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Molecular Detection of Canine Ehrlichiosis in Baghdad, Iraq

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Abstract

Background: Ehrlichiosis is a widespread disease transmitted from dog to dog by ticks and caused by specific rickettsial bacterial disease in dogs by three main species, including *Ehrlichia canis, Ehrlichia chaffeensis*, and *Ehrlichia ewingii*. **Aim:** Molecular investigation of canine ehrlichiosis (*Ehrlichia canis*, *E. ewingii*, and *E. chaffeensis*) in Baghdad province. **Methods:** The study was conducted in Baghdad, between October 2023 and March 2024 on 155 dogs. All samples were examined by nested PCR, and cases were positive when producing product fragment about 396 bp for each species in the second round of PCR of each *Ehrlichia* species. **Results:** The results revealed that the infection rate per dog was 7.74% as follows: 12 dogs had an overall infection rate, 12 dogs had *E. canis* infection, 7 dogs had *E. chaffeensis* infection, and 3 dogs had *E. ewingii* infection. The study documented instances of mixed infections caused by multiple *Ehrlichia* species among the 7 cases. However, this study showed no association between canine ehrlichiosis infection and age, as well as the sex of dogs while significant in huskies. The analysis of the 16S rRNA gene of *E. canis* revealed that the Iraqi isolates exhibited the highest homology with isolates from countries, such as Turkey, Japan, India, and China. The sequences of the 16S rRNA gene of *E. chaffeensis* were grouped within clades; the first clade was very similar to isolates from Mexico, South Korea, and Argentina, while the second clade clustered with isolates from China, Egypt, South Korea, and other parts of the world. Phylogenetic analysis for *E. ewingii* obtained in the present study revealed the high homogeneity of the Iraqi isolate to clade 1 of *E. ewingii* with Mexico, Grenada, and China, while clade 2 included another Iraqi isolate of *E. ewingii* with the isolates from India and the USA. **Conclusion:** The study concluded that this study detected the infection rate of *Ehrlichia* species in dogs in Baghdad, and concluded from phylogenetic trees that presented a significant multiple hosts of these pathogens, including dogs, cats, ticks and humans, indicated that had the zoonotic potential of *Ehrlichia* species. This study contributed in the study epidemiological factors, and genetic diversity of *Ehrlichia* species in dogs.

Keywords: Molecular, *Ehrlichia canis, E. chaffeensis, E. ewingii*, Iraq.
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Introduction

Ehrlichiosis is a widespread disease transmitted from dog to dog by ticks and caused by specific rickettsial bacterial disease in dogs by *Ehrlichia* species. This disease doesn't just infect dogs; it also can be a threat to humans as well as various other animals. The gram-negative and intracellular bacteria specifically target the blood-forming cells in the host. In dogs, there are three main species responsible for this condition, including *Ehrlichia canis*, *Ehrlichia* chaffeensis, and *Ehrlichia* ewingii (1).

Ehrlichia infections in dogs are transmitted mainly through the salivary secretions of attached ticks, specifically, *Rhipicephalus sanguineus* tick, which is commonly known as the brown dog tick. In addition to tick transmission, *Ehrlichia* species can also trans through blood transfusions (2). Hematological changes associated with canine ehrlichiosis were moderate to severe thrombocytopenia, along with non-

regenerative and normocytic normochromic anemia, leukocytosis, neutropenia, eosinophilia and lymphopenia. The detection of *Ehrlichia* organisms in the form of morulae is quite rare, observed in only about 4–6% of cases, although this method can help a diagnosis of ehrlichiosis, but it has low sensitivity (1). The 16S rRNA gene was used for detecting and taxonomic multiple types of bacteria (3-5).

Polymerase chain reaction (PCR) is a highly sensitive technique for detecting *Ehrlichia* infections early, typically between 4 and 10 days after the initial exposure (6). PCR is particularly useful for identifying early infections that serological tests might miss. For *E. canis*, several molecular assays have been developed that focus on species-specific genes, such as the p30 and 16S rRNA genes, to ensure precise identification (7). PCR assays for detecting *E. canis* DNA are more effective for early diagnosis of canine monocytic ehrlichiosis (CME) compared to IFAT or ELISA tests, especially in dogs with acute infections. During the acute phase, nested PCR (nPCR) can identify *E. canis* DNA before serological tests are able to detect anti-*E. canis* antibodies, making it a superior method for early detection (8). The polymerase chain reaction is a sensitive and specific diagnostic test widely employed to diagnose canine ehrlichiosis (9).

In Iraq, between April and September 2019, the molecular examinations were carried out on 200 dogs in Baghdad; the results indicated an infection rate of 3.5% depending on molecular analysis (10). Previous report in Iraq focused only on *E. canis*, while the present study was designed for molecularly investigation and sequencing of *Ehrlichia* species.

Materials and methods

Ethical approval

All procedures were performed with the approval of the animal care and use committee (ACUC) of the college of veterinary medicine/university of Baghdad (number PIG-1291).

Study period and location

The study was conducted in the college of veterinary medicine, University of Baghdad, between October 2023 and March 2024.

Animals and collection

The present study was conducted on 155 dogs (included 95 males and 60 females, aged from one month to ten years): 66 German shepherded, 29 Malinois, 22 crossbreed, 12 huskies, 18 Terriers, 3 Rottweilers, 2 LoLo foxes, 2 Pomeranian dogs, 1 Pointer dog. A round of 2 ml of blood samples was obtained by puncturing the cephalic vein and collected in a sterile tube containing EDTA (ethylenediaminetetraacetic acid). Giemsa staining was used for the blood count and blood smear process. All samples were stored at -20 °C until molecular assay was performed.

DNA extraction

Total genomic DNA was extracted from 200 µL of EDTA-anticoagulant blood samples using an extraction kit (blood genomic extraction kit, Geneaid, Taiwan) according to the manufacturer's instructions. DNA concentration and purity were measured by using nanodrop apparatus (Thermofisher, USA). Extracted DNA was stored at -20 °C further used for polymerase chain reaction (PCR).

Polymerase chain reaction (PCR)

All samples were examined by nested PCR. The first round was tested by using universal primers of the 16S rRNA gene *Ehrlichia* species ECC (AGA ACG AAC GCT GGC GGC AAG C) and ECB (CGT ATT ACC GCG GCT GCT GGC

A). All products in the first round was amplified with the second round with specific species primers for *E. canis* F-ECAN (CAA TTA TTT ATA GCC TCT GGC TAT AGG A) and R-HE3 (TAT AGG TAC CGT CAT TAT CTT CCC TAT), for *E. chaffeensis* F-HE1 (CAA TTG CTT ATA ACC TTT TGG TTA TAA AT) and R-HE3, and for *E. ewingii* F-EE52 (CGA ACA ATT CCT AAA TAG TCT CTG AC) and R-HE3 and give positive products at 396 bp for each species. All primers in this study were designed by Murphy *et al.* (11). The thermocycle protocol of the PCR was presented in the Table (1) and Table (2) according to Murphy *et al*. (11). The master mix reaction was performed for PCR was showed in Table (3).

Table (1). Thermocycle first round of universal primers of *Ehrlichia* spp. (ECC and ECB primers).

Steps	Temperature (C°)	Time	No. of cycles
Initial denaturation	94	5 min.	1
Denaturation	94	1 min.	30
Annealing	65	2 min..	
Extension	72	2 min..	
Final extension	72	5 min.	1

Table (2). Thermocycle second round of specific primers of *E. canis* (ECAN5 and HE3*), E. chaffeensis* (HE1 and HE3), and *E. ewingii* (EE52 and HE3).

Steps	Temperature (C°)	Time	No. of cycles
Initial denaturation	94	5 min.	1
Denaturation	94	1min.	37
Annealing	55	2min.	
Extension	72	1.5 min.	
Final extension	72	5 min,	1

Table (3). Master mix composition protocol for conventional PCR of *Ehrlichia*.

PCR reaction components		Volume
Master mix reagent		12.5 µl
Primer (10 picoml)	Forward	1 µl
	Reverse	1 µl
Template DNA in 1st round or product of the 1st in the second round		3µl
Nuclease-free water		7.5 µl
Total reaction		25 µl

The 16S rRNA gene for 2 positive samples for each *Ehrlichia* species was sequenced by Macrogen / Korea. Phylogenetic analysis constructed after sequence alignment by nucleotide BLAST/NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) . The gene bank recorded positive samples under accession numbers *E. canis* (accession numbers: PP966958.1 and PP966958.1), *E. ewingii* (accession numbers: PP966960.1 and PP966961.1), and *E. chaffeensis* (accession numbers: PP966962.1 and PP966963.1). Multiple isolates were selected from the NCBI database for generated multiple sequence alignment with high identity isolates. The UPGMA method was used for estimating the evolutionary relationship between isolates. The evolutionary distance and phylogenetic tree were constructed by using the maximum composite likelihood method and substitutions per site as units of the phylogenetic tree. MEGA 6 software was used for phylogenetic analysis.

Statistical analysis

The program SPSS, version 20, was used for estimating all studied data. The one-way ANOVA method was used for calculating the significant difference of the mean of the values in the study, while the coefficient of the logistic regression statistical method was odds ratio confidence interval (CI) for estimating the risk factors of the data in the study. Differences were considered significant at level ($P \leq 0.05$).

Results

Microscopic diagnosis

The current study revealed the infection rate of *Ehrlichia* species in 10/155 (6.4%) dogs, as determined by microscopic examination using the Giemsa stain. All positive samples exhibited large or small morula, similar to intracytoplasmic inclusion bodies (Figure 1).

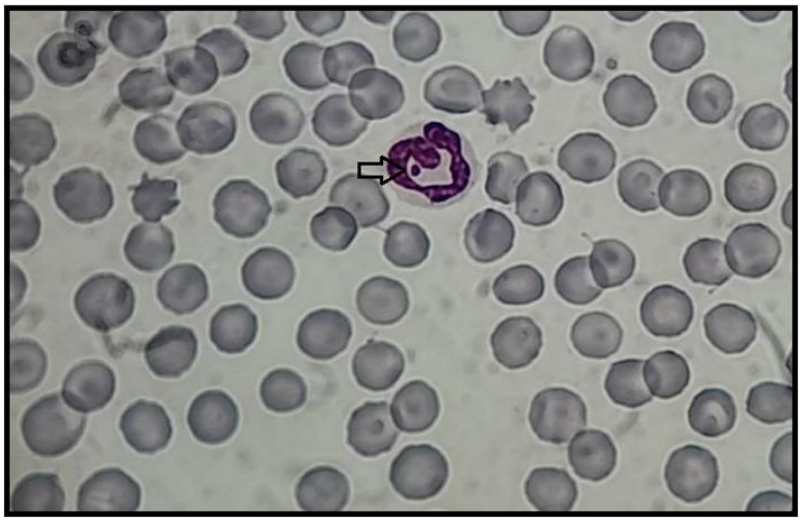

Figure (1): Blood Smear Stained with Giemsa Showing Morula of *Ehrlichia* Species Inside Neutrophil (light microscope [100X] zoom 1.5 by camera)

Molecular diagnosis

All extracted DNA was subjected to amplification by nested PCR with universal primers of *Ehrlichia* species and specific primer sets and gave positive products at 396 bp for each species of *E. canis*, *E. chaffeensis*, and *E. ewingii*. According to the PCR results in this study, the total infection rate per dog of canine ehrlichiosis was 12 out of 155 dogs (7.74%), including 12 dogs had *E. canis* infection (Figure 2), 7 dogs had *E. chaffeensis* infection (Figure 3), and 3 dogs had *E. ewingii* infection (Figure 4).

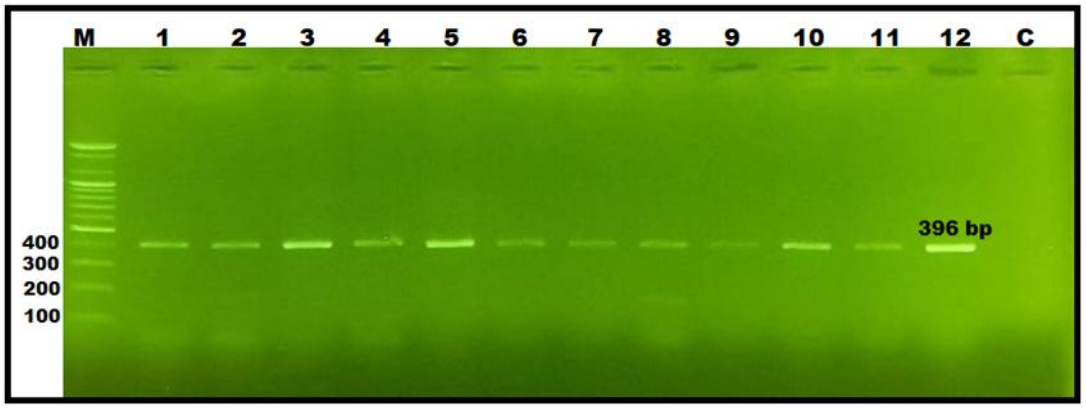

Figure (2): Agarose gel electrophoresis 1.2% with red safe stain showing amplification of 396bp fragment of 16S rRNA gene (ECAN5 and HE3primers), numbers 1-12 cases positive of *E. canis* in dogs.

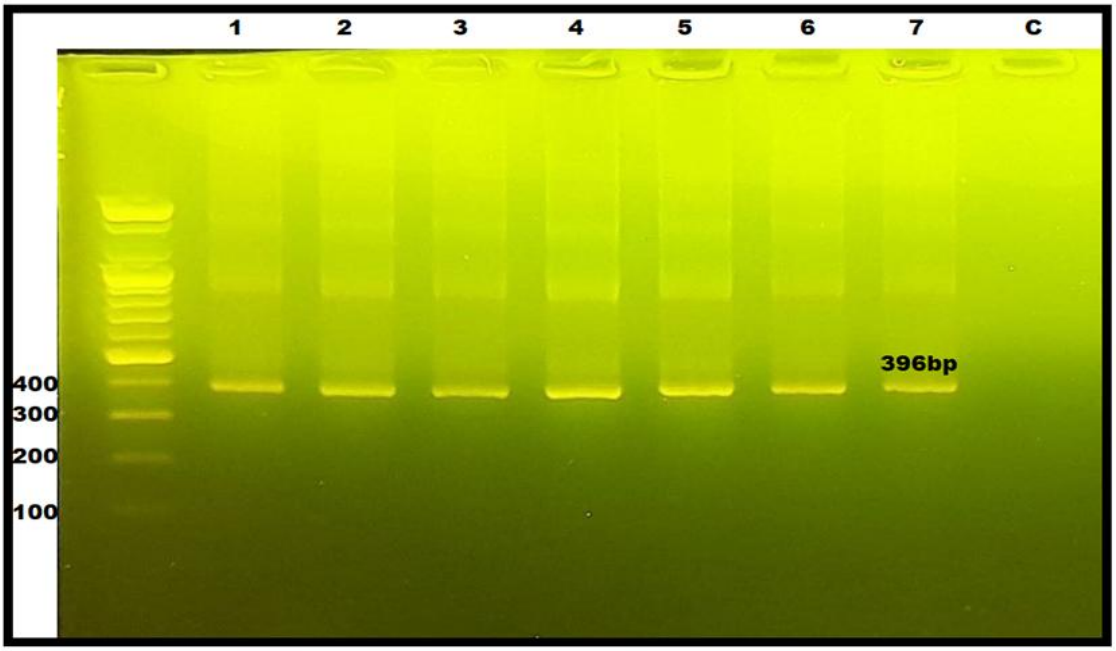

Figure (3): Agarose gel electrophoresis 1.2% with red safe stain showing amplification of 396bp fragment of 16S rRNA gene (HE1 and HE3primers), numbers 1-7 cases positive of *E. chaffeensis* in dogs.

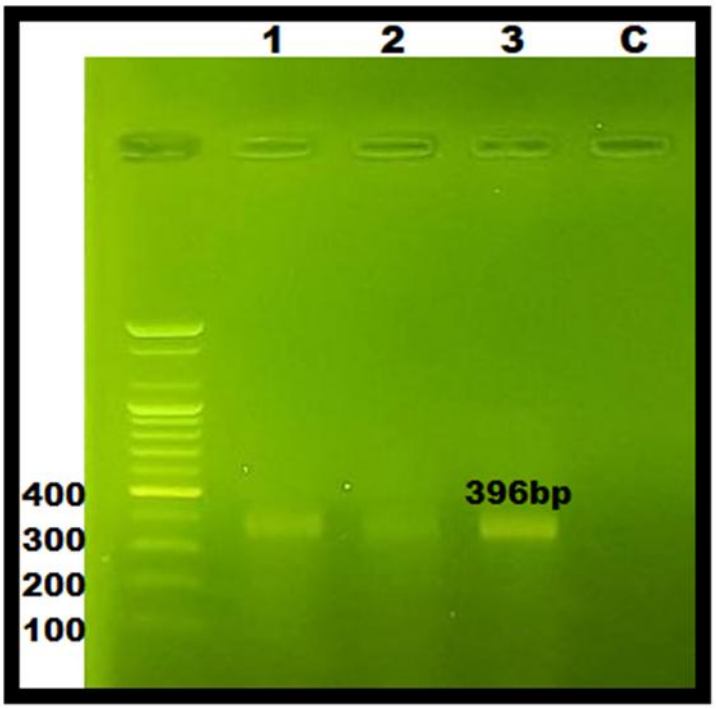

Figure (4): Agarose gel electrophoresis 1.2% with red safe stain showing amplification of 396bp fragment of 16S rRNA gene (EE52 and HE3primers), numbers 1-3 cases positive of *E. ewingii*in dogs.

The study documented the presence of mixed infections by multiple *Ehrlichia* species in 8 dogs among 12 infected dogs, including five dogs infected by *E. canis* and *E. chaffeensis*, one dog infected by *E. canis* and *E. ewingii*, and two dogs infected by *E. canis*, *E. chaffeensis* and *E. ewingii* (Table 4).

Table (4): Cases numbers of *Ehrlichia* species show the mixed infection between species.

No.	*E. canis*	*E.chaffeensis*	*E. ewingii*
1	+ve	+ve	
2	+ve		
3	+ve	+ve	+ve
4	+ve	+ve	
5	+ve		
6	+ve		
7	+ve	+ve	+ve
8	+ve	+ve	
9	+ve	+ve	
10	+ve		+ve
11	+ve		
12	+ve	+ve	
	12	7	3

The risk factors

The results revealed that non-significant increased of the risk of the infection in dogs equal and less than one year, and females' dogs, while breed incidence of the canine ehrlichiosis was

significantly higher in huskies (25%) when compared to other dog breeds: Malinois (10.3%), Terrier (16%), crossbreed (4.9%), and German shepherd (2.9%) (Table 5).

The results showed no association between canine ehrlichiosis infection and age, as well as the sex of dogs.

Table (5): The infection rate of canine ehrlichiosis according risk factors.

Factors	group	infected dogs	Odds ratio(CI)
Age	≥1year	7/64 (10.9%)	2.11
	< 1year	5/91 (5.49%)	(0.63-6.98)
Sex	Males	7/95 (7.63%)	1.14
	Females	5/60 (8.33%)	(0.34-3.78)
Breed	German-Shepherd	2/67 (2.9%)	
	Malinois	3/29 (10.3%)	
	Crossbreed	1/22 (4.5%)	*10.83
	Husky	3/12 (25%) *	(1.85-73.9)
	Terrier	3/18 (16.6%)	
	Rottweiler	0/3	
	Lolo fox	0/2	
	Pomeranian	0/2	
	Pointer	0/1	
***Significant differences (P≤ 0.05). CI: Confidence Intervals 95%**			

The phylogenetic analysis

The phylogenetic analysis of the sequence data revealed the presence of *E. canis* isolates within other world isolates. Furthermore, the analysis of the 16S rRNA gene of *E. canis* revealed that the Iraqi isolates (accession numbers: PP966958.1 and PP966959.1) exhibited the highest homology with isolates from neighboring countries, such as Turkey, Japan, India, and China (Figure 5).

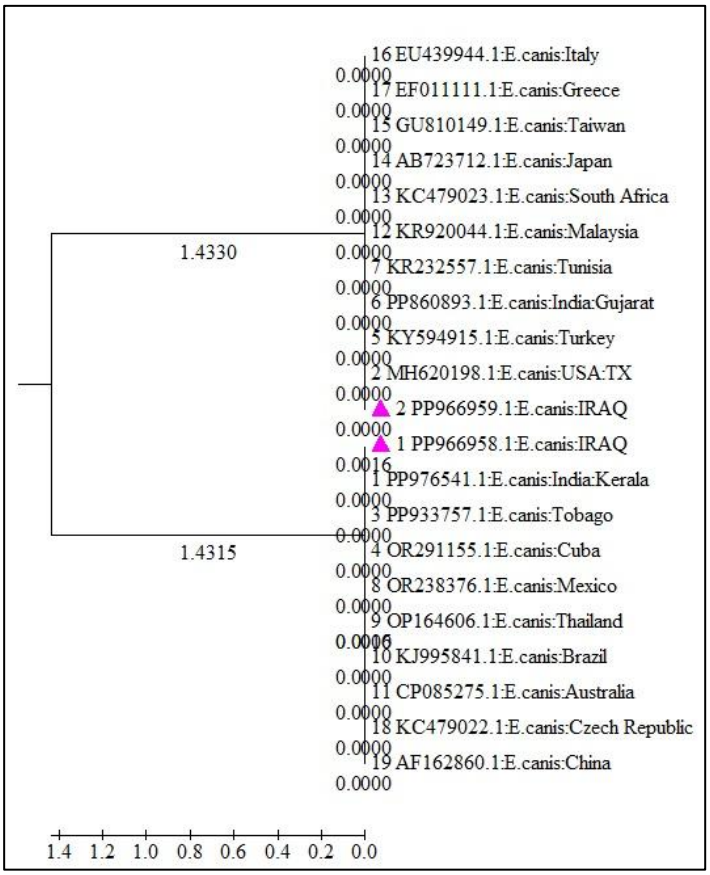

Figure (5): Phylogenetic Tree of Partial Sequences of the 16s RNA Gene Of *E. Canis* Infection.

The sequences of the 16S rRNA gene of *E. chaffeensis* when compared to the other high-identical isolates in the gene bank, the results indicated that the *E. chaffeensis* sequences in dogs from Iraq were grouped within two clades; the Iraqi isolate (accession numbers: PP966962.1) in the first clade was very similar to isolates from Mexico, South Korea, and Argentina, while the isolate in the second clade (accession numbers: PP966963.1) clustered with isolates from China, Egypt, South Korea, and other parts of the world (Figure 6).

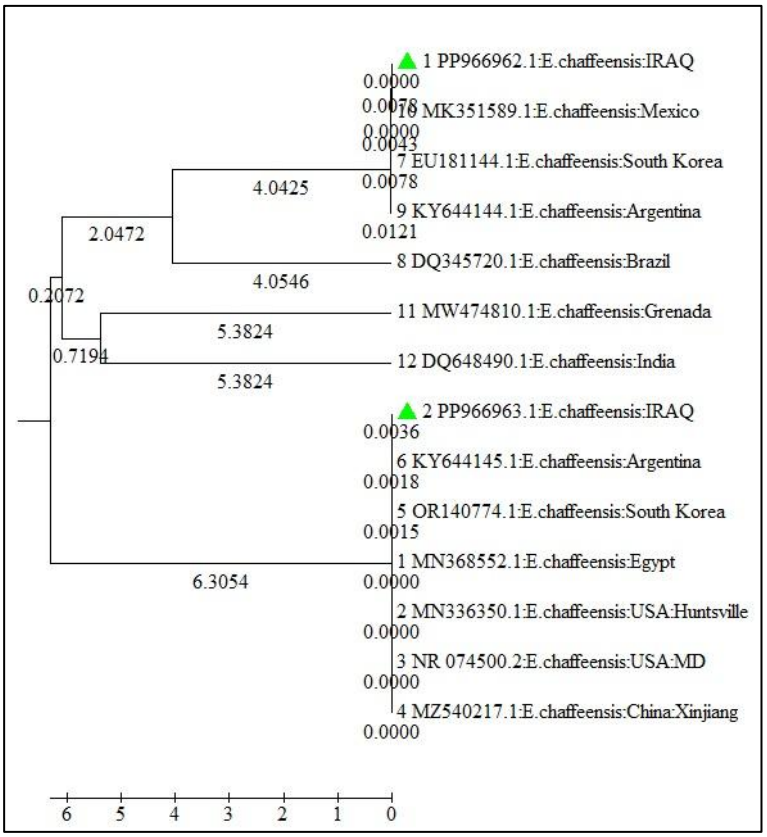

Figure (6): Phylogenetic Tree of Partial Sequences of the 16s rRNA Gene of *E. Chaffeensis* Infection.

The phylogenetic analysis for *E. ewingii* obtained in the present study revealed the high homogeneity of the Iraqi isolate (accession numbers: PP966960.1) clade 1 of *E. ewingii* with Mexico in dogs (accession number: OR238378.1), Grenada, and China in *Haemophysalis flava* ticks (accession number: MN148614.1). Furthermore, the sequence of 16s rRNA of another Iraqi isolate of *E. ewingii* (accession numbers: PP966961.1) in second clade resembled that of the isolates from blood of dog in 2024 at India (accession numbers: PP957601.1), and isolate from blood dogs in 2017 at USA (accession numbers: MF893272.1) (Figure 7).

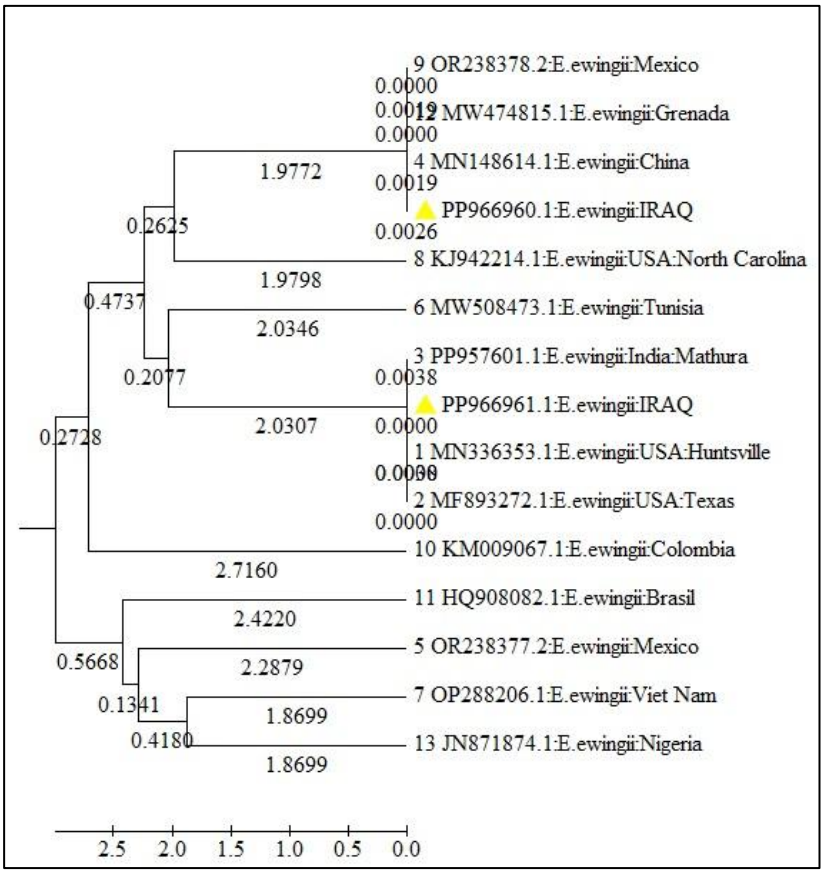

Figure (7): Phylogenetic Tree of Partial Sequences of the 16s rRNA Gene of *E. Ewingii* Infection.

Discussion

The finding of the percentage of the microscopic examination in the present study was different from other studies in some countries as South Korea 0% (12), India (14.28%) (13), in Bangladesh (8%) (14) and in Maharashtra (5%) (15), while only one study in Iraq in 2022 by Badawi *et al.* detected 3.5% of morula in the microscopic examination (10). The differences in the microscopic examination between studies of *Ehrlichia* species are occurred because of limitations of microscopic examination and no differentiation between species, as well as requiring some expertise to diagnose (16).

The infection rate according PCR results was 7.74%, and in line with previous studies in some countries, such as India (8%) (17). The infection rate was lower than present study in In Taiwan, South Korea, Japan, and China (3.6%, 3%, 1.5%, and 1.4%, respectively) cited by Aziz *et al.* (7), and the previous study in Iraq in 2022 reported prevalence of *E. canis* infection (3.5%), which is lower than the present study finding (10). while other reports observed the infection rate to be higher than our observation as in Maharashtra (16.66%) (15), India (30%) (16), Nepal (27.1%) (17), Pakistan (28%) (18), and China (12.12%) (19). Molecular assay is a more sensitive method for *Ehrlichia* sp. infection; the variation in the infection rate between low and high prevalence can depend on the molecular method that is used (20), climate conditions, vector distribution, and population of dogs, as well as control strategies of ticks, all these factors play a role in the infection rate with ehrlichiosis in dogs (7).

The mixed infection in dogs can be occurred due to *Ehrlichia* species belong under Anaplasmataceae family and transmitted by same type of tick species, Nair *et al.* documented possibility of mixed infection by *E. canis* and *E. chaffeensis* in dogs (----), and coinfection of tick-borne diseases was very possible especially when the pathogens are transmitted by same tick species (**Santiago Sanchez-Vicente)**

The risk factors were estimated. The current study found that the risk factors were higher in huskies and did not significantly affect females or young dogs under one year. In contrast, in our observation, one study recorded a high frequency of risk in males and dogs older than one year (21). The results were consistent, with some reports indicating a high-risk factor in huskies (22, 23), less than one year (18), and a high prevalence in German Shepherds (21). Some studies demonstrated that there was no correlation evidence between infection rate with sex and age (24, 25).

A study conducted in Iraq had a high similarity with the Turkish isolate in dogs from Elazig (accession number: KY594915.1). This finding agrees with the study in Baghdad of Badawi *et al.* (10), who also reported a high similarity for the same isolate from Turkey. The phylogenetic tree of *E. canis* in the results had isolate from Japan (accession number: AB723712.1), which was obtained from wild cats between 2002 and 2011, with a prevalence rate of 12% (25).

The phylogenetic tree *E. chaffeensis* sequences in dogs were included a sample from Mexico (accession number: MK351589.1) that was isolated from human blood and amplified by specific primers (26). In the same cluster included an isolate from South Korea (accession number: EU181144.1), which was isolated from *Haemaphysalis longicornis* ticks (27). In the second cluster, an Egyptian isolate of *E. chaffeensis* (accession number: MN368552.1) was extracted from *Hyalomma excavatum* ticks in 2019, there are presented a significant multiple hosts of *E. chaffeensis*, including dogs, cats, ticks and humans.

Ehrlichiosis is important disease in dogs and endemic in India, and Indian isolates had minor differences to the isolates from USA, Turkey and other countries, this indicated a global distribution of the *Ehrlichia* species and its vectors (Mitesh Mittal). *Ehrlichia ewingii* caused infection in both humans and dogs; it is considered a common *Ehrlichia* species in dogs from the United States (Starkey). Many police dogs in Iraq were exported from USA dogs. The *E. ewingii* can be developed to persist and cause asymptomatic infection in the dogs and become a source of infection to humans and other dogs (Barbara). The increased incidence of *Ehrlichia ewingii* and *E. Chaffeensis* infection and case fatality in humans, especially in children (Heitman), this increased the necessity that researchers should conduct further studies on the *Ehrlichia* species in Iraq in humans and other animals.

This molecular study emphasizes the wide range of *Ehrlichia* hosts, which include dogs, humans, and several tick species. The variation depends on the host source of the isolates across different countries, as shown in the phylogenetic trees of this study. *Ehrlichia* is one of the rickettsial pathogens classified under the *Anaplasmataceae* family (28). In Iraq, many studies were conducted on the *Anaplasma* species in different animals (29–32).

Conclusion

In conclusion, this study detected the prevalence rate of *Ehrlichia* species in dogs, and revealing 7.7% through six month according molecular assays. The results are consistent from first molecular detection of some *Ehrlichia* species in dogs in Iraq *E. chaffeensis* and *E. ewingii*. Also, phylogenetic analysis of the *Ehrlichia* species showed a strong relationship between Iraqi isolates and isolates from other countries, such as Turkey, India, and China. The phylogenetic analysis of *E. canis*, *E. chaffeensis*, and *E. ewingii* demonstrated that presented a significant multiple hosts of these pathogens, including dogs, cats, ticks and humans, and different countries, indicated that had the zoonotic potential of these *Ehrlichia* species. This study contributed in the study epidemiological factors, and genetic diversity of *Ehrlichia* species in dogs.

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