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For the Degree of Master of Veterinary Medicine

A Retrospective Epidemiological Study of the Prevalence of *Babesia* spp. and *Hepatozoon* spp. in Dogs in Jeju Island

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GRADUATE SCHOOL
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Abstract

A Retrospective Epidemiological Study of the Prevalence of *Babesia* spp. and *Hepatozoon* spp. in Dogs in Jeju Island

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Hepatozoon spp. and *Babesia spp.* are tick-borne protozoan parasites that infect a variety of animals, including domestic dogs. The objective of this study was the investigation of prevalence of tick-borne pathogens in dogs in Jeju island, South Korea, including *Hepatozoon spp.* and *Babesia spp.*.

Blood samples were collected from 346 dogs, and complete blood count (CBC) and blood smear analyses were performed. Primer sets that amplify partial sequences of the *Babesia* and *Hepatozoon* 18S rRNA gene were used for polymerase chain reaction (PCR) and the DNA sequences of the PCR products were determined. Of the 346 dogs, 66 (19.1%) were *Hepatozoon*-positive, 68 (19.7%) were *Babesia*-positive, and 32 (9.2%) were positive for coinfection. The analysis of the sequenced samples demonstrated >99% similarity with other 18s rRNA gene sequences of *Hepatozoon canis* and *Babesia gibsoni* in GenBank.

The data in this study provides the importance of understanding the high prevalence of *Hepatozoon* spp. and *Babesia* spp. infections of dogs in Jeju Island, which will assist in the management and treatment of these blood-borne parasites in dogs.

Keywords : Tick-borne diseases, *Babesia* spp., *Hepatozoon* spp., PCR, Dogs

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I. Introduction

Hepatozoon species are tick-borne protozoan parasites, classified in the phylum apicomplexa and they may cause hepatozoonosis when dogs eat infected ticks [1]. *H. canis* infects leukocytes and causes clinical signs such as anemia, emaciation, anorexia and intermittent fever [2]. The two species of *Hepatozoon*, *H. canis* and *H. americanum* are important in domestic dogs with distinct clinical, pathological, genetic, and antigenic aspects and vectors of transmission. The distribution of *H. americanum* has been reported only in North America. *H. canis* is more widely distributed in many countries of the Americas, Europe, Africa, and Asia [3, 4, 5].

Babesia species are tick-borne apicomplexan parasites infecting various animals, including dogs [6]. *B. canis canis, B. canis vogeli, B. canis rossi,* and *B. gibsoni* are canine piroplasms. *B. gibsoni* is prevalent in Europe, North America, Africa and Asia, including South Korea [7, 8, 9]. *Babesia* species cause clinical signs such as anorexia, fever, pale mucous membrane, dark brown urine, splenomegaly, and icterus [10].

Tick-borne canine blood parasites can cause severe infection in dogs and humans. Dogs may carry parasites that are transmissible to human, playing an important role as the reservoirs in the transmission cycles [11, 12].

Hepatozoonosis and babesiosis can be diagnosed by the observation of intracellular gametocytes or oocytes in Giemsa-stained peripheral blood smears [13, 14]. Recently, molecular techniques such as polymerase chain reaction (PCR) and sequence analysis have been widely used for the diagnosis of *Hepatozoon* spp. and *Babesia* spp. infections and specification of their isolates, respectively [15, 16, 17].

To date, there have been few reports of tick-borne pathogen infections in dogs in South Korea. The objective of this study was the investigation of prevalence of *Hepatozoon* spp. and *Babesia* spp. infections in dogs in Jeju Island. In this study, PCR methods were performed to evaluate the presense of tick-borne pathogens in 346 blood samples from dogs in Jeju Island.

II. Materials and Methods

1. Animals and ethical statement

A total of 346 domestic dogs that presented to the Jeju Veterinary Medicine Teaching Hospital for a health examination between February 2022 and October 2022 were included in this retrospective study. The breed, age, and gender were recorded. Permission for performing the blood collection and using the collected blood in the study was obtained from the owners.

An institutional animal ethics committee approval was not required to collect the blood samples, as all samples were collected health examinations and a retrospective study was conducted. However, the study was conducted in accordance with the principles of the World Medical Association Declaration of Helsinki's "Ethical Principles for Medical Research Involving Human Subjects."

Blood samples (2 mL) were collected by puncture of the cephalic vein or jugular vein, not providing for any segregation or stress of the animal. Blood sample was divided into 0.5-mL portions and stored in potassium ethylene diamine tetra-acetic acid (EDTA) tubes.

2. Hematological analysis

Within 2 hours of blood collection, Complete Blood Count (CBC) was performed using MEK 6420P (Nihon Kohden, Japan). The hematological parameters evaluated include hematocrit (HCT) and total white blood cell (WBC) count.

3. Microscopic examination

Blood smears were fixed in methanol and stained with Giemsa and Diff-quik solution. *Babesia* spp. and *Hepatozoon* spp. were observed using a CX-31 microscope (Olympus, Japan) with oil immersion at a final magnification of 1000×.

4. Chromatographic immunoassay

Canine Babesia Ab Test kit (Bionote, South Korea) was performed according to the manufacturer's instructions.

5. DNA extraction and Polymerase chain reaction (PCR)

1) DNA extraction

DNA was extracted from 300 μL of a whole blood sample using a G-DEX ™ IIb genomic DNA extraction kit (iNtRON Biotechnology, South Korea), according to the manufacturer's instructions. Concentrations of total DNA were determined by NanoVue Plus spectrophotometer (GE Healthcare, IL, USA). The eluted DNA was stored at -20°C before PCR was performed.

2) PCR

A single conventional PCR was used for the detection of the 18S rRNA genes of *Hepatozoon* spp. and *Babesia* spp., performed in a T100™ thermal cycler (Bio-Rad, CA, USA), total reaction volume of 20 μL containing 1 μL (~100 ng/μL) of DNA, 1 μL each primer, 10 μL AccuPower Taq PCR Master

Mix (Bioneer, Daejeon, South Korea), and 7 μL RNase-free water (DEPC-treated water) (Welgene Inc., South Korea).

Table 1 PCR amplification protocol

	Temperature		Time	number of cycle
	Hep18-S	Babe18-S		
initial denaturation	95℃	95℃	5 min	1
denaturation	95℃	95℃	30 sec	
annealing	50℃	58℃	30 sec	30
extension	72℃	72℃	30 sec	
final extension	72℃	72℃	5 min	1

For *Hepatozoon* spp. detection, primers Hep18S-F (5′-GGTAATTCTAG AGCTAATACATGAGC-3′) and Hep18S-R (5′-ACAATAAAGTAAAAAACA YTTCAAAG-3′) which amplify a fragment of the 18S ribosomal RNA (rRNA) gene of *Hepatozoon* spp. were used, following conditions described by Almeida *et al.* (2012) [18]. The amplification protocol was 95 °C for 5 min followed by 35 cycles at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s, and final extension step of 72°C for 5 min was also used (Table 1).

For the *Babesia* spp. detection, a fragment of the 18S rRNA gene was amplified by PCR, using the primers Babe18S-F (5′-CCGTGCTAATTGTAG GGCTAATACA-3′) and Babe18S-R (5′-GCTTGAAACACTCTARTTTTCTC AAAG-3′), following conditions described by Almeida *et al.* (2012). The amplification protocol was 95°C for 5 min followed by 35 cycles at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. A final extension step of 72°C for 5 min was also used (Table 1). DNA amplicons were electrophoresed using a QIAxcel Advanced System (Qiagen, Hilden, Germany).

6. Sequencing

Among all positive amplicons, two *Babesia*-positive samples and two *Hepatozoon*-positive samples with high DNA concentrations selected and submitted for sequencing. For purification, PCR products were electrophoresed on 1% agarose gels stained with ethidium bromide and observed under UV illumination. Amplicons were purified using NucleoSpin Gel and PCR Clean up (Machery-Nagel, Düren, Germany). The purified 18S rRNA gene product was sent to sequencing company (Solgent, South Korea), determined in both directions using the same primers individually as for the PCR. Partial sequences obtained were submitted to BLAST analysis (Altschul *et al.*, 1990) to determine the closest similarities in GenBank.

III. Results

Of the 346 total dogs that visited Jeju Veterinary Medicine Teaching Hospital for health checkups and were included in this retrospective study, 143 (41.3%) were male, and 203 (58.7%) were female. One hundred eighty-two of the 346 dogs (52.6%) were younger than 1 year, 156 (45.1%) were aged 1 - 7 years, and 11 (3.2%) were older than 8 years. One hundred and ninety-nine dogs (57.5%) were crossbreeds, and 147 (42.5%) were purebred.

Through blood smear samples, both *Hepatozoon* spp. and *Babesia* spp. were observed by microscope. *Hepatozoon* spp. was observable with both diff quick staining and Giemsa staining, but *Babesia* spp. could not be observed under a microscope with diff-quick staining and could be identified only when Giemsa staining was performed.

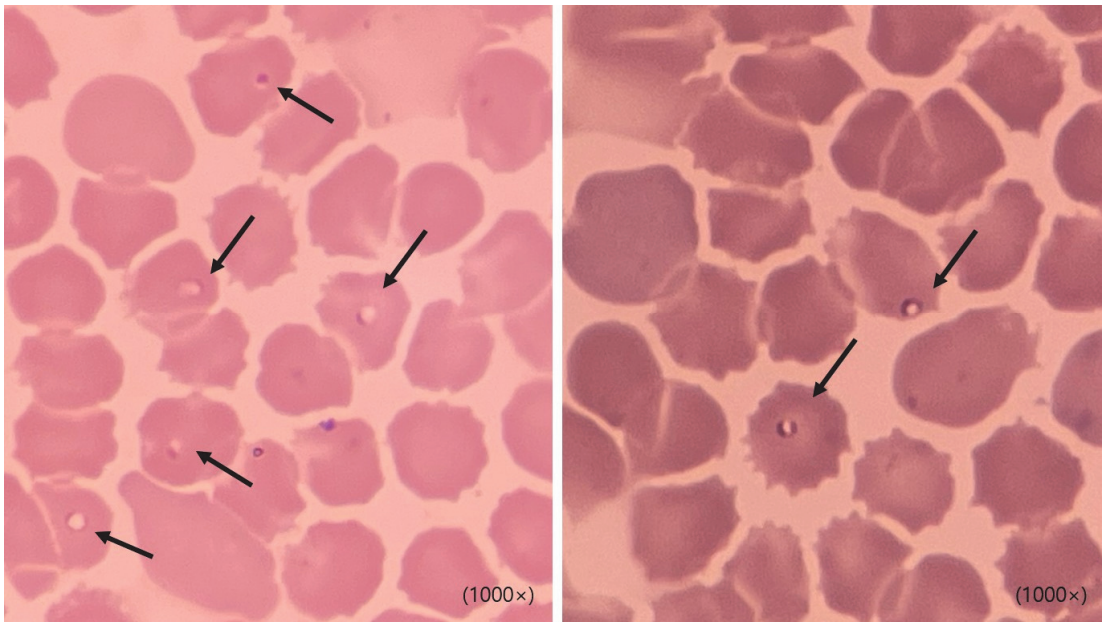

Figure 1. Giemsa stained peripheral blood smear, *Babesia* spp. observed. Slides were examined with CX-31 (Olympus, Japan) at 1000× magnification.

Ring-shaped *Babesia* spp. trophozoite in erythrocyte was identified by Giemsa-stained blood smear slides (Figure 1). Diff-quik-stained or Giemsa-stained peripheral blood smear showed an ellipsoidal-shaped gamont of *Hepatozoon* spp. in the leukocyte cytoplasm (Figure 2 and Figure 3).

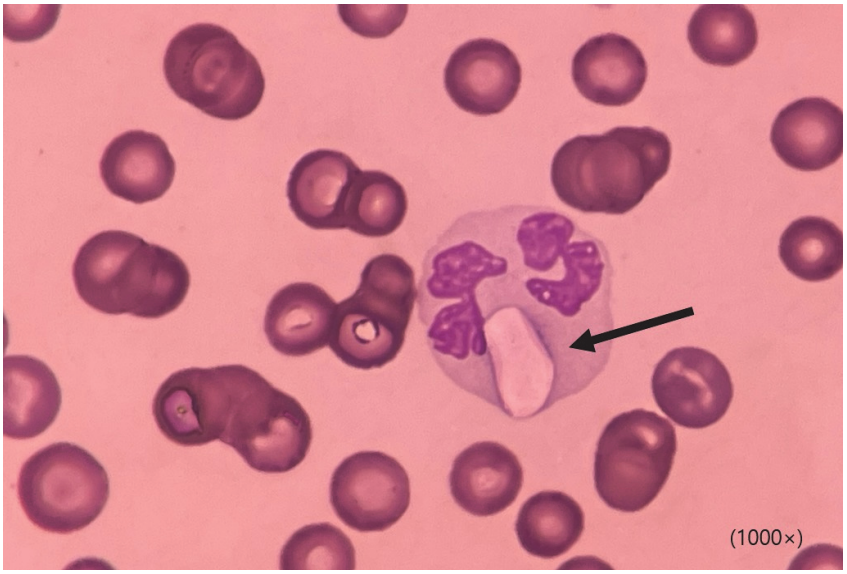

Figure 2. Diff quik stained peripheral blood smear shows an ellipsoidal-shaped gamont of *Hepatozoon* spp. in the leukocyte cytoplasm. The slide was examined with CX-31 (Olympus, Japan) at 1000× magnification.

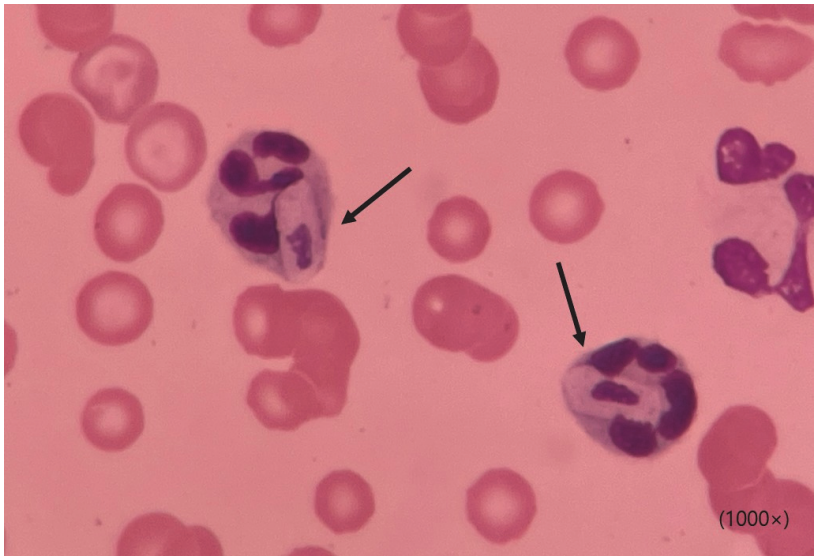

Figure 3. Giemsa stained peripheral blood smear. A *Hepatozoon spp.* gamont is observed. The slide was examined with CX-31 (Olympus, Japan) at 1000× magnification.

With PCR, 19.1% (66/346) of dogs were positive for *Hepatozoon* spp., 19.7% (68/346) were positive for *Babesia* spp., and 9.2% (32/346) were positive for both. 70.4% (244/346) were negative for both *Hepatozoon* spp. and *Babesia* spp. The positivity rates for *Hepatozoon* spp. and *Babesia* spp. according to month, gender, age, and breed are listed in Table 2 and Table 3.

Table 2 *Hepatozoon* spp. and *Babesia* spp. infection frequencies by month

Month	n	*Hepatozoon* spp. PCR positive No(%)	*Babesia* spp. PCR positive No(%)	Co-infection No(%)
February	20	8(40%)	6(30%)	6(30%)
March	42	7(16.7%)	5(11.9%)	4(9.5%)
April	29	2(6.9%)	4(13.8%)	2(6.9%)
May	29	6(20.7%)	4(13.8%)	2(6.9%)
June	29	8(27.6%)	2(6.9%)	2(6.9%)
July	23	5(21.7%)	6(26.1%)	1(4.3%)
August	24	3(12.5%)	2(8.3%)	0
September	103	21(20.4%)	26(25.2%)	11(10.7%)
October	47	6(12.8%)	13(27.7%)	4(8.5%)

Table 3 *Hepatozoon* spp. and *Babesia* spp. positivity rates by PCR in relation to gender, age, and breed

Risk factors		n	*Hepatozoon* spp. PCR positive No(%)	*Babesia* spp. PCR positive No(%)	Co-infection No(%)
Gender	Male	143	28(19.6%)	32(22.4%)	15(10.5%)
	Female	203	38(18.3%)	36(17.7%)	17(8.4%)
Age	<1 year	182	41(22.5%)	51(28.0%)	26(14.3%)
	1-7 year	156	23(14.7%)	15(9.6%)	5(3.2%)
	≥8 year	11	2(18.2%)	2(18.2%)	1(9.1%)
Breed	Mixed	199	48(24.1)	54(27.1%)	26(13.1%)
	Jindo	31	6(19.4%)	8(25.8%)	4(12.9%)
	Maltese	20	5(25%)	1(5%)	1(5%)
	Poodle	13	1(7.7%)	1(7.7%)	0
	Pointer	12	1(8.3%)	1(8.3%)	0
	Labrador retriever	12	1(8.3%)	0	0
	Border collie	10	1(10%)	0	0
	Golden retriever	5	0	2(40%)	0
	Others	44	3(6.8%)	1(2.3%)	1(2.3%)

Comparisons of the *Babesia* spp. immunoassay results and PCR results are shown in Table 4. The false positive rate was 10.9%, and the false negative rate was 6.5%.

Table 4 Comparisons of *Babesia* spp. immunoassay results and PCR results

	Immunoassay positive	Immunoassay negative
PCR positive	49	19
PCR negative	6	291

Among the complete blood cell count results, the relationship between HCT and *Hepatozoon* spp. and *Babesia* spp. Infections was investigated (Table 5). There were *Babesia* single infected or coinfected dogs who had 26 or less HCT value. However, there were no dogs with HCT of less than 26 when infected with *Hepatozoon* alone in this study.

Table 5 Relationship between the hematocrit (HCT) of 346 dogs and presence of *Hepatozoon* spp. and *Babesia* spp. infections

		Non-infection No(%)	*Hepatozoon* spp. PCR positive No(%)	*Babesia* spp. PCR positive No(%)	Co-infection No(%)
HCT	Min -Max	26.2-84.6	34.3-76.9	27.1-70.7	11.1-84.9
	<26	0	0	8(22.2%)	5(15.6%)
	26-37	65(26.6%)	9(26.5%)	16(44.4%)	15(46.9%)
	>37	179(73.4%)	25(73.5%)	12(33.3%)	12(37.5%)

The correlation between WBC and *Hepatozoon* spp. and *Babesia* spp. infections was also investigated (Table 6).

Table 6 Relationship between the white blood cell (WBC) count of 346 dogs and presence of *Hepatozoon* spp. and *Babesia* spp. infections in 346 dogs

		Non-infection No(%)	*Hepatozoon* spp. PCR positiveNo(%)	*Babesia* spp. PCR positiveNo(%)	Co-infection No(%)
WBC	Min-Max	3.2-59.1	4-33	2.3-21.3	3.6-29.8
	<6	15(6.1%)	3(8.8%)	3(8.3%)	2(6.7%)
	6-17	169(69.3%)	23(67.6%)	26(72.2%)	17(56.7%)
	>17	60(24.6%)	8(23.5%)	7(19.4%)	11(36.7%)

Two *Babesia* spp.-positive samples on PCR and two *Hepatozoon* spp.-positive samples on PCR were each requested for sequencing and were confirmed by 18S rRNA gene sequence comparisons in Genbank. *Babesia* spp.-positive samples on PCR were both confirmed that a similarity of more than 99% with *B. gibsoni* (GenBank accesion MN134517.1, MN134516.1, MN134509.1, MN134508.1, MN134504.1, MG604347.1, KY524483.1, KP666167.1, LC012793.1, LC012792.1, KJ142323.1, KF878945.1). The score was 918 bits (497), identities were 501/503 (99%), and gaps was 1/503 (0%).

In the case of two *Hepatozoon* spp.-positive samples on PCR, both had a similarity of more than 99% with *H. canis* (GenBank accession MN393910.1. MK091087.1, LC331052.1, MG758124.1, MG062866.1, KX712127.1, KX712126.1, KU893127.1, KU893126.1, KX776324.1, KX776323.1, MW295531.1). The score was 616 bits (333) identities were 333/333 (100%), and gaps was 0/333 (0%).

IV. Discussion

In South Korea, reports and surveys on hepatozoonozis have been started with a first PCR-based detection in 2008 [19], and the first case report was published in 2017 [20]. Genetic characterization revealed that isolates of *Hepatozoon* spp. in South Korea are closely related to *H. canis.*, and as the sequencing results in this survey matched with the results of previous studies, the *Hepatozoon* spp. prevalent in South Korea was thought to be *H. canis,* not *H. americanum.* PCR-based detection and survey of *B. gibsoni* infection in South Korea has been reported [21, 22]. To date, the sequencing results in this survey are consistent with the results of previous studies.

An immunoassay kit, the canine babesia antibody test kit (Bionote, South Korea), and a PCR test were performed together. The sensitivity of the immunoassay kit was 72.1%, and the specificity was 98.0%. In the case of blood smear tests, although accurate values were not obtained, parasites were often not seen in blood smears of PCR-positive individuals. Karagenc *et al.* (2006) compared the sensibility of different techniques in the diagnosis of *Hepatozoon* spp. The parasitological exam by the technique of blood smears detected 10.6% of dogs infected by *H. canis*, whereas the technique of PCR detected 25.8% [5]. Rucksaken *et al.* (2019) also compared the sensitivity of blood smear test. There was one individual who was *Babesia*-positive on PCR, but it was not confirmed on a blood smear. In the case of hepatozoonosis, there were two PCR-positive individuals, but only one was confirmed on a blood smear [23]. Although a blood smear can be used as a simple blood parasite diagnostic technique, since the sensitivity is very low, it is recommended to use the PCR diagnostic technique.

In this study, sensitive and specific primers were used for *Babesia* and *Hepatozoon*, respectively, and sequencing confirmed that *Hepatozoon* and *Babesia* isolates. In present studies, a single PCR was successfully used to detect *Babesia* and *Hepatozoon* simultaneously in canine blood samples. This provided an easy screening method for the detection and accurate information about the infectious agents in combination with subsequent sequencing analysis. It is theoretically possible that a dog may be infected with more than one subspecies of *Babesia* or *Hepatozoon* at the same time. In such a case, the results of subsequent sequence analysis would be more difficult to interpret, because the results of the direct sequencing of the PCR products could not be read accurately. Although there were no difficulties in determining the subspecies of *Babesia* or the species of *Hepatozoon* in the sequencing analysis in the present study, a species-specific PCR for *Hepatozoon* and a subspecies-specific PCR for *Babesia* would be required to evaluate the infection rate with better accuracy in those cases.

Compared with those of other breeds, the *Hepatozoon* spp. and *Babesia* spp. infection rates of mixed dogs were relatively high. In another study, it was reported that the *Hepatozoon* infection rate in pet dogs (10.4%; 95% CI: 6.2 - 16.2) was significantly lower than that in shelter dogs (25.7%; 95% CI: 20.7 - 31.1) and that in stray dogs (26.3%; 95% CI: 20.9 - 32.3) [25]. Indoor dogs are more frequently treated with insecticides to eliminate ticks. It is thought that mixed dogs in Jeju Island are mainly raised in the yard, and outdoor activities are frequent. In addition, although information was not obtained in this survey, ectoparasite prevention is also thought to be related to the infection rate. Moreover, many factors, such as the susceptibility of the host, infectability of the parasite, and environment, are responsible for the succession of tick-borne pathogen infections [26].

There were *Babesia*-single infected or coinfected dogs who had 26 or less HCT value. However, there were no dogs with an HCT value of less than 26 when infected with *Hepatozoon* alone in this study. Pathogenesis of *H. canis* is thought to be weak. *H. canis* infection may range from an asymptomatic state to severe clinical symptoms of fever, lethargy, anemia, and emaciation [27]. According to a previous study, it is difficult to show clinical symptoms by the single infection with *H. canis*, and PCR should be performed in dogs with clinical symptoms because of the possibility of coinfection with various tick-borne pathogens [5]. In this survey, infection with *Hepatozoon* spp. alone does not seem to cause severe anemia, which is consistent with various previous studies. Therefore, it is reasonable to conduct *Hepatozoon* spp. PCR test together with other vector borne pathogens such as *Babesia* spp. and *Anaplasma* spp.

Because the elimination of gametocytes of *Hepatozoon* spp. from the peripheral blood is slow, an 8-week treatment is always required [1]. Atovaquone in combination with azithromycin for 10 days was the first described treatment combination to eliminate *B. gibsoni* in the majority of dogs with no adverse reactions [28]. The treatment period of *Hepatozoon* is longer than that of *Babesia*. In the case of individuals coinfected with *Babesia* and *Hepatozoon*, when only *Babesia* infection has been confirmed, long-term treatment may not be available as needed. Therefore, despite the low pathogenesis of *Hepatozoon*, it is thought to be necessary to confirm the presence of infection.

Hepatozoonosis is one of the other tick-borne diseases and poses a great risk to domestic and wild canines worldwide. Therefore, epidemiological investigations are essential to explain regional and regional infection trends for specific pathogens and their vectors. Knowledge of vector-borne infections at the local level allows veterinarians to recognize pathogens that may affect patients, facilitating a rapid diagnosis and treatment [29]. Therefore, this molecular survey is significant in that it confirmed the prevalence of canine hepatozoonosis and babesiosis in South Korea. Veterinarians may need to perform ectoparasite treatment in dogs residing in South Korea that present with fever, anemia, and possible exposure to infectious ticks, bearing in mind the possibility of *Hepatozoon* and *Babesia* infections.

V. Conclusion

In conclusion, the present study determined the prevalence of *Hepatozoon spp.* and *Babesia spp.* infections among dogs in Jeju Island. The data in this study provide the importance of understanding the high prevalence of *Hepatozoon* and *Babesia* spp. infections in dogs in Jeju island, which will assist in the management and treatment of these blood-borne parasites.

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국문초록

제주도 개에서 바베시아와 헤파토준 유병률에 대한 후향적 역학 연구

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Hepatozoon spp. 및 *Babesia spp.*는 개를 포함하여 다양한 동물을 감염시키는 진드기 매개 원생동물 기생충이다. 본 연구의 목적은 *Hepatozoon spp., Babesia spp.*를 포함한 제주도 개에서 진드기 매개 병원체의 유병률을 조사하는 것이다. 개 346마리로부터 혈액 샘플을 채취하여 CBC(Complete Blood Count) 분석 및 혈액 도말 검사를 실시한 후, Hep-18S, Babe-18S 프라이머를 사용하여 *Hepatozoon spp.*와 *Babesia spp,* 종합효소연쇄반응검사를 진행하였다. 346마리의 개 중 66마리(19.1%)는 헤파토준 양성, 68마리(19.7%)는 바베시아 양성, 32마리(9.2%)는 중복 감염 되었다. 시퀀싱된 샘플의 분석은 기존에 연구되어 Genbank에 저장된 Hepatozoon canis 및 Babesia gibsoni의 18s rRNA 유전자 시퀀스와 99% 이상의 유사성을 가진 것으로 확인되었다. 이번 감염 조사는 수의사가 제주도에서의 헤파토준, 바베시아의 높은 유병률을 인지하고, 혈액 내 기생충을 예방, 치료하는 데에 도움이 될 것으로 기대된다.

주제어 : 진드기매개질병, 바베시아, 헤파토준, PCR, 개