RESEARCH ARTICLE

IJVAR *International Journal of Veterinary and Animal Research*
E-ISSN: 2651-3609 6(1): 17-22, 2023
DOI: 10.5281/zenodo.7769666

The Effect of Different Diseases (*Hepatozoon canis*, Distemper and *Babesia canis canis*) on Serum Haptoglobin, Ceruloplasmin and Albumin Levels in Dogs

Cemalettin Ayvazoğlu[1,a,*], Şemiştan Kızıltepe[2,b], Nilgün Aydın[3,c]

[1]Ardahan University, Nihat Delibalta Göle Vocational High School, Ardahan, Türkiye
[2]Iğdır University, Tuzluca Vocational School, Tuzluca, 76100, Iğdır, Türkiye
[3]Kafkas University, Faculty of Veterinary Medicine, Department of Parasitology, Kars, Türkiye

[a]ORCID: 0000-0003-2064-0657; [b]ORCID: 0000-0002-3288-2058; [c]ORCID: 0000-0002-0571-7882

*Corresponding Author
E-mail: cemayvazoglu@hotmail.com

Received: December 12, 2022
Accepted: March 02, 2023

Abstract

In this study; it was aimed to determine serum Hp, Cp, and Alb levels in dogs infected with *Hepatazoon canis*, Distemper and *Babesia canis canis*. The material of the study; 45 dogs infected with *H. canis* (n=15), *B. c. canis* (n=15) by PCR analysis, and canine distemper (n=15) with the rapid diagnosis kit, which were brought to Iğdır University Tuzluca Animal Hospital formed. In addition, 15 healthy dogs were used for control purposes in the study. The serum Hp, Cp, and Alb levels of dogs with *H. canis* were determined as 1.8712±0.003 mg/mL, 9.1746±1.504 mg/dL, and 3.1067±0.073 g/dL, respectively. The serum Hp, Cp, and Alb levels of dogs with distemper were 1.8787±0.005 mg/mL, 7.3016±1.439 mg/dL, and 2.9667±0.080 g/dL, respectively. The serum Hp, Cp, and Alb levels of dogs with *B. c. canis* were measured as 1.8780±0.002 mg/mL, 7.8456±2.092 mg/dL, and 3.2467±0.129 g/dL, respectively. Whereas, serum Hp, Cp, and Alb concentrations of healthy dogs were determined as 1.8662±0.003 mg/mL, 2.9745±0.343 mg/dL, and 2.9600±0.108 g/dL, respectively. While Cp concentration of sick animals were higher than healthy animals ($P<0.05$), there was no statistically significant difference in Hp and Alb concentrations ($P>0.05$). As a result, it was determined that serum Cp concentration increased in dogs with *H. canis*, distemper and *B. c. canis* compared to healthy dogs, while Hp and Alb concentrations did not change.

Keywords: Albumin, *Babesia canis canis,* ceruloplasmin, distemper, dog, haptoglobin, *Hepatazoon canis.*

INTRODUCTION

The stimulation of the neuro-immuno-humoral system as a result of tissue damage and the restoration of the damaged tissue is called acute phase response (APR) (Milanović et al., 2019; Kırmızıgül et al., 2020). In short, APR can also be considered as an early warning system that informs the state of the body (Sevgisunar and Şahinduran, 2014). Proteins synthesized as a result of APR are called acute phase proteins (APP) and are generally produced in hepatocytes and some extrahepatic tissues and organs (adipose tissue, testicular tissue, uterus, ovary, mammary glands, lung, digestive system) (Gökce and Bozukluhan, 2009; Tuna and Ulutaş, 2015). APP are proteins whose serum concentrations increase (positive APP) or decrease (negative APP) by at least 25% in response to injury (Hacımustafaoğlu, 2017). Positive APP's Serum Amyloid A (SAA), Haptoglobin (Hp), C-Reactive Protein (CRP), α-1 Acid Glycoprotein (AGP), Ceruloplasmin (Cp), and Fibrinogen (Fb); Negative APP's are reported as Albumin (Alb), transthyretin (TTR/prealbumin), cortisol binding globulin, retinol-binding protein (RBP) and transferrin (Tf) (Tothova et al., 2014; Iliev and Georgieva, 2018; Erkılıç, 2019).

Haptoglobin is synthesized by the liver and its function is to prevent iron loss by forming stable complexes with free hemoglobin in the blood (Sevgisunar and Şahinduran, 2014; Tuna and Ulutaş, 2015). As a result, Hp limits the availability of iron required for bacterial growth and has a bacteriostatic effect. Also Hp; shows anti-inflammatory properties by binding hemoglobin and integrins (main receptors on the cell wall of leukocytes) (Sevgisunar and Şahinduran, 2014). Serum Hp concentration**s** increase in dogs in cases of inflammation, trauma, and infection (Mcgrotty et al., 2003; Kırmızıgül et al., 2020). Cp is synthesized in the liver and extrahepatic tissues; it's a protein responsible for the transport of copper in the plasma and also protects the tissues from damage caused by free radicals containing iron. In addition, CP shows antioxidant and cell protective activity (Sevgisunar and Şahinduran, 2014; Erkılıç, 2019). In acute phase reactions; Alb, which is one of the negative acute phase proteins, is synthesized by the liver and its most important task is to keep the plasma oncotic pressure in balance (Tuna and Ulutaş, 2015; Kırmızıgül et al., 2020).

Hepatozoon canis is an apicomplexan parasite of the family Hepatozoidae and is transmitted by the brown dog ticks, *Rhipicephalus sanguineus* (*R. sangeineus*) (Baneth, 2011; Aktaş et al., 2015). *H. canis* causes long-term parasitemia clinically (Baneth, 2011). In cases of high parasitemia, symptoms such as fever, anorexia, weight loss, anemia, ocular discharge, and paralysis of the hind legs are occured (Baneth et al., 2001).

Canine distemper; it's a highly contagious, multisystemic, and often fatal viral disease that affects domestic and wild dogs as well as other terrestrial and aquatic carnivores (Gallina et al., 2011). Canine distemper

Cite this article as: Ayvazoğlu C, Kızıltepe A, Aydın A. 2023. The Effect of Different Diseases (*Hepatozoon canis*, Distemper and *Babesia canis canis*) on Serum Haptoglobin, Ceruloplasmin and Albumin Levels in Dogs. International Journal of Veterinary and Animal Research, 6(1): 17-22.

virus (CDV) can infect animals in all ages, but animals 0-6 months old are more susceptible (Çalışkan and Burgu, 2007).

Canine babesiosis is a protozoan disease that is transmitted to dogs by ticks, caused by protozoa of the Babesia species, and can cause fatal results (Boozer and Macintire, 2003; Kırmızıgül et al., 2020). Disease agents settle into the erythrocytes and cause the lysis of erythrocytes. Infection occurs in domestic dogs and wild carnivores and is common throughout the world (Gökçe et al., 2013; Sudhakara Reddy et al., 2016; Kırmızıgül et al., 2020). Canine Babesia species are classified into two groups as large and small, according to the morphology of their pyroplasmic forms (Vichova et al., 2016). Large Babesia species have been identified *B. c. canis, B. c. rossi* and *B. c. vogeli* (Uilenberg, 1989; Hauschild, 1995). In addition, an unnamed Babesia species that is closely related to *B. bigemina* has been described in North Carolina in the United States (Boozer and Macintire, 2003). Small Babesia species can infect canines, *B. gibsoni, B. conradae* and *B. microti-like spp.* (Zahler et al., 2000; Baneth et al., 2015). The clinical features of canine babesiosis often include hyperthermia, anemia, hemoglobinuria, lethargy, and anorexia (Bourdoiseau, 2006), but clinical signs in dogs may vary depending on the pathogen (Schetters et al., 1997) and host immunity (Brandão et al., 2003).

In this study; it was aimed to determine serum Hp, Cp and Alb concentrations in dogs with *H. canis*, Distemper and *B. c. canis*. In this way, it will be possible to present the changes in Hp, Cp and Alb concentrations in different diseases seen in dogs.

MATERIALS AND METHODS

Animals

The material of the study consisted of 45 dogs diagnosed with *H. canis* (n=15), *B. c. canis* (n=15), Canine Distemper (n=15) and 15 healthy dogs were brought to Iğdır University Tuzluca Animal Hospital. 8 mL of blood was collected from *V. cephalica* of sick and healthy animals into EDTA and serum tubes. Serum was used for serological and virological analysis, and whole blood sample was used for molecular analysis. For serological analysis, blood samples were centrifuged at 3000 x g for 10 minutes, and serums were separated and stored at -20ºC until Hp, Cp, and Alb concentrations were measured.

Genomic DNA isolation

DNA extractions were performed using the blood DNA extraction kit (Quick-DNATM Miniprep Kit, Zymo Research, USA) according to the manufacturer's instructions. The isolated gDNA was stored at -20ºC until analysis.

Diagnosis of dogs infected with H. canis by PCR

PCR was performed using *H. canis* specific primers HepF (5'-ATA CAT GAG CAA AAT CTC AAC-3') and HepR (5'-CTT ATT ATT CCA TGC TGC AG-3'), which amplify the 666 bp fragment of the 18S rRNA gene (Inokuma et al., 2002; Aktaş et al., 2015; Barati and Razmi, 2018; Akyüz et al., 2020). A 25 µL solution (8,5 µL nuclease-free water, 12,5 µL master mix (Mytaq, Bioline), forward (Hep F) and reverse (Hep R) primers (20 pmol/µL) 1 µL, and 2 µL template DNA) was used for PCR using a thermal cycler (Biometra, Analytik Jena, USA). Positive and negative control DNA samples were used for each reaction. PCR conditions; an initial 5 min denaturation at 95ºC; 34 repeated cycles of denaturation (95ºC for 30 s), annealing (57ºC for 30 s), and extension (72ºC for 90 s); followed by a 5 min extension at 72ºC (Barati and Razmi, 2018; Akyüz et al., 2020).

Diagnosis of dogs infected with B. c. canis by PCR

Confirmation of the species in the cases examined was performed by PCR using *B. c. canis* specific primers. The Bab1(5'-GTG AAC CTT ATC ACT TAA AGG-3') and Bab3 (5'-CTA CAC AGA GCA CAC AGC C-3') primers belong to 18S rRNA gene region (Duarte et al., 2008) which amplify the 746 bp region were used in PCR. A 25 µL solution (8,5 µL nuclease-free water, 12,5 µL master mix (Mytaq, Bioline), forward (Bab1) and reverse (Bab3) primers (20 pmol/µL) 1 µL, and 2 µL template DNA) was used for PCR using a thermal cycler (Biometra, Analytik Jena, USA). Positive and negative control DNA samples were used for each reaction. PCR conditions; an initial 2 min denaturation at 94ºC; 35 repeated cycles of denaturation (94ºC for 30 s), annealing (56°C for 30 s), and extension (72ºC for 45 s); followed by a 10 min extension at 72ºC (Duarte et al., 2008; Gokce et al., 2013).

Running out and Visualizing

PCR products were run out on a 1.5% agarose gel using 0.5X TAE and visualized by ethidium bromide (0.5 µg/mL) stain under ultraviolet light.

Diagnosing dogs with canine Distemper

A rapid diagnosis kit (ASAN Easy Test, Canine Distemper Virus Antigen Test, Cat. No: 022321, Korea, relative sensitivity: 97,96%) was used to identify dogs infected with distemper. The test kit was used in according to the manufacturer's recommendations. Eye conjunctival secretion and nasal discharge samples were taken from dogs suspected of distemper with a swab. The samples were mixed with the dilution solution until dissolved. 3-4 drops (approximately 100 µL) of the solution thoroughly mixed with the samples were dropped into the sample chamber of the disposable cassette. The rapid test kit result was evaluated within 5-10 minutes. In the area following the reservoir; those with control and test lines were evaluated as distemper positive, and those with only control lines were evaluated as distemper negative.

Serological analysis

Hp and Cp concentrations in serum samples were determined using the ELISA device (Thermo Scientific Multiscan GO, TYPE: 1510) and the commercial Elisa kit (BT LAB, China). Alb level was determined with a fully automatic analyzer device (Mindray BS-120).

Statistical analysis

SPSS 20 package program was used for statistical analysis of the obtained data. Shapiro-Wilk test ($n<50$) was used to test whether the data showed normal distribution and it was determined that it showed normal distribution ($P>0.05$). One-way Analysis of Variance (ANOVA), a parametric test, was used to determine whether there was a difference between the groups, and the Turkey test was used to determine the difference between the groups. All results were given as Mean±SE.

RESULTS

Haptoglobin, Cp and Alb concentrations in the serum of dogs infected *H. canis*, Distemper, *B. c. canis*, and healthy dogs are given in Table 1.

Table 1 Serum Hp, Cp and Alb Concentrations of *H. canis*, Distemper and *B. c. canis* in Dogs (n=15)

Parameters	Group	Mean±SE (Min-Max)	F/P
Hp (mg/mL)	Distemper	1.8787±0.005[a] (1.85-1.93)	F=2.381 P=0.079 P>0.05
	H. canis	1.8712±0.003[a] (1.84-1.89)	
	B. c. canis	1.8780±0.002[a] (1.86-1.90)	
	Healthy	1.8662±0.003[a] (1.85-1.90)	
Cp (mg/dL)	Distemper	7.3016±1.439[b] (1.47-16.06)	F=3.264 P=0.028 P<0.05
	H. canis	9.1746±1.504[b] (2.58-25.43)	
	B. c. canis	7.8456±2.092[b] (1.75-25.43)	
	Healthy	2.9745±0.343[a] (0.05-4.93)	
Alb (g/dL)	Distemper	2.9667±0.080[a] (2.50-3.50)	F=1.832 P=0.152 P>0.05
	H. canis	3.1067±0.073[a] (2.60-3.50)	
	B. c. canis	3.2467±0.129[a] (2.40-4.50)	
	Healthy	2.9600±0.108[a] (2.10-3.70)	

[a,b] The difference between the means shown with different letters in the same column is significant (P<0.05)

The Hp (mg/mL) concentration of canine distemper, *H. canis, B. c. canis* and healthy dogs were determined as 1.8787±0.005, 1.8712±0.003, 1.8780±0.002 and 1.8662±0.003, respectively (Table 1). However, there was no significant difference in Hp concentration between healthy dogs and sick dogs (P>0.05). Likewise, when the Alb (g/dL) concentration of sick and healthy dogs were examined, the averages were 2.9667±0.080, 3.1067±0.073, 3.2467±0.129 and 2.9600±0.108, respectively (Table 1). Nevertheless, there was no significant difference between healthy dogs and sick dogs in terms of Alb concentration (P>0.05).

In the analysis, the Cp (mg/dL) concentration of the sick dogs were 7.3016±1.439, 9.1746±1.504, 7.8456±2.092, respectively and no statistically significant difference was found between the serum Cp concentration of the dogs with canine distemper, *H. canis* and *B. c. canis*. On the other hand, serum Cp concentration of 2.9745±0.343 in healthy dogs was lower than that of sick dogs, and the difference was statistically significant (P<0.05).

DISCUSSION AND CONCLUSION

Acute phase proteins are used in the diagnosis, differential diagnosis, and prognosis of diseases as well as in determining the efficacy of treatment. The concentrations of APP's, which are not specific to the disease, but whose concentrations increase rapidly in cases of tissue destruction and inflammation, decrease with effective treatment. The concentration of the increase in plasma concentrations of APP's correlates with the size and activity of the inflammation. Therefore, the determination of the circulating concentrations of these proteins provides information about the ongoing inflammatory reaction. Profile, synthesis, secretion, and excretion of APP's differ between animal species (Murata et al., 2004; Cecilliani et al., 2012).

Hepatozoon canis; it's transmitted by *R. sanguineus* ticks and causes clinically long-term parasitemia. The disease is endemic in temperate climate zone, tropical and subtropical regions where vector ticks are active (Baneth, 2011). In studies conducted in different parts of the world, the prevalence of *H. canis* has been reported to vary between 7.5-52%. (Rojas et al., 2014; Maia et al.,2015; Piratae et al., 2015; Hamel et al., 2016). In a study, it was reported that the Hp concentration in dogs with *H. canis* was similar to the control group. In the same study, it was reported that the Cp concentration increased statistically significantly (Ulutaş et al., 2007). In another study, it was reported that the concentration of Alb decreased in dogs with *H. canis* (Paşa et al., 2009). In addition, increased Hp concentration have been reported in cats infected with *H. felis* (Vilhena et al., 2017). Moreover; according to the increase levels, positive AFPs are evaluated in 3 groups as those that increase approximately 50% (Cp and complement factor 3), those that increase 2-3 (Hp, fibrinogen, α-globulins with antiprotease activity and lipopolysaccharide binding protein) times and those that increase 5-1000 (CRP and SAA) times (Ulutaş et al., 2008). In the study, it was determined that there was no statistically significant change in Hp and Alb concentrations in dogs with *H. canis*, while the Cp concentration increased approximately 4 times. According to our results in study, although Hp and Cp levels are parallel to the literature data, there is a difference in Alb level. This situation; it can be said that the disease is caused by the severity, care and feeding differences. Because; the entire life cycle of *H. canis* is completed in a total of 81 days in ticks and dogs (Baneth et al., 2007). Moreover; it's possible to encounter the agent in the lung, heart, skeletal muscles, liver, spleen and lymph nodes in the schizogonic period, and low parasitemia is the most common form of infection in which less than 5% of neutrophils are infected (Baneth, 2011).

Canine distemper; it is a highly contagious, multisystemic and often fatal viral disease (Gallina et al., 2011). It has been reported that the prevalence of CDV, which is endemic globally, is up to 71% (Fischer et al., 2016; DiGandi et al., 2019). In a study, it was reported that there was a decrease in the concentration of Alb in dogs infected with CDV, but this situation was not statistically significant (Değirmençay et al., 2021). Kogika et al., (2003); in his study on CDV, he reported slight increases in Hp and Cp levels. In another study, it was reported that Hp and Cp concentrations increased and Alb concentration decreased in CDV-infected dogs (Kocatürk et al., 2011). Moreover, it has been reported that the increase in Cp level in dogs with canine distemper is an important prognostic indicator of the disease (Erogowda et al., 2020). In our study, it was determined that the Cp concentration increased (approximately 2,5 times) significantly parallel with the literature. This suggests that high Cp level protects against hepatic production and tissue damage (Schmidt et al., 2015).However, although there were very small numerical increases in Hp and Alb concentrations, no statistically significant change was observed. The numerical increase in Hp and Alb levels in sick dogs compared to healthy dogs suggests that this may be related to racial differences or nutrition. Moreover; clinical symptoms of CDV infections with an incubation period of 1-3 weeks; it depends on the host's immune system, the state of secondary bacterial infection, and particularly the strain of the virus (De Almeida, 2009; Ludlow et al., 2012).

Babesiosis, the prevalence of which varies between 2.2-67.8% in the world; It is a protozoan disease transmitted to dogs by ticks and can lead to fatal consequences (Boozer and Mcintyre, 2003; Watanabe et al., 2004; Rodriguez-Vivas et al., 2005; Davoust et al., 2006; Tsachev et al., 2006; Kırmızıgül et al., 2020). In a study, it was reported that Alb and Hp concentrations decreased in dogs with *B. c. canis* compared to healthy dogs, while Cp concentration increased (Erkılıç, 2019; Kırmızıgül et al., 2020). In our study, it was determined that there was no statistical change in Alb and Hp concentrations in dogs with *B. c. canis,* while the Cp concentration increased approximately 3 times. It was thought that the reason for the higher Cp concentration in sick dogs compared to healthy dogs was due to the inflammatory changes that occurred. In our study; Hp and Alb concentrations in sick dogs showed slight increases numerically compared to healthy dogs. This situation in Hp and Alb concentrations can be explained as being in the early stages of the disease. Because; it has been reported that Hp and Alb levels decrease as a result of hemolysis in advanced stages of babesiosis (Kırmızıgül et al., 2020).

As a result, with this study; Species-specific Hp, Cp and Alb levels, whose importance in veterinary medicine are increasing day by day, have been determined for diagnosis and differential diagnosis in different diseases (*H. canis*, distemper and *B. c. canis*) in dogs. It was determined that while Cp concentration increased in dogs with *H. canis,* distemper and *B. c. canis* compared to healthy ones, Hp and Alb concentrations did not change. It can be said that the Hp and Alb concentrations in these diseases are similar to those of healthy dogs, which is related to the severity of the disease. Higher Cp concentration in dogs with *H. canis*, distemper, and *B. c. canis* compared to healthy dogs; It is thought to be due to oxidative damage and inflammatory changes. However, it is thought that detailed studies are needed to determine the clinical importance of the prognosis and treatment efficacy of these diseases.

Conflict of Interest
The authors declare that they have no competing interests.

Authorship contributions
Concept: C.A., Design: C.A., Data Collection or Processing: C.A., Ş.K., N.A., Analysis or Interpretation: C.A., Literature Search: C.A., Writing: C.A., Ş.K., N.A.

Financial Support
This research received no grant from any funding agency/sector.

Ethical Approval
This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: 2022-090).

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