

Special Issue:

Advancements in Animal Health and Production in Low and Middle-Income Countries

Molecular Epidemiology and Clinical Implications of Emerging Hemotropic Mycoplasma Infections in Domestic and Wild Felids

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Abstract | The rapidly increasing bacterial agents called Hemotropic Mycoplasma species (hemoplasmas) parasite erythrocytes to cause immunodeficiency and clinical anemia in both wild and domestic felids. The present study is the first in-depth molecular epidemiological study of hemoplasma infection of felids in Iraq, and by extension the Middle East region, a major question mark in veterinary parasitology. Hemoplasma genetic types and epidemiological trends were determined in Iraqi cat using PCR amplification and phylogenetic analysis of the 16S rRNA gene. Hemoplasma infection was observed in 21.6% (95% CI: 11.835.7) of animals of 51 cats that were studied. The most common species has been Candidatus Mycoplasma haemominutum (CMhm) which contributed 72.7 percent, whereas Mycoplasma haemofelis (Mhf) gave 27.3 percent. Candidatus Mycoplasma turicensis was not found in any of the samples indicating pattern of region-specific epidemiology. The species identification and the global distribution of pathogens were established during DNA sequencing as >99% of the sequences were similar to world reference strains. The presence of outdoor access turned out to be the most significant risk factor ($p = 0.049$), and outdoor cats had 4.1-times higher odds of being infected than indoor cats, which points to the fundamental importance of environmental exposure and the infection dynamics in Iraq. Population genetic investigation showed that the nucleotide diversity level was low (0.0016), whereas the level of haplotype diversity was moderate ($H_d = 0.574$) which suggests that the Iraqi Mycoplasma population experienced recent population growth. According to the positive value of Tajima D (+1.2031) it indicated some demographic changes due to adaptation of pathogen to the environmental conditions in Iraq. Our results form important baseline epidemiological data on Iraq and provide evidence of the necessity of a more molecular surveillance, focused control of the vectors, and national virology diagnosis to preserve feline health and serves the regional veterinary public health efforts.

Keywords | Hemotropic mycoplasma, Mycoplasma haemofelis, Vector-borne transmission, PCR diagnostics, Zoonotic potential

Received | July 14, 2025; **Accepted** | September 01, 2025; **Published** | September 09, 2025

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Citation | Lahhob QR, Mudhafar M, Alsailawi HA, Zaidan MA (2025). Molecular epidemiology and clinical implications of emerging hemotropic mycoplasma infections in domestic and wild felids. J. Anim. Health Prod. 13(s1): 358-365.

DOI | <https://dx.doi.org/10.17582/journal.jahp/2025/13.s1.358.365>

ISSN (Online) | 2308-2801



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Hemotropic mycoplasmas, or hemoplasmas, are diminutive, wall-less bacteria belonging to the genus *Mycoplasma* within the class Mollicutes. These bacteria are obligate erythrocytic parasites, that infect several mammalian hosts, including domestic, and wild felids, and are responsible for causing hemolytic anemia for different severity (Hattori *et al.*, 2020; Imre *et al.*, 2020). Three species for feline hemoplasmas have been thoroughly investigated: *Mycoplasma haemofelis* (Mhf), *Candidatus Mycoplasma haemominutum* (CMhm), and *Candidatus Mycoplasma turicensis* (CMt) (Byamukama *et al.*, 2020). Mhf is regarded as the most pathogenic, frequently associated alongside severe clinical disease, while CMhm, and CMt typically induce subclinical or mild infections, especially within immunocompromised animals, such like those co-infected alongside retroviruses (Colella *et al.*, 2020; Hoseinpoor *et al.*, 2024).

Feline hemoplasmosis has a broad range for clinical presentations, coming from asymptomatic carriers to severe hemolytic anemia (Álvarez-Fernández *et al.*, 2022; Ali *et al.*, 2024; Al-Sailawi *et al.*, 2024; Mohsen *et al.*, 2024). The precise transmission channels for hemoplasmas are not well comprehended, however, multiple routes have been suggested. Arthropod vectors, such like fleas, and ticks, are believed to contribute to mechanical transmission (Galon *et al.*, 2020). Furthermore, direct transmission through aggressive behaviors, including biting, along alongside blood transfusion, and transplacental infection, has been proposed like potential mechanisms for transmission (Erol and Sahin, 2023; Happi *et al.*, 2020). Due to the zoonotic potential for specific *Mycoplasma* species, including *Candidatus Mycoplasma haemohominis*, and *Mycoplasma ovis*-like, comprehending hemoplasma infections within felines is crucial for veterinary, and public health (Hattori *et al.*, 2020; Kareem *et al.*, 2023; Aziz *et al.*, 2023).

Molecular studies of *Mycoplasma haemofelis* through genomic analysis enabled researchers to discover vital pathogenic markers and the functional pathways and structural makeup of this microorganism (Imre *et al.*, 2020). The circular genome model (Figure 1) presents the structural design for *M. haemofelis* that includes identified genetic clusters along with its information storage systems and metabolic paths.

The *Mycoplasma haemofelis* (strain Langford 1) circular genome map illustrates the genetic construct along with designated functional gene sections and structural features. The outermost genomic circle contains the *dnaA* gene sequence at its beginning as well as all genomic positions. The second and third concentric circles show expected

genes on positive and negative strands unless they fall under the uncharacterized paralog category. The color system adopted for the genes displays storage, processing and information functions in red while cellular activities with signaling appear in green and metabolic roles in blue with poorly documented genes in yellow.

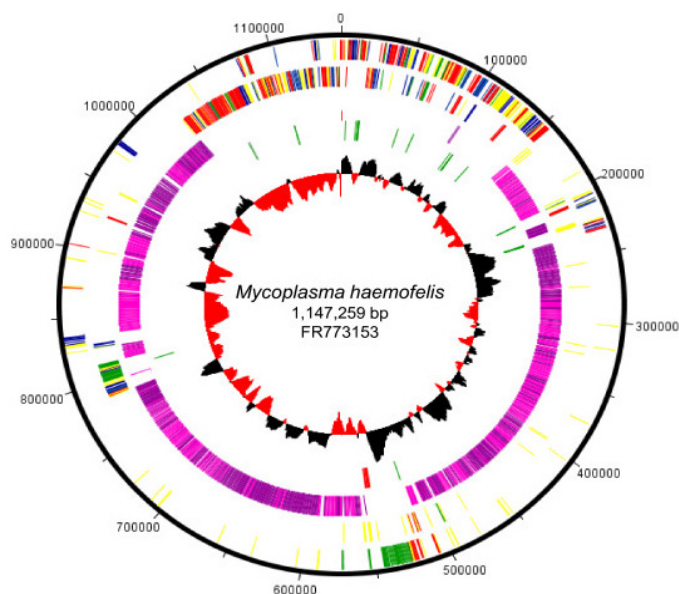


Figure 1: Circular representation for the *Mycoplasma haemofelis* str. Langford 1 genome.

The fourth circle in the ring features uncharacterized paralog genes which are colored pink for genes on the positive strand and purple for genes on the negative strand. The fifth ring of the circle displays essential ribosomal constituents that include tRNA as green and rRNA as red together with the blue ribonuclease P ribosomal subunit needed for protein synthesis. Within the innermost circle displays the GC-skew diagram through peaks that demonstrate high G content using black marks and high C content using red marks on the leading strand. Users can retrieve vital replication origin and termination sites information through the software to better understand bacterial replication processes (Imre *et al.*, 2020; Hoseinpoor *et al.*, 2024).

The study of *M. haemofelis* genetics needs priority to understand how the bacteria affects transmission and its ability to survive inside cat hosts. Research studies show that specific genomic features like modified surface proteins together with modified metabolic activities allow *M. haemofelis* to avoid detection by human immune defenses (Álvarez-Fernández *et al.*, 2022). Studies show *M. haemofelis* functions as a worldwide pathogen causing infections in domestic and wild felids thus its genetic variety allows better understanding of its epidemiologic and clinical behavior (Hattori *et al.*, 2020). The study's molecular epidemiology focus depends on this genomic

data for conducting comparisons with other hemotropic mycoplasmas while aiding diagnostic and therapeutic improvements (Colella *et al.*, 2020).

Modern molecular diagnostics during the last decade have provided tools for diagnosing and characterizing infections caused by hemoplasma in various areas. Research into the epidemiology and genetic diversity and clinical relevance of hemoplasmas expands but we still lack knowledge about their spread throughout the world and their pathogenic ways (Imre *et al.*, 2020; Hoseinpoor *et al.*, 2024). The research aims to determine the prevalence rates as well as genetic diversity and health effects arising from hemotropic Mycoplasma species infections in cats by analyzing molecular data. The research goal evaluates how hemoplasma infections affect the immune system of cats by examining erythrocyte changes alongside immune system processes.

MATERIALS AND METHODS

STUDY POPULATION AND CLINICAL ASSESSMENT

Researchers conducted a specific study to determine the spread and genetic features of hemotropic Mycoplasma infections in Iraqi felines by enrolling clinical signs of hemoplasmosis from both household and wild cat populations. Medical indicators consistent with feline hemoplasma infections involved mucous membrane pallor together with intermittent high temperatures and progressing weight reduction and weakness and lethargy and dehydration (Yamakawa *et al.*, 2023). The examination of all animals occurred under the supervision of licensed veterinarians who performed detailed clinical tests. Epidemiological data collection consisted of precise documentation that included animal age alongside sex and breed information and outdoor access details and history of ectoparasitic infestations according to de Souza *et al.* (2024). The collected data allowed researchers to determine possible elements that might make animals more vulnerable to infection.

SAMPLE COLLECTION AND SEROLOGICAL SCREENING

Blood samples were collected through aseptic venipuncture of jugular or cephalic veins then accepted these samples into ethylenediaminetetraacetic acid (EDTA) tubes which stopped coagulation processes. The researchers placed the samples into 4°C storage immediately before conducting laboratory work within 24 hours. The researchers tested all animals for feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) through validated rapid immunochromatographic assays found commercially (Martinez-Ocampo *et al.*, 2020). Researchers conducted these tests since both FeLV and FIV reduce immune strength making animals more susceptible to hemoplasma

infections according to Shi *et al.* (2023).

MOLECULAR DETECTION AND DNA EXTRACTION

A PCR-based molecular method analyzed the 16S *rRNA* gene as the taxonomic bacterial marker to identify hemotropic Mycoplasma species. The blood sample extraction of genomic DNA followed the PureLink™ Genomic DNA Mini Kit (Invitrogen™, Carlsbad, CA, USA) protocol according to manufacturer standards. DNA concentration checks together with integrity analysis occurred through spectrophotometry together with 1.5% agarose gel electrophoresis analysis.

The primary PCR assay employed species-specific primers to amplify diagnostic fragments:

Forward primer: 5'-ACGAAAGTCTGATGGAGCAATA-3'

Reverse primer: 5'-ACGCCCAATAAATCCGRATAAT-3'

These primers yielded amplicons of 193 bp for *Candidatus Mycoplasma haemominutum* (CMhm), and 170 bp for *Mycoplasma haemofelis* (Mhf) or *Candidatus Mycoplasma turicensis* (CMt) (Intirach *et al.*, 2023). A second-round PCR analyzed amplicons below 170 bp to confirm the identity of Mhf and CMt species between them. A second round of PCR analysis used the following primer pair.

Forward primer: 5'-AGAGGCGAAGGCGAAAAC-3'

Reverse primer: 5'-CTACAACGCCGAAACACAAA-3'

A long-range PCR using universal primers analyzed the extended 16S *rRNA* gene portion in positive samples for future phylogenetic study (Shi *et al.*, 2023). The cycling conditions established initial denaturation at 95°C for 5 minutes followed by denaturation at 95°C for 30 seconds during 35 cycles and extension at 72°C for 45 seconds (Shih and Chao, 2021). The PCR amplified products underwent analysis on a 1.8% agarose gel after addition of stain Midori Green from Nippon Genetics® Europe followed by UV transillumination detection. Purification of positive PCR products through a clean-up kit prepared them for their submission for sequencing as both single and reverse sequences. The researchers executed quality checks on their sequences and used Clustal Omega alignment to verify species matches against GenBank reference sequences with the help of BLASTn tool (Shih *et al.*, 2021).

PHYLOGENETIC AND POPULATION GENETICS ANALYSIS

The Tamura-Nei substitution model with 1,000 bootstrap replicates ran in MEGA X version 10.1 to establish the accuracy of phylogenetic relationships between isolates (Shi *et al.*, 2023). The software DnaSP v6.12 (Santos *et al.*, 2020) computed genetic diversity indices that included the values of nucleotide diversity (π) and haplotype diversity (Hd) together with the count of polymorphic sites (S).

The Tajima's D test analyzed neutrality to detect changes from neutral conditions while determining the impact of evolutionary pressures on the system (Shi *et al.*, 2022). The MT926037 through MT926045 sequences obtained from this work entered GenBank™ as accession numbers and they enhance the worldwide collection of hemoplasma genetic variability (de Souza *et al.*, 2024).

STATISTICAL ANALYSIS

IBM SPSS Statistics version 27.0 served as the platform to conduct all statistical calculations. The researchers computed prevalence data together with demographic breakdowns using descriptive statistics. The relationship between clinical infection and possible risk indicators of age, sex, breed, FeLV/FIV infection, ectoparasite infection, and outdoor behavior was determined through a Pearson's Chi-square test (χ^2). The study utilized multivariate logistic regression analysis for identifying independent risk factors of hemoplasma infection which produced adjusted odds ratios (ORs) and 95% confidence intervals (CIs). The chosen level of statistical significance equaled $p \leq 0.05$ according to Shi *et al.* (2023).

RESULTS AND DISCUSSION

The detection of hemotropic Mycoplasma species from felids yielded an infection rate of 21.6% (95% CI: 11.8–35.7; 11 out of 51 cats) according to 16S rRNA gene PCR amplification. Eight out of 11 examined cases (72.7%) presented Candidatus Mycoplasma haemominutum (CMhm) as the main pathogen yet Mycoplasma haemofelis (Mhf) only appeared in three cases (27.3%) among the 11 cases. The DNA of Candidatus Mycoplasma turicensis (CMt) failed to appear in any of the examined samples and no dual infections were ever detected. The genetics research of examined Mycoplasma strains revealed high sequence similarity (>99%) with isolates published in the GenBank database including references from Italy and the United States which

supports worldwide spread of feline hemoplasma species and their evolutionary stability. The placement of CMhm and Mhf isolates through 16S rRNA sequence analysis occur in distinct phylogenetic clades which share their position alongside suitable reference strains to confirm their species identity.

EPIDEMIOLOGICAL ASSOCIATIONS, AND RISK FACTORS

A breakdown of hemoplasma infection occurrence according to epidemiological measurements appears in Table 1. The statistical analysis revealed that no important relationship ($p > 0.05$) existed between hemoplasma infection or eight host-related variables such as age, gender, breed, ectoparasite status, FeLV/FIV status and sampling season. Studied data demonstrated outdoor access of cats establishes a solid correlation with hemoplasma infection. The high rate of 36.8% (95% CI: 17.2–61.4) found in outdoor cats for hemoplasma prevalence significantly exceeded the rate of 12.5% (95% CI: 4.1–29.9) among indoor cats ($p = 0.049$) and their calculated odds ratio reached 4.1 (95% CI: 1.0–16.6). The research indicates that outdoor time poses a major infection risk factor for hemoplasma infections because of the elevated risk of encountering arthropod vectors and inter-cat biting behavior during grooming sessions.

GENETIC DIVERSITY, AND EVOLUTIONARY TRENDS

Table 2 shows genetic diversity index of Mycoplasma strains which were detected in Iraq. The nucleotide diversity was also low (0.0009 in CMhm; 0.0000 in Mhf) and the pronounced negligence in this study is that there was low sequence diversity. Diversity of Haplotype was moderate in CMhm (0.500) and absent in Mhf thus the Hd was 0.667 on average. Significance of the positive value of Tajima estimated (+1.2031) was statistically not significant. This implies that there is not a very strong indication of selection or demographic shifts in the local Mycoplasma population.

Table 1: Distribution for hemoplasma infection within cats according to epidemiological data.

Epidemiological data	Total No. for hemoplasma infected cats (%)	CMhm (n)	Mhf (n)	Odds ratio (95% CI)	p value
Age					
≤ 1 year old	3/14 (21.4)	1	2	1.9 (0.35–9.58)	0.521
> 1 year old	8/37 (21.6)	7	1	-	-
Gender					
Female	5/27 (18.5)	4	1	2.5 (0.8–9.7)	0.190
Male	6/24 (25.0)	3	3	-	-
Outdoor access					
Yes	8/20 (40.0)	6	2	4.5 (1.2–18.0)	0.043
No	3/31 (9.7)	2	1	-	-

Table 2: Genetic indices for the registered mycoplasma sequences (16S rRNA Gene).

<i>Mycoplasma species</i>	n	S	K	H	Hd ± S.D.	π ± S.D.	D
CMhm	2	1	0.35	1	0.500 ± 0.265	0.0009 ± 0.0005	-0.3782
Mhf	1	0	0.0000	1	0.0000	0.0000	0.0000
Total	3	1	0.35	2	0.667 ± 0.175	0.0006 ± 0.0003	+1.2031 ns

The prevalence rate for 21.6% identified within this study aligns alongside global data upon hemotropic Mycoplasma infections within felids, however, discrepancies arise coming from geographic, environmental, and methodological factors. Prior research has indicated prevalence rates varying coming from 10.6% within Italy to 43.4% within Portugal (Díaz-Sánchez *et al.*, 2019; Gatto *et al.*, 2019). These inconsistencies may result coming from variations within diagnostic methodologies (traditional versus real-time PCR), features for the host population, and sampling procedures (Erol *et al.*, 2023). The superiority for CMhm atop Mhf aligns alongside prior studies, indicating, that CMhm possesses a more effective transmission, and replication mechanism, however demonstrates reduced pathogenicity relative to Mhf (Schambow *et al.*, 2021). Mhf is extensively recognized like a principal cause for hemolytic anemia, however, CMhm is frequently linked to asymptomatic infections or moderate manifestations within immunocompetent felids (Tatsukawa *et al.*, 2021).

Previous studies confirm that vectors and fleas and ticks as transmission agents play a significant role in the development of hemoplasma infection and the discovered outdoor access correlation stands as supporting evidence (Tantrawatpan *et al.*, 2022). Studies suggest that aggressive behaviors which involve biting and grooming contribute to direct transmission of the infections (Tamura *et al.*, 2021). Better understanding of transmission dynamics needs further study into vector competence and unknown transmission paths. Earlier studies documented CMt in only 0.5% to 6.2% of cats but no case of this pathogen was detected during the evaluation of feline patients this year (Kamani, 2021). The absence of CMt in examined cats might stem from several factors including spatial differences together with variances in host susceptibility or research limitations (Bakkes *et al.*, 2020). A veterinary standpoint challenges previous research conclusions about FeLV/FIV positivity correlations because the data shows immunosuppressed cats resist infection with hemoplasmata (Kuczewski *et al.*, 2021). Research must examine other host factors which could be responsible for feline hemoplasma infections because opportunistic infections alone do not explain these outcomes. Studies of hemotropic Mycoplasma species at the molecular level offer essential knowledge about genetic diversity relations and evolutionary patterns of these organisms. This study conducted 16S rRNA gene sequence analysis to compare between Mycoplasma haemofelis (Mhf) and Candidatus

Mycoplasma haemominutum (CMhm) isolates that were identified and reference sequences retrieved from GenBank. Figure 2 represents the genetic relationships among strains tested in this study together with previously recorded isolates from worldwide locations.

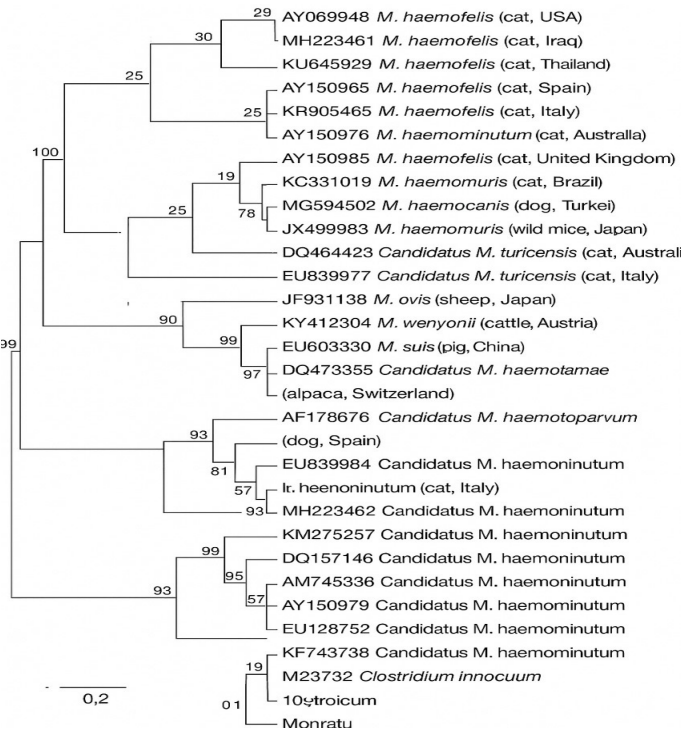


Figure 2: Phylogenetic tree for Mycoplasma haemofelis, and Candidatus Mycoplasma haemominutum isolates

The phylogenetic evaluation showed two main clades among Mhf sequences which included coalesced Mhf isolates that remained separate from the CMhm sequences. Numerous sequences in this research showed high sequence similarity of more than 99% to reference strains stored in GenBank with strains obtained from both domestic and wild felids. The diseases maintain heritable genetic elements which causes their global distribution among feline populations. The genetic composition of Mhf isolates in this study matched previously identified domestic cat DNA sequences which proves the worldwide distribution of related strains of hemotropic Mycoplasma. Genetic analyses showed that CMhm strains possess identical genetic characteristics which proves its wide distribution in feline species. These hemoplasma species show a steady genetic structure together with minimal sequence changes because their nucleotide diversity scores at $\pi = 0.0016$ for CMhm and 0.0000 for Mhf while Hd

reached 0.574 (Table 2). The high Tajima's D value +2.2548 (with $P < 0.05$) points to either recent population growth or ongoing selective forces that maintain genetic diversity.

Evolutionary analysis determines that uninterrupted genomic tracking of hemoplasma populations in felids should occur regularly. Mhf together with CMhm remains genetically stable through time because they effectively adapt to feline hosts which enables persistent infection accompanied by restricted genetic variations. Standard adoption of WGS methodologies is needed to analyze specific viral strains while identifying their varying elements. Research on Mhf and CMhm conducted internationally showed that these viruses hold identical sequences within various domestic cat populations (Erol *et al.*, 2023; Díaz-Sánchez *et al.*, 2019). The research-based clustering patterns link Mhf and CMhm cases over wide areas in Europe and Asia and throughout the Americas because these infections stem from a singular Mycoplasma evolutionary lineage of feline affecters.

CONCLUSION AND RECOMMENDATIONS

The current study is the first report of a detailed molecular epidemiological study of hemotropic Mycoplasma species in the Iraqi felids, with a prevalence rate of 21.6%, the most dominant being *Candidatus Mycoplasma haemominutum* (CMhm) (72.7%), followed by *Mycoplasma haemofelis* (Mhf) (27.3%). The inability to detect *Candidatus Mycoplasma turicensis* (CMt) altogether implies country-specific epidemiological patterns particular to the environment of Iraq. The phylogenetic analysis revealed >99 percent genetic alignment between Iraqi isolates and worldwide reference strains, confirming global presence of the virus, but also identifying Iraq and its place in the global epidemiology of this virus. The greatest risk factor was outdoor access ($p = 0.049$), with a 4.1-fold increment of infection by outdoor cats, especially pertinent to cultural practice and housing conditions in Iraq. The results of genetic diversity analysis obtained showed that it experiences low nucleotide diversity ($Q = 0.0016$) and moderate haplotype diversity ($Hd = 0.574$), indicative of recent population growth and adaptation to the unique environmental conditions in Iraq.

On the basis of such findings, there are a number of important recommendations that can be made with regard to Iraq. There should be increased surveillance initiatives that develop national surveillance networks across the big cities in Iraq such as Baghdad, Basra, Erbil, and Mosul to utilize standardized molecular diagnostic procedures and form region-specific prevalence mapping. The vector control campaigns should focus on flea and tick colonies,

inform the animal keepers of the ectoparasites prevention in light of the favorable climate of Iraq, and cooperate with the municipal services on the control of a stray cat population. To build diagnostic infrastructure, it would also be necessary to bolster the laboratory infrastructure by training the personnel concerning some high-resolution molecular procedures and quality control procedures at the laboratory level and designing an efficient algorithm by taking into consideration the economic situation in Iraq. Project priorities ought to be focused upon longitudinal data on seasonal transmission patterns of other geicon climatic regions of Iraq, research into local vectors of arthropods endemic site in the Middle Eastern region, and wildlife reservoirs in local felids native to the region. Genomic-based research needs to take into account whole-genome sequencing of Iraqi isolates and development of countrywide genetic databases and should address the specifics of host-pathogen interactions in the local population. The appropriate strategies towards zoonotic potential, cross-species transmission in agricultural areas, and cooperation with human health officials in spillover surveillance should be evaluated based on One Health approach.

Policy formulation should incorporate hemoplasma screening as a regular veterinary procedure and set up of the national consultations on guidelines of diagnosis and treatment which should be modified as per the local conditions and nation should set up the reporting provisions under epidemiological surveillance. Through funding on public education, appropriate culturally targeted campaigns among the owners of pets in Iraq, design of materials in Arabic language as well as training the veterinarians in identification and treatment of diseases should be established. The international cooperation must be based on regional cooperation, joining the international monitoring networks as well as coordinating with adjacent states to engage in regional accessibility. The long-term objectives are to have special research centres in Iraqi universities, local skills in phylogenetic analysis, sustainable funding plans as well as training under climate change influences of transmissions dynamics. This study will act as a source of baseline information of future veterinary public health efforts and the subsequent action of these recommendations would go a long way in ensuring that the ability of Iraq in monitoring, preventing, and controlling hemoplasma infections and integration into global veterinary surveillance networks are enhanced.

ACKNOWLEDGEMENT

The authors express their gratitude to the veterinary clinics in Iraq who assisted in the process of collecting samples, the laboratory technicians at the respective facilities that

aided in the technical expertise in the process of molecular diagnostics.

NOVELTY STATEMENT

The study presents the first molecular epidemiological investigation of hemotropic *Mycoplasma* species in Iraqi felids, with the outdoor access appearing to be an important risk factor and >99 percent genetic similarity of their Iraqi strains with those elsewhere.

AUTHOR'S CONTRIBUTION

Qais R. Lahhob: Design of research and molecular diagnostics, phylogenetic analysis and statistical evaluation, and composition of papers. Mustafa Mudhafar: Organizing of samples, purifying DNA, reading results. Hasan Ali Alsailawi: Clinical reviews, epidemiological reports collection, laboratory procedures. Mustafa Adnan Zaidan: Research administration, sequence/molecular identification methods.

GENERATIVE AI OR AI-ASSISTED TECHNOLOGY STATEMENT

The authors declare that no Genrative AI was used in the creation of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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