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Unveiling a hidden threat: First report of clinical canine hepatozoonosis and Babesia canis vogeli co-infection in Jordan

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Abstract

Canine hepatozoonosis is an important tick-borne parasitic disease of dogs with increasing significance in veterinary medicine due to its impact on canine health. The disease is primarily caused by *Hepatozoon canis*, and unlike many tick-borne infections, transmission occurs through ingestion of infected ticks. This study reports fifteen cases of clinical canine hepatozoonosis in Jordan, including four cases with confirmed co-infection by *Babesia canis vogeli*, highlighting the diagnostic challenges posed by dual hemoparasitic infections. Diagnosis was based on clinical findings, hematological alterations, and microscopic examination of blood smears demonstrating characteristic gamonts within neutrophils, and was confirmed by PCR targeting the 18S rRNA gene yielded amplicons of approximately ~660 bp for *Hepatozoon canis* and ~490 bp for *Babesia canis vogeli.* Phylogenetic analysis showed that the obtained sequences clustered with reference strains of *H. canis* and *Babesia canis vogeli*. The cases were identified during a one-year screening of 200 canine blood samples submitted to our diagnostic clinical pathology laboratory, with 15 samples testing positive for one or more hemoparasites. The findings provide insight into the clinical manifestations, hematological alterations, diagnostic complexities, and the potential impact of co-infection on disease severity and prognosis. While *H. canis* infection has been previously reported in Jordan (20), this study represents, to the best of our knowledge, the first report of co-infection with *B. canis vogeli* in the country. By integrating clinical, laboratory, and molecular approaches, it contributes valuable information on the epidemiology, diagnosis, and management of hemoparasitic infections in Jordanian dogs, with implications for veterinary practice and disease surveillance.

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Introduction

Canine hepatozoonosis is an important veterinary disease, which mainly results from infection by a protozoan of the genus *Hepatozoon* (H.). The etiology of this disease is mainly due to two species: *H. canis* and *H. americanum*. *Hepatozoon canis*, the more widely distributed species, is commonly reported in tropical and subtropical regions, with numerous studies documenting its occurrence across Asia, Africa, the Middle East, and parts of Europe. (1). Pathogenic potential ranges from subclinical infections to very severe and potentially life-threatening forms, depending on the immunity of the host and the presence of other infections. On the other hand, *H. americanum* was first described mainly in the United States and is characterized by a more exacerbated clinical picture, which can result in much more severe and incapacitating symptoms (2).

In the Middle East, including Jordan, canine vector-borne diseases are emerging disorders; however, specific data on hepatozoonosis are scarce. The clinical diagnosis of canine

hepatozoonosis is made through detailed observation of the symptoms and signs, history, and physical examination of the infected dog. Usually, infected dogs show a spectrum of clinical signs, from mild to severe, which includes depression, emaciation, and chronic subnormal temperature, in addition to more specific presentations such as ocular discharge and cyclic neutrophilia (3). Yet, the non-uniform and non-specific nature of these signs mandates a comprehensive differential diagnosis for successful differentiation of hepatozoonosis from other diseases in Jordan.

The absence of complete epidemiological information on canine hepatozoonosis in the area represents an obstacle to diagnosis, control, and treatment. Moreover, urban/rural interfaces and the transit of domestic animals across frontiers compound the dynamics of infection. Investigating the involvement of local vectors, the distribution, and the seasonal activity of local vectors is essential to understand the transmission cycles of *Hepatozoon* species in Jordan. Therefore, this study aims to lay a foundation for subsequent needed research by illuminating the need for focused investigation into the prevalence, vector ecology, and control measures specific to Jordan. Moreover, it aims to document the occurrence of canine hepatozoonosis in Jordan and to describe associated clinical and laboratory findings, including co-infection with another significant tick-borne hemoprotozoan, *B. canis*.

Materials and Methods

Ethical approval

Ethical approval was not required for this study because it involved retrospective analysis of blood samples submitted to the laboratory for routine diagnostic purposes, with no direct animal involvement or additional interventions.

Case Selection and Blood Sample Collection

This study was conducted as a retrospective case series using purposive sampling of dogs that tested positive during initial screening. Over a one-year period, 200 canine blood smear samples referred to our diagnostic laboratory were screened for hemoparasites using light microscopy of stained peripheral blood smears. Fifteen samples tested positive for one or more hemoparasites and were subsequently selected for detailed investigation. All 15 positive dogs were local breeds, female, aged 1–7 years, and presented with a history of lethargy, weakness, decreased appetite for several weeks, dull hair coat, mild to heavy tick infestation, and vomiting. On clinical examination, all dogs were febrile, mucous membranes ranged from normal to pale, and an increased respiratory rate was observed.

Blood samples were collected from the jugular vein into EDTA tubes and submitted for CBC determination. Blood smears were prepared from each sample and stained with Diff-Quik (Diff-Three) stain, as previously described in standard hematology protocols (4). Each stained blood smear was examined carefully for thirty minutes under 20× and 100× magnifications by a board-certified veterinary clinical pathologist.

DNA Extraction

The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was utilized to extract genomic DNA from 100 µL of the collected blood in EDTA tubes. The manufacturer's protocol was followed, except for the final elution step. DNA was eluted in 50 µL of elution buffer for a final high yield. The concentration and purity of the tested samples were checked using a BioTek Gen5 microplate reader, then stored at −20 °C until the PCR assay was performed.

Polymerase Chain Reaction (PCR)

Applying the PCR technique, the 18S ribosomal RNA gene (18S rRNA) of *Hepatozoon* and *Babesia* were targeted for amplification in the infected samples. For *Hepatozoon* spp., a 666 bp region was amplified using HepF (5′-ATACATGAGCAAAATCTCAAC-3′) and HepR (5′-CTTATTATTCCATGCTGCAG-3′) primers (5). The accuracy of these primers had been previously reported (6,7).

For piroplasmid (Babesia, *Theileria*, and *Cytauxzoon* spp.) infection detection, TB-F (5′-CTTCAGCACCTTGAGAGAAAT-3′) and TB-R (5′-TCDATCCCCRWCACGATGCRBAC-3′) primers were used to amplify nearly 490 bp of the targeted region (8).

In total, 25 µL PCR mixtures were prepared containing 12.5 µL of 2× GoTaq® Green Master Mix (Promega, Wisconsin, USA), 4 µL of genomic DNA, 1 µL of each forward and reverse primer (10 µM), 1 µL of $MgCl_2$ (25 µM), and nuclease-free water to complete the final volume. In the negative control, nuclease-free water was added instead of DNA. Gene amplification was achieved using the following program: 1 cycle at 95 °C for 2 min; (30 cycles for *Hepatozoon* / 40 cycles for piroplasmids) of 95 °C for 30 s, (54 °C for *Hepatozoon* / 60 °C for piroplasmids) for 30 s, and 72 °C for 30 s; one final cycle at 72 °C for 5 min, then cooling at 4 °C. PCR results were visualized on 1.5% agarose gels previously stained with ethidium bromide.

Sequencing

Up to 20 µL of *Hepatozoon* and piroplasmid 18S rRNA PCR amplicons were sent to the commercial service Macrogen (South Korea) for purification and Sanger sequencing. Sequencing reactions were performed using forward primers and run in an automated sequencer. Chromatogram reads were visually checked and analyzed using UGENE v38.1 software (Unipro) and then uploaded for homology search to the deposited sequences in the BLASTn database (Nucleotide Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov).

Alignment analyses (18S rRNA data)

To determine the homology between the obtained *Hepatozoon canis* sequence and other related taxa deposited in GenBank (NCBI, National Center for Biotechnology Information), the sequences were aligned and analyzed using the MEGA11 program to obtain a phylogenetic tree. For phylogenetic analysis, 18S rRNA gene sequences of *Hepatozoon* spp. were retrieved from the NCBI GenBank database. These included isolates of *H. canis* from Turkey (AY150067), Brazil (KU903841), Spain (KX685374), and Japan (AB181504), as well as *H. americanum* (AF176836) and *H. ayorgbor* (AY600625) for outgroup comparison. The references were selected based on high sequence similarity in BLASTn searches to represent global genetic diversity for *H. canis*. Maximum Likelihood (ML) and Neighbor-Joining (NJ) trees were constructed based on the Kimura 2-parameter model with 1000 bootstrap replicates (9).

Results

Out of 200 examined blood smears, fifteen samples showed few to many neutrophils containing intracytoplasmic pale, elliptical, 11 × 5 µm capsular structures with and without nuclei, consistent with Hepatozoon gametocytes (Figure 1). Few erythrocytes contained intracellular purple, 4–5 µm, tear-drop-like structures consistent with Babesia piroplasms in four dogs (Figure 2). Four dogs had concurrent hepatozoonosis and babesiosis. Stained blood smears examined showed that Hepatozoon gametocytes were present in low numbers in eleven dogs and in high numbers in four dogs.

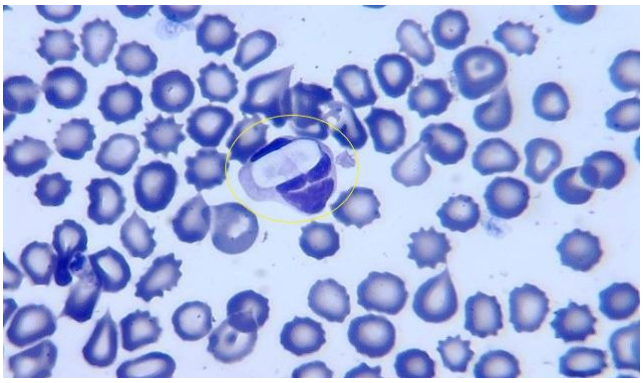

Figure 1: Blood smear showed a neutrophil containing intracytoplasmic pale, elliptical, 11x 5 micrometer in diameter structures consistent with hepatozoon gametocytes. Diff Quick Stain. Magnification 100X.

The CBC results generated by the automated analyzer for all 15 dogs are summarized in Table 1. CBC of eight dogs revealed microcytic, normochromic to hypochromic anemia without regeneration, most likely supporting iron deficiency anemia due to external blood loss by ticks. Many hypochromic target cells, ovalocytes, few schistocytes, burr cells, microcytes, and hypochromic erythrocytes were seen in the blood smears of the anemic dogs. Nine affected dogs showed mild to severe thrombocytopenia.

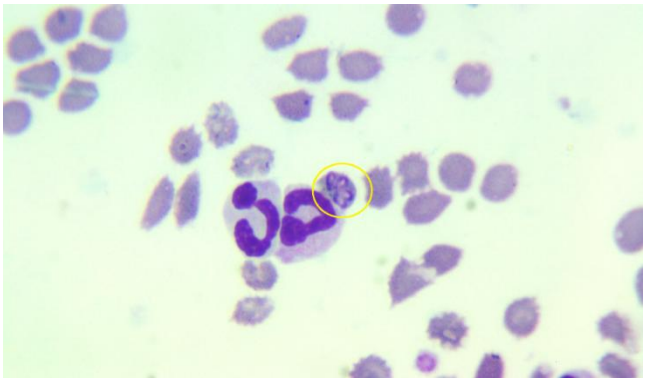

Figure 2: Blood smear showed an erythrocyte containing intracellular purple, 4-5 micrometer in length, tear- drop like structures, consistent with Babesia Periplasms. Diff Quick stain. Magnification 100X.

Leukogram changes in twelve dogs showed mild to severe leukocytosis characterized by neutrophilia (8 cases) with left shift (5 cases). Mild to severe lymphocytosis was seen in seven dogs, and many lymphocytes showed mild to severe lymphoid reactivity characterized by increased cytoplasmic basophilia. Four dogs showed monocytosis, and eight dogs showed peripheral eosinophilia. For further identification of the detected infectious agents, the 15 positive samples were sent for PCR for further specification.

PCR revealed that all 15 dog samples were infected with Hepatozoon, and four of these samples were co-infected with Babesia (Figure 3). PCR amplification produced clear bands at the expected sizes of approximately 666 bp for *Hepatozoon* spp. and ~490 bp for piroplasmids, confirming the specificity and successful amplification of the target genes**.** The constructed Neighbor-Joining phylogenetic tree for Hepatozoon revealed that the sequence of D1-Hep HepF clusters within the *Hepatozoon canis* clade. The results showed high similarity (93–95%) with various known *H. canis* isolates. This high level of sequence similarity likely reflects the close evolutionary relationship among *H. canis* isolates, indicating that they share a common ancestor and exhibit limited genetic divergence within this species**.** This isolate was phylogenetically distinct from other Hepatozoon species such as *H. americanum* and *H. ayorgbor* (Figure 4). The phylogenetic tree for the piroplasmid sequence showed 99% similarity to *Babesia canis vogeli*, clearly distinguishing it from *B. canis rossi* and *B. canis canis*, confirming subspecies-level resolution of the NJ method (Figure 5).

Table 1. Complete blood count and blood smear findings in 15 dogs

Case#	RBC	PLT	HB	Hct	MCV	MCHC	WBC	N	BN	L	M	E	*B. canis*	*H. canis*
RR	4.95–7.87	211–621	11.9–18.9	35–57	66–77	32–36.3	5–14.1	2.9–12	0–0.45	0.4–2.9	0.1–1.4	0–1.3	-	-
1	5.24	147	13.7	41	77.4	33.1	20.3	8.12	0	5.68	0.61	5.28	–	+
2	5.02	215	12	36	71.1	33	16.5	8.09	0	5.78	0.99	1.65	–	+
3	3.78	192	8.4	25	65.1	33.6	39.6	29.7	2.38	6.34	1.19	0	–	++
4	6.82	115	16	48	69.8	33.2	10.8	5.72	0	3.46	0.86	0.76	–	+
5	7.08	99	13.9	44	61.4	31.7	29.11	7.28	0	20.7	1.16	0	–	+
6	4.4	29	9.7	29	65.4	33.3	32	18.56	5.76	3.84	3.84	0	–	++
7	6.65	126	14.6	44	66.7	36.7	76.99	33.88	0.77	40	0.77	1.54	–	+
8	6.32	286	13.7	41	65.2	35.7	45	34	0	6	2	3	–	+
9	6.49	207	14.2	43	66.7	32.6	25.92	12.7	0	4.41	2.33	6.48	–	+
10	5.27	270	10.1	32	60.4	32.7	18.04	13.17	0	1.26	0.72	2.89	–	+
11	4.57	32	10	30	65	21.9	15.9	11.13	0	1.59	0.37	1.91	+	+
12	4.75	376	9	26	57.3	18	32.9	26.98	0	1.65	0.66	4.28	+	++
13	4.18	248	8.1	24.4	58.3	33.4	9.17	5.23	0.64	1.93	1.65	0.46	–	+
14	4.2	37	9.6	28.1	66.9	34.2	8.1	3.48	0	3.08	0.41	1.13	++	++
15	1.7	46	4.9	10.4	61.2	47.1	28.4	24.71	1.14	1.42	1.14	1.14	++	+

Abbreviations: RR, Reference range; RBC ($\times 10^6$/µl), red blood cells; PLT($\times 10^3$/µl), platelets; HB (g/dl), hemoglobin; HCT (%), hematocrit; MCV(fl), mean corpuscular volume; MCHC(g/dl), mean corpuscular hemoglobin concentration; WBC ($\times 10^3$/µl), white blood cells; N ($\times 10^3$/µl), neutrophils; BN ($\times 10^3$/µl), Band neutrophil, L ($\times 10^3$/µl), lymphocytes; M ($\times 10^3$/µl), monocytes; E ($\times 10^3$/µl), eosinophils; B. canis, Babesia canis; H. canis, Hepatozoon canis. +, low numbers; ++ high numbers.

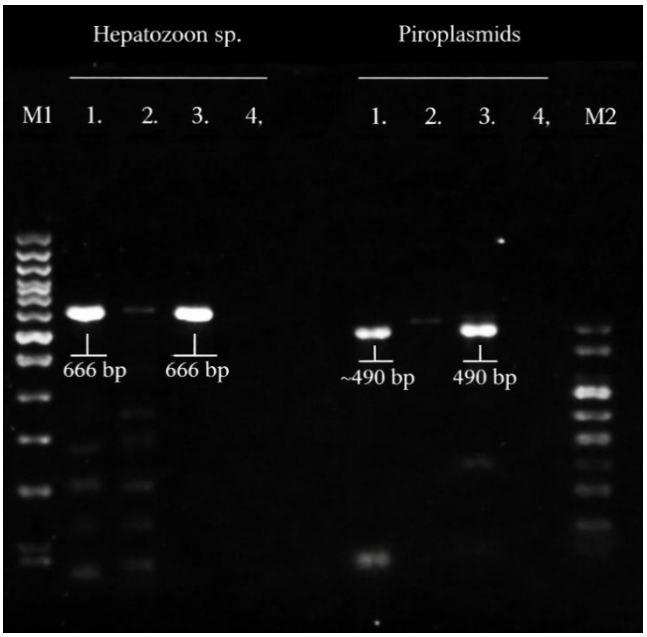

Figure 3: 18s rRNA PCR-gel of Hepatozoon and piroplasmid infection in dog blood samples. Lane M1; ELK 100 bp DNA ladders (ELK biotechnology), Lane 1; dog1 sample, Lane 2; dog2 sample, Lane 3; dog3 sample, Lane 4; negative control (nuclease-free water (NFW)). The expected amplicon size in Hepatozoon PCR is 666 bp, and nearly 490 bp in piroplasmid PCR.

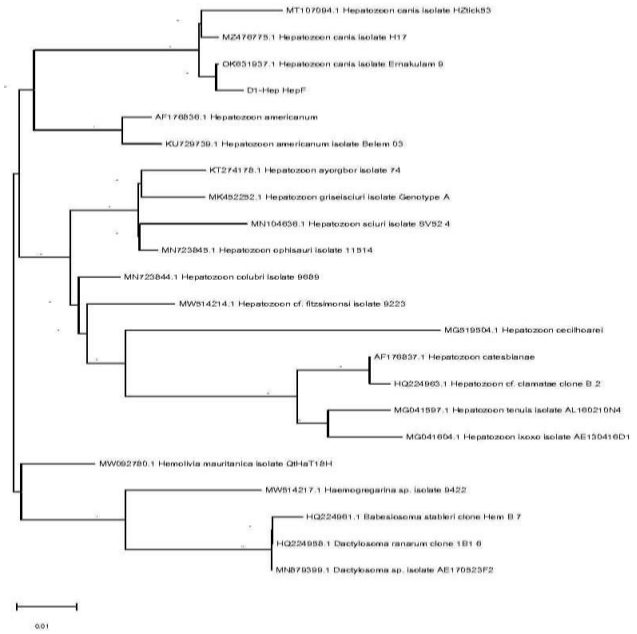

Figure 4: Neighbor-joining (NJ) phylogenetic tree for Hepatozoon and piroplasmid 18s rRNA gene. Phylogenetic trees were constructed using the Kimura 2-parameter model with 1000 bootstraps and the maximum composite likelihood method. (a) Hepatozoon phylogenetic tree. Reference isolates taken from the BLAST-NCBI database were included.

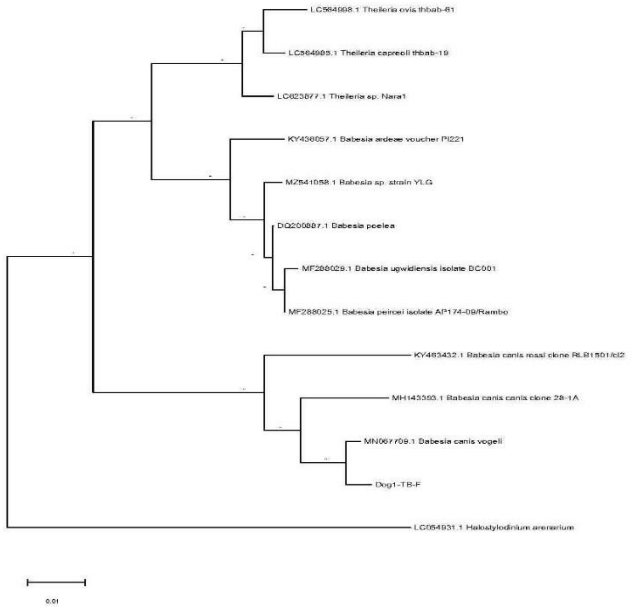

Figure 5: Neighbor-joining (NJ) phylogenetic tree for Hepatozoon and piroplasmid 18s rRNA gene. Phylogenetic trees were constructed using the Kimura 2-parameter model with 1000 bootstraps and the maximum composite likelihood method. (b) piroplasmid phylogenetic tree. Reference isolates taken from the BLAST-NCBI database were included.

Discussion

This study provides preliminary evidence that clinical cases of canine hepatozoonosis are present in Jordan. The occurrence of co-infection with *Babesia canis* in the studied dogs may be explained by shared transmission routes, particularly via tick vectors such as *Rhipicephalus sanguineus*, in addition to factors such as inadequate tick control, close contact in breeding facilities, and the importation of dogs from endemic regions, all of which may increase exposure to multiple tick-borne pathogens. The identification of *B. canis* co-infection in four dogs suggests that such dual infections may exacerbate clinical signs and complicate diagnosis and treatment. To the best of the authors' knowledge, this is the first documented report in Jordan of clinical H. canis infection with concurrent B. canis co-infection, along with associated clinical and hematological findings. The hematological abnormalities observed, including mild anemia, leukocytosis, and occasional thrombocytopenia, reflected the systemic impact of these infections and supported the clinical observations of lethargy, pale mucous membranes, and weakness. The high phylogenetic similarity observed among *Hepatozoon canis* isolates is likely due to their close evolutionary relationship, indicating that these isolates share a common ancestor and exhibit limited genetic divergence, which is consistent with the conserved nature of the 18S rRNA gene used for molecular analysis.

Similar studies in the region have reported related findings. For instance, a study in northern Jordan conducted on 45 dog carcasses revealed *H. canis* in 28.9% of examined DNA samples (10). Moreover, two other hemopathogens, *Anaplasma phagocytophilum* and piroplasmid DNA, were detected in 39% and 7.9% of the samples, respectively (10). In Palestine, a molecular study detected *H. canis* in *Rhipicephalus sanguineus* ticks, with a significant association between the pathogen and ticks collected from dogs (11). The same study detected other vector-borne pathogens—*Theileria ovis*, *Babesia ovis*, and *B. vogeli*—in 5.4%, 0.6%, and 0.4% of examined ticks, respectively (11).

These findings align with the present study and highlight the importance of considering multiple vector-borne pathogens in the clinical assessment of dogs in this geographic area. In contrast to those studies, which relied solely on molecular detection (10,11), the current study combined clinical, hematological, and molecular approaches. The difference in detection rates at the molecular level between *H. canis* and *B. canis vogeli* in this study reflects both biological differences between the two pathogens and limitations of conventional PCR methodology. *H. canis* gamonts persistently circulate within neutrophils, keeping detectable parasitemia for long periods, thus allowing consistent amplification by the primer set HepF/HepR that targets the 18S rRNA gene (5,6). On the other hand, *B. canis vogeli* has cyclic parasitemia with numbers of piroplasms often fluctuating below the detection thresholds for both microscopy and conventional PCR (12,13). Although effective for broad piroplasmid detection (8), TB-F/TB-R primers may have reduced sensitivity when parasitemia is low. Therefore, PCR confirmed Babesia infection only in those four dogs that had microscopically visible piroplasms consistent with previous observations that conventional PCR would not pick up subpatent infections unless nested or real-time approaches were applied (14). This clearly shows how microscopy and PCR complement each other in diagnosing hemoparasite co-infections.

Unlike previous studies that primarily sampled non-clinical or deceased stray dogs, the subjects in this study were client-owned dogs undergoing health assessments at private veterinary clinics in Jordan. Although these dogs were pets, they could still have been exposed to ticks through outdoor activities such as walks, visits to parks, or contact with other animals infested with ticks. Tick exposure in Jordan is not limited to stray dogs, as ticks are common in the environment, particularly in areas with vegetation or where other domestic or stray animals are present. Therefore, even well-cared-for pets can acquire ticks and subsequently become infected with tick-borne pathogens such as H. canis and B. canis.

The concurrent occurrence of canine hepatozoonosis and *B. canis* infection represents a growing concern, adding complexity to diagnosis and treatment, increasing the severity of clinical signs, and introducing possible drug interactions. Co-infection can worsen pathogenicity in the host (7), and treatment often requires combination therapy.

Co-infections can modify the host immune response, making recovery more complex, particularly in endemic regions (8). Co-infections are defined as simultaneous infection of the same host by two or more different, and sometimes unrelated, pathogens that elicit heightened clinical signs, diagnostic challenges, and treatment difficulties (15). These pathogens not only share epidemiological niches but can also influence one another's clinical course, with evidence suggesting synergistic pathogenicity that worsens overall prognosis (16).

Clinically, canine hepatozoonosis can range from subclinical to overt forms depending on infective dose, underlying disease, or immune suppression (17). Most cases are subclinical or show mild signs such as lethargy and anorexia (18). Severe infections manifest as fever, weight loss, and myalgia/myositis due to parasite localization in muscle tissue (18). Advanced cases may cause marked anemia and leukocytosis due to periosteal reactions observable radiographically (2,6). The clinical signs observed in our 15 cases (lethargy, weakness, anorexia, vomiting, fever, and pale mucous membranes) were consistent with previous reports from endemic regions (19,20).

Transmission of *H. canis* occurs not through direct contact or tick bites, but through ingestion of infected ticks, most commonly *R. sanguineus* (21). In contrast, *B. canis* is transmitted through bites of infected ticks such as *Dermacentor reticulatus* and occasionally *Rhipicephalus* species (22). Dogs may also contract infection through ingestion of infected ticks or prey animals (23). These vectors are widespread in the Middle East, where climate and the abundance of stray dogs perpetuate tick populations (10,24-26).

The ecological association of these pathogens with their vectors underscores the need for effective tick control and continued surveillance of tick-borne pathogens in Jordan.

This study has some limitations. The geographical scope was restricted to cases presented at private clinics, which may affect the observed dynamics of vector–pathogen interaction and co-infection prevalence. Additionally, the 15 cases described here were identified from a larger screening of 200 samples, providing a snapshot of positivity but not a true prevalence rate. The scarcity of data on *H. canis* and *B. canis* vector distribution across Jordan limits generalizability. Therefore, further broader studies encompassing diverse regions of Jordan are warranted.

Conclusion

In Jordan, hepatozoonosis in canids is present and should be included in the differential diagnosis of dogs presenting with compatible clinical signs, particularly those with tick exposure. Clinical severity may be exacerbated by co-infection with *B. canis*, warranting a more comprehensive diagnostic and therapeutic approach. This study underscores that a complete diagnostic workup for tick-borne illnesses must include blood film examination, as it remains the only method for visually identifying specific hemoparasites such as *Hepatozoon* and *Babesia*, which are undetectable by automated hematology analyzers. Additionally, PCR results provided confirmatory evidence for the presence of these hemoparasites in the reported cases, reinforcing the importance of integrating molecular diagnostics with conventional microscopy for accurate diagnosis

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Availability of Data and Materials

All data supporting this study are included in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authorship Contribution Statement

Raida Al-Rukibat: Writing – review and editing, writing – original draft, methodology. Wael Hananeh: Writing – review, methodology. Rami Mukbel: Methodology.

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داء الهيباتوزون الكلبي وبابيزيا كانيس لدى الكلاب في الأردن: أول دراسة لحالات الإصابة المشتركة

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الخلاصة

داء الهيباتوزون الكلبي هو مرض طفيلي مهم ينقله القراد ويؤثر بشكل متزايد على صحة الكلاب، مما يجعله ذا أهمية متنامية في الطب البيطري. يسببه أساسًا الطفيلي *Hepatozoon canis*، وتحدث العدوى على عكس معظم الأمراض المنقولة بالقراد عن طريق ابتلاع القراد المصاب. تستعرض هذه الدراسة ١٥ حالة سريرية في الأردن، من بينها ٤ حالات بعدوى مشتركة مع *Babesia canis vogeli*، مما يبرز التحديات التشخيصية المرتبطة بالعدوى المزدوجة بالطفيليات الدموية. تم التشخيص اعتمادًا على العلامات السريرية، والتغيرات الدموية، والفحص المجهري لمسحات الدم التي أظهرت الأشكال المميزة داخل الخلايا المتعادلة، إضافة إلى تأكيد النتائج باستخدام تقنية PCR باستهداف جين ١٨S rRNA، حيث بلغ حجم نواتج التضخيم نحو ~٦٦٠ زوج قاعدي لـ *H. canis* و~٤٩٠ زوج قاعدي لـ *B. canis vogeli*. أظهر التحليل الوراثي التطوري أن التسلسلات الناتجة تتطابق مع السلالات المرجعية المعروفة. وقد تم الكشف عن هذه الحالات خلال فحص ٢٠٠ عينة دم لكلاب على مدى عام، حيث كانت ١٥ عينة إيجابية لطفيلي دموي واحد أو أكثر. تسلط النتائج الضوء على المظاهر السريرية والتغيرات الدموية والتعقيدات التشخيصية، إضافة إلى تأثير العدوى المشتركة على شدة المرض والتكهن به. ورغم تسجيل *H. canis* سابقًا في الأردن، تُعد هذه الدراسة أول توثيق للعدوى المشتركة مع *B. canis vogeli*. وتسهم هذه النتائج في تحسين فهم وبائيات وتشخيص وإدارة هذه العدوى في الكلاب في الأردن.