



First Serological and Molecular Phylogenetic Confirmation of Feline Leishmaniasis in Iraq

Amal Hassan Abed¹, Abbas H.K. Sray², Haider H. Alsaedi²,
Israa M. Essa³, Hasanain A.J. Gharban⁴

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ABSTRACT

Background: Leishmaniasis, caused by different etiological *Leishmania* species such as *L. infantum*, represents an important zoonotic disease of various potential hosts as cat whose roles are largely unclear. This present study aims to serological investigation of leishmaniasis and molecular phylogeny of *L. infantum*.

Materials: Blood samples were collected from totally 91 adult pet cats in Wasit province (Iraq) and examined serologically by the qualitative ELISA, molecularly through PCR and then, the positive DNAs were sequenced and analysed phylogenetically.

Result: Findings of serological ELISA revealed that 15.38% of study cats were positive to leishmaniasis at a cut off value of ≥ 0.230 . Respectively, the OD values of seropositively and seronegatively samples were 0.369857 ± 0.025774 and 0.172896 ± 0.003906 , respectively. Targeting the *ITS1* and *5.8S rRNA* gene, molecular PCR reported that 9.89% of study cats were infected with *L. infantum*. Significant prevalence of leishmaniasis was significantly higher by ELISA than PCR with a level of sensitivity and specificity being 100% and 93.9% respectively. Phylogenetically, the study *L. infantum* isolates were documented in the NCBI-GenBank database (PQ327040.1, PQ327041.1, PQ327042.1, PQ327043.1, PQ327044.1, PQ327045.1, PQ327046.1, PQ327047.1 and PQ327048.1) and comparative analysis with the NCBI-BLAST *L. infantum* isolates detected the marked significant identity with the Iranian (ID: KF705514.1) and Brazilian (ID: MH231229.1) isolates obtained from dogs.

Key words: Cat parasitic diseases, *Felis catus*, *Leishmania infantum*, Neglected tropical diseases (Ntds), Visceral leishmaniasis.

INTRODUCTION

Leishmaniasis is a protozoan parasitic disease caused by the genus *Leishmania* that belongs to Trypanosomatidae family in Kinetoplastea class of Euglenozoa phylum (Silveira *et al.*, 2025). Globally, more than thirty *Leishmania* species are endemic in nearly 100 countries in Asia, Africa and America (Lorenzetti *et al.*, 2026). Predominantly, *Leishmania* is acquired throughout infected phlebotomine sandflies that transmit parasitic forms to mammalian hosts; but rarely by alternative routes as congenital, blood transfusion, organ transplantation and laboratory accidents (Shaw, 2025). However, the lifecycle encompasses two main forms are flagellated promastigote that thrives midgut of invertebrate vector and aflagellated amastigote that proliferates within the phagolysosomes of vertebrate (mammalian) macrophages (Arone-Farfán *et al.*, 2024; Liévin-Le and Loiseau, 2016).

In tropical and subtropical areas where the incidence is elevated continuously, *Leishmania* infections present a significant public health challenge (Pal *et al.*, 2022). This directly influenced by accurate detection of *Leishmania* species and active prevention/treatment (Abbasi, 2025; Cosma *et al.*, 2024). Direct microscopic visualization of amastigotes in smears of bone marrow, lymph nodes, liver and spleen considered as gold standard of confirmation but sensitivity is limited and possible risk of hemorrhage (Pena *et al.*, 2020). Culture can be applied to increasing diagnostic yield; however, it's time-consuming and prone to bacterial contamination (de Oliveira Filho *et al.*, 2024).

¹Department of Clinical Laboratory Sciences, College of Pharmacy, University of Al-Qadisiyah, Al-Qadisiyah, Iraq.

²Department of Parasitology, College of Veterinary Medicine, University of Wasit, Wasit, 52001, Iraq.

³Department of Public Health, College of Veterinary Medicine, University of Basrah, Basra, 61001, Iraq.

⁴Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Wasit, 52001, Iraq.

Corresponding Author: Hasanain A.J. Gharban, Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Wasit, 52001, Iraq.
Email: hghirban@uowasit.edu.iq

ORCIDs: 0000-0002-7189-9733, 0000-0003-0076-0600, 0009-0002-7824-1480, 0000-0002-7379-7503, 0000-0002-6438-1450

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Lastly, molecular PCR-based assays have offered a high sensitivity and specificity in determining *Leishmania* species in various specimens with enabling quantification of parasite burden (Gow *et al.*, 2022; Castelli *et al.*, 2021). Subsequently, phylogenetic analysis demonstrates an efficacy in diagnosis and epidemiological tracking of infections, which essentially help in understanding

transmission dynamics and predicting clinical outcomes (Naz *et al.*, 2024; Glidden *et al.*, 2023).

In Iraq, several studies mentioned that leishmaniasis is endemic in human; while in animals, online searching detected that rare recent studies have performed on dogs (Fadhil and Ali, 2025; Alseady and Al-Dabbagh, 2024; Alobaidii and Almashhadany, 2023), with lack of such reports in cats. Therefore, the present study was carried out to serological and molecular investigation of leishmaniasis (*L. infantum* infection) and phylogenetic analysis of study isolates.

MATERIALS AND METHODS

Samples

A total of 91 adult pet cats of different ages and sexes were admitted to private veterinary clinics in Wasit province (Iraq) during November (2023) to July (2024). Five ml of venous blood was drained from each cat, divided equally into without and with-anticoagulant tubes and transported to the Laboratory of Clinical Pathology (College of Veterinary Medicine, University of Wasit). The obtained sera and whole blood were kept frozen to be used later for serological and molecular testing (Archana *et al.*, 2026; Al-Eodawee *et al.*, 2024; Al-Abedi *et al.*, 2018; Suzan *et al.*, 2012).

Serological examination

Qualitative Feline Leishmaniasis ELISA Kit (Cat.No.SL0039Fe, SunLong Biotech, China) was served to screening anti-*Leishmania* antibodies. Briefly, the serum samples were prepared, processed and optical density (OD) was read. Post evaluation of test effectiveness, calculation of critical value (Cutoff) was done based on the of Negative Control to identify positive samples at $\geq$ Cutoff value.

Molecular examination

Initially, the whole blood samples were thawed at room temperature using the water bath and Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) was utilized to extract the DNAs. Post estimation purity and concentration of DNAs, Master Mix tubes were prepared at a final volume of 25ml using the Accu Power® PCR Pre Mix Kit (Bioneer, Korea) and one set of designated primers [HaF: 5'-CAT CAA GGC CGG ACG GAT AG-3' and HaR: 5'-CCA AGT CAT CCA TCG CGA CA-3'] based on the internal transcribed spacer 1 (*ITS1*) and 5.8S *rRNA* gene of the NCBI-GenBank *L. infantum* isolate (ID: PQ454617.1). Then, the Master Mix tubes were transferred to Thermal Cycler system and subjected to the modified conditions (Table 1). Electrophoresis of PCR products was done in Agarose-gel (1.5%) for 90 minutes at 100 Volts and 80 Am and positive amplified DNAs were observed under UV transilluminator at 291 bp.

Phylogenetic analysis

The positive DNAs in addition to the study primers were sequenced by the Sanger method and study isolates were named and documented in NCBI-GenBank database. Alignment of sequence data of study isolates was

constructed through the Clustal W alignment and phylogenetic analysis with the NCBI-BLAST *L. infantum* isolates was done by the multiple sequence alignment (MSA) in the NCBI website and MEGA-11 Software as well as using the homology sequence identity and phylogenetic tree analysis in MEGA-11 Software.

Statistical analysis

Among study groups, data were tested statistically by the *t*-test and One-Way ANOVA in the GraphPad Prism Software to indicate significant differences between study values at $p < 0.05$; whereas, confidence interval 95% (CI95%) was also calculated among different study groups (Al-Hamed *et al.*, 2026; Gharban, 2022; Ajaj *et al.*, 2021).

RESULTS AND DISCUSSION

Serological detection of leishmaniasis

After calculation of cut off value at ≥ 0.230 , the findings of qualitative ELISA detected that 15.38% (14/91) of study cats were serologically positive to leishmaniasis whereas 84.62% were seronegatives (Fig 1).

According to their titers, values seropositives (0.369857 ± 0.025774) were significantly ($p < 0.0001$; 95%CI: 0.9799 to 15.23) higher than recorded for seronegatives (0.172896 ± 0.003906) (Fig 2).

In some endemic areas, serological surveys have been carried out in cats using indirect hemagglutination antibody test (IHAT) in Egypt, Western blot (WB) in France and indirect immunofluorescence antibody test (IFAT) in Italy (Mancianti, 2004). In Egypt, serological testing using an ELISA technique was performed on 104 cats living in Jerusalem area and the seropositive findings were observed in 6.7% (Nasereddin *et al.*, 2008). In Spain, Ayllon *et al.* (2008) observed that the prevalence rate of leishmaniasis in blood samples of 233 cats tested by IFAT was 4.29% (10/233), which involving 1.29% (3/233) under the cutoff 1:100 and 3% (7/233). In another recent Spanish document, Alcover *et al.* (2021) revealed leishmaniasis in blood samples of 2.2% (4/179) stray cats tested serologically by ELISA and 14.5% (26/179) by WB. Among different Brazilian reports, Bezerra *et al.* (2019) detected that the serological prevalence rates of *leishmaniasis* in diseased cats with feline immunodeficiency virus and feline leukemia virus were 15.38% and 28.57%, respectively. On other hand, Costa-Val *et al.* (2020) found 29.80% positive

Table 1: Conditions for amplification of DNAs.

Number of cycle(s)	Step	Time	Temperature (°C)
1	Initial denaturation	5min	95°C
35	Denaturation	30sec	95°C
	Annealing	3 sec	58°C
	Extension	2min	72°C
1	Final extension	5min	72°C

cats among 64 blood samples tested serologically by ELISA; while, Leonel *et al.* (2020) recorded that the seropositive rates of *leishmaniasis* in blood samples of 94 cats were 31.91% and 29.79% through ELISA and IFAT respectively. In Northern Italy, 12.5% of 152 cats were shown a positive reactivity using IFAT with a titer of $\geq 1:80$ (Urbani *et al.*, 2020). In Iran, a cross-sectional descriptive study involved the testing of blood samples of 60 stray cats by ELISA and the findings recorded that the prevalence of *L. infantum* was 6.7% (Akhtardanesh *et al.*, 2017). In Turkey, Aksulu *et al.* (2021) indicated that an overall of 47 out 301 (15.6%) cats were having the anti-Leishmania antibodies using the IFAT. Based on these data, considerable variation among the findings of various studies might be attributed to differences in the parasite species (strains), host immune response, specific test platform used, targeted antigen and geographical endemicity of disease.

Molecular identification of *L. infantum*

Targeting the *ITS1* and *5.8S rRNA* gene, the findings shown that 9.89% (9/91) of study cats were infected with *L. infantum* while 90.11% (82/91) were negatives (Fig 3).

In a previous review article, Mancianti (2004) mentioned that the typing of clinical cases described in several European countries as France, Italy, Portugal and Spain identified *L. infantum* in all reported cases. Comparatively, *L. infantum* infection was detected molecularly in 0.43% (Ayllon *et al.*, 2008) and 5.6% (Alcover *et al.*, 2021) of Spanish cats, 16.7% of Iranian stray cats (Akhtardanesh *et al.*, 2017), 10% of Brazilian cats (Costa-Val *et al.*, 2020) and 2.3% of Turkish cats (Aksulu *et al.*, 2021). Among the various molecular targets, the *ITS1* and *5.8S rRNA* gene has emerged as particularly valuable due to its high variability and conserved flanking regions, which facilitate species-specific amplification and differentiation (Gunderina and Katokhin, 2020). Also, the inherent genetic diversity within the *ITS1* region coupled with the stability of the *5.8S rRNA* gene enables the development of highly sensitive and specific PCR-based assays capable of identifying *Leishmania* at the genus and species level, even in samples with low parasite loads (Usmael *et al.*, 2022). This makes them superior to other targets like the *18S rRNA* gene that, despite its abundance, requires more intricate amplification methods and often presents

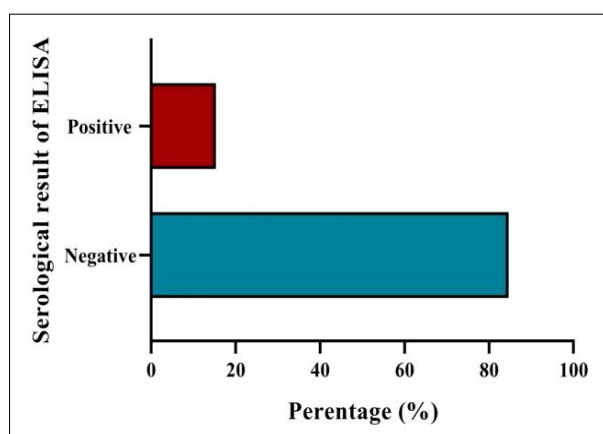


Fig 1: Serological prevalence of leishmaniasis among 91 pet cats.

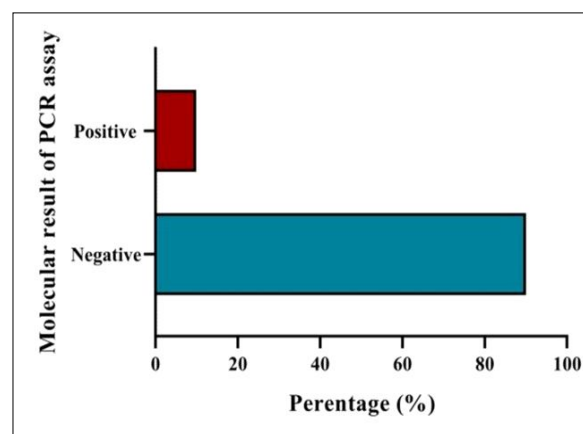


Fig 3: Molecular prevalence of leishmaniasis (*L. infantum*) among 91 pet cats.

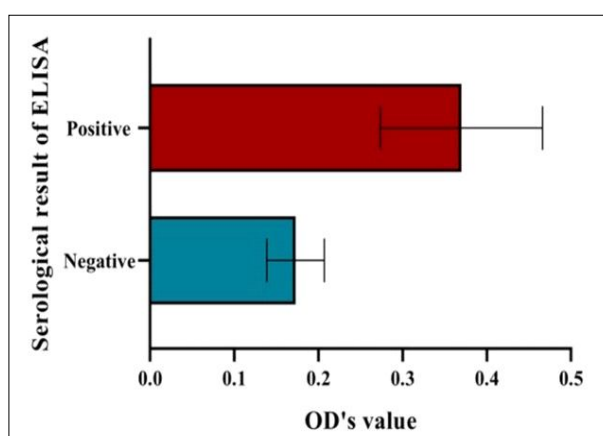


Fig 2: OD's levels among the seropositively and seronegatively study of cats.

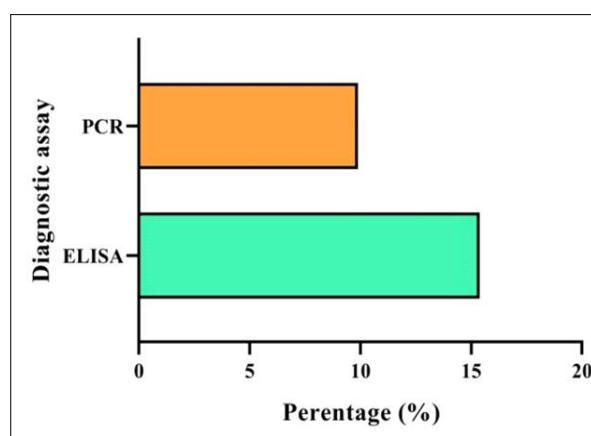


Fig 4: Positive serological and molecular results of feline leishmaniasis.

challenges in achieving species-level discrimination (Alsulaimany, 2025). Moreover, the tandem repeat organization of rDNA units, which can be present in tens to hundreds of copies per *Leishmania* cell, significantly enhances the sensitivity of *ITS1*-based assays, allowing for robust detection even in clinical samples with limited parasite DNA (Salloum *et al.*, 2016).

Table 2: Sensitivity and specificity of serology assay (ELISA) according to molecular (PCR) technique.

Molecular	Serology		Total
	Positive	Negative	
Positive	9	0	9
Negative	5	77	82
Total	14	77	91
Sensitivity		100%	
Specificity	93.9%		

Comparative accuracy of diagnostic assays

This study found significantly that the number serologically positive samples (15.38%) was higher ($p < 0.0474$; 95%CI: 22.24 to 47.51) than reported by molecular PCR assay (9.89%), which accordingly indicated that the levels of sensitivity and specificity for ELISA were 100% and 93.9% respectively (Fig 4, Table 2).

In contrast to our data, Akhtardanesh *et al.* (2017) obtained that the prevalence rates of positive *Leishmania* infection by ELISA and PCR were 6.7% and 16.7%, respectively. However, this high sensitivity and specificity might be attributed to the high efficacy of ELISA's kit applied in current study or to the role of testing similar sample sources in a study. Sherry *et al.* (2011) determined that serology and PCR results are positively associated and moderate agreement was found between *Leishmania* ELISA and PCR since the total rate of *L. infantum* infection derived from seroreactivity and/or positive PCR was

Table 3: Homology sequence identity for study cattle *L. infantum* isolates and NCBI-BLAST *L. infantum* isolates.

Local isolate	NCBI isolate					Identity
Name	Access No.	Access No.	Source	Host	Country	(%)
Feline1	PQ327040.1	KF705514.1	Ticks	Dog	Iran	99.49
Feline2	PQ327041.1	KF705514.1	Ticks	Dog	Iran	98.94
Feline3	PQ327042.1	MH231229.1	Conjunctive	Dog	Brazil	98.27
Feline4	PQ327043.1	MH231229.1	Conjunctive	Dog	Brazil	99.15
Feline5	PQ327044.1	MH231229.1	Conjunctive	Dog	Brazil	99.11
Feline6	PQ327045.1	KF705514.1	Ticks	Dog	Iran	99.44
Feline7	PQ327046.1	KF705514.1	Ticks	Dog	Iran	98.63
Feline8	PQ327047.1	MH231229.1	Conjunctive	Dog	Brazil	98.95
Feline9	PQ327048.1	KF705514.1	Ticks	Dog	Iran	99.01

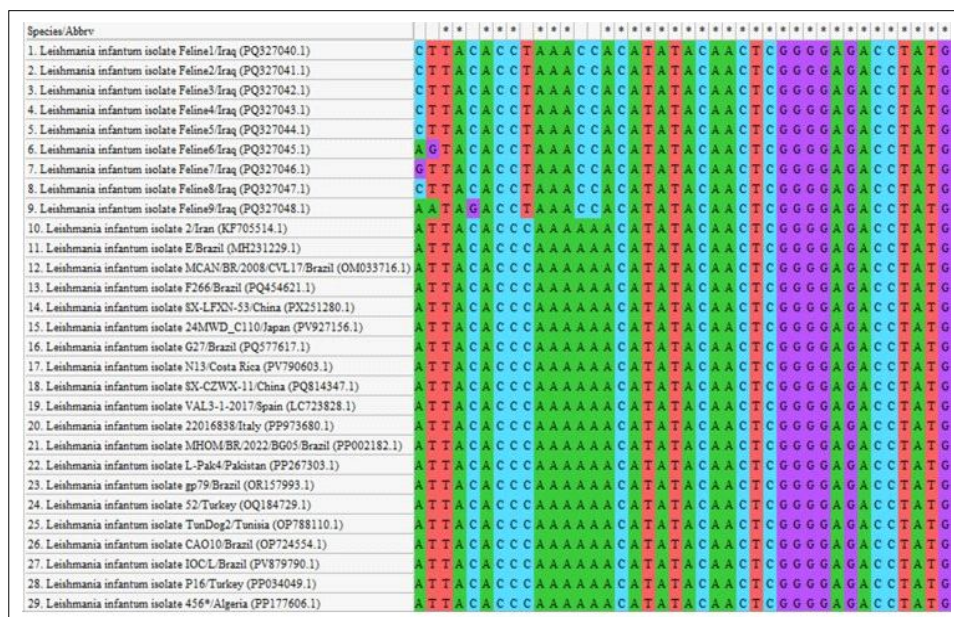


Fig 5: MSA showing similarity (*) and differences between the local study and NCBI-GenBank *L. infantum* isolates using the MEGA-11 software.

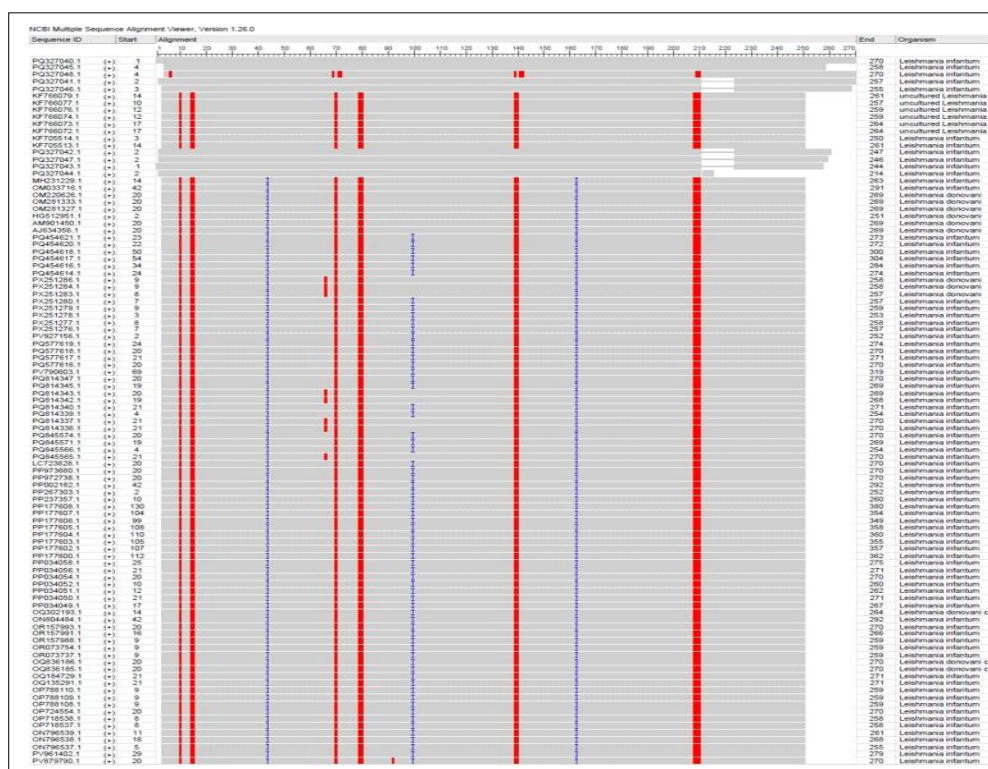


Fig 6: MSA showing differences between the local study and NCBI-GenBank *L. infantum* isolates using the NCBI MSA viewer.

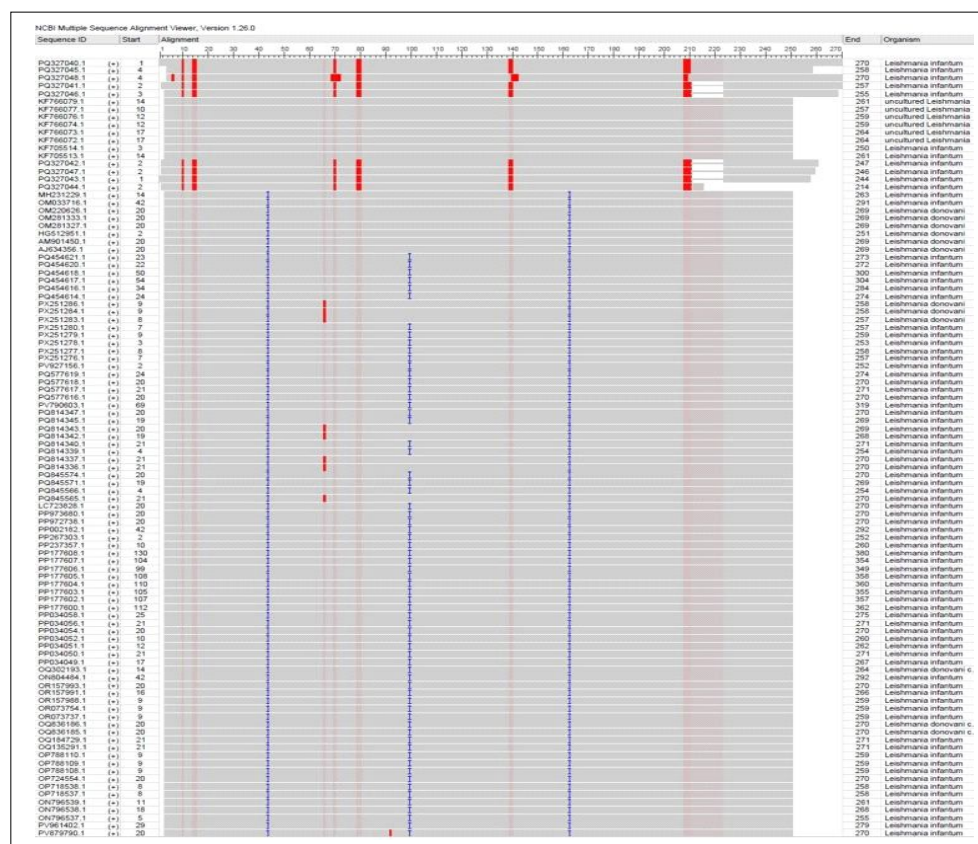


Fig 7: MSA showing frequency-based differences between the local study and NCBI-GenBank *L. infantum* isolates using the NCBI MSA viewer.

15.4%. Alcover *et al.* (2021) found that the overall rate of leishmaniasis among 179 stray cats was 15.6% and only two cats tested positive by all the diagnostic methods (IFAT, ELISA, WB and PCR). However, several studies have been reported inconsistent results when using different types of tissue samples for PCR-based diagnosis compared with the parasitological and serological data indicating that sampling from different tissues together with serological analysis is required when screening for leishmaniasis (Rihs *et al.*, 2025; Gow *et al.*, 2022; Castelli *et al.*, 2021; Costa-Val *et al.*, 2020).

Phylogenetic analysis of study *L. infantum* isolates

Sequencing data of study positive *L. infantum* isolates were named as Feline isolates and documented in the NCBI-GenBank database following specified accession numbers (PQ327040.1, PQ327041.1, PQ327042.1,

PQ327043.1, PQ327044.1, PQ327045.1, PQ327046.1, PQ327047.1 and PQ327048.1). Comparative analysis of study *L. infantum* isolates with the NCBI-BLAST *L. infantum* isolates detected an existence of nucleotide alignment similarities (*) ranged 89-100% and substitution mutations ranged 0.000-0.0583%. However, the marked significant identity was observed between the study isolates named Felin1, Feline 2, Feline6, Feline7 and Feline9 with the NCBI-GenBank *L. infantum* Iranian (ID: KF705514.1) isolate that identified in ticks of naturally infected dogs; while, the study isolates named Feline3, Feline4, Feline5 and Feline8 were identical with the NCBI-GenBank *L. infantum* Brazilian (ID: MH231229.1) isolate obtained from the conjunctive of naturally infected dogs (Table 3, Fig 5-9).

In the only available study in Iraqi animals, phylogenetic analysis of *L. infantum* isolates identified in dogs revealed a significant identity to Brazilian, French and American.



Fig 8: MSA showing nucleic acid similarity and differences between the local study and NCBI-GenBank *L. infantum* isolates using the NCBI MSA viewer.

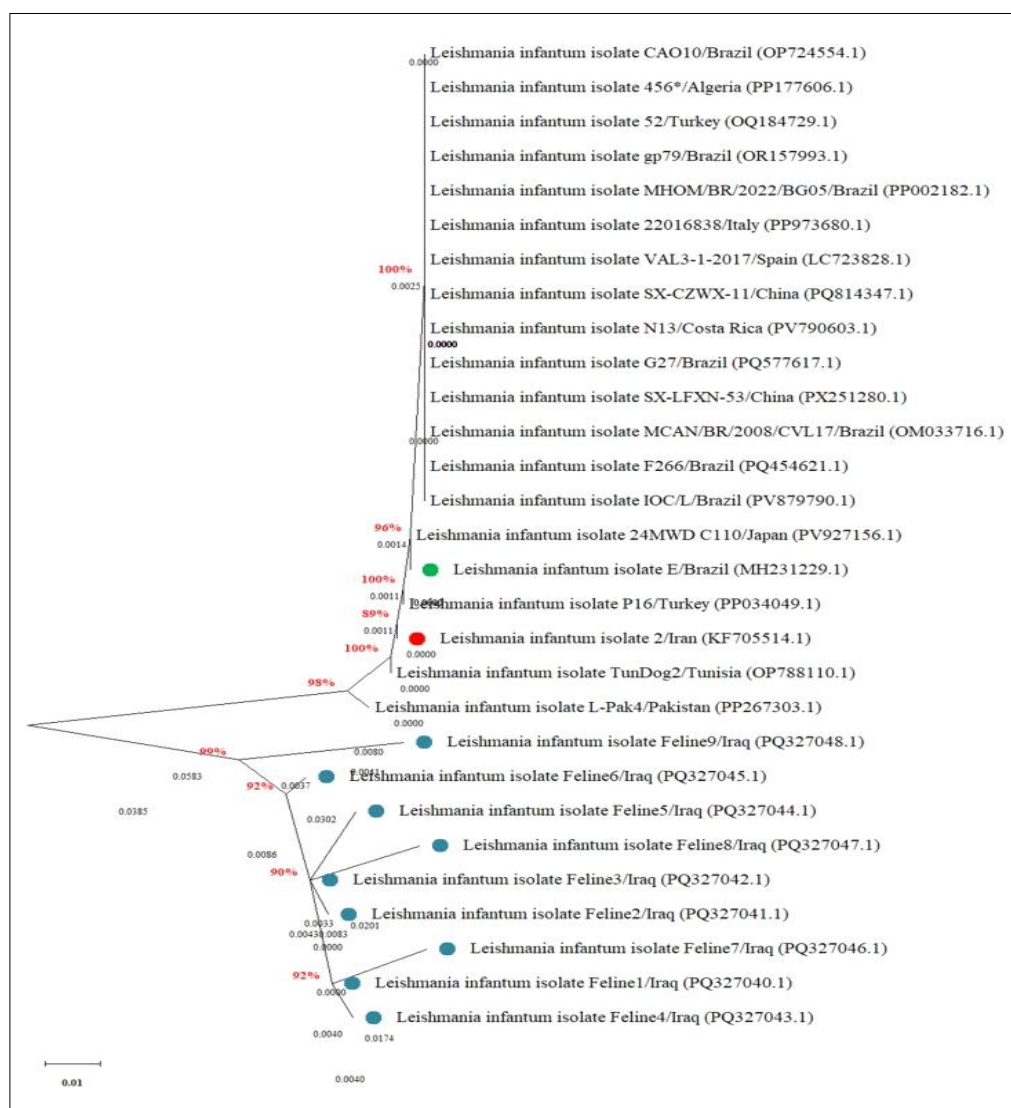


Fig 9: Phylogenetic tree analysis of the local study and NCBI-GenBank *L. infantum* isolates using the MEGA-11 software.

This demonstrates that many strains of *L. infantum* might be circulated in Iraq particularly Brazilian ones suggesting that cats could attract their infection from dogs (Fadhil and Ali, 2025). Also, low levels of biosecurity in Iraq allows to introduction of various animals and breeders which may act as a reservoir to infection or infected asymptotically. Nonetheless, Costa-Val *et al.* (2020) refer to the genetic complexity of *Leishmania* spp. populations which circulate in a different geographical area but the same species.

CONCLUSION

For our knowledge, this might represents the first study in Iraq highlighting feline leishmaniasis serologically and molecular phylogenetically. Based on our data, pet cats may carry leishmaniasis (*L. infantum*) as either a reservoir or an actively asymptomatic suggesting the importance of furthermore epidemiological studies. Additionally, combination of serological ELISA and molecular PCR

techniques with phylogenetic analysis by targeting the *ITS1* and *5.8S rRNA* gene revealed a high efficacy in investigation of feline leishmaniasis and the great role of these diagnostic techniques in detection of such infections. Phylogenetic analysis of study isolates determined a close-relationship with two global Iranian and Brazilian strains of dog source indicating the role of dogs in transmission of infection to cats and other animals or even humans.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are

responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures were approved by the Scientific Committee in the College of Veterinary Medicine at University of Wasit (UW.CVM.2023.237) dated 14/11/2023.

Conflict of interest

All authors declare that they have no conflict of interest.

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