143±0.014	T=1.222
		Male	5	0.0930±0.007	P>0.05
	H. canis	Female	9	0.2972±0.061	T=0.191
		Male	6	0.3182±0.099	P>0.05

Of the examined dogs, 59.3 % (16/27) were female, and 40.7 % (11/27) were male. In *H. canis* infected female and male dogs, the hs-cTnT levels were 0.2972±0.061, and 0.3182±0.099 ng mL^{-1} respectively, and 0.1143±0.014, and 0.0930±0.007 ng mL^{-1} in healthy dogs with no statistically significant difference between the groups (P>0.05, Table 2). In *H. canis* infected female and male dogs, the hs-cTnI levels were 0.3854±0.086, and 0.5985±0.117 ng mL^{-1} respectively, and 0.1351±0.013, and 0.1138±0.007 ng mL^{-1} in healthy dogs with no statistically significant difference between the groups (P>0.05, Table 3).

Table 3. Changes in hs-cTnI levels in healthy and *H. canis* infected dogs by sex

Parameter	Group	Gender	N	Mean±SE	T/P
hs-cTnI (ng mL^{-1})	Healthy	Female	7	0.1351±0.013	T=1.257
		Male	5	0.1138±0.007	P>0.05
	H. canis	Female	9	0.3854±0.086	T=1.496
		Male	6	0.5985±0.117	P>0.05

Table 4. Changes in hs-cTnT levels in healthy and *H. canis* infected dogs by age

Parameter	Group	Age	N	Mean±SE	T/P
hs-cTnT (ng mL^{-1})	Healthy	≤4	7	0.0984±0.011	T=0.939
		≥5	5	0.1153±0.014	P>0.05
	H. canis	≤4	8	0.3040±0.058	T=0.031
		≥5	7	0.3074±0.095	P>0.05

Of the dogs, 55.6 % (15/27) were 4 years old and under, and 44.4 % (12/27) were 5 years old and over. In *H. canis* infected and healthy dogs aged 4 years and younger, the hs-cTnT levels were 0.3040±0.058, and 0.0984±0.011 ng mL^{-1} and 0.3074±0.095, and 0.1153±0.014 ng mL^{-1} in dogs 5 years and older, respectively with no statistically significant difference between the groups (P>0.05, Table 4). In *H. canis* infected and healthy dogs aged 4 years and younger, the hs-cTnI levels were 0.4005±0.084, and 0.1193±0.011 ng mL^{-1} respectively, and 0.5508±0.122, and 0.1360±0.014 ng mL^{-1} in dogs 5 years and older, respectively with no statistically significant difference between the groups (P>0.05, Table 5).

Table 5. Changes in hs-cTnI levels in healthy and *H. canis* infected dogs by age

Parameter	Group	Age	N	Mean±SE	T/P
hs-cTnI (ng mL^{-1})	Healthy	≤4	7	0.1193±0.011	T=0.956
		≥5	5	0.1360±0.014	P>0.05
	H. canis	≤4	8	0.4005±0.084	T=1.033
		≥5	7	0,5508±0,122	P>0.05

Independent t-test was used to revealed differences in ALT, AST, LDH, ALP, Albumin, cholesterol, and calcium values between healthy and infected dogs (regardless of sex and age). The analysis revealed a statistically significant difference between healthy and infected dogs for the ALT ($t(18)=-5.19$, $p<0.01$), AST ($t(18)= -6.00$, $p<0.05$), ALP ($t(18)= -2.93$, $p<0.05$), LDH ($t(18)= -3.14$, $p<0.05$) and albumin ($t(18)= 4.81$, $p<0.01$) values. However, the difference between the groups for cholesterol ($t(18)= -0.90$, $P>0.05$) and calcium ($t(18)= -0.21$, $P>0.05$) values was not statistically significant. (Table 6).

Table 6. Serum Biochemistry results

Parameters	Group	N	Mean±SE	T/P
ALT (U L^{-1})	Healthy	10	12.85±1.28	T=-5.19 P<0.01
	H. canis	10	30.01±3.05	
AST (U L^{-1})	Healthy	10	17.12±1.65	T=-6.00 P<0.05
	H. canis	10	35.80±2.63	
LDH (U L^{-1})	Healthy	10	168.78±33.87	T=-3.14 P<0.05
	H. canis	10	356.23±49.19	
Cholesterol (mg dL^{-1})	Healthy	10	146.73±16.48	T=-0.90 P>0.05
	H. canis	10	168.73±18.02	
Calcium (mmol L^{-1})	Healthy	10	4.61±0.36	T=-0.21 P>0.05
	H. canis	10	4.70±0.25	
Albumin (g Dl^{-1})	Healthy	10	3.57±0.11	T=4.81 P<0.01
	H. canis	10	2.97±0.06	
ALP (U L^{-1})	Healthy	10	23.10±2.46	T=-2.93 P<0.05
	H. canis	10	36.80±3.98	

H. canis infection, which usually progresses subclinically with mild symptoms, is transmitted by ticks. It may create long-term parasitemia leading to a severe and fatal clinical presentation with fever, lethargy, and anaemia. The agent has also been shown to cause myocardial lesions (Dixit et al., 2023; Tresamol and Vincy, 2023).

A small proportion of troponin molecules, which are mostly attached to the intracellular contractile apparatus, are cytosolic. Although the cytosolic troponin released immediately after myocardial injury causes a sudden increase in the blood serum sample, there is also a long and slow-term release from the intracellular apparatus (Wu, 2017; Coscarella et al., 2023). Since it is difficult to distinguish between cytosolic or dependent release, as in the aforementioned case, molecules with complex mechanisms of release are not used for this type of distinction in blood studies (Langhorn and Willesen, 2016).

It has been reported that cTnT, a specific biomarker of cardiac muscle, is secreted at a minimum level in skeletal muscle (Langhorn and Willesen, 2016). Result from different studies that the cTnT level ranges from <0.01 to <0.05 ng mL^{-1} in healthy dogs (Shaw et al., 2004). In our study, the hs-cTnT level was 0.1054±0.009 ng mL^{-1} in the control group. Despite being healthy in terms of *H.canis*, the reason for this difference may be due possible pre-post capillary pulmonary hypertension (Guglielmini et al., 2010), acute chronic disease (Ricchiuti et al., 1998), necrosis, apoptosis, necroptosis, cellular injury, reduced clearance (Hammarsten et al., 2018), a measurement made with high sensitivity.

Different studies report an increase in cTnT in cardiac injury in dogs(Burgener et al., 2006; Çakıroğlu et al., 2009), however, there is no study on cTnT levels in dogs infected with *H. canis*. Results from this study, showed that the hs-cTnT level was significantly higher in *H. canis* infected dogs than in healthy ones (P<0.01). This situation suggests heart damage.

Mclaurin et al. (1997) reported that cTnI is more specific than cTnT among cardiac troponins. This specificity was attributed to the lower molecular weight of cTnI (McLaurin et al., 1997; Missov and De Marco, 1999). Therefore, cTnI is considered to be a more sensitive and specific marker than cTnT for the detection of minor myocardial injury (Langhorn and Willesen, 2016). It has been reported in different studies that cTnI levels vary between 0.004 and 0.136 ng mL^{-1} in healthy dogs (Langhorn et al., 2013). The hs-cTnI levels in healthy dogs in our study were similar to previous studies.

Several studies on dogs reported that cTnI levels increase in cardiac injury (Oyama and Sisson, 2004; Bakirel and Gunes, 2009). However, literature on *H. canis* infected dogs lacks high-sensitivity studies on cTnT levels. In our study, the hs-cTnI level was found to be significantly higher in dogs with *H. canis* compared to healthy ones (P<0.01).

Since the cTnI gene sequence in dogs and cats is 95-96 % homologous to humans (Rishniw et al., 2004), a similar difference was expected in dogs, but our study found that hs-cTnT and hs-cTnI levels in dogs did not differ by sex (P>0.05). Although there was no statistically significant difference between the sexes, there was a numerical decrease in hs-cTnT and hs-cTnI levels in female dogs compared to male dogs (P>0.05). This may be caused by the oestrous cycle and estrogen. Studies have reported that estrogen provides myocardial protection due to its antioxidant activity (Kim et al., 1996; Knowlton and Lee, 2012).

In a human study, slight increases in cTnI levels were reported due to increased myocardial remodelling with possible cardiomyocyte loss characterized by ageing (Reiter et al., 2011). A study on dogs reported a significant correlation between age and cTnI level (Oyama and Sisson, 2004). In the present study, although hs-cTnT and hs-cTnI levels were found to be higher in dogs aged 5 years and older than in dogs aged 4 years and younger, the difference was not statistically significant (P>0.05).

The asexual phase (merogony) of the biological cycle of *H. canis* takes place in the canine epithelium, liver, and muscle tissue (Ewing and Panciera, 2003). In this case, it is crucial to consider that potentially unvaccinated dogs can cause worsening liver disease. Vaccinated dogs may show a milder inflammatory response. In addition, today, some are still discussing systemic diseases and clinical pathological implications that may

occur in this infection and mentioning its zoonotic potential. Previous studies have reported that the role of this infection in systemic diseases may be reasonable and clinical manifestations may vary (Baneth, 2011; De Bonis et al., 2021). Myositis and myocarditis have been shown in recent cases of hepatozoonosis *(*Gökçe et al., 2013; Simonato et al., 2022). These studies documented increasing AST, ALT, ALP, and LDH activities (Gökçe et al., 2013; De Bonis et al., 2021), decreasing albumin levels (hypoalbuminemia), and no significant difference in cholesterol and calcium levels (Maguire et al., 2011). Similar to the literature, this research determined a significant increase in cytosolic enzyme activities [ALT ($p<0.01$), AST ($p<0.05$), LDH ($p<0.05$), and ALP ($p<0.05$)]. While there was a significant decrease in serum albumin levels ($p<0.01$), there was no difference in cholesterol and calcium values ($P>0.05$). Some studies mention the effectiveness of cholesterol in the occurrence of myocardial damage(Sakamoto et al., 1991). Some studies have stated that high cholesterol increases the likelihood of having an infarction(Sakamoto et al., 1991; Yöntem et al., 2017). The fact that serum cholesterol levels were insignificant in our study suggested that the increase in troponin levels in control and infected dogs was not due to a cholesterol-induced infarction. In our study, no significance was observed in serum calcium levels. This situation is similar to the studies in the literature showing that calcium values are not significant in the development of infarctions (Khoshnegah et al., 2009; Maguire et al., 2011).

4. Conclusions

Although our study contributed to the expansion of the knowledge in the literature regarding the myocardial injury in *H. canis* infected dogs with findings on troponin (cTnI and cTnT), it was not sufficient to fully define the specificity or make it less complex. In our current study, the significant increase in Troponin levels in the serum of dogs with *H. canis* (in accordance with the literature) suggests that it is an effective biomarker. However, the fact that the increase in the healthy group did not match the literature data suggested that troponin levels may have increased due to many different reasons (low clearance) and even distant myocardial ischaemic events (acute, pulmonary) in dogs who were supposed to be healthy. Due to the uncertainty of these partial causes, more studies on troponin levels of dogs infected with *H. canis* should contribute to the literature. In this context, our recommendation for future studies is to increase the number of samples according to age and gender, and to evaluate whether there are other diseases other than *H.canis* infection by researchers.

Our aim was to determine the relationship between dogs with *H.canis* and serum troponin levels. The small number of samples and the scarcity of literature studies in this context constitute the limitations of our discussion and study. It is important to increase the number of samples in each group in future studies. Our results suggest that troponin levels may be a marker in infected dogs. In clinical practice, the veterinarian can evaluate the possible relationship between troponin levels and *H. canis* infection.

Declaration of Author Contributions

ÜY, CA; analysis, methodology, conceptualization, writing and original draft preparation; ZGY; analysis, writing, review and editing; ŞK; methodology, analysis; NA; writing, PCR analysis

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

Ethical Committee Approval

This study was conducted by obtaining approval from the Animal Experiments Local Ethics Committee of Kafkas University (Kars, Turkey, KAÜ-HADYEK\2022-056).

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