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Identification and Sequences of Two *Leishmania*Species Isolated From Human Cutaneous Lesion in Iraq

[*]AbdulKareemAakoolAl-Tamemy [(1)]; Noor Naeem Shakir Al-Qurashi [(2)]
(1) (College ofScience,DepartmentofBiology)WasitUniversity, Iraq
(2) (College ofScience,DepartmentofBiology) WasitUniversity, Iraq
Corresponding Author: Abdul Kareem Aakool Al-Tamemy

Abstract:*Total 80 specimens collected passively from patients with cutaneous ulcers suggestive of leishmaniasis attending hospital and health centers in all parts of Iraq for diagnosis were included in the study. Microscopic examination of dermal Giemsa-stained smears scrapings revealed LD bodies in 60 (75%)samples gave positive results whereas Nested PCR detectLeishmaniaparasite in 68(85%) samples;the cutaneousleishmaniasis (CL) in Iraq are caused by two species of Leishmania.There are only seven positive cases was detected as L. Tropicathat distribute in city center and sixty one is L. major in different area of Wasit-Iraq.Nested PCRwas depend as molecular technique to differentiate parasites species depend on molecular weight the fragment with 560bp indicated Leishmania major and 750 bp indicated L. tropica, . Phylogenetic analysis grouped the Leishmania isolates into two main clades, representing L. major, and L. tropica.*

Keywords: *cutaneous leishmaniasis, Sequence.Phylogenetic tree. Nested PCR.*

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I. Introduction

Leishmaniasis, is a third of the most important vector-borne diseases, it is caused by a protozoan intracellular obligatory parasite of the genus Leishmania, which transmitted by the bite of sand flies, Leishmaniasis is one of the most neglected tropical diseases with current high worldwide incidence [1]. It is endemic in 88 countries with an estimated of 12 million cases worldwide in addition to about 2 million new cases annually; 90% of which occur in Afghanistan, Algeria, Brazil, Iraq, Peru, Saudi Arabia, Syria and Iran[2,3].

In molecular biology the polymerase chain reaction (PCR) is a diagnostic tool with a higher sensitivity compared to serologic methods in identifying parasite in tissue; molecular techniques such as PCR and Real-time PCR can determine the species of the parasite with only a small amount of DNA from the infected tissue[4]. Specifically identify *Leishmania* species by PCR via targeting different regions of kinetoplast and nuclear DNA [5]. Kinetoplast minicircles DNA were considered good candidates for detecting *Leishmania* spp. because of their high copy numbers [6]. The kDNA PCR is considered to be the most sensitive method for diagnosing leishmaniasis since there are ~10,000 minicircles per parasite[7]. This method has shown the highest sensitivity (98.7%) for the diagnosis of CL in other studies too[7, 8].Nested PCR approach was applied for the detection and identification of the *Leishmania* parasites according to the Noyes *et al.* (1998)method [9]. This test used to identify the *Leishmania* species. Nested PCR is a one of the best parts of the parasite genome for sequencing to identify different *Leishmania* species[9, 10]. Genomic sequencing of a wide variety of organisms enables scientists to compare and explore the similarities and peculiarities among different species at the molecular level[11, 12]. The trypanosomatids are protozoan parasites of medical relevance that have evolved a unique organization of their genetic information, the control of gene expression in these organisms differs from most of the eukaryotes mainly in the lack of transcriptional control and in the absence of canonical RNA polymerase II promoters and of typical transcription factors [13,14]. As with most other single celled organisms, evolutionary relationships within Trypanosomatidae were very poorly known prior to the availability of molecular data because there are few morphological characters documenting relationships within this family. The advent of molecular sequence data provided many additional characters for phylogenetic analysis, but so far evolutionary relationships within the family remain poorly resolved even by molecular data[15, 16].

The study aimed todetection of genetic characteristics of *Leishmaniatropica* and *L. major*and identify sequences ofIraqiisolates.The origin of the parasite causing the disease, and the geographical location and the compatibility of the genetic traits with the strains spread throughout the world by phylogenetic tree.

II. Materials And Methods

1.Samples collection:

Eighty specimens collectedfrom different cases suspected cutaneous leishmaniasis in Al-Zehraa Teaching hospital and Karamah Teaching Hospitals in Iraq (Figure 1). The patients complained from skin lesion in exposed part of the body mostly in the face, leg, and arm and diagnosed clinically by special dermatologist as cutaneous leishmaniasis. Samples divided into two partition, small amount to preparing staining smear and the other preserved in deepfreeze (-20 °C) for molecular analysis.

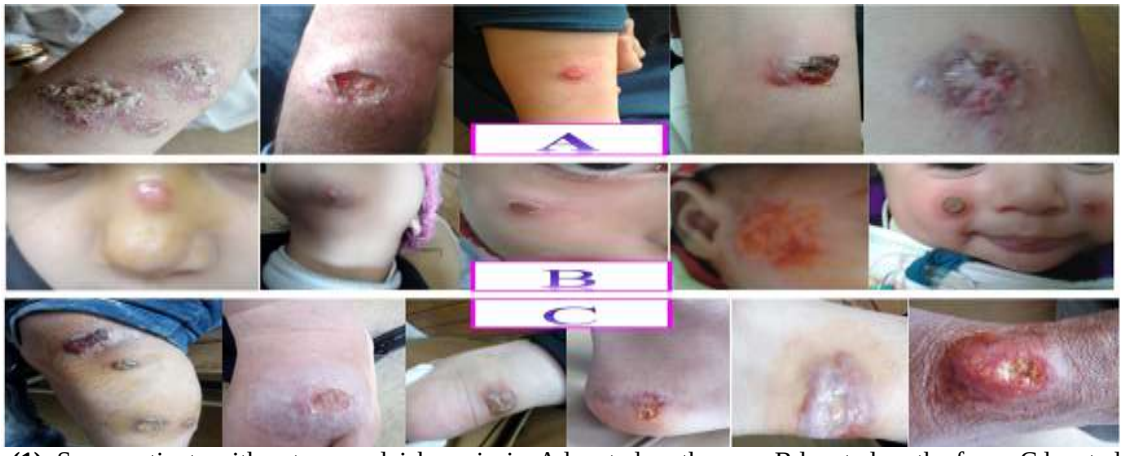

Fig. (1): Some patients with cutaneous leishmaniasis, A located on the arm, B located on the face , C located on the leg

2- Microscopic Examination

Direct examination of Giemsa staining smears ofskin scraping to detect LD bodies. If at least one intra- or extracellular amastigote with adistinctive kinetoplast was found the smear was declaredpositive.

3- Genomic DNA Extraction:

Genomic DNA was extracted from specimen of skin lesion by using AccuPrep® Genomic DNA Extraction Kit according to the manufacturer's instructions (Geneaid, USA). The purified DNA. Being stored at -20 °C for later analysis.

4- Nested PCR

Nested PCR approach was applied for the detection and identification of the *Leishmania* species,Thevariable minicircles kinetoplast DNA (kDNA) was the target of this test,the primers for *L. major* and *L. tropica* were design by [9]and provided by (Bioneer company, Korea)**(table 1).**

Table (1):primers sequence of Nested PCR.

Primer	**Sequence**	
Primary primer	**F** (CSB2XF**)**	CGAGTAGCAGAAACTCCCGTTCA
	R (CSB1XF**)**	ATTTTTCGCGATTTTCGCAGAACG
Secondary primer	**F**(13Z**)**	ACTGGGGGTTGGTGTAAAATAG
	R(LiR)	TCGCAGAACGCCCCT

The Nested PCR was performed according to [9], the totalvolume of component of primary reaction was 20 µL, including 5µL. Genomic DNA, 1.5µL. of each primers and 12 µL. of master mix. The total volume of component of secondary reaction also 20µL. including the, 3µL. Primary round PCR product, 1.5µL. of each primers and 14 µL. of master mix, the PCR PreMix as lyophilized materials containing all other components needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and tracking dye).Cycling of two rounds was performed in a thermocycler (MULTIGENE $_{\text{Labnet}}$) with the following conditions: an initial denaturation step at 5 min at 95˚c, thirty cycles of denaturation, 30 second at 95˚c, annealing 30second at 55˚c, elongation 1 min at 72˚c followed with, finally, an extension step at 5 min at 72 ˚c.

5- Gel electrophoresis

PCR products were analyzed by loading in 1% Agarose gel containing 3µL of ethidium bromide and electric current was performed at 100 volt and 80 mA for 1hour. PCR products were visualized by using ultraviolet transilluminator.

6- DNA sequencing

Twenty µLof a second round Nested PCR products were sequencedUPGMA method. By NCBI site was used to draw the phylogeny trees of *L. major* and *L. tropica.*

III. Results And Discussion

The microscopic examination of 80 staining slide to detect LD body, there are 60(75%) positiveslides, figure (2), but the results of PCR directed to kDNAwas 68(85%). These resultscompatible with [17] whenexaminationof the 93 samples obtained from direct smears of suspectedpatients, 81 were positive in parasitological examination while 84 were positive in PCR directed to kDNA. The results also agreement with other studies such as [18]that have prepared about 200 slides in which 35 were positive but in PCR techniques about 55 were positive from a total cases.

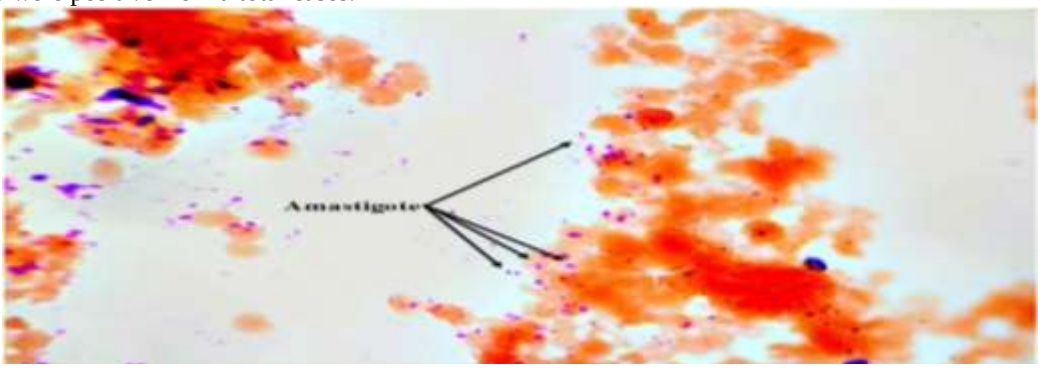

Fig.(2): Amastigote with kDNA by direct skin smear (Giemsa stain,1000x).

1- Nested PCR

The eighty samples that tested by this technique, only 68 samples was positive and the other were negative, the bands of DNA that appear in figure (3) with different size depending on species of parasite. There are seven samples of 68 appear to be *L. tropica* in which the DNA bands of *L. tropica* was 750bp,the other 61 samples was *L. major*that showing 560 bp molecular weight. Nested-PCR based method that permits both very sensitive detection and high resolution identification of *Leishmania* parasites directly from clinical samples[9] .The present study was revealed that the highest infection (89.7%) caused by *L. major* than *L. tropica* (10.2%), several studies found that the cause of CL in Iraq is *L. major* more than *L. tropica*[19] .This results also agreement with[20,21,22] where it study the diagnosis of two species of *Leishmania* in Iraq by using PCR technique.

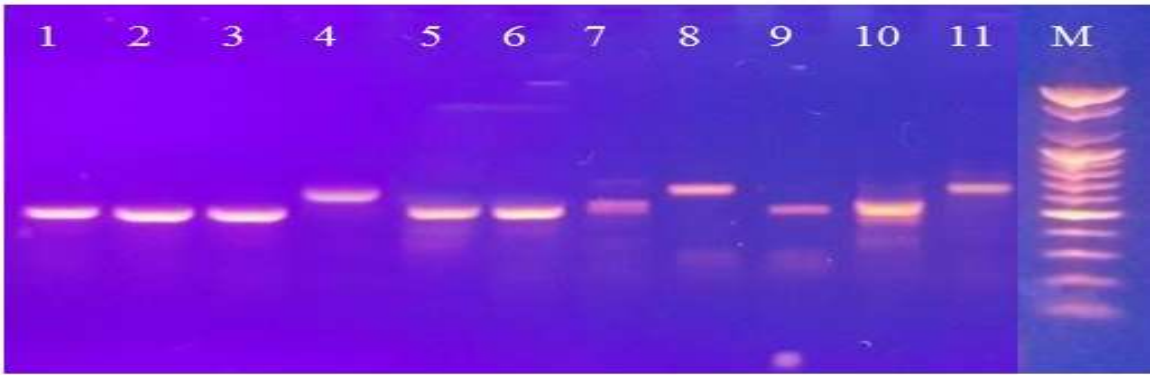

Fig. (3): gel electrophoresis of DNA fragment the produced by PCR product M DNA size marker, lane4, 8and 11*L. tropica* size 750 and all remained *L. major* size 560.

The seven positive cases of *L. tropica* distribute in city center where is no reservoir host vertebrate animals while the high distribution of ZCL may be due to the presence of reservoir animals in large numbers in rural region [23, 24, 25].Leishmania species distinguished depending on molecular weight of DNA fragments that produced by using kinetoplast DNA specific primer in PCR, the large DNA fragment of *L. tropica* (750)pb and smaller PCR product (560) bp could be identified as derived from *L. major* in this result compatible with [26,27,28].This results also agreement with [29] when used conventional PCR technique by specific primer targeting kDNA to differentiate the species of parasites that causes cutaneous leishmaniasis in different regions of Iraq , the molecular weight of DNA fragment (650) bp indicated *L. major* and (800) bp indicated *L.* tropica.

2- Nucleotide sequence and Phylogenetic tree

Nucleotide sequence data reported here have been submitted to the GenBank database with accession numbers, *L. tropica* KM491168.1 and *L. major*KC869688.1. The phylogenytrees of these two species are shown in Figure (4).Phylogenetic tree analysis based on the partial of kinetoplast minicircle DNA that used for confirmative genetic detection. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and Molecular Evolutionary Genetic (MEGA 6.0 version). Local *Leishmania major* isolate No.1-No.5 were show closed related to NCBI-Blast *Leishmania major* isolate kinetoplast minicircle DNA (KC869688.1), local *Leishmania tropica* isolate No.1-No.5 by were show closed related to NCBI-Blast *Leishmania tropica* isolate kinetoplast minicircle DNA (KM491168.1), with different identical 98-100% and other NCBI-Blast *Leishmania spp*. were show more different and out of tree at total genetic change (0.1-0.7%).The present study is the first in Iraq in the molecular analysis of cutaneous leishmaniasis and the magnitude of genetic attraction between them and the global strains depending on the genetic tree where the DNA sequence of samples of *Leishmania* parasite was determined. Analysis of the sequence of the nitrogen bases of the polymerase chain reaction products of the Leishmania parasite gene showed that all the samples studied were consistent with those recorded at the National center Biotechnology information (NCBI).The sequences L. major (KC869688.1) and L. tropica (KM491168.1), were similar to the Pakistan ,Iran . *L. tropica* sequence deposited in GenBank under method to detect *Leishmania* DNA[30], and identity to the published isolate These 2 isolates showed 99% identity to the published isolates from UK, Egypt, and Iran (nos. AF308689, X84845, and KM491168) [31]The phylogenetic analysis revealed that the positive sample in this research was closely related to the Iraqi *L. tropica* kDNA with accession number MF166796, MF166797, MF166798, MF166799, MF166800 .the rustle compatibly with [32] in study *L . tropica* in dog in Iran with accession number KM491168.And the sequences of fivestock of *L. tropica* showed 100% identity to the [30] published isolateIran(KM491168).The phylogenetic analysis is also revealed that the positive samples in this study was closely related to the Iraqi *L. major* kDNA with accession number MF166791 , MF166792, MF166793, MF166794, MF166795.

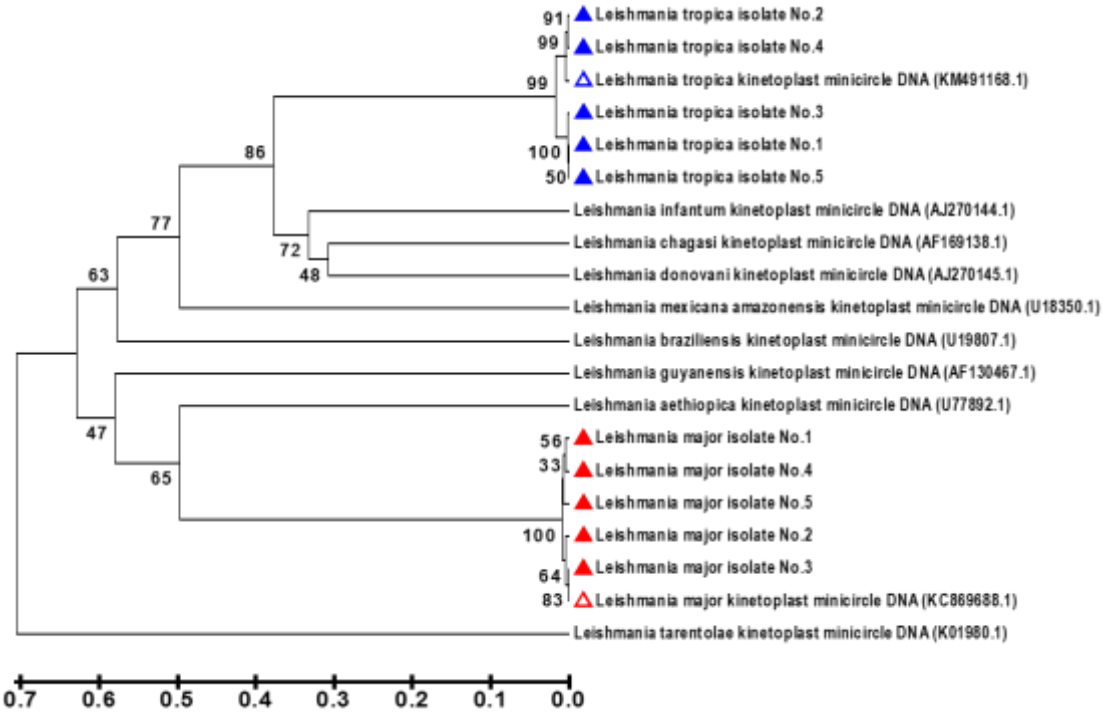

Figure (4): Phylogenetic tree analysis based on the partial of kinetoplast minicircle DNA that used for confirmative genetic detection.

IV. Conclusion

Both *L. tropica* and *L. major* are the causative agents of cutaneous leishmaniasis in Iraq *L. major is* the dominant *Leishmania* species in Iraq. The Nested PCR is a very reliable technique to species differentiation and identification. In addition, record the isolate strain in gen bank as the first phylogenetic tree of *Leishmania*parasite in Iraq.

References

[1] de Vries, H. J., Reedijk, S. H., &Schallig, H. D. (2015). Cutaneous leishmaniasis: recent developments in diagnosis and management. American journal of clinical dermatology, 16(2), 99.

[2] World Health Organization. Control of the leishmaniasis. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniasis, 2010, Geneva, 22-26 March 2010. WHO technical report series 949. Geneva: World Health Organization; 2010. [Online] Available from: http://apps.who.int/iris/bitstream/10665/44412/1/WHO_TRS_949_eng. pdf [Accessed on 14th January, 2016

[3] World Health Organization. Leishmaniasis. Fact sheet N°375 (updated February 2015). Geneva: World Health Organization; 2015. [Online] Available from: http://www.who.int/mediacentre/factsheets/fs375/en/ [Accessed on 14th January, 2016]

[4] Cruz, I., Millet, A., Carrillo, E., Chenik, M., Salotra, P., Verma, S&Arévalo, J. (2013). An approach for interlaboratory comparison of conventional and real-time PCR assays for diagnosis of human leishmaniasis. Experimental parasitology, 134(3), 281-

[5] Ready, P. D. (2014). Epidemiology of visceral leishmaniasis. Clinical epidemiology, 6, 147.289.

[6] Ozerdem, D., Eroglu, F., Genc, A., Demirkazik, M., &Koltas, I. S. (2009). Comparison of microscopic examination, rK39, and PCR for visceral leishmaniasis diagnosis in Turkey. Parasitology research, 106(1), 197-200.

[7] Ghasemloo H, Rasti S, Delavari M, Doroodgar A(2016). Molecular Diagnosis of Clinical Isolates of Cutaneous Leishmaniasis Using ITS1 and KDNA Genes and Genetic Polymorphism of Leishmania in Kashan, Iran. Pak. J. Boil. Sci. 2016; 19(3): 136- 142.].

[8] Bensoussan, E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL(2006). Comparison of PCR Assays for Diagnosis of Cutaneous Leishmaniasis. J ClinMicrobiol. 2006; 44(4): 1435–1439

[9] Noyes, H. A., Reyburn, H., Bailey, J. W., & Smith, D. (1998). A nested-PCR-based schizodeme method for identifying Leishmania kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of Leishmania tropica in Pakistan. Journal of clinical microbiology, 36(10), 2877-2881

[10] Aransay, A. M., Scoulica, E., &Tselentis, Y. (2000). Detection and identification of Leishmania DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. Applied and environmental microbiology, 66(5), 1933-1938

[11] Delsuc F, Brinkmann H, et al. (2005): Phylogenomics and the reconstruction of the tree of life. Nat Rev Genet 6(5):361–375.

[12] Snel B, Huynen MA, et al. (2005): Genome trees and the nature of genome evolution. Annu Rev Microbiol 59:191–209.)

[13] LeBowitz JH, Smith HQ, Rusche L, Beverley SM(1993).Coupling of poly(A) site selection and trans-splicing in Leishmania. Genes Dev 1993;7:996–1007

[14] Vanhamme, L., & Pays, E. (1995). Control of gene expression in trypanosomes. Microbiological reviews, 59(2), 223-240.

[15] Wright, A. D. G., Li, S., Feng, S., Martin, D. S., & Lynn, D. H. (1999). Phylogenetic position of the kinetoplastids, Cryptobiabullocki, Cryptobiacatostomi, and Cryptobiasalmositica and monophyly of the genus Trypanosoma inferred from small subunit ribosomal RNA sequences. Molecular and biochemical parasitology, 99(1), 69-76.

[16] Stevens, J. R., Noyes, H. A., Schofield, C. J., & Gibson, W. (2001). The molecular evolution of Trypanosomatidae. Advances in parasitology, 48, 1-53.

[17] Rezai, A., Moghaddas, E., Bagherpor, M. R., Naseri, A., &Shamsian, S. A. (2017). Identification of Leishmania Species for Cutaneous leishmaniasis in Gonabad, Bardaskan and Kashmar, Central Khorasan, 2015. Jundishapur Journal of Microbiology, (In press).

[18] Rehman, A. U., Rehman, H. U., Ayaz, S., Gul, H., Tahseen, M., Ishaq, M &Jehan, S. (2016). Molecular detection of cutaneous leishmaniasis in human at district Hangu Khyber Pakhtunkhwa, Pakistan.Journal of Entomology and Zoology Studies 4(3): 94-97

[19] Ali, M. A., Rahi, A. A., &Khamesipour, A. (2015). Species Typing with PCR–RFLP from Cutaneous Leishmaniasis Patients in Iraq. Donnish Journal of Medicine and Medical Sciences, 2(3), 026-031.

[20] Qader, A. M., Abood, M. K., &Bakir, T. Y. (2009). Identification of Leishmania parasites in clinical samples obtained from Cutaneous Leishmaniasis patients using PCR technique in Iraq. Iraqi Journal of Science, 50(1), 32-6.

[21] Alimoradi, S., Hajjaran, H., Mohebali, M., &Mansouri, F. (2009). Molecular identification of Leishmania species isolated from human cutaneous leishmaniasis by RAPD-PCR. Iranian Journal of Public Health, 38(2), 44-50

[22] Rahi, A. A., Nsaif, S., Hassoni, J. J., Ali, M. A., & Hamza, H. A. (2013). Comparison of diagnostic methods in Cutaneous Leishmaniasis in Iraq. Am J BioSci, 1(1), 1-5.

[23] Al Samarai, A.M. and AlObaidi,H.S.(2009). Cutaneous leishmaniasis in Iraq. J. Infect Developing Countries; 3(2):123-129

[24] World Health Organization (2000) Leishmaniasis and Leishmania / HIV co- infection. In: WHO report on global surveillance of epidemic prone infectious diseases, WHO/CDS/CSR/ISR/2000.1.PP:121-127.

[25] Philippon E, Gramond C, Chouc C. (2005) Leishmaniasis. Med Trop 65:419-420.

[26] Vazirianzadeh, B., Saki, J., Jahanifard, E., Zarean, M., Amraee, K., & Pour, S. N. (2013). Isolation and Identification of Leishmania Species From Sandflies and Rodents Collected From Roffaye District, Khuzestan Province, South west of Iran. Jundishapur Journal of Microbiology, 6(6).

[27] Haddad, M. H. F., Ghasemi, E., Maraghi, S., &Tavala, M. (2016). Identification of Leishmania species isolated from human cutaneous leishmaniasis in Mehran, Western Iran using nested PCR. Iranian journal of parasitology, 11(1), 65.

[28] Akhtardanesh, B., Sharifi, I., Mohammadi, A., Mostafavi, M., Hakimmipour, M., &Pourafshar, N. G. (2017). Feline visceral leishmaniasis in Kerman, southeast of Iran: Serological and molecular study. Journal of Vector Borne Diseases, 54(1), 96.

[29] Al tamemy Abdul Kareem; May H. Kawan , Ihsan M. Al-Saqur (2012)Viscerotropic Leishmaniasis; Diagnosis and species detection using the polymerase chain reaction with kinetoplast DNA-specific primers in Balb/c mice Wasit Journal for Science && Medicine 5 (1): (32 - 48)

[30] Mohammadpour, I., Motazedian, M. H., Handjani, F., &Hatam, G. R. (2017). Lip leishmaniasis: a case series with molecular identification and literature review. BMC infectious diseases, 17(1), 96.

[31] Mohammadpour, I., Motazedian, M. H., Handjani, F., &Hatam, G. R. (2016). Cutaneous Leishmaniasis of the Eyelids: A Case Series with Molecular Identification and Literature Review. The Korean journal of parasitology, 54(6), 787.

[32] Bamorovat, M., Sharifi, I., Dabiri, S., Mohammadi, M. A., Harandi, M. F., Mohebali, M., ...&Keyhani, A. (2015). Leishmania tropica in stray dogs in southeast Iran. Iranian journal of public health, 44(10), 1359.

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