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# Molecular Study of Blood Parasite Infection in Dogs Referred to Veterinary Hospitals in Tehran


## Abstract

Dogs are sensitive to numerous diseases, nutritional problems, and internal and external toxins and parasites. Parasitic infections in dogs are typically common and make life problematic for this animal. This research aimed to investigate blood parasites in dogs referred to veterinary hospitals in Tehran. One hundred blood samples were collected from 10 veterinary hospitals in Tehran (10 samples from each hospital) from dogs referred to them with the dog owners' permission and with the hospital authorities' cooperation. After microscopic examination (for the separation of samples with blood parasites from samples without blood parasites), positive samples (with blood parasites) were sent to the laboratory for molecular analysis to recognize the type of blood parasite. Out of all the samples collected, 15 were microscopically positive (1 to 15).

The results of the microscopic examination of the samples revealed that samples No. 4-6-7-9-10-12-14-15 were infected with Hepatozoon and samples No. 1-3-5-11 with Babesia, and samples No. 2-8-13 with Ehrlichia. Fifteen samples were also suffering from anemia, and no contamination with blood parasites was observed by microscopic examination.


**Keywords:** *Blood parasite, Hepatozoon, Babesia, Ehrlichia, Dog*


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## Introduction

Dogs are sensitive to numerous diseases, nutritional issues, internal and external toxins, and parasites. Typically, parasitic infections are common in dogs and make life challenging for this animal. Some chief internal parasites, including worms (cestodes and nematodes) and protozoa, can cause anemia in dogs. Notable blood parasites in dogs include microfilaria and adult worms of Dirofilaria immitis (dog heartworm) and microfilaria of Dipetalonema reconditum from the group of nematodes, Babesia, Trypanosoma, Hepatozoon, and Leishmania from the group of protozoa, Ehrlichia from the group of Rickettsia and Haemobartonella from the group of mycoplasmas. The mature form of Diptalonema reconditum is not pathogenic, but the rest of these factors cause diseases in dogs. Babesia, Haemobartonella, Hepatozoon, Leishmania, and Ehrlichia are intracellular parasites. Babesia and Haemobartonella are parasites inside red blood cells that cause compensable anemia by directly destroying red blood cells. Hepatozoon, Leishmania, and Ehrlichia are also parasites inside white blood cells, and they attack the cells of some other tissues, such as the liver and lymph nodes. The intermediate hosts of these parasites are arthropods, among which mosquitoes and ticks play a more significant role. Due to the fact that these intermediate hosts are found more in the free environment, the abundance of these parasites is higher in the herd and stray dogs than in domestic dogs. Likewise, regarding the care and observance of animal health and the fact that they are more in the home environment, domestic dogs are less likely to be bitten by arthropods, and thus they are less affected by internal and external parasites.

Hamidi et al. (1982) reported the prevalence of visceral leishmaniasis in domestic and stray dogs in northern Iran to be 64.17 (1). Mazloumi et al. (2008) reported that the seroprevalence of canine visceral leishmaniasis in the Meshkin Shahr area was 4.4 (3). Farzam et al. (2008) reported the seroprevalence of visceral leishmaniasis in dogs of Sarab city as 9% (5). Fakhkhar et al. (2010) reported seroprevalence as 5.5% (6). Fallah et al. (2010) reported the prevalence of visceral leishmaniasis in domestic dogs in the Sarab district as 1.79 (7). Mahmudovand et al. (2011) found the prevalence of visceral leishmaniasis in domestic dogs to be 23% in the Baft district of Kerman province (8). Haddadzadeh et al. (2012) reported seroprevalence in domestic and stray dogs as 6.3% (9). Barati et al. (2013) reported the seroprevalence of visceral leishmaniasis in Meshkin city as 4.23 (10), Bamrot et al. (2014) reported 25.11% seroprevalence of visceral leishmaniasis in Kerman city (11). Qarakhani et al. (2014) reported the seroprevalence of visceral leishmaniasis in Hamedan province as 95.3 (12).

Pozio et al. (1981) reported the prevalence of visceral leishmaniasis in Italy by indirect immunofluorescence as 9.9% (13), Mancianti et al. reported the prevalence of visceral leishmaniasis by indirect immunofluorescence method as 48.14% (15), Dantas Torres et al. (2006) reported prevalence of leishmaniasis in domestic dogs in Brazil by indirect immunofluorescence serology method as 40.3% (16). Atasui et al. (2010) reported seroprevalence of visceral leishmaniasis in Turkey as 9-76 (17).

Vehini et al. (2008) studied 150 dogs and reported that 36 were infected with cephalus sanguineus ticks (18). Gabrieli et al. (2010) isolated 290 ticks from 119 studied foxes, most of

which were rhipicephalus sanguineus (19). O Yumada et al. (2005) reported 61 cases of tick infection out of 78 studied dogs, and 44 of these 61 tick species (72.1%) were rhipicephalus sanguineus (20). Paya et al. (2008) investigated ten dogs suffering from Hepatozoon canis, and out of these ten dogs, 7 had rhipicephalus sanguineus (21).

Among parasites, Leishmania is of distinctive importance. This parasite is common between dogs and humans and causes the dangerous disease visceral leishmaniasis (VL) or kala-azar and cutaneous leishmaniasis. Similarly, dogs can act as disease reservoirs and spread the disease agent in human societies. Consequently, a timely and precise diagnosis of this disease in dogs can be very valuable in avoiding its occurrence in humans. There are diverse methods for identifying dog blood parasites. The most accessible method is the direct observation of the blood-stained spread by the Giemsa method by an optical microscope.

Regarding the negative effect of these parasites on the normal life of dogs, causing chronic disease and gradual loss of the animal's natural conditions, there is a need for rapid and timely diagnosis. Serological and PCR methods are available for diagnosing some parasites with good sensitivity and specificity. Regarding the stated requirements, the current research aims to study the molecular level of blood parasites in dogs referred to veterinary hospitals in Tehran.

**Materials and Methods**

**1. Sample collection**

One hundred blood samples were collected from 10 veterinary hospitals in Tehran (10 from each hospital). The dogs were referred to them with the permission of the dog owners and with the cooperation of the hospital authorities, and after the microscopic examination (to isolate the samples with blood parasites from samples without blood parasites); positive samples (with blood parasites) were sent to the laboratory for molecular analysis to identify the type of blood parasite. Two ccs of blood were taken from the hand's cephalic vein from each dog using a sterile syringe and stored in tubes containing EDTA anticoagulant.

**2. Parasitological diagnosis method**

Parasites in the blood are detected by testing the stained peripheral blood smears. The blood smears should be prepared immediately and dried in the air. To maintain the blood smear quality, before staining them with absolute ethanol alcohol, the researchers fix 10% formalin or 70% ethanol (22). To identify the stained slides, the researchers observed them under 100x magnification. Then, to see the details of the parasite, the researchers changed the magnification to high dry magnification (450x) or (1000x) magnification with immersion oil.

**For the coverglass method**, the researchers put a drop of blood on a coverglass and put another coverglass on it. After spreading the blood, the researchers separate the two coverglasses. This method causes more blood cells to spread.

To stain blood smears, the researchers put the dried blood smear in absolute methanol for about 10 minutes, took out the sample, and dried it in the vicinity of the air. The researchers stained the dried slide with Giemsa dye for 20 to 60 minutes. The researchers washed the stained slide in distilled water to remove the excess dye and let the stained spread dry.

To concentrate whole blood, the researchers transferred one milliliter of fresh blood into a 15-milliliter graduated centrifuge tube. The researchers added a milliliter of 3% acetic acid and mix well. The researchers centrifuged it at 1500 rpm for 5 minutes. The researchers discarded the supernatant. The researchers made the sediment into suspension and measured its volume. The researchers put a measured amount of the suspension on the slide, placed a coverglass, and tested it with low magnification (x40).

For PCR analysis, the samples in which the blood parasite test was positive in the parasitology laboratory were transferred to a particular container. The pieces were kept in a freezer at -20 degrees Celsius until DNA isolation.

**3. Method of molecular testing of positive samples**

First, for DNA isolation, whole blood samples containing anticoagulants were taken out of the freezer, and after melting, they were placed in the sample racks. We used a commercial kit (ViralGene-spin Viral DNA / RNA Extraction Kit) for DNA isolation (VetTek, South Korea).

To check the contamination with blood parasites, pipetting was done after each DNA sample was melted. Then the rapid PCR kit (VeTeK EHR Detection Kit, made in South Korea) was used. After each DNA sample was melted, pipetting was done, then two microliters of the DNA sample were added to the PCR premix tube. Then 18 microliters of DNase/RNas-free water was added to each tube.

The desired bands embedded in the negative control (sample without DNA) were examined, and the samples with the bands in Table (1) were considered positive.

**Table 1:** Control bands of blood parasites

| Blood parasite | Desired band |
|---|---|
| Dirofilaria immitis | 432bp |
| Leishmania infantum | 720bp |
| Hepatozoon canis | 356bp |
| Babesia canis | 235bp |
| Ehrlichia canis | 336bp |

**Findings**

In this research, done on 100 blood samples from 100 dogs from Tehran veterinary hospitals to examine blood parasites, fifteen samples were microscopically positive (numbers 1 to 15). The results of the microscopic examination of the samples

revealed that samples No. 4-6-7-9-10-12-14-15 belong to Hepatozoon and samples No. 1-3-5-11 belong to Babesia, and samples No. 2- 8-13 were infected with Ehrlichia. Fifteen samples were also suffering from anemia, and no contamination with blood parasites was observed by microscopic examination. In Chart (1), the contamination with blood parasites is shown by the type of parasite (microscopic examination).

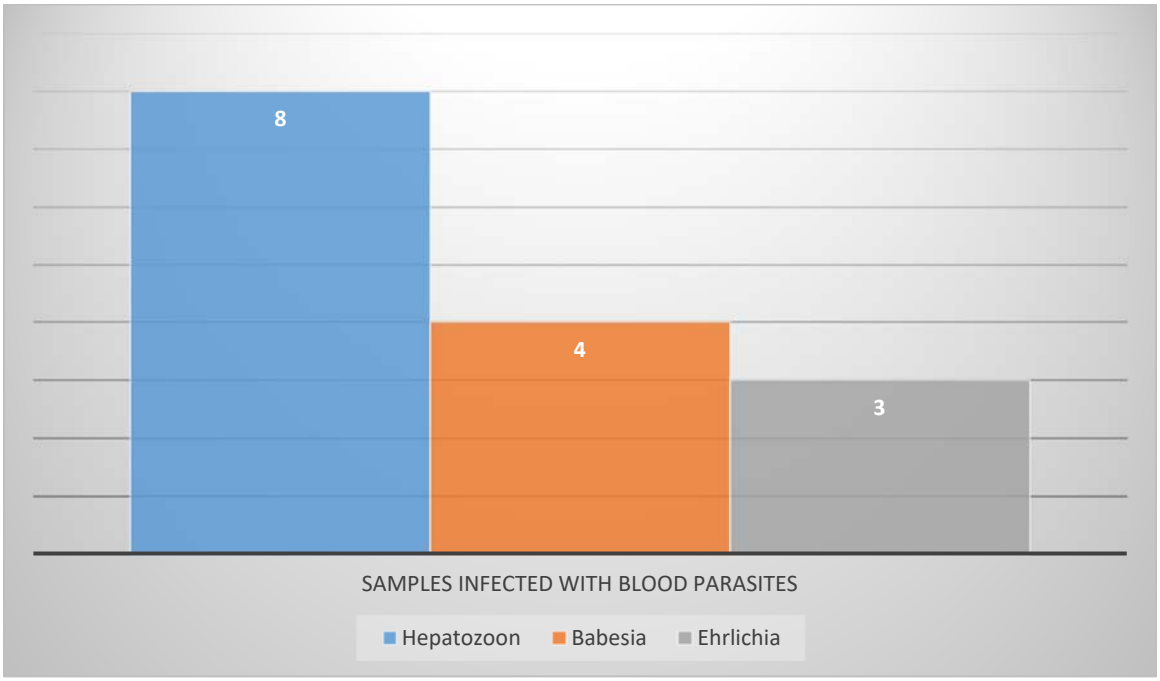


**Chart 1:** Infection with blood parasites by type of parasite (results from microscopic examination)

The positive samples were sent to the laboratory through microscopic examination, and a PCR test found that none of the samples were infected with blood parasites.

**Table 2:** Sequencing table to check the sent samples

| SinaClon Primer Synthesis Order Form | | | | | | |
|---|---|---|---|---|---|---|
| Name: | mehrnoosh naderi | | | | | |
| Email: | www.mehrnoosh.naderi73@gmail.com | | | | | |
| Tel: | 9120518860 | | | | | |
| | | Oligo sequences 5'→3' | | | | |
| Number | Primer Name | Sequence 5'→ 3' (10-50 bp)* | Length | Inosien | OD | Purification |
| | | DO NOT USE ANY SPACE AND CHARACTERS BETWEEN BASES. | | | | |
| 1 | Hepatozoon 18 s-F | GGTAATTCTAGAGCTAATACATGAGC | 26 | 0 | 2-4 | Choose |
| 2 | Hepatozoon 18 s-R | ACAATAAAGTAAAAAACAYTTCAAAG | 26 | 0 | 2-4 | Choose |
| 3 | Babesia 18s-F | CCGTGCTAATTGTAGGGCTAATACA | 25 | 0 | 2-4 | Choose |
| 4 | Babesia 18s-R | GCTTGAAACACTCTARTTTTCTCAAAG | 27 | 0 | 2-4 | Choose |
| 5 | Ehrlichia 16s-F | TCGCTATTAGATGAGCCTACGT | 22 | 0 | 2-4 | Choose |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6 | Ehrlichia 16s-R | GAGTCTGGACCGTATCTCAGT | 21 | 0 | 2-4 | Choose |

Below is the image obtained from the PCR of the samples sent to the laboratory.

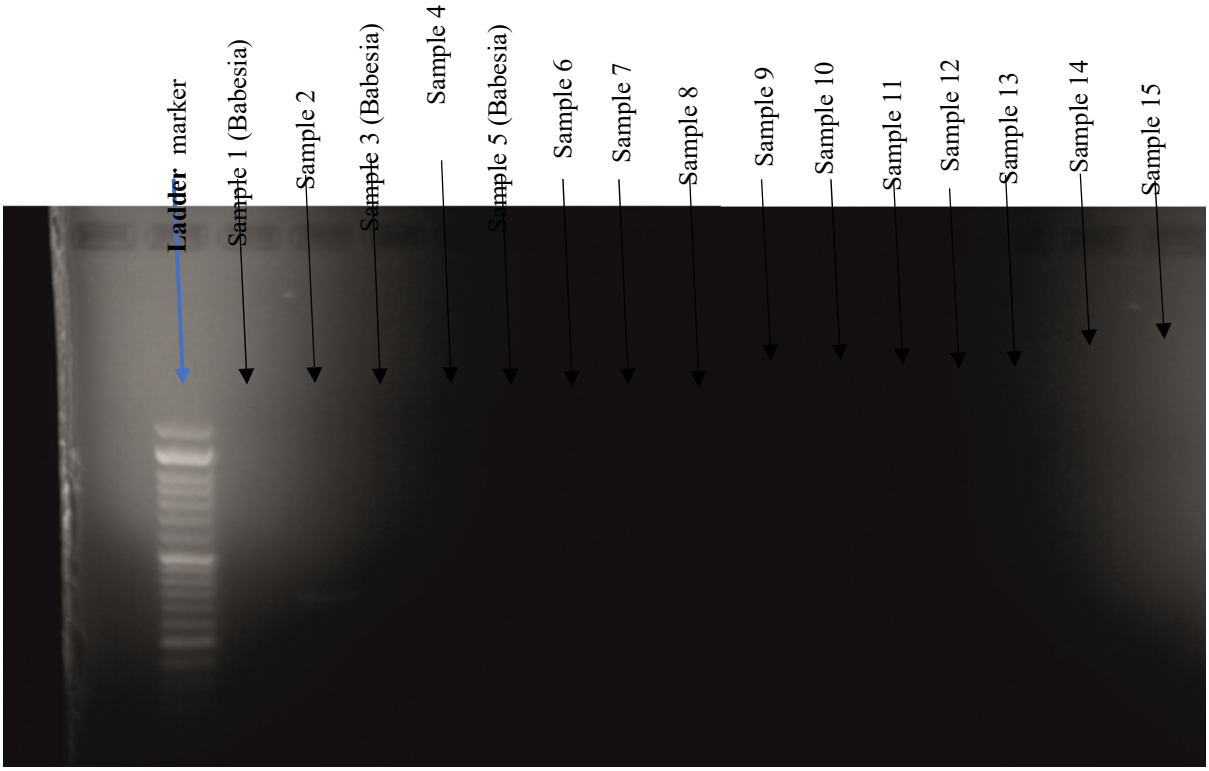


**Figure 1**: PCR result of the samples

All the samples sent to the laboratory were negative by PCR test.

**Discussion**

The results revealed that none of the 15 anemic samples were infected with blood parasites through microscopic examination. Via scrutinizing the samples through microscopic observation, out of 15 contaminated samples, eight samples (53.4%) were suspected of Hepatozoon, four samples (26.6%) were suspected of Babesia, and three samples (20%) were suspected of Ehrlichia. These 15 samples, suspected to be infected by microscopic examination, were sent to the laboratory for PCR. By conducting PCR, none of the samples were found positive concerning contamination with blood parasites. The noteworthy point is that we did not have access to positive control in the present study. There may be an error in PCR without a positive control, but this possibility seems unlikely considering the primer and the high reproducibility achieved.

Jafari et al. (1997), when studying 180 dogs in Shiraz, reported infection of 17 dogs (44.9%) with E. canis (23). In Brazil, they showed that heartworm infection in male dogs is 7.89%, much higher than in females 30.10%, which can be attributed to the effect of sex hormones on infection. In 1990, it was shown that one of the effective factors in the epidemiology of Dirofilaria immitis is the place where the dog is kept, and the rate of infection in dogs that live outside the doghouse is twice as much as those that are kept indoors.

Vehini et al. (2008) reported 36 dogs infected with cephalus sanguineus ticks from 150 studied dogs (18). Gabrieli et al. (2010) isolated 290 ticks from 119 studied foxes, most of which were Rhipicephalus sanguineus (19). O Yumada et al. (2005) reported 61 cases of tick infection out of 78 studied dogs, of which 44 tick species (721%) were Rhipicephalus sanguineus (21). Paya et al. (2008) examined ten dogs suffering from Hepatozoon canis, and out of these ten dogs, 7 had Rhipicephalus sanguineus (20).

In another study conducted by Akhtar Danst et al. (2009) in Kerman city (south of Iran), the prevalence of E. Canlis in the population of 124 dog collars was positive in 63.14 percent (17 positive dogs) referred to the clinic of the Faculty of Veterinary Medicine of Kerman University. In this study, two laboratory methods, IFA and ICA, have been used. Escanis serum prevalence with these two methods has been reported as 1368% and 8.10%, respectively. In this study, E. canis morulae

were observed in monocytes in 16,166% (2 dogs) of infected dogs (24). Avizah et al. (2010) reported the prevalence of E. canis using the immunochromatography method on 198 dogs, as 6.9% (19 dogs) were referred to Shahid Chamran University Hospital in Ahvaz (25). Santos et al. (2009) reported the most frequent finding observed in blood tests of dogs infected with E. canis as Thrombocytopenia (26). The significant difference between E. canis with negative cases and the average number of platelets of dogs with a positive seropositive result were stated by Rodrigues et al. (2005) (27).

Majlanova et al. (2007) reported the Hepatozoon canis parasite from the foxes of this country for the first time in an area known for the abundant presence of rhipicephalus sanguineus (28). Kunishio et al. (2008) investigated the prevalence of Babzia gibsoni in Japan through the ELISA method, and 6.10% of the sera were infected. In the history of dog bites by ticks, it was found that the bite is a risk factor for Babzia gibsoni infection, while age, gender, and breed were not considered statistical risk factors (29). Gary et al. (2006) investigated the seroprevalence of tick-borne pathogens in dogs in North America. The samples were transferred to the University College of Veterinary Medicine in North Carolina for serology. The samples were studied in terms of Ehrlichia canis, Anaplasma phagocytophilum, Babzia canis, Bartonella henselae, Borrelia burgdorferi and Rickettsia rickettsii. This research recorded information about the breed and the city with the state where the dog belonged (30).

In a study by Meshki et al. (2001), in Tabriz city, on 357 dogs to investigate the epidemiology of blood filariasis, 4.8% of dogs were infected with Dirofilaria immitis. In the study of Albarzi et al. (2006), the blood samples of 100 dogs were positive by modified Knott test, direct expansion, and checking the presence of antigen (by immunochromatography kit) to 1% by direct expansion, 5% by modified Knott test and 6% were positive with the antigen detection kit. In addition, in one percent of infected dogs, Dirofilaria immitis was secretly identified, the level of contamination with Dirofilaria immitis in the examined dogs was one case and only one male worm of Dirofilaria immitis from the pulmonary artery of an infected dog (Female, 3/5 years old) was separated. In the study of Brati et al. (2013) (9), Miranda et al. (2007) (31), and Brabin et al. (1992) (32), the prevalence of infection in males was reported to be higher than in females, this pattern may be due to the higher number of male dogs (9).

Fakhkhar et al. (2011) investigated leishmaniasis infection in 110 blood samples of dogs in Fars province using the molecular PCR method of Buffy coat and LIN primers. A total of 25 cases (/23) were positive by PCR. Only 3 cases were positive with both serological and molecular methods, and the correlation between positive serum and positive PCR was weak (7).

In the study by Razzaghi Menesh et al. (2012) in the Ardestan region, 1.8% of 184 pet dogs were positive by PCR (33). Leontides et al. (2002), in Greece to investigate Leishmania species, investigated 73 healthy hunting dogs living in an endemic area; and found 63 positives by PCR method of bone marrow (34). In the study by Bahadri et al. in Khuzestan province, out of 119 blood samples collected from dogs in different regions of Khuzestan province with the Modified Knott test, 15 dogs (61.12%) contained microfilaria, and the highest contamination was in 7-year-old male dogs, and the highest level of infection was in Dezful city. In the research done in Mazandaran, Gilan, and Golestan, out of 200 samples, 5.25% of samples (n=51) Dirofilaria immitis Microfilariae was observed. There was 51.42% contamination in Gilan, 38.15% in Golestan, and 7.69% in Mazandaran.

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