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Original Research Article

Microscopic and Molecular Diagnosis *Leishmania donovani* of Dog in Babylon Province

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Abstract: Leishmaniasis is a vector-borne parasitic disease caused by protozoa of the genus Leishmania, transmitted primarily through the bites of infected female sand flies (Phlebotomus spp. in the Old World and *Lutzomyia spp*. in the New World). It presents in several clinical forms, including cutaneous, mucocutaneous, and the visceral form known as kala-azar, the latter being the most severe. Dogs are considered a primary reservoir for Leishmania, the main causative agent of zoonotic visceral leishmaniasis (ZVL), contributing to the human-animal transmission cycle. This study aimed to diagnose the disease by this protozoan molecular and microscopically in stray and domestic dogs using a staining by gimsa stain. In the current study, a total of 75 blood samples of dogs (different in sex and age) were collected from other areas of Babylon City during the period from December 2024 to February 2025. The microscopic findings found that dogs infection by *leishmania donovani* the infection rate 41.33% (31 out of 75). A molecular study using PCR and sequencing techniques identified gene of leishmania donovani in dog sample. The sequencing technique demonstrated the species of *L.donovani* in this study. The molecular results demonstrated that 41 out of 75 dogs (54.66%). According to sex, no significant difference (p =0.669) was found in the infection, where it was 53.19% (25 out of 45) in males, while 57.14% (16 out of 28) in females. For the age groups, there was no significant difference (p=0.073), which has a high incidence of illness in canines less than 1 years 22/34(64.70%) compared to other age groups. Current data also revealed that the infection rate in rural areas was slightly higher than in urban areas, where it recorded 15/19 (78.94%) and 12/19 (63.15%), respectively, with significant differences (P = 0.025).

Keywords: *Leishmania*, *Donovani*, Dogs, Kala-Azar, PCR, Cutaneous.

INTRODUCTION

Leishmania is a parasitic protozoan, a single-celled organism of the genus *Leishmania* that is responsible for the disease leishmaniasis (Wang *et al.,* 2015) they are spread by type of sandflies of the genus *Phlebotomus* in the Old World, and genus *Lutzomyia* in the New World. Their primary hosts are vertebrates; *Leishmania* can infects hyraxes, canids, rodents, and humans and occurs most frequently in the tropics and sub-tropics of Africa, Asia, the Americas, and southern Europe (Ansari *et al.,* 2015). The disease can present in three main ways:cutaneous, mucocutaneous and visceral, the cutaneous form presents with skin ulcers, while the mucocutaneous form presents with ulcers of the skin, mouth, and nose. The visceral form starts with skin ulcers and later presents with fever, low red blood cell count, and enlarged spleen and liver. (Sundar and Chakravarty, 2013). About 200 million people in Asia, Africa, South and Central America, and southern Europe live in areas where the disease is common (Barrett and Croft, 2012). The World Health Organization has obtained discounts on some medications to treat the disease. It is classified as a neglected tropical disease (Ansari *et al.,* 2015) the disease may occur in a number of other animals, including dogs and rodents, and the type of infection (Mathison *et al.,* 2021). *Leishmania* have two major different cell morphologies, exemplified by the promastigote morphology in the sand fly and the amastigote morphology in the mammalian host. Most cases of VL occur in rural and suburban areas of low socioeconomic status in Bangladesh, Ethiopia, Brazil, India, Sudan, and South Sudan. Cutaneous leishmaniasis is more widespread, with nearly 75% of the cases

occurring in Afghanistan, Algeria, Brazil, Ethiopia, Iran, Peru, Sudan, Costa Rica, Colombia, and Syria. Mucocutaneous leishmaniasis is most commonly seen in Bolivia, Brazil, Peru, and Ethiopia, as well as Thailand (Lazar and Abass, 2020).

MATERIAL AND METHODS

Collection of Blood Samples

This study included the collection and examination of (75) blood samples from dog with different ages, different sex and different areas of the Babylon province: (Al. Qassime, Al-Hashemite, Al-Hilla, and Mahaweel Districts). The field study included weekly visits to regular veterinary hospital and primary centers and from owners farms in the city of Hilla, Babil province, clinics, veterinary ubiquitous within which surveyed in the province for the purpose of collecting blood samples from dog. A sample of 5 ml of blood was taken from each dog from the intermediate antebrachial vein and jugular vein by a sterile syringe. A sample of 5 ml of blood was taken, 3 ml blood sample for Molecular test, and 2m blood sample for direction thine smear examine with a microscope. These samples were then transported in a cool box to the Parasitology Laboratory at the College of Veterinary Medicine at AlQasim Green University for the microscopic examination using the traditional diagnosis methods (staining by Giemsa).

Laboratory Examination of Blood Samples (Giemsa Stain)

Giemsa stain is one of the synthetic dyes commonly used to differentiate intracellular parasites, such as *Leishmania spp*., in blood and tissue smears (Barcia, 2007; Elmahallawy *et al*., 2014). The procedure involves the following steps:

1. Prepare a thin blood film on a clean glass slide, allow it to air-dry completely, and place it on a staining rack.
2. Fix the dried smear in absolute methanol for 3–5 minutes.
3. Stain the smear with diluted Giemsa stain (usually 1:20 dilution with buffered water, pH 7.2) for 15–30 minutes at room temperature.
4. Rinse gently with running tap water to remove excess stain.
5. Allow the slide to dry in air, and examine microscopically under oil immersion (×1000 magnification).

Molecular Detection

A / Genomic DNA Extraction

All blood samples were prepared in order to extract the genomic DNA by using Genaid DNA purification kit and following manufacturer's instructions:-

1. Amount of 0.5 ml blood was taken in 1.5 ml centrifuge tube then adding 500 µl of RBC-lysis buffer for 10 minute.
2. The sample was then centrifuged at 12000 rpm for 2 minutes. The supernatant was transferred to another 1.5 ml centrifuge tube.
3. 100 µl of binding buffer (GT) and 20 µl of Proteinase K were added to the supernatant and mixed well by vortex and then 500 µl of GB buffer was added and mixed well by flipping then Incubated at room temperature for 10 minute.
4. Transferring the whole solution into GD column and spinning for 30 sec at 12000 rpm, discarding the flow through and adding 500 µl of wash Buffer and spinning for 30 sec at 12000 rpm as well, this step was repeated twice.
5. Last step was transferring the GD column into 1.5 ml collection tube then adding 50 µl of elution buffer. The eluted solution was store in -20 °C until needed.

B / PCR Sample Preparation

Amplification of kRNA was carried out in a final volume of 25 µl reaction mixture following the manufacturer instructions, after that all sample was set in PRC machine plate to be ready for running using a proper setting. The forward primer sequence was 5'- AAATCGGCTCCGAGGCGGGAAAC -3', while the reverse primer sequence was 5'-GGTACACTCTATCAGTAGCAC-3'.

C / Sequence of Leishmania Species

The resolved PCR amplicons were commercially sequenced from termini, forward and reverse, according to instruction manuals of sequencing company (Souel University). Only, evident chromatographs acquire from Applied Biosystem extension (ABI) sequence files were moreover analyzed, to improve that the annotation and diversity are not because of PCR or sequencing artifacts. By match the observed DNA sequences of Iraqi localisolates with the reference DNA sequences of N. caninum., the actual location and other specifics of the retrieved PCR fragments were put on.

D / Statistical Analysis

The data were analyzed using SPSS software. Chi-square test was applied compare between the factors that affect the infection rate and values $P \leq 0.05$ were considered as statistically significant.

RESULTS

Microscopic Examination

The microscopic examination of blood samples revealed that 31 out of 75 (41.33%) dogs were positive for leishmania species. The results of blood examination using staining showed (Figure 1) and (Table 1):

Table 1: Rate of *leshmania* spp. Infection in dog determined by Microscopic examination

Total No. Samples	No. of positive samples	Percentage
75	31	41.33%

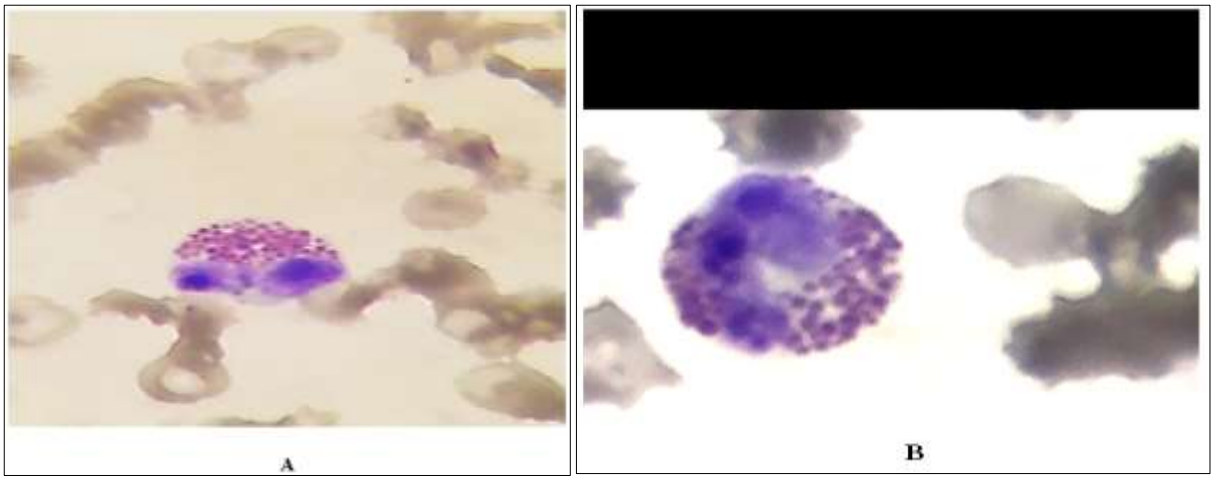

Figure 1: leishmania Isolated from the infected dog by Giemsa stain under oil immersion (1000X). A-inter macrophage cell (Eosinophils), B- Neutrophils cell infection of leishmania spp.

Molecular Result

Conventional PCR

For confirmation, DNA extraction was performed for 75 blood samples of dog, including microscopically positive and negative samples. The results showed the amplicons of dogs targeting a partial region within the 18SrRNA gene in isolated dogs' samples which found that the infection rate in the samples was 54.66% (41 out of 75), as show in (Table 2) and (Figure 2).

Table 2: Rate of *leshmania donovani* Infection in dog determined by PCR

Total No. Samples	No. of positive samples	Percentage
75	41	54.66%

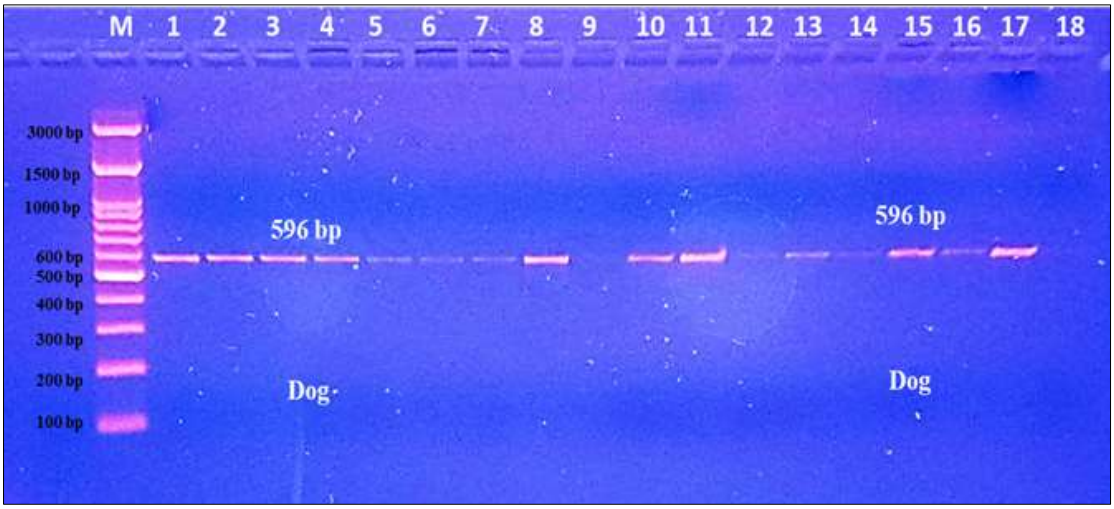

Figure 2: Shows the PCR amplification results of 18s rRNA gene of *leishmania Donovani* isolated from Dogs. Agarose gel picture appears the PCR produce bands with molecular weight of 596bp.(M) refers to (3000bp) DNA ladder, (1,2,3,4) positive control, (5,6,7) Negative control. PCR results of blood samples.

Infection Rates of *Leishmania Donovani* According to Area

The findings revealed the distribution of infection rates of *leishmania donovani* infection in the examined of *leishmania* according to the present study region, where they found that in Al-Hamaza al-gharbi (78.94%), Al-Qassim (63.15%), Al-Mahaweel (38.88%), and Al-Hilla (36.84%) (Table 3).

Table 3: Rate of the *leishmania donovani* infection in dogs determined by (PCR) according to the geographical area

Geographical area	Total No.	Positive cases	%
Al-Mahaweel	18	7	38.88c
Al-Qassim	19	12	63.15ab
Al-Hamaza al-gharbi	19	15	78.94a
Al-Hilla	19	7	36.84c
Total No.	75	41	54.66b
Calculated X^2		**9.31**	
Calculated P value		**0.025 (significant difference)**	

Different letters between any two percent denote to the significant difference

Infection Rates of *Leishmania Donovani* According to Age

The results demonstrated the distribution of infection rates of leishmania infection in the examined dogs according to age groups as follows in the <1 year age group which was 70.58% (24 out of 34)., while in the age group 1-3 year which was 48.14% (13 out of 27) and the >3 years age group which was 28.57% (4 out of 14). The statistical analysis reveals that there was no significant (P>0.05) difference in the prevalence of parasite between age intervals Table (4).

Table 4: Rate of the *leishmania donovani* infection in dogs determined by PCR according to the age group

Age	No. of examined samples	No. of positive samples	Percentage of positive samples
<1 year	34	24	70.58%
1-3 year	27	13	48.14%
>3 year	14	4	28.57%
Calculated X^2		**7.78**	
Calculated P value		**0.02(No significant difference)**	

Infection Rates of *Leishmania Donovani* According to Sex

This current study concluded the rate of infected female dogs with Leishmania D. was 60.71% (17 out of 28), and males was 51.06 % (24 out of 47) Through the above, we found that the percentage of infection in was females greater than males, as shown in the table (5). When results statistical analyzed showed there are no significant effect on in infected rate at (P>0.05) on sex,as Table (5).

Table 5: Rate of the *leishmania donovani* infection in dogs determined by PCR according to sex

Sex	Total of sample	No. of postive sample	%
Male	47	24	**51.06%**
Female	28	17	**60.71%**
total	75	41	**54.66%**
Calculated X^2		**0.659**	
Calculated P value		**0.669(No significant difference)**	

 Similar letters denote to the no significant difference at P<0.05.

Phylogenetic Tree

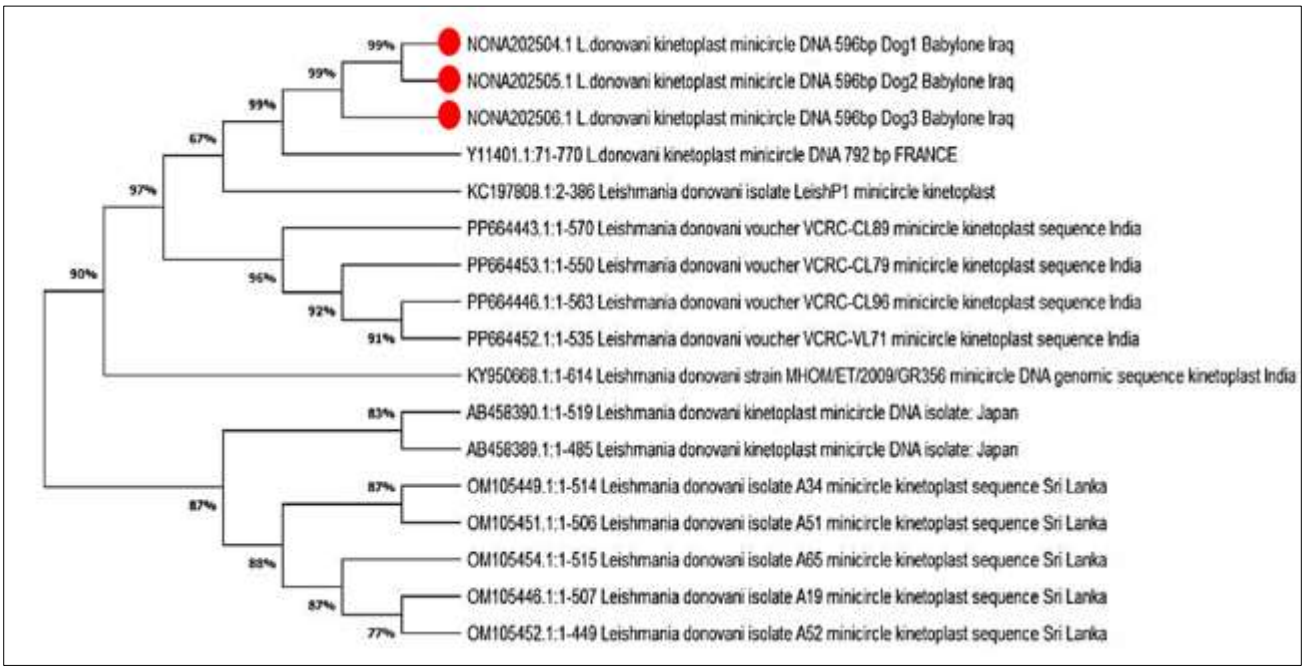

Figure 3: The comprehensive phylogenetic tree of genetic variants of 18S rRNA gene fragment leishmania D. isolate. The red color refers to the sequenced GF1, GF2, GF3 variants, while the Black line refer to the related referring NCBI leishmania D. deposited species.

DISCUSSION

Microscopic examination of blood samples indicated that 31 out of 75 dogs (41.33%) tested positive for Leishmania spp. This confirms the high prevalence of leishmaniasis among dogs, which serve as the definitive host and reservoir for the parasite transmitted by sand flies. The absence of vector control measures and the limited use of insecticides contribute to the proliferation of sand flies, particularly in rural areas characterized by favorable conditions such as high temperatures, humidity, dense vegetation, and large populations of stray dogs. These factors create an optimal environment for vector propagation. These findings are consistent with those reported by (Alsaad, 2021) in Maysan Governorate but contrast with the results from (Abass *et al*., 2024) in Sudan. PCR analysis targeting the 28S rRNA gene revealed Leishmania donovani infection in 54.66% of the tested dogs, with 41 positive cases. These results are in line with those of (Rezaei *et al*., 2022), who reported a prevalence of 51.4% in southern Iran. However, the prevalence was higher than that observed by (Wang *et al*., 2011) in China (36.79%) and (Fadhil and Ali, 2025) in Al-Qadisiyah, Iraq (20%). In contrast, a notably higher prevalence of 70.3% was found in a study conducted by (Ahmed *et al*., 2024) in Sudan. The current study also assessed the geographical distribution of L. donovani infections. Prevalence rates were found to be 78.94% in Al-Hamza Al-Gharbi, 63.15% in Al-Qassim, 38.88% in Al-Mahaweel, and 36.84% in Al-Hilla. The data highlight significant geographical variation in infection rates. The higher prevalence in Al-Hamza and Al-Qassim is likely due to a combination of environmental, biological, and social factors. These areas offer suitable conditions for sand fly reproduction, including favorable climatic conditions (moderate temperatures and humidity), the presence of mud houses, and extensive agricultural land. The abundance of stray dogs, which act as natural reservoirs, further facilitates the parasite's transmission cycle (Al-Hayali and Muntsir, 2021). These results align with molecular findings reported by (Abdalla *et al*., 2024) in Sudan, but differ from those of (Vilas-Boas *et al*., 2024) in Tunisia.Infection rates by age group were as follows: 70.58% (24 out of 34) in dogs under 1 year, 48.14% (13 out of 27) in dogs aged 1–3 years, and 28.57% (4 out of 14) in dogs older than 3 years. Statistical analysis revealed no significant difference in infection rates across age groups ($P > 0.05$). Younger dogs (≤1 year) may be more susceptible to L. donovani due to immature immune systems, which limit their ability to control infection (CAPC, 2023). Additionally, vertical transmission may result in early infection prior to vector exposure, and puppies in endemic areas are at increased risk due to environmental exposure (Silva *et al*., 2021).Regarding sex, the infection rate in female dogs was 60.71% (17 out of 28), while in males it was 51.06% (24 out of 47). Although the infection rate was higher in females, statistical analysis indicated no significant difference based on sex ($P > 0.05$). These findings are consistent with those of Pekajrbash *et al*., (2022). Several factors may explain the observed difference, including hormonal influences—especially estrogen and progesterone—which may modulate immune responses and favor a Th2-type response, less effective against intracellular pathogens such as Leishmania spp. Additionally, physiological states such as pregnancy or lactation may induce temporary immunosuppression, increasing vulnerability to infection. These findings contrast with those of (Fakhar *et al*., 2022).

CONCLUSION

According to the results of this study, the conclusions as follows:

1. Molecular techniques are a reliable method for diagnosing and identifying Leishmania species and can be applied in epidemiological investigations.
2. Leishmaniasis is a parasite that is widespread in all regions, especially rural areas with environmental conditions suitable for its growth.
3. The vector host is sandflies and dogs, which are considered the definitive host and reservoir host until the parasite completes its life cycle and is then transmitted to humans.
4. The response rate of those infected with leishmaniasis depends on the nature of their work and where they sleep in the summer.

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