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Original Research Paper

Detection of Visceral Leishmaniasis by *Leishmania infantum* using kDNA

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Abstract: Visceral Leishmaniasis is considered one of the endemic parasitic diseases transferred via sand fly *Phlebotomus alexandri* bite that can be transferred *Leishmania infantum*. This study aimed to detect visceral leishmaniasis in peripheral blood for patients using IFA test and kDNA polymerase chain reaction. Eighty samples were collected in the period (September 2022 -December 2023) from patients (1-10 years of age) suspected of VL and admitted to hospitals at several governorates in Iraq. IFAT showed positive results for 65 out of 80 samples with visceral leishmaniasis by L.infantum (81%). While kDNA results showed 60 out of 80 samples with visceral leishmaniasis by L.infantum (75%). , the sensitivity and specificity of IFA were 92% and 100% comparison with PCR. Concerning the results sequencing of PCR products, there was one isolate of L.infantum that differed from the global isolates in the NCBI It was found variation sequences in position substitution including deletion, transition, and transversion in the local isolate registered with accession number OR886069.1 . Moreover, it was observed in the phylogenetic tree analysis, there were the highest and lowest similarity distances of the Iraqi isolate with other isolates that including the French isolate, and the Iranian isolate.

Keywords: Visceral Leishmaniasis , *L. infantum*, IFAT, kDNA,PCR

1. Introduction

Leishmaniasis is reported as an epidemic infection for children in ninety countries. The rate of infection was 1500000 per year [1]. Three forms of leishmaniasis exist today, including cutaneous, visceral, and mucocutaneous Leishmaniasis. In the ancient world, Leishmaniasis occurred due to infection by one of each species involved*: Leishmania major*, *L.infantum*, and *L.tropica* [2].

In Iraq, Visceral Leishmaniasis is considered one of the endemic parasitic diseases transferred via sand fly Phlebotomus alexandri bite that can be transferred Leishmania infantum [3]. While in Iraq, asymptomatic infection was when contact with children infected with VL, it was 34%(86/250) of seropositive to Leishmania [4]. In previous years, it was identified visceral Leishmaniasis as an asymptomatic infection and prevalent in endemic regions with ratios of infection-to-disease range from (50–100:1year) in Spain, (8.9:1year)in Nepal, and India , (13:1year) in Iran and (6.5:1year) to (89:1year) in Brazil [3].

According to the WHO Leishmaniasis 2020 program, There are 87% of cases of VL were announced in eight nations, including Brazil, Ethiopia, Eritrea, India, Somalia, Kenya, Sudan, and South Sudan . Also, it was reported in 2020 that seven nations had more than 5000 cases of cutaneous Leishmaniasis (CL), including Algeria, Colombia, Afghanistan, Brazil, Pakistan, Iraq, and Syria, representing 80% of recorded cutaneous leishmaniasis incidence globally. Further, it was reported that 880 CL cases and 99 VL cases were imported globally [5,6]. Visceral Leishmaniasis (VL) is

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distinguished through its chronic and systemic prevalence capacity, and it is also fatal if the therapy is not delivered. VL needs to be diagnosed correctly. Therefore, laboratory methods that have high sensitivity are applied [7]. VL diagnosis techniques represented as gold standards include microscopic screening for bone marrow, spleen exudation specimens to observe the amastigote on smears, and promastigotes isolation in culture [8]. IFA (immunofluorescence antibody) test has been applied for VL diagnosis since 1970s, which was used most frequently to diagnose L. infantum [9]. Freire and colleagues [10] showed that the IFA test had high specificity in patients. Although IFA is not considered a typical test, but used frequently as a reference analysis for the proportional confirmation of modern diagnostic assays [11]. As a result of the different protocols applied for IFA and the global standards absence, therefore it is complicated to contrast information from various studies. The positive threshold can differ (1/40) in some studies, and (1/80) in other studies in population studies and set-up of assay [12,13].

Peripheral blood specimens represent one of the biological materials that are collected easily and utilized in very sensitive trials to check such as DNA amplification DNA for Leishmania via PCR conducted in the whole blood and serological screening to antibodies for anti-Leishmania [7]. This study aimed to detect visceral leishmaniasis in peripheral blood for patients using IFA test and kDNA polymerase chain reaction.

2. Methodology

Collection specimens

Eighty samples were collected from patients with visceral leishmaniasis.The samples were collected in the period (September 2022 -December 2023) from patients (1-10 years of age) suspected of VL and admitted to hospitals at several governorates in Iraq including Baghdad, Diyala, Wasit, Thi-Qar, Salah Al-Din, Anbar, Kirkuk, Babil, Karbala and Al-Qadisiyah province). Five ml of blood sample was divided into two aliquots: One was put in a plain tube to separate serum and the second aliquot was put in an EDTA tube stored at -60 C for genetic analysis.

Detection of Leishmania infantum IgG by IFA

A serum sample of patients was used to detect IgG L. infantum using IFA according to the manufacturer company (Vircell Microbiologist company /Spain). The method is dependent on antibodies in samples that react with adsorbed antigen on the slide surface [14].

Detection of kDNA using PCR

DNA extraction of frozen blood samples in EDTA TUBE using G spin DNA extraction iNTRON biotechnology Kit(Catalog: 17045). Nanodrop applied to determine the concentration of DNA.Primer of kDNA (Macrogen company /Korea)was used according to [7] as shown in Table 1. PCR analysis was applied to detect the kDNA gene .To reach the final volume of 25 µl, the PCR mixture reaction required 5 µl of the Maxime Taq PCR premix PCR (2X) (iNTRON /Korea), 1 µl of each of the forward and reverse primers with 10 picomoles/µl), 1.5 µl of DNA sample, and 16.5 µl of free nuclease water. The kDNA gene PCR settings are given in (Table 2). Electrophoresis was conducted with 2% agarose gel staining with Safe Red stain to detect PCR amplicons using UV.

Table 1:Sequence of kDNA primer

Primer	**Sequence**	**Tm (°C)**	**GC (%)**	**Product size**
Forward	5′-GGGKAGGGGCGTTCTSCGAA -3′	58.6	54.5 %	**120 base pair**
Reverse	**5′-SSSWCTATWTTACACCAACCCC -3′**	**52.3**	**40.9 %**	

Table2: PCR condition of kDNA

Phase	**Tm (°C)**	**Time**	**No. of cycle**
Initial Denaturation	94°C	5min.	**1 cycle**
Denaturation 2	94°C	1min.	**40 cycle**
Annealing	58 °C	1min.	
Extension1	72°C	1min.	
Extension 2	72°C	7 min.	**1 cycle**

Sequencing of kDNA gene

PCR amplicons sequencing was carried out for kDNA gene for L.infantum isolates by (Macrogen Company/Korea). It was the alignment of nucleotide sequences for gene with international isolates by NCBI Basic Local Alignment Search Tool Bio Edit program.

Phylogenetic tree analysis

The phylogenetic tree was analyzed using Neighbor-Joining to determine evolution distances by Jukes-Cantor model in MEGA11 [1**5**].

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3. Results

Detection of Leishmania infantum IgG by IFA

The results of IFAT showed positive results for 65 out of 80 samples with visceral leishmaniasis by L.infantum (81%)as shown in Figure (1). The FA test depended on antibodies in the sample reacting with an antigen labeled with fluorescein-labeled anti-human IgG and forming an antigen-antibody complex with fluorescent-labeled anti-human globulin. In addition, the sensitivity and specificity of IFA were 92% and 100% in comparison with PCR (Table3).

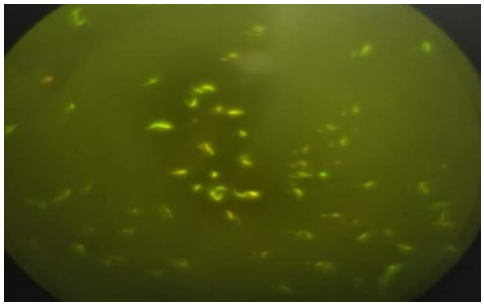

Fig. 1. IFAT positive IgG for Visceral Leishmaniasis by Leishmania infantum

Table 3: Comparison of IFA test with PCR in the detection of *L.infantum*

		IFA		**Total**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	Accuracy
		+ve	-ve						
PCR	+ve	60	0	60/80 (75%)	92.31%)82.95-97.46%	100% (78.20-100%)	100%)94.04 - 100%)	75%)56.38-87.44%(	93.8%)86.01 - 97.94%)
Total	-ve	5	15						
		65/80 (81.3%)	15						

Genetic detection of kDNA of L.infantum

The results showed kDNA found in 60 out of 80 samples with visceral leishmaniasis by L.infantum (75%)as shown in Figure 2.

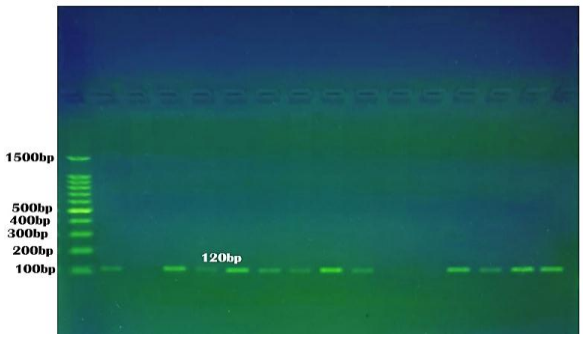

Fig. 2. Electrophoresis of PCR amplicons (120bp(for kDNA of some samples with *L. infantum* on 2% agarose at 5 volt/cm2 and 1x TBE buffer for one hour.DNA ladder (1500bp).

Sequencing and phylogenetic tree analysis of L.infantum

After aligning the isolates, PCR amplicon sequencing revealed that one isolate of L. infantum was different from the isolates that were registered with the NCBI. It was found variation sequences in position substitution including deletion, transition, and transversion in the local isolate registered with accession number OR886069.1 that compared with the global isolates in Gene Bank when alignment by BLAST program (Table 4). According to phylogenetic tree evolution, it was observed the compatibility for Iraqi local isolate registered was (87-90%) with isolates registered in Gene Bank for Tunisia,USA,France,Spain, and China (Figure 3).Table (5) shows the comparison of distance evolution for Iraqi local isolate (OR886069.1) with global isolates. The highest and lowest similarity distance of the Iraqi isolate with other isolates in Gene Bank was 0.115942029 with the French isolate and 0.4776119403 with the Iranian isolate.

Table4:Alignment of Leishmania infantum isolate registered in NCBI

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No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
17	Transversion	34	G/T	ID: HQ585885.1	Leishmania infantum isolate MCAN/CN/90/SC clone III minicircle, partial sequence;	74%
	Gap	42	G			
	Transversion	45	(C,T) Y/A			
	Transition / Transversion	46	(C,T) Y/T			
	Transversion	50	(G,T) K/G			
	Transition / Transversion	52	(C,T) Y/T			
	Transversion	55	A/T			
	Transversion	56	(A,T) W/T			
	Transition / Transversion	59	(C,T) Y/T			
	Transversion	61	(C,G) S/G			
	Gap	64	C			
	Gap	65	G			
	Gap	70-71	C			
	Transversion	75	A/T			
	Transversion	76	(A,T) W/T			
	Transversion	77	(A,T) W/T			
	Transversion	80	(C,G) S/G			
	Transversion	82	(C,G) S/C			
	Transition / Transversion	84	(C,T) Y/T			
	Transversion	91	(C,G) S/C			
	Transition	94	A/G			
	Transversion	109	(G,T) K/T			
	Transversion	111	(A,T) w/T			

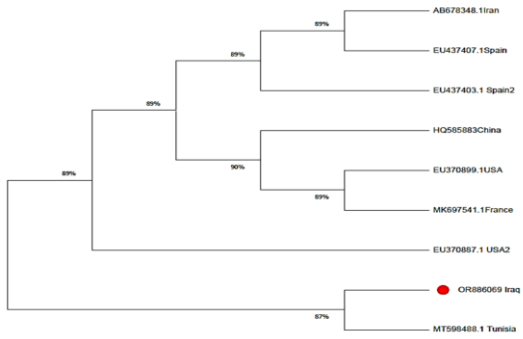

Fig.3.Phylogenetic analysis for the local isolate of L.infantum .The evolution was concluded via Neighbour-Joining method

Table 5:Comparative distance of local isolate for *L. infantum* with global isolate

	AB678348.1Iran	EU437407.1Spain	HQ585883China	EU370899.1USA	MK697541.1France	OR886069_Iraq	EU437403.1_Spain2	MT598488.1_Tunisia	EU370887.1_USA2
AB678348.1Iran									
EU437407.1Spain	0.0000000000								
HQ585883China	0.4177215190	0.4177215190							
EU370899.1USA	0.4430379747	0.4430379747	0.0240963855						
MK697541.1France	0.4358974359	0.4358974359	0.0121951220	0.0121951220					
OR886069_Iraq	0.4776119403	0.4776119403	0.1285714286	0.1428571429	0.1159420290				
EU437403.1_Spain2	0.1097560976	0.1097560976	0.4556962025	0.4683544304	0.4743589744	0.5373134328			
MT598488.1_Tunisia	0.6296296296	0.6296296296	0.5000000000	0.5250000000	0.5063291139	0.4637681159	0.6172839506		
EU370887.1_USA2	0.4556962025	0.4556962025	0.1686746988	0.1927710843	0.1707317073	0.1571428571	0.4936708861	0.5000000000	

4. Discussion

VL diagnosis remains the confirmation of the parasite through direct techniques such as microscopy, culture, and PCR, despite the need for accurate methods and high proficiency personnel and tools limiting their utilization. Besides, indirect diagnosis methods, that depend on serology, remain widely used result of their easy and comparatively low cost. There are many serological procedures available for diagnosis of VL such as IFAT, ICT, ELISA, latex agglutination, and direct agglutination[16]. The current study was agreed with another study conducted by Maritati et al. [6] showed in their study IFA test appeared sensitivity of 100% and specificity of 100% in the diagnosis of VL in patients, thus emphasizing that this test reveals the actual infection.

Moreover, our outcomes concur with previous reports about IFAT that showed its sensitivity and specificity for identification of VL ranging (78.8-100%) in immunocompetent individuals [17,10].

El Hamouchi et al.[18] shown a large number of studies looking on the potential role of parasites in clinical symptoms of leishmaniasis showed the relation between genotypes of Leishmania and clinical form, and illustrated that genotyping of parasites has an important role in the estimation of clinical and epidemiological hazard.Falih et al.[19] showed in their study the amplification of kDNA(kinetoplast DNA)minicircles by PCR and sequencing shows the genetic variation between isolates of Leishmania.

Concerning phylogenetic tree analysis, one study showed that sequencing was used to evaluate the genetic variation in the six isolates L. infantum discovered. The analysis of the phylogenetic tree for L. infantum depends on kDNA illustrated the genetic variation level and correlation between host, the geographical origin of parasite, and its genetic variation [20]. Higher genetic variation is considered one of characteristic of kDNA, as indicated through many investigators. Cortes et al. [21] showed a high level polymorphism for kDNA gene in L. infantum isolates. Kariyawasam et al. [22] indicated high diversity of haplotype for L. donovani in Sri Lanka and Portugal. This characteristic was observed in Leishmania species [23].

Conclusions

In sum, the outcomes showed that VL represents important health issues in the research areas and must be monitored continuously by health professionals.The outcomes appeared that 65 positive samples of VL were diagnosed with IFAT and there are 60 positive samples of VL were diagnosed by kDNA .The current study showed the efficiency of these tests in the diagnosis.

Besides, it was registered local Iraqi isolates in GenBank with accession number OR886069.1

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None

Author's Contributions

Zeina Anwar Jaffar:Designing the experiment, collecting samples, analyzing samples (software), data analysis, and writing the manuscript.

Amani Mohammed Jasim and Ghanim Husien Majeed: Designing the experiment, supervision, and writing-reviewing of the manuscript.

Ethics

This study was approved by the ethics committee of Middle Technical University / Health and Medical Technology College to perform this study. Also, ethical approval was obtained for the sample collection from the ethics committee in the Ministry of Health /Iraq (Ref:144498 dated 11/9/2022). Moreover, it was obtained oral consent from patients to collect blood samples.

. References

1. Al-Naimy, A. F., & Al-Waaly, A. B. (2021). Investigation of Cutaneous Leishmaniasis Cases in Baghdad Province, Iraq. Prof.(Dr) RK Sharma, 21(1), 65. https://doi.org/10.37506/mlu.v21i1.2282.

2. Sundar, S., & Singh, O. P. (2018). Molecular diagnosis of visceral leishmaniasis. Molecular diagnosis & therapy, 22(4), 443-457. https://www.ncbi.nlm.nih.gov/pmc/ articles/ PMC6301112

3. Mody, R. M., Lakhal-Naouar, I., Sherwood, J. E., Koles, N. L., Shaw, D., Bigley, D. P., Co, E. A., Copeland, N. K., Jagodzinski, L. L., Mukbel, R. M., Smiley, R. A., Duncan, R. C., Kamhawi, S., Jeronimo, S. M. B., DeFraites, R. F., & Aronson, N. E. (2019). Asymptomatic Visceral Leishmania infantum Infection in US Soldiers Deployed to Iraq. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, *68*(12), 2036–2044. https://doi.org/10.1093/cid/ciy811

4. Hantosh, H. A., & Al Lami, F. H. (2013). Prevalence of asymptomatic visceral leishmaniasis among under 5 years contacts of confirmed cases in Thiqar Governorate, 2012. *J Infect Dis Ther*, *1*(122), 2332-0877.

5. World Health Organization (2022) The global health observatory. Available: https://www.who.int/data/gho/data/themes/topics/gho-ntd-leishmaniasis . Accessed: 29 October 2022.

6. Maritati, M., Trentini, A., Michel, G., Hanau, S., Guarino, M., De Giorgio, R., ... & Contini, C. (2023). Performance of five serological tests in the diagnosis of visceral and cryptic leishmaniasis: a comparative study. *The Journal of Infection in Developing Countries*, *17*(05), 693-699.

7. de Godoy, N. S., Andrino, M. L. A., de Souza, R. M., Gakiya, E., Amato, V. S., Lindoso, J. Â. L., & Almeida Braz, L. M. (2016). Could kDNA-PCR in peripheral blood replace the examination of bone marrow for the diagnosis of visceral leishmaniasis?. *Journal of Parasitology Research*, *2016*.

8. Gonçalves, C., Borges, A., Dias, V., Marques, J., Aguiar, B., Costa, C., & Silva, R. (2023). Detection of Human Visceral Leishmaniasis Parasites in Microscopy Images from Bone Marrow Parasitological Examination. *Applied Sciences*, *13*(14), 8076.

9. Lévêque, M. F., Lachaud, L., Simon, L., Battery, E., Marty, P., & Pomares, C. (2020). Place of serology in the diagnosis of zoonotic leishmaniases with a focus on visceral leishmaniasis due to Leishmania infantum. *Frontiers in Cellular and Infection Microbiology*, *10*, 67.

10. Freire, M. L., Machado de Assis, T., Oliveira, E., Moreira de Avelar, D., Siqueira, I. C., Barral, A., ... & Cota, G. (2019). Performance of serological tests available in Brazil for the diagnosis of human visceral leishmaniasis. *PLoS neglected tropical diseases*, *13*(7), e0007484.

11. Adel, A., Berkvens, D., Abatih, E., Soukehal, A., Bianchini, J., & Saegerman, C. (2016). Evaluation of Immunofluorescence Antibody Test Used for the Diagnosis of Canine Leishmaniasis in the Mediterranean Basin: A Systematic Review and Meta-Analysis. *PloS one*, *11*(8), e0161051. https://doi.org/10.1371/journal.pone.0161051

12. Maia, Z., Lírio, M., Mistro, S., Mendes, C. M. C., Mehta, S. R., & Badaro, R. (2012). Comparative study of rK39 Leishmania antigen for serodiagnosis of visceral leishmaniasis: systematic review with meta-analysis. *PLoS neglected tropical diseases*, *6*(1), e1484.

13. Bangert, M., Flores-Chavez, M. D., Llanes-Acevedo, I. P., Arcones, C., Chicharro, C., Garcia, E., ... & Cruz, I. (2018). Validation of rK39 immunochromatographic test and direct agglutination test for the diagnosis of Mediterranean visceral leishmaniasis in Spain. *PLoS neglected tropical diseases*, *12*(3), e0006277.

14. Alvar, J., Canavate, C., Gutierrez-Solar, B., Jimenez, M., Laguna, F., Lopez-Velez, R., ... & Moreno, J. (1997). Leishmania and human immunodeficiency virus coinfection: the first 10 years. *Clinical microbiology reviews*, *10*(2), 298-319.

15. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011 Oct;28(10):2731-9. doi: 10.1093/molbev/msr121.

16. Asfaram, S., Teshnizi, S. H., Fakhar, M., Banimostafavi, E. S., & Soosaraei, M. (2018). Is urine a reliable clinical sample for the diagnosis of human visceral leishmaniasis? A systematic review and meta-analysis. *Parasitology international*, *67*(5), 575-583.

17. Krepis, P., Krepi, A., Argyri, I., Aggelis, A., Soldatou, A., Papaevangelou, V., & Tsolia, M. (2018). Childhood visceral leishmaniasis: distinctive features and diagnosis of a re-emerging disease. An 11-year experience from a tertiary referral center in Athens, Greece. *The Pediatric Infectious Disease Journal*, *37*(5), 419-423.

18. El Hamouchi, A., El Kacem, S., Ejghal, R., & Lemrani, M. (2018). Genetic polymorphism in Leishmania infantum isolates from human and animals determined by nagt PCR-RFLP. *Infectious Diseases of Poverty*, *7*, 1-8.

19. Flaih, M. H., Al-Abady, F. A., & Hussein, K. R. (2021). Phylogenetic analysis of kinetoplast DNA: kDNA of Leishmania tropica in Thi-Qar province, Iraq. *Comparative Immunology, Microbiology and Infectious Diseases*, *78*, 101696.

20. Behniafar, H., Taghipour, N., Spotin, A., Zare, Z., Tabaei, S. J. S., Kazemirad, E., Moin Vaziri, V., & Mohebali, M. (2024). Detection and phylogenetic analysis of kinetoplast DNA of Leishmania infantum infected humans, domestic dogs and sandflies in Northwest Iran. *PloS one*, *19*(3), e0296777.

21. Cortes, S., Mauricio, I., Almeida, A., Cristovão, J. M., Pratlong, F., Dedet, J. P., & Campino, L. (2006). Application of kDNA as a molecular marker to analyse Leishmania infantum diversity in Portugal. *Parasitology international*, *55*(4), 277-283.

22. Kariyawasam, U. L., Selvapandiyan, A., Rai, K., Wani, T. H., Ahuja, K., Beg, M. A., ... & Karunaweera, N. D. (2017). Genetic diversity of Leishmania donovani that causes cutaneous leishmaniasis in Sri Lanka: a cross sectional study with regional comparisons. *BMC Infectious Diseases*, *17*, 1-11.

23. Oryan, A., Shirian, S., Tabandeh, M. R., Hatam, G. R., Randau, G., & Daneshbod, Y. (2013). Genetic diversity of Leishmania major strains isolated from different clinical forms of cutaneous leishmaniasis in southern Iran based on minicircle kDNA. *Infection, Genetics and Evolution*, *19*, 226-231.