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Original Article

First Molecular Detection and Phylogenetic Analysis of *Ehrlichia canis* in Dogs from Baghdad, Iraq

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Abstract

Ehrlichia canis is a pathogen considered a disease in both dogs and breeders, a tropical and non-tropical disease caused by an intracellular pathogen. For the first time, the study aimed at hematological and molecular detection and phylogenetic analysis of *Erlchcia canis* in dogs in Baghdad, Iraq. Two hundred dogs were clinically examined from April to September 2019 at the Veterinary Hospital in Baghdad. Blood samples with EDTA tubes were used for microscopic examination, complete blood count (CBC) and polymerase chain reaction (PCR). The study's results revealed that the infection rate was 3.5% when analyzed using microscopic or molecular methods. Ataxia, posterior recumbency, and occasionally vision problems were identified as the clinical characteristics that were distinguished in this study. The hematological values showed no significant differences between infected and uninfected dogs ($P>0.05$). However, the study did show that infected dogs had neutrophilia and monocytosis. Four samples were sent to the sequencers, and NCBI accession numbers were assigned to two isolates of the *Ehrlichia canis* 16s rRNA gene (MN227483.1 and MN227484.1). This study showed that 99% of the isolates matched those found in other countries. The study concluded that microscopic examination is not the best method for diagnosing *Ehrlichia* in dogs because it requires the ability to differentiate microscopically between intracellular inclusion bodies and the included morula of *Ehrlichia* and may produce incorrect results. Instead, molecular tests are used to confirm an *Ehrlichia* diagnosis.

Keywords: Hematology, *Ehrlichia canis*, Polymerase Chain Reaction

1. Introduction

Ehrlichia is one of the rickettsia belonging to the Anaplasmataceae family (1). *Ehrlichia canis* is a pathogen considered a zoonotic disease in animals and significantly affects the public health of both dogs and breeders (2). The disease was also known as canine monocytic ehrlichiosis, a tropical and non-tropical disease caused by an intracellular pathogen (3, 4). *Ehrlichia* contains many species, such as *E. canis*, *E. muris–like*, *E. ewingii*, and *E. chaffeensis* (5, 6). Clinical signs of ehrlichiosis in dogs include fever, pale mucous, lethargy, epistaxis with thrombocytopenia and petechiae, hepato-splenomegaly, lymphadonmegaly, and neurological signs (7). The seroprevalence of *E. canis* varies from 33.1% to 74.3% in some areas of Mexico (5, 6). *Ehrlichia canis* is spread transstadially by ticks; *Rhipicephalussanguineus* is one of the dogs' most important transmission carriers. Also, all stages of the tick can transmit the infection, usually after the adult tick and nymph are separated from the infected host (8).

The blood smear is often used to diagnose ehrlichiosis, especially in the early stages, but it is not very sensitive and requires experience. On the other

hand, the polymerase chain reaction is one of the most sensitive and accurate tests for diagnosing ehrlichiosis in all clinical phases (9, 10). There are a number of molecular studies that have studied tick-borne diseases in Iraq (11-13), but there is still an insufficient amount of research on *Ehrlichia canis* as well as other *Ehrlichia* species in a number of geographical areas, including Iraq. The study aimed to investigate the prevalence, molecular detection, and phylogenetic analysis of *Erlchcia canis* in dogs in Baghdad, Iraq, and to detect haematological and clinical changes linked to canine ehrlichiosis.

2. Materials and Methods

2.1. Samples Collection, Clinical and Hematological Examinations

Two hundred dogs were clinically examined (temperature, pulse, and respiratory rate), and other clinical signs were evaluated from April to September 2019 at the Veterinary Hospital in Baghdad. The study included 122 males and 78 females, including 106 dogs aged three years or less and 94 dogs aged three years or more. 114 German Shepherds, 46 Malinois, seven huskies, 12 crossbreds, six terriers, four Rottweilers, two Boo-dogs, two Pekingese, two Lolo foxes, two Labrador retrievers, two Sheep-dogs, and one Husher-dog were the dog breeds included in the survey. Two mL of blood taken directly from the cephalic vein and put in EDTA tubes were used to do a blood smear with Giemsa stain and microscopic examination, as well as a complete blood count (CBC). After the haematological assays were completed, DNA extraction and polymerase chain reaction (PCR) was performed on the blood.

2.2. Molecular Diagnosis

2.2.1. DNA Extraction

From 200 µl of EDTA blood, DNA was isolated using a genomic DNA isolation kit (Promega, USA). An agarose gel (1.5%) was loaded with RedSafe nucleic dye (iNtRON, Korea), and template DNA was evaluated using the NanoDrop system (Thermo Scientific, USA). DNA purity at 260/280 nm ratios varied from 1.7 to 1.9.

2.2.2. PCR Test

The forward primer EC9 (5-TACCTTGTTACGACTT-3) and reverse primer EC12 (5-TGATCCTGGCTCAGAACGAACGAACG-3) developed by Badawi and Yousif (14) were used to amplify a fragment of the *Ehrlichia canis* 16S rRNA gene of about 1400-bp. The master mix (12.5 µL) (Promega, USA) contains Taq DNA polymerase (50 units/mL), MgCl2 (3 mM), each dNTP (400 µM), each primer (1 µL) with concentration (10 pmol), template DNA (3 µL), and nuclease-free water (8.5 µL) for a total volume of 25 µL for the PCR reaction. The thermal cycling protocol was used after estimating the annealing temperature using the (ThermoFisher Scientific website, Tm Calculator): initial denaturation (94°C for 5 minutes), 40 cycles of denaturation (94°C for 40 seconds), annealing (51.5°C for 40 seconds), extension (72°C for 30 seconds), final extension (72°C for 5 minutes), and holding (4°C for 10 minutes). PCR electrophoresis (Bio Basic, Canada) and agarose gel (1.5 %) with RedSafe nucleic dye (Bio Basic, Canada) were used to identify PCR results and visualize them under UV light.

2.3. Sequencing

The forward primer EC9 was used to sequence the PCR products submitted to Macrogen, Korea. The sequences were then compared to other 16S rRNA genes of *E. canis* sequences using the BLAST of NCBI data on the website (http://www.ncbi.nlm.nih.gov/BLAST/). The Phylogenetic Tree and Multiple Sequence Alignment analyses were done with the help of the Molecular Evolutionary Genetics Analysis (MEGA) 6.0 software.

2.4. Statistical Analysis

The SPSS programme version 20 was used for statistical analysis, and Analysis of Variance (ANOVA) at level ($P \leq 0.05$) was used to estimate significant differences.

3. Results

3.1. The Clinical Examination and Hematological Study

The results of clinical and hematological examinations revealed that 7 of the 200 dogs studied were infected with *Ehrlichia canis*. This means that 3.5% of the dogs were infected. The most significant clinical signs were prolonged inappetence, a body temperature between 38.5 and 39.6 °C, vomiting, and dehydration. The initial neurological clinical signs were ataxia and posterior recumbency. Two of the dogs lost much weight after being infected for months without treatment, lay on their sides at the end of the disease, showed signs of nervousness like lack of attention, tremors, and shifting their legs, affected one eye with uveitis, and died of respiratory failure (Figure 1).

Figure 1. Dogs with *Ehrlichia canis* infection suffered from severe emaciation, recumbency, and neurological signs

Examination of infected dogs' blood smears revealed that the *Ehrlichia canis* morulae were spherical and inside the neutrophils (Figure 2). Many *Ehrlichia canis* extracellular morulae spread along the microscopic field outside the white cells that can phagocyte them in severe or late-stage infections (Figure 3). The main hemogram values reveal a non-significant difference (P>0.05) in the (Table 1) of blood values: Red Blood Cells (RBC), Hemoglobin (Hb), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC). There was no significant difference (P>0.05) in the total white blood cell (WBC) and differential white blood cell counts. However, there were no significant differences (P>0.05) between *Ehrlichia canis* infected and non-infected dogs, there was an apparent non-significant increase (P>0.05) in the Mean±SE of neutrophils at 75±2.45 and 69.26±0.99, and monocytes at 4.57±2.37 and 2.59±0.27 of infected and non-infected dogs, respectively.

3.2. Molecular Detection of *Ehrlichia canis* by 16S rRNA

The PCR test revealed that seven of the 200 dogs were affected (3.5%). A specific primer for *Ehrlichia canis* in PCR was used to confirm blood smear staining. Figure 4 shows the loading of the products of the 16rRNA gene in the agarose medium (1400bp). The affected dogs ranged in age from one to five years old in five cases, with two dogs aged six and eight. Five German Shepherds and two Malinois, four females and three males were also among the dogs affected.

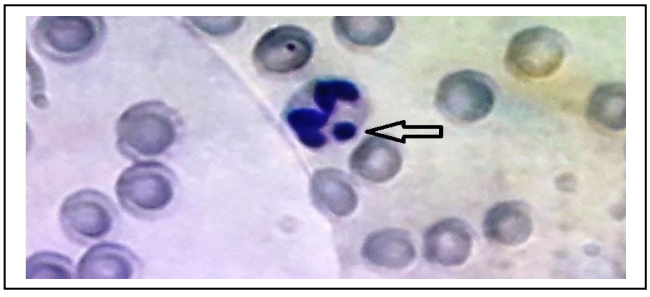

Figure 2. *Ehrlichia* morulae with neutrophil, Giemsa stain, under a light microscope (100×)

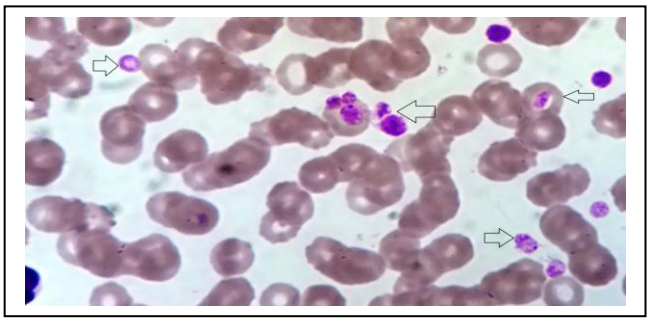

Figure 3. Extracellular morulae of dogs with *Ehrlichia canis* infection, Giemsa stain, under a light microscope (100×)

Table 1. The hematological values of infected and non-infected dogs with *Ehrlichia canis* were compared (Mean+SE)

Tests	Infected (n=7)	Non- infected (n=193)
RBC ($\times 10^6/\mu$ L)	4.54-7.32 (6.17±0.35)^A	4.07-8.84 (5.99±0.08)^A
Hb (g/dl)	10.40-17 (14.77±0.83)^A	8.1-19.7 (14.3±0.2)^A
HCT (%)	30.50-51.78 (43.74±2.78)^A	24.56-61.69 (42.14±0.61)^A
MCV (fl)	67-76 (70.57±1.23)^A	60-80 (70.34±0.24)^A
MCH (pg)	21.3-27.7 (24.01±0.77)^A	19.3-30.8 (23.92±0.13)^A
MCHC (g/dl)	31.3-37.8 (33.88±0.75)^A	27.9-43.5 (33.99±0.13)^A
WBC	6.27-17.5 (10.14±1.48)^A	4.9-22.5 (10.91±0.42)^A
Lymphocytes %	8-28 (15±3.12)^A	8-64 (22.67±0.92) A
Neutrophils %	66-83 (75±2.45)^A	28-88 (69.26±0.99)^A
Monocytes %	0-16 (4.57±2.37)^A	0-25 (2.59±0.27)^A
Eosinophils %	0-16 (5.43±1.98)^A	0-24 (5.47±0.44)^A
Basophils %	0 A	0-1 (0.01±0.006)^A
Platelets ($\times 10^{3/\mu L}$)	279-378 (328±13.84)^A	202-654 (343±7.78)^A

The differences in capital letters horizontally refer to significant differences at ($P \leq 0.05$)

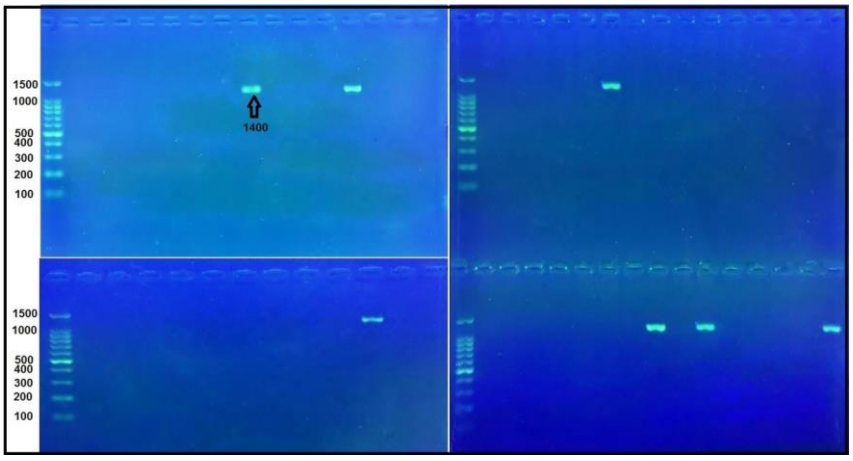

Figure 4. *Ehrlichia* canis PCR amplicons of 16S rRNA gene, 7 positive dogs at 1400bp EC9 and EC12 primers (Agarose 1.5% with safe red stain), DNA ladder in the first lane, 100 bp

3.3. DNA Sequencing

The nucleotide sequences of the four samples were sent to Korea Macrogene, and the NCBI database was used to confirm that *Ehrlichia canis* was present by comparing the sequence results to other sequences. The results of phylogenetic analysis are shown in the phylogenetic tree (Figure 5), which shows that these four isolates are in two different groups, or "clades." In the first sister clade, isolate A5 was an exact match for isolate A21. Furthermore, second sister clade isolates A52 and A53 were 100% similar and Chinese isolate CP025749.1, although the similarity between these two clades was 99%. All of the isolates in this study were 99% matched to isolates from other countries. For documentation, two isolates, A5 and A21, were registered in the NCBI Gene Bank. The accession numbers MN227483.1 and MN227484.1 were assigned to them, respectively.

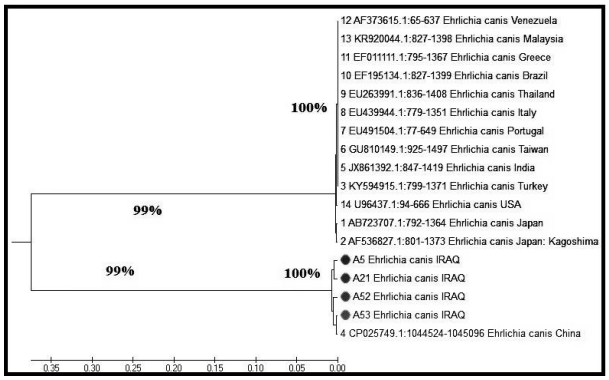

Figure 5. The phylogenetic tree of *Ehrlichia canis* isolates from Iraq and other countries

4. Discussion

Canine ehrlichiosis is usually caused by the rickettsia *Ehrlichia canis* and transmitted by a brown dog tick infected with the *Ehrlichia canis*. Several of the dogs in this research showed clinical signs of a slight temperature increase, a lack of appetite, vomiting, and dehydration. These signs are common for a wide range of illnesses, making it challenging to identify a specific disease from them. Malik, Qamar (15) observed these signs, noting that blood tests are required for a thorough diagnosis, which most cases are considered subclinical, and that unique signs appear in the advanced stages of the disease (16). The current study indicates that some of the most important signs for a dog with canine ehrlichiosis are lying down, its back legs being weak or partially paralyzed, and having trouble seeing. Canine ehrlichiosis causes ataxia, limb paralysis, and other neurological symptoms due to a brain lesion that includes T2-hyperintense and punctate in the spinal cord and brain (15). Dogs with *E. canis* have been reported to have neurological and ophthalmological abnormalities (17). Parashar, Sudan (18) showed that the different ocular signs might reach 43.47% in 46 dogs that were confirmed to be infected with *Ehrlichia canis*.

Although red blood cells and platelets did not change significantly between infected and uninfected dogs, neutrophils and monocytes showed non-significant increases. In contrast, another study indicated that dogs with *E. canis* infection had more eosinophils and fewer PCV and platelets (19). Other investigations found a decrease in white blood cells, red blood cells, hemoglobin, and platelets, with no difference in the differential count of white blood cells between infected and uninfected dogs (18, 20).

The study found the infection rate to be 3.5%, which is close to what has been reported elsewhere in the world. The prevalence rate of *Ehrlichia canis* infection was 2% in a study of 500 dogs in Malaysia (21). While in another study, after analyzing three distinct regions in Pakistan, it was discovered that there was a very high infection incidence of canine ehrlichiosis, 28% (22). Infection rates were affected by a number of things, like geographic region and season, as well as the type and method of diagnosis.

The study found that the genetic profile of *Ehrlichia canis*, which was detected in Iraq, had a high level of similarity, with a limit of 99%, with some worldwide isolates. One of these isolates had about 1433bp of the 16S rRNA gene of *Ehrlichia canis* in the blood of the dogs and was identical to Iraq isolates with the

accession number (KR920044.1) from Malaysia (23). Also, a Taiwanese isolate (GU810149.1) was similar to the isolates of this study. Using a whole genome blood extraction method, 1620bp of the same gene for *Ehrlichia canis* was found in the blood of dogs from Taiwan (24).

Also, one study from India found that using doxycycline reduced the severity of canine ehrlichiosis and its pathogenicity (25), with this study showing genetic similarity to current isolates from Iraq and recording 1478bp of the 16S rRNA of *Ehrlichia canis* in NCBI under accession number (JX861392.1) (which is not the case in Iraq, where the use of doxycycline has gone down, and there is a decline in the diagnostic tools for canine ehrlichiosis in veterinary clinics in Iraq). Several global isolates with genetic affinity to Iraq isolates were fixed in dogs, including Turkish isolate (KY594915.1) with 1380bp of the 16S rRNA gene of *Ehrlichia canis*, Japanese isolate (AB723707.1) with 1369bp of the 16S rRNA gene of *Ehrlichia canis* in cats of the same gene, and Chinese isolate (CP025749.1) in dogs. All of these global isolates were taken from whole blood for PCR, which was confirmed as the best way to determine if *Ehrlichia* infection occurred.

According to the study, this was the first molecular detection of *Ehrlichia canis* in Iraq. Neurological symptoms and posterior recumbency are identifying characteristics of canine ehrlichiosis. The study concluded that microscopic examination is not the best method for diagnosing *Ehrlichia* in dogs because it requires the ability to differentiate microscopically between intracellular inclusion bodies and the included morula of *Ehrlichia* and may produce incorrect results. Instead, molecular tests are used to confirm an *Ehrlichia* diagnosis.

Authors' Contribution

The research idea, doing molecular tests and writing the research: N. M. B.
Contribute to sample collection and clinical examination: M. M. Q.
Interpretation of the results: M. A. A.
Analysis of the sequences: J. M. K.
Supervisor and revision: A. A. Y.

Ethics

The ethics Committee and Scientific Committee of the Department of Internal and Preventive Veterinary Medicine has to approve this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

1. Chomel B. Tick-borne infections in dogs—an emerging infectious threat. Vet Parasitol. 2011;179(4):294-301.
2. Otranto D, Dantas-Torres F, Weigl S, Latrofa MS, Stanneck D, Decaprariis D, et al. Diagnosis of Hepatozoon canis in young dogs by cytology and PCR. Parasit Vectors. 2011;4(1):1-6.
3. Dumler JS, Barbet AF, Bekker C, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and'HGE agent'as subjective synonyms of Ehrlichia phagocytophila. Int J Syst Evol Microbiol. 2001;51(6):2145-65.
4. Kelly PJ, Xu C, Lucas H, Loftis A, Abete J, Zeoli F, et al. Ehrlichiosis, babesiosis, anaplasmosis and hepatozoonosis in dogs from St. Kitts, West Indies. PLoS One. 2013;8(1):e53450.
5. Rodriguez-Vivas R, Albornoz R, Bolio G. Ehrlichia canis in dogs in Yucatan, Mexico: seroprevalence, prevalence of infection and associated factors. Vet Parasitol. 2005;127(1):75-9.

6. Sosa-Gutierrez CG, Quintero Martinez MT, Gaxiola Camacho SM, Cota Guajardo S, Esteve-Gassent MD, Gordillo-Pérez M-G. Frequency and clinical epidemiology of canine monocytic ehrlichiosis in dogs infested with ticks from Sinaloa, Mexico. J Vet Med. 2013;2013.
7. Sainz Á, Roura X, Miró G, Estrada-Peña A, Kohn B, Harrus S, et al. Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe. Parasit Vectors. 2015;8(1):1-20.
8. Nicholson WL, Allen KE, McQuiston JH, Breitschwerdt EB, Little SE. The increasing recognition of rickettsial pathogens in dogs and people. Trends Parasitol. 2010;26(4):205-12.
9. Little SE, O'Connor TP, Hempstead J, Saucier J, Reichard MV, Meinkoth K, et al. Ehrlichia ewingii infection and exposure rates in dogs from the southcentral United States. Vet Parasitol. 2010;172(3-4):355-60.
10. Neer T. Canine monocytic and granulocytic ehrlichiosis. Infectious Diseases of the Dog and Cat, 2nd ed Philadelphia, PA: WB Saunders. 1998:139-47.
11. Al-Abedi G, Al-Amery A. Molecular Diagnosis and Phylogenetic Analysis of Babesia Species Isolated from Ticks of Infested Cattle in Wasit Governorate, Iraq. Iraqi J Agric Sci. 2020;51(5):1249-60.
12. Al–Obaidi SSA, Al-Ani JMK, Al-Shammari NB. Molecular detection of some anaplasma species in blood of dogs in Baghdad province, Iraq. Iraqi J Vet Med. 2020;44(1):39-45.
13. Abdullah SH, Ali SA. Molecular study and phylogeny of babesia spp. in native sheep from sulaimani govenorate/northern Iraq. Iraqi J Agric Sci. 2021;52(5):1077-83.
14. Badawi NM, Yousif AA. Babesia canis spp. in dogs in Baghdad Province, Iraq: First molecular identification and clinical and epidemiological study. Vet World. 2020;13(3):579.
15. Malik MI, Qamar M, Ain Q, Hussain MF, Dahmani M, Ayaz M, et al. Molecular detection of Ehrlichia canis in dogs from three districts in Punjab (Pakistan). Vet Med Sci. 2018;4(2):126-32.
16. Kawahara M, Rikihisa Y, Lin Q, Isogai E, Tahara K, Itagaki A, et al. Novel genetic variants of Anaplasma phagocytophilum, Anaplasma bovis, Anaplasma centrale, and a novel Ehrlichia sp. in wild deer and ticks on two major islands in Japan. Appl Environ Microbiol. 2006;72(2):1102-9.
17. Gaunt S, Beall M, Stillman B, Lorentzen L, Diniz P, Chandrashekar R, et al. Experimental infection and co-infection of dogs with Anaplasma platys and Ehrlichia canis: hematologic, serologic and molecular findings. Parasit Vectors. 2010;3(1):1-10.
18. Parashar R, Sudan V, Jaiswal AK, Srivastava A, Shanker D. Evaluation of clinical, biochemical and haematological markers in natural infection of canine monocytic ehrlichiosis. J Parasit Dis. 2016;40(4):1351-4.
19. Harrus S, Waner T. Diagnosis of canine monocytotropic ehrlichiosis (Ehrlichia canis): an overview. Vet J. 2011;187(3):292-6.
20. Lang LG, Griffin JF, Levine JM, Breitschwerdt EB. Magnetic resonance imaging lesions in the central nervous system of a dog with canine monocytic ehrlichiosis. Case Rep Vet Med. 2011;2011.
21. Moonarmart W, Sungpradit S, Rawangchue T, Suphaphiphat K, Suksusieng S, Jirapattharasate C. Clinical history and hematological findings among canines with monocytic ehrlichiosis. Southeast Asian J Trop Med Public Health. 2014;45(1):157.
22. Badawi NM, Yousif AA. Survey and Molecular Study of Babesia gibsoni in Dogs of Baghdad Province, Iraq. Iraqi J Vet Med. 2020;44:34-41.
23. Petrov EA, Ulcar I, Celeska I, Ilievska K, Trenkovska PS, Novakov T, et al. Effects of doxycycline treatment on hematological and blood biochemical parameters in dogs naturally infected with Ehrlichia canis. Maced Vet Rev. 2018;41(1):99-105.
24. Nazari M, Lim SY, Watanabe M, Sharma RS, Cheng NA, Watanabe M. Molecular detection of Ehrlichia canis in dogs in Malaysia. PLoS Negl Trop Dis. 2013;7(1):1982.
25. Koh FX, Panchadcharam C, Tay ST. Vector-borne diseases in stray dogs in peninsular Malaysia and molecular detection of Anaplasma and Ehrlichia spp. from Rhipicephalus sanguineus (Acari: Ixodidae) ticks. J Med Entomol. 2016;53(1):183-7.