

## Molecular investigation of *Hepatozoon canis* in dogs in Mosul city

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### Abstract

This study is the first molecular detection of *Hepatozoon canis* in dogs in Mosul city. One hundred twenty blood samples were collected from stray and household dogs of different ages, sexes, breeds, and breeding, suffering from emaciation, anemia, paleness in mucous membranes and the presence of ticks in the ear. The DNA was extracted using a commercial kit (Qiagen, Germany). Polymerase Chain Reaction was used to detect the parasite targeting a partial region of 18S rRNA gene. The polymerase chain reaction produced positive results, as bands in electrophoresis at 737 bp. The total positive sample was 33 dogs out of 120 dogs (27.5%) the relationship between the infection with the parasite and the age of animals recorded a high prevalence of infection in dogs less than 6 months while the lowest prevalence of infection in animals more than 2 years old, The male dogs showed a high prevalence of infection when compared with female dog, The large breeds dogs recorded high prevalence of infection than small breeds dogs, The outdoor dogs showed a high prevalence of infection Animals which suffer from paleness in mucous membrane showed high prevalence, while healthy animals recorded lowest prevalence of infection, gene sequence showed there were only three isolates out of the total samples sent for genetic sequencing.

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### Introduction

There is less information regarding the *Hepatozoon canis* (Adeleorina: Hepatozoidae), which causes canine hepatozoonosis (1). The tick serves as the vector in the life cycle of *H. canis* in the caindea family (2). In inequality to other tick-borne protozoa (3), The parasite is spread by canines ingesting ticks (4), which carry mature oocysts and affect white blood cells and specific organs. (5) The sporozoites then spread throughout the body to the organs (liver, kidney, spleen, bone marrow, and lung) via the lymphatic and vascular systems (6). Organs produce meronts, followed by numerous merogony cycles that release merozoites, invading white blood cells to make gamats. The primary tick species that carry *H. canis* are numerous, such as the brown dog tick (*Rhipicephalus*

*sanguineus*, Ixodida: Ixodidae) (7). Though numerous surveys on canine hepatozoonosis are recorded in various countries, *H. canis* is a canine vector-borne disease (CVBD) (8). Clinical symptoms like anemia, lymphadenomegaly, fever, lethargy, anorexia (9), and weight loss can also be brought on by it (10). One standard method for detecting hepatozoonosis is the IFAT, which is intended to identify anti-*H. canis* immunoglobulins, and microscopic examination in a blood smear to identify intracytoplasmic ellipsoidal-shaped gamonts (11). In the last ten years, molecular diagnosis has been developed (12), which has significantly advanced our knowledge of how these protozoan spreads throughout canine populations (13). While PCR is considered the most accurate method of detecting parasites, microscopic analysis of smears is a straightforward method often employed to diagnose this

infection (14). However, not much research has compared these approaches (15). Therefore, the gold standard for diagnosis needs to be clarified. Similarly, more is needed about the validity of various tissues for molecular detection (16). Lastly, the prevalence of hepatozoonosis in dogs needs to be understood (17). The study aimed to ascertain the most efficient method of diagnosis of *H. canis* infection in a population of dogs by examining the relationships between the presence of *H. canis* and the dog population.

## Materials and methods

### Ethical approval

The scientific board of the College of Veterinary Medicine at Mosul University in Mosul, Iraq, approved this study; the approval number is UM. VET. 2023. 096. 120.

### Blood samples

Blood samples were collected from stray and household dogs of different ages, sexes, breeds, and breeding management, which suffer from emaciation, anemia, paleness in mucous membranes and, in some cases, the presence of ticks in the ear (18).

### DNA extraction

The DNA was extracted using a commercial kit (Qiagen, Germany). And the DNA concentration was measured using (Nanodrop).

### Polymerase chain reaction

The primers (Forward: HEP-F5'-CCT GGC TAT ACA TGA GCA AAA TCT CAA CTT-3' and Reverse: HEP-R-5'CCA ACT GTC CCT AAT CAT TAA AGC-3') targeted a partial region of the 18S rRNA gene (19). 5.0 µL of template DNA was used in the PCR to detect the parasite in a 25 µL volume containing 2.5 µL of buffer, 200 µM of dNTP, 2.0 mM of MgCl<sub>2</sub>, 1.5 U of Taq DNA polymerase. The DNA was amplified using a thermocycler. One cycle of five minutes at 94 °C was used for initial denaturation. After that, denaturation was conducted for thirty seconds at 94 °C, annealing for thirty seconds at 67 °C, extension for thirty seconds at 72 °C, and final extension for five minutes at 72 °C. The final amplified DNA products were recognized and displayed using a gel-documentation system using electrophoresis in a 1.5% agarose gel. DNA sequencing and GenBank submission. The positive samples' PCR products were shipped to Macrogen, Korea, for Sanger sequencing. The matching primer was sent with 25 µl of the target gene's PCR product. Sequencing results were acquired as text files in the FASTA format. A particular accession number and registration for a subset of strains were obtained by sending them to GenBank, NCBI. Using Mega X software, the chosen sequences were examined to compare the evolutionary relationships among the *H. canis*.

## Results

The results of the polymerase chain reaction showed positive results, which showed as bands in electrophoresis at 737 bp (Figure 1).

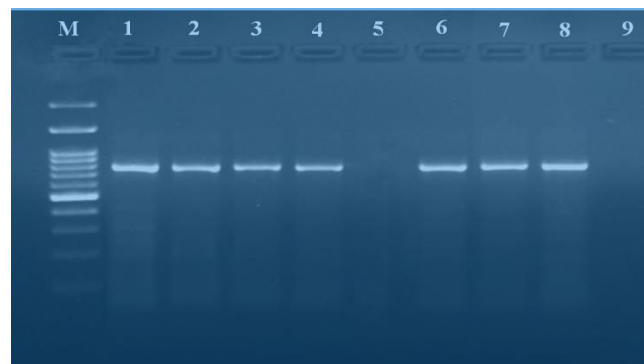


Figure 1: Gel electrophoresis image showing: Lane (M) DNA ladder; Lanes (5,9) negative samples to *H. canis* DNA; Lanes (1-4,6-8) positive samples to *H. canis* DNA in approximately band size 737 bp.

Of 120 dogs, 33 (27.5%) were the positive sample overall. The association between animal age and parasite infection revealed that animals older than two years old had the lowest incidence of infection. In contrast, canines younger than six months had the highest prevalence (Table 1). Male dogs showed a higher prevalence of infection than female dogs (Table 2). The large breed of dogs recorded a higher prevalence of infection than the small breed of dogs (Table 3). Outdoor dogs showed a higher prevalence of infection than indoor dogs (Table 4). Animals which suffer from paleness in mucous membranes showed a high prevalence of infection with the parasites, while healthy animals recorded the lowest prevalence of infection (Table 5).

Table 1: Shows the relationship between the infection with *H. canis* and the age of dogs

	Sample (n)	Positive n (%)
Less than 1 year	41	18 (43.9)
< 1 year to 2 years	39	9 (23.0)
More than 2 years	40	6 (15.0)
Total	120	33 (27.5)

Table 2: Shows the relation between the infection with *H. canis* and the gender of dogs

	Sample (n)	Positive n (%)
Male	67	23 (34.3)
Female	53	10 (18.8)
Total	120	33 (27.5)

Table 3: Shows the relation between the infection with *H. canis* and with the breed of dogs

	Sample (n)	Positive n (%)
Large breed	53	20 (37.7)
Small breed	67	13 (19.4)
Total	120	33 (27.5)

Table 4: Shows the relationship between the infection with *H. canis* and the breed management of dogs

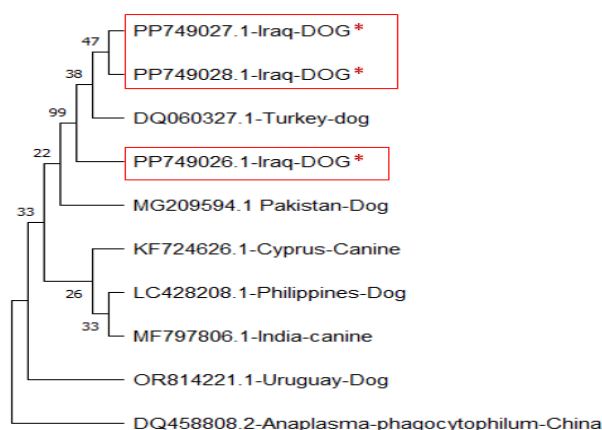
	Sample (n)	Positive n (%)
Outdoor	44	23 (52.2)
Indoor	76	10 (13.1)
Total	120	33 (27.5)

Table 5: Shows the relation between the infection with *H. canis* and the clinical signs of dogs

	Sample (n)	Positive n (%)
Pale mucous membrane	44	21 (47.7)
Loss of weight	38	15 (39.4)
Loss of appetite	29	11 (37.9)
Ticks in the ear	27	11 (40.7)
Health	35	8 (22.8)

Table 6: Using NCBI BLASTn, determine the degree of similarity between the local sequences of *H. canis* 18S rRNA gene and other sequences in GeneBank

Local	Query Cover %	Similarity %	GenBank Accession Number	Country
	100	99.77	DQ060327.1	Turkey
PP749026.1	100	96.34	LC428208.1	Philippines
PP749027.1	100	96.34	MF797806.1	India
PP749028.1	100	96.34	MG209594.1	Pakistan
	100	96.34	KF724626.1	Cyprus
	100	96.34	OR814221.1	Uruguay

Figure 2: Phylogenetic tree of the partial sequences of the 18S rRNA gene of *H. canis* in Mosul city, Iraq (\*), with the outgroup *Anaplasma phagocytophilum* DQ458808, China.

The *H. canis* 18S rRNA gene genetic sequencing results revealed that the samples supplied to Mosul had a genetic sequence. Out of all the samples sent for genetic sequencing, only three isolates were present, and they were assigned a serial number in GenBank PP749026. 1, PP749027. 1, PP749028. 1. Results of the molecular nucleotide sequence of the 18S rRNA gene of the *H. canis* in the database. The results showed the presence of gene sequences that have a percentage of similarity to the nucleotide sequence of the 18S rRNA gene for each of the *H. canis* that were diagnosed in the study when BLAST was performed in the database compared to the nucleotide sequences of the international strains, which were identified during this study and which were used for the purpose of constructing the genetic phylogenetic tree. The results showed that there was a similarity rate between the local isolates, where the highest similarity rate was reached. 99. 77% with the strain registered in the World GenBank under the name DQ060327. 1 and isolated in Turkey, followed in terms of similarity by the strain under the genetic name LC428208, reaching 96. 34 % and also isolated in Philippines. The percentage of similarity with the local strain reached 96. 34% in India; it reached 96. 34 % for the isolates in Pakistan, strains under the names KF724626. 1, OR814221. 1, reaching 96. 34% (Table 6 and Figure 2).

## Discussion

According to the investigation, *H. canis* was found in the study region for the first time. They may be risky because they can expose domestic dog populations to hematophagous vector bites and related arthropod-borne illnesses (20,21). Unlike domestic dogs, wild animals do not exhibit clinical symptoms, where the illness can cause subclinical to severe symptoms that damage many organs and cause anemia and lethargy (22). However, studies on wildlife are difficult because there is little information about wild canids because of their untamed nature. At the moment, *H. canis* infects a variety of carnivorous hosts across the globe, such as domestic cats, dogs, jackals, foxes, and opossums (23).

Polymerase chain reaction was utilized in this investigation to identify the presence of *H. canis* in the blood of dogs in the Nineveh area the positive sample shows a band in electrophoresis at 737 bp, and the total positivity in

animals was 33 from 120 animals 27.5%. This percentage of infection was low compared to other studies like (10), which recorded 100% of infection, while a 51% prevalence of infection with the parasites in a dog shelter in Italy (24). In contrast, other studies recorded a low prevalence of infection 18% (25). The reason for differences in the prevalence of infection is due to variations in the number of samples, the using different diagnostic techniques, the specification of the geographic area of the study, and the presence or absence of biological vectors, given that *H. canis* is harmful in natural hosts, its presence warrants consideration (3). The disease was finally recorded in the state after a lengthy absence of more than ten years. An increase in pet tourism and travel may also contribute to the international spread of disease. This study showed a higher prevalence of infection in the puppy than in the adults once (25).

It documented *H. canis* infection in dogs younger than five years old compared to dogs older than ten and dogs between the ages of one and five. We cannot compare these results with those of other papers that involved a larger number of samples because few samples were looked at in this instance, and only a descriptive statistical analysis was done. However, conflicting information about the incidence of *H. canis* in the canine population in the literature. Other studies showed that the rate of *H. canis* infection in the dog population was not significantly impacted by age (26,27). Twenty-seven other studies found that older dogs had a higher infection prevalence than younger pups. The immunological state of the animals and the vertical transmission of parasites from moms to puppies are further causes of these discrepancies (28).

This study recorded a higher prevalence in males than females, and these results did not agree with another study which shows that the prevalence of hepatozoonosis in female dogs is more than in male dogs. These findings contrasted with earlier research that did not highlight a sex propensity toward hepatozoonosis in infected dogs (29). However, these variations could be brought about by the dogs being in various surroundings, exposing them to more ticks. Male dogs get into constant fights and are often used to defend farms and herds, which makes them more vulnerable to contact with wild animals (30). This reason is also related to the breed of dogs, which is recorded higher in large breeds than small ones; small breeds often breed in indoor houses, and this difference is because many large breeds will use outdoor management (31). This is because they are large, and this is not suitable for raising them inside homes, as well as for the work of some of them because they are used for guarding or protecting fields and herds.

Another reason for the increase of infection in outdoor dogs when compared with indoor ones is the exposure of outdoor dogs to vectors (32). The tick is the principal carrier of these infections (33). Recent studies have only recently

shown *Amblyomma*, *Haemaphysalis*, and *Rhipicephalus*; several species are now considered possible hosts of this parasite (34). The most common species of tick is *Rhipicephalus sanguineus*, which multiple studies have shown to be prevalent in northern Sardinia and central and southern Sardinia (35). Consequently, when the ticks are most active, pet dogs may be exposed to *R. sanguineus* ticks and at a higher risk of contracting *H. canis*. Thus, this exploratory study aimed to ascertain whether a subgroup of Sardinian domestic dogs carried *Hepatozoon* species DNA (33). Animals that suffer from paleness in mucous membranes show a high prevalence of infection with parasites. In contrast, healthy animals recorded the lowest infection prevalence,

and severe clinical manifestations may arise in cases of high parasitemia or co-infection of the vertebrate hosts with other vector-borne infectious pathogens (36). are among the severe symptoms of the illness (37). While some studies demonstrated non-specific clinical symptoms, others did not exhibit any clinical indicators. This feature can indicate the existence of mild or subclinical infections, which are difficult to diagnose, as previously mentioned. In most documented cases, the diagnosis is made only after problems arise, and clinical indications appear only when the parasitemic load is high or when the same host has been found to be coinfecting with *H. canis* (38). *R. sanguineus* is a common species in Sardinia because the island's ideal climate permits growth. Furthermore, this study was conducted because we could not conclude the variables due to the small number of samples (39).

Although most of Despite having received antiparasitic medication, which shields them from infections and reduces their spread, Sardinian dogs were nevertheless at significant risk of contracting, several studies confirm that a higher percentage of dogs—both owned and stray—do not receive sufficient preventative care to shield them against arthropod infestations (40). It raises the possibility of disease transmission, ectoparasite infestations, and contaminated arthropods in residential settings. Additionally, the lack of a viable treatment means that therapy for dogs afflicted with hepatozoonosis remains a significant issue (41). Hepatozoonosis is actually usually thought to be a lifetime illness in dogs, and as of right now.

## Conclusions

Canines are afflicted with *H. canis*, and Nineveh province has a higher prevalence of parasites.

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## Conflict of interest

The authors declare no conflict of interest in the manuscript.

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## التقصي الجزيئي لطفيلي الهيباتوزون الكلب في الكلاب في مدينة الموصل

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### الخلاصة

تعتبر هذه الدراسة الأولى للكشف الجزيئي لطفيلي الهيباتوزون الكلب في الكلاب في مدينة الموصل. تم جمع ١٢٠ عينة دم من مختلف الأعمار والأجناس والسلالات ونظم التربية والتي يعاني البعض منها من الهزال وفقر الدم وشحوب في الأغشية المخاطية وفي بعض الحالات وجود القراد في الأذن، حيث تم استخلاص الحمض النووي باستخدام عدة استخلاص خاصة بذلك، ومن ثم تم تطبيق تفاعل البلمرة المتسلسل باستخدام بادئات استهدفت 18r SRNA. أظهرت نتائج تفاعل البوليميراز المتسلسل نتائج إيجابية حيث أظهرت حزم في الترحيل الكهربائي عند ٧٣٧، كانت عدد العينات الإيجابية ٣٣ كلباً مصاباً من مجموع ١٢٠ كلباً وبنسبة مئوية (٢٧,٥%)، وسجلت علاقة الإصابة بالطفيلي مع عمر الحيوان ارتفاعاً في انتشار الإصابة في الكلاب التي أعمارها أقل من ٦ أشهر بينما سجلت أقل نسبة للإصابة في الحيوانات بعمر أكثر من سنتين، أظهرت الكلاب الذكور نسبة انتشار عالية للإصابة مقارنة بالإناث، وسجلت الكلاب ذات السلالات الكبيرة معدل انتشار أعلى للإصابة مقارنة بالكلاب الصغيرة، أظهرت الكلاب ذات نظم التربية الخارجية انتشاراً عالياً للإصابة مقارنة بالكلاب المنزلية، وأظهرت الحيوانات التي تعاني من شحوب في الغشاء المخاطي ارتفاعاً في معدل الإصابة بالطفيلي بينما سجلت الحيوانات السليمة أقل نسبة إصابة، وأظهر التسلسل الجيني وجود ثلاث عزلات فقط من أصل ٢٠ عزلة.