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RESEARCH ARTICLE

The best method for Diagnosis of Cutaneous Leishmaniasis and identification of the causative Leishmania species in Al-Najaf governorate by using PCR assay

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Abstract

Background: The diagnosis of cutaneous leishmaniasis (CL) might be difficult, in particular in endemic areas where different species of Leishmania can cause lesions of very similar appearance and where other skin diseases with similar clinical symptoms occur. Even today, the parasitological diagnosis of CL remains the gold standard and it is based on the direct identification of amastigotes in microscopy smears and/or culture of promastigotes from infected tissues. Although these techniques are highly specific, they are not sensitive enough.

Objective: The present study aimed to choose the best method and best medium in isolation and cultivation of cutaneous leishmania parasite that gives rapid recovery of parasite in the first inoculation from the patients and determine the effectiveness of a polymerase chain reaction (PCR) technique for identification of Leishmania species in Al-Najaf governorate by PCR assay and differentiation of the cutaneous leishmania parasites in clinical sample collected from lesion exudates patients.

Methods: A total of 138 patients presenting with cutaneous lesions suggestive of CL were sampled for parasitological diagnosis by direct examination (DE), cutaneous leishmania parasites were cultured in tow different media include Novy-Mac Neal-Nicolle (NNN) medium and Roswell Park Memorial Institute medium (RPMI)1640 with fetal calf serum then DNA isolation. The DNA of the promastigote were amplified by PCR including primers selected on repetitive KDNA for identification of leishmania species. The data was analyzed by using frequency and percentage.

Results: It's found that NNN medium was the best medium in maintenance and survival growth of parasite and RPMI1640 was the best medium in increasing parasite number gtfas a same peak growth period, which only one day. The recovery time of isolated cutaneous leishmania from RPMI1640 with FCS was the most rapid medium in leishmania parasite isolation (seven days) in comparison with NNN medium (twenty one to thirty days**).** On other part comparison done between both isolation of cutaneous leishmania parasite and cultivation of an old cultured cutaneous leishmania parasite from NNN media in RPMI1640 with 10% FCS there is difference in growth peak number and in recovery time .The PCR result showed that two species of *Leishmania* (*Leishmania major* with 620 bp and *Leishmania tropica* with 760 bp) exist .

Conclusions: : The PCR technique has high specificity and sensitivity during the differentiation between Cutaneous Leishmanial species compared to the conventional methods.

INTRODUCTION

Leishmaniases are vector-borne parasitic diseases caused by protozoa of the genus Leishmania (Kinetoplastida: Trypanosomatidae) [1]. The clinical spectrum of these diseases range from self- resolving cutaneous lesions to visceral forms , fatal if left untreated. Leishmaniases remain one of the world's most devastating neglected tropical diseases. According to the World Health Organization (WHO), up to 350 million people are at risk in 98 countries around the world. It is considered that approximately 12 million people are currently infected, and 2 million new infections occur every year, of which an estimated 1.5 million cases are cutaneous cutaneous leishmaniasi**s** and 500,000 cases of visceral leishmaniasis [2,3,4].

The leishmaniasis is manifest itself in verity of clinical forms, ranging from self-healing cutaneous lesion to the more serious, potentially fatal visceralizing form, and include the metastasize muco-cutaneouse form and the post kala-azar dermal leishmaniasis [5].

The diagnosis of CL can be difficult, especially in endemic areas where different species of Leishmania can cause lesions of very similar appearance and where other skin diseases with similar clinical symptoms occur[6]. Presumptive diagnosis of CL is based on clinical symptoms. Even today, the parasitological diagnosis remains the gold standard and it is based on the direct identification of amastigotes in microscopy smears and/or culture of promastigotes from infected tissues [7]. Although these techniques are highly specific and only way available to primary health care in infested areas, they are not sensitive enough, generally time-consuming and all types of leishmaniasis are similar in form and cannot distinguish between them [3,8].In addition, culture should be kept for at least one month before definitive negative result [9].

Even today, the diagnosis of CL in Iraq is mainly based on clinical symptoms and microscopic examination of smears; culture is carried out only in some specialized laboratories , there is few trials used only NNN media and semi-solid media for Isolation and cultivation of leishmania then a RBLM Media [10].Thus, the diversity of the species associated with human CL and the changing epidemiological situation in Al-Najaf highlights the need for the development of a method allowing the diagnosis and the characterization of the infective species: this method has to be preferably simple, cheap, and less time consuming than the gold standard. In this frame, PCR-based methods offer an alternative approach to traditional techniques [11]. A crude search in the Pub- Med database revealed that since 1989, more than 700 articles on PCR diagnosis of leishmaniases have been published, in which a multitude of gene targets, protocols, and applications are described, including genus and/or species-specific PCR, ranging from low- tech to high-tech approaches [7]. The PCR-based assays currently constitute the main molecular diagnostic approach for the detection and identification of Leishmania parasites in clinical samples [7]. PCR primers targeting the kinetoplast and ribosomal DNA genes are amongst the most commonly used for the diagnosis and/or identification of Leishmania species in the Old World and they provide good results [11-18]. In Iraq ,Abdullah et al. 2009 have shown that the encoded gene of DNA that called Kinetoplast DNA (kDNA) from metukndria of leishmania parasite in technique PCR has a high sensitivity and specificity[19].

Thus, given the diversity of the clinical presentations and the circulation of potentially responsible species of CL in Al-Najaf , the objective of this study is to contribute to improving the diagnosis of the disease and the identification of the causative Leishmania species by PCR assays applied directly on dermal samples.

Materials and Methods

Patients and Samples

Amongst the patients consulting at the Department of Dermatology Al-Seder and Al-Hakim Hospitals during September 2013 to September 2014 , 138 presented with cutaneous lesions suggestive of CL ; were investigated to diagnose and identify the causative agent of the disease. The following diagnostic investigations were performed for each case:

1. Microscopic examination of Geimsa-stained smears from the ulcer border;
2. Culture from skin lesion scrapes;
3. PCR .

The dermal syringe-sucked fluid was collected under sterile conditions from the border of active skin lesions from each patient as follows: the lesions were cleaned with alcohol, and 0.1 to 0.2 ml of sterile saline solution was injected using a syringe (1-ml, 25-gauge needle) into the nodule and the needle was rotated gently several times. A small amount of saline solution was injected into the tissue, and then aspirated. The samples were split in three parts: The first part for culture, the 2nd put on slides for the purpose of direct microscopic examination and the 3ed Part put in 200 Maekerolatr of PBS (Phosphate buffered saline) used in the PCR. The sample meant for the PCR was conserved at −30°C until processing.

Direct examination (DE)

For making stained smears, tissues were taken using a disposable lancet. A small incision was made in the cleaned margin of the nodules and lesions with the point of the blade. The blade was turned 90 degrees and scraped along the cut edge of the incision to remove and pick up the skin tissue, which was smeared on a clean glass microscope slide. After the smears dried completely, they were fixed with 100% methanol, allowed to dry again, and stained with Geimsa stain for microscopic examination . The whole slide was analyzed with a 100× immersion objective , all the slides were inspected for the presence of amastigote forms. At least two specimens were prepared for each case. One was stained and the other stored to be applied in the next appropriate time if necessary.

Parasite culture

The syringe-sucked dermal fluid was inoculated in sterile conditions to NNN medium and RPMI1640 medium with fetal calf serum, and then incubated at 24-28°C. The supernatant was examined for parasite growth by light microscopy every three days until promastigotes were seen and subcultured once a week for 6 weeks before they were reported as negative, then cultivation of an old cultured cutaneous leishmania parasite from NNN media in RPMI1640 with 10% FCS . The cultures were made at least in duplicates for each case . Positive cultures were transferred to RPMI-1640 supplemented with 10% fetal calf serum for mass culturing.

DNA extraction

DNA was extracted from each clinical sample (100 µl volume) preserved at −30°C and from the reference strains culture (volumes corresponding to1 × 10^7 parasites) using the Pure Link™ Genomic DNA Mini Kit (Geneaid company) according to the manufacturer's instructions. The DNA was kept at −20°C until PCR processing.

PCR amplification

The PCR was performed on all 138 methanol fixed and/or Giemsa-stained samples. The PCR used to amplify the variable area of the minicircle kinetoplastic DNA of any *Leishmania* in the smears was a slight modification [20] of that described by Rodgers et al. (1990). The forward length 22 [5'- GGGGTTGGTGTTAAAATAGGCC -3 '] and reverse length 19 [5'- CTAGTTTCCCGCCTCCGAG -3 ']. We use Ready kit from Geneaid company ,each 25- µl reaction mixture contained [12.5µl Go Taq Green Master Mix, 1µl Forward primer, 1µl Reverse primer,5µl DNA , 19.5µl Nuclease-Free Water]. After the completion of all additions samples centrifuged, then samples were transferred to PCR Agilent Sure Cycler 8800 and adjust the program of work as follows: Preincubation at 94ºC for5 min, Denaturation at94ºC for 1 min , Annealing at80 sec for 48ºC, Extended at 72ºC for1 min , Extraincubation at 72ºC for 10 min,the number of cycles 35-45 cycles. A 5- µl sample of each PCR product was subjected to electrophoresis in 1.5% agarose gel. The bands were then stained with ethidium bromide and visualized under ultra-violet trans-illumination. The parasites were identified by the presence of the parasite DNA size of 760 bp and 620 bp compared with the DNA Ladder.

Statistical analysis

SPSS was Used program of statistics by which analyze the data by using mean and stander error for growth mean, the results of the various diagnostic techniques were analyzed If P was<0.05, the difference was considered significant.

Results

A total of 138 patients with a clinical suggestion of CL were investigated in a routine setting. Direct microscopy, culture, and PCR were the diagnostic techniques performed and according to our consensus criteria,95 (68.84%) samples were true positive in at least 1 of the 3 performed techniques, out of these patients, 39(41.05 %) were men and 56(58.94%) were women with an average age of 20 ± 24 years patients were mostly younger than 12 years and older than 20 years. Nine (9.47%),25 (26.31%) and 61 (64.21%) of cases had 1, 2 and 3 or more than 3 lesions respectively. The most frequently affected sites were the upper extremities (51%).

Of the 95 cases, 72(75.7%), 55(57.8%) and 90(94.7%) were positive by direct microscopy, cultivation and PCR, respectively. All the three diagnostic tests were positive for only 39 of the 95 cases. PCR and microscopy showed more correlation (*P*= 0.014).

Growth of old cultured cutaneous leishmania parasite from NNN media in RPMI 1640 with 10% FCS in 26°C show curve ,which the peak reach at fourth day of inoculation with mean of number $2.50 \times 10^6 \pm 25$, then decline occurred for eight days from the peak till reach the inoculums dose at thirteenth days of inoculation (Fig. 1).

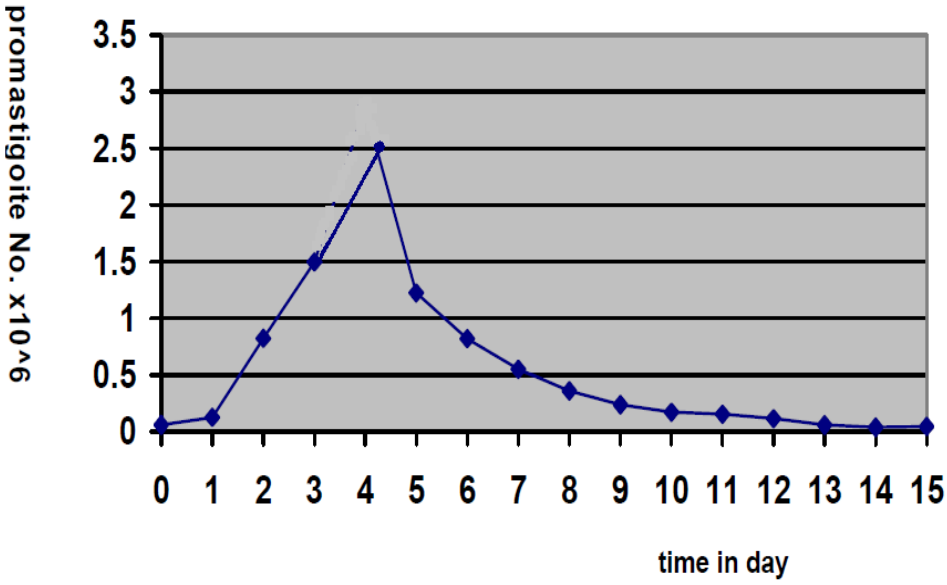

Figure (1): growth curve of old cultured cutaneous leishmania parasite in RPMI 1640 with10% FCS.

Growth of Isolated Cutaneous Leishmania parasite from Patient in RPMI 1640 with 10 % FCS in 26°C show Growth curve with the recovery time at seven day from inoculums time, peak reach with mean of number 1.549×10^6 at thirteenth day from inoculation time then decline occurred for seven days from the peak till reach to zero in number at twenty one day of inoculation(Fig.2).

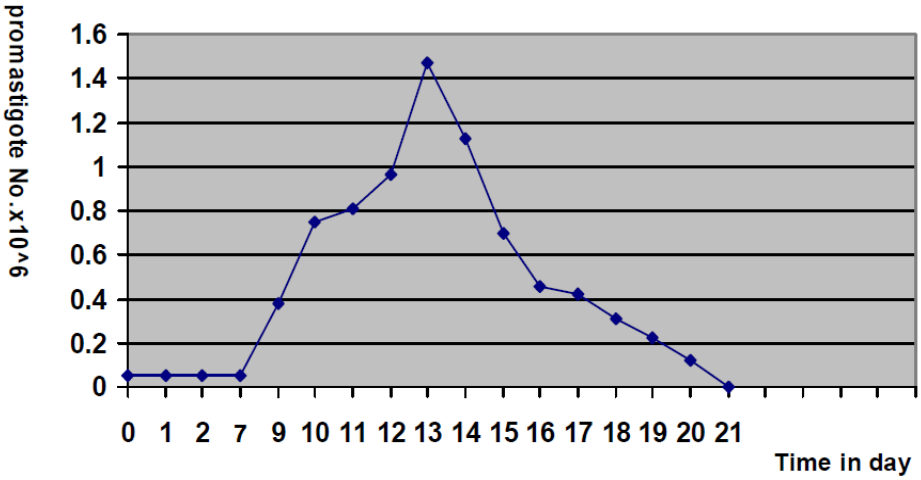

Figure (2): growth curve of isolated cutaneous leishmania parasite in RPMI 1640 with 10 % FCS

Ninety five percent and 5% of isolates were identified as *L. major*, *L. tropica*, respectively (Fig. 3).

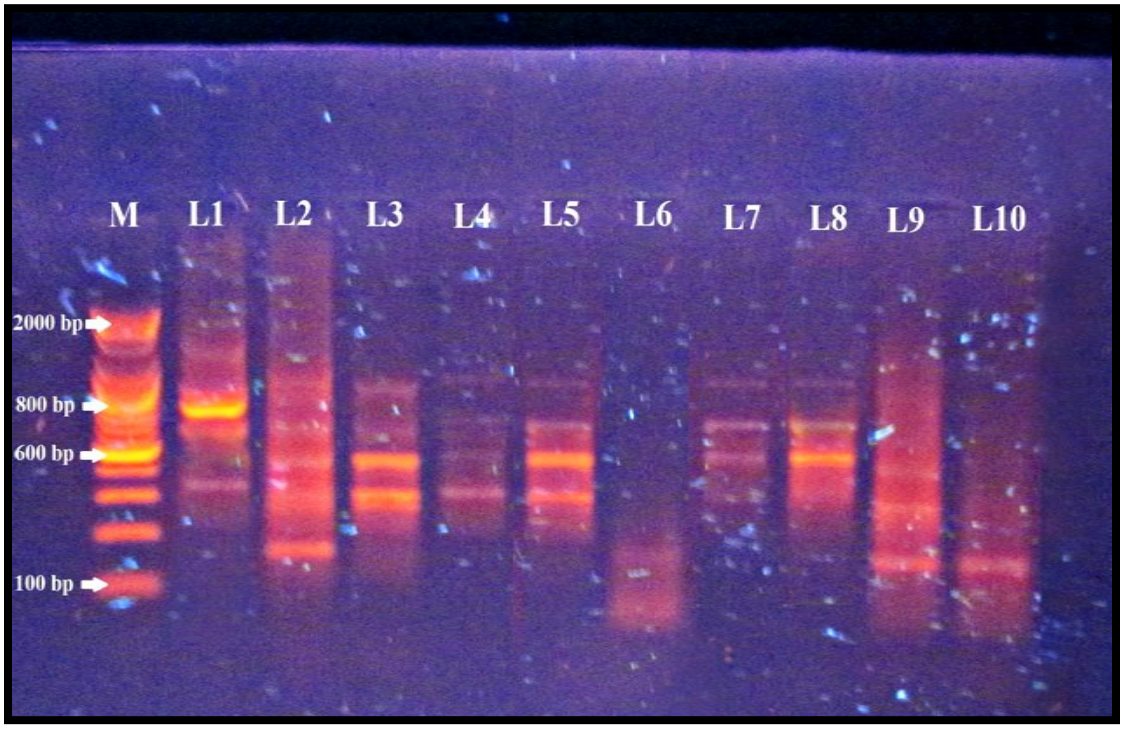

Fig. 3: The result of the PCR-based amplification of kinetoplast DNA extracted from skin lesions of the patients. The bands shown on 1.5% agarose gel stained with ethidium bromide correspond to molecular weight markers. Lanes M: ladder markers; lanes 1 : *L. tropica* (760 bp) ; lanes 3, 5, 8 and 9 : *L. major* (620 bp) .

Discussion

CL disease represents a major public health problem in Iraq, According to the WHO, women and children are the most affected groups ; this fact was consistent with our study where women were almost more affected with CL than men (p = 0.036). They may be more vulnerable because during the hot season they mostly sleep indoors at night, while men sleep outside the home [3,17,19,21].

Appropriate diagnosis and characterization of the particular parasite is important for evaluating prognosis and prescribing appropriate treatment [22] .Until recently, diagnosis of CL was based primarily on clinical symptoms, microscopic observation of the parasites in stained tissue smears, and/or culture of promastigotes from tissue [22]. Even today, microscopic identification and parasite cultivation are still primary diagnostic tools employed in many regions where leishmaniasis is endemic. Culture of promastigotes from the infected tissues and/or direct identification of amastigotes in microscope smears have long been considered as the standard for diagnosis. While these techniques are highly specific for diagnosing leishmaniasis, they are not sensitive [15] .

Growth of old cultured cutaneous leishmania parasite from NNN media in RPMI 1640 with 10% FCS as in Figure (1) of the present study shows that the growth peak of cultivated cutaneous leishmania parasite was 2.5x106 at fourth day from inoculums time as the inoculums dose was 6x104 that corresponding with[23] the peak of growth was 2.8 x106 that was faster than result of [24] who use RPMI1640 and found the peak of growth was 19x106 at ten day of inoculums time of L. tropical as the inoculums dose was 105 cell/ ml could be due to different inoculums dose**.**

Growth of Isolated Cutaneous Leishmania parasite from Patient in RPMI 1640 with 10 % FCS as in Figure (2) of this study proves that hat growth peak of isolated cutaneous leishmania was at thirteen day of inoculation which is longer to the time of result of Limoncu *et al.* who use RPMI1640 and found that the growth peak of isolated cutaneous leishmania was at seven day and this difference may be due to different strain of this parasite or different culturing environment [25]. The same figure shows that the recovery time of leishmania parasite was seven day and this time is shorter than Mobarak (2008) , but longer to the result of Andrea *et al.* who found the median of isolation time in RPMI 1640with FCS was six day, this could be due to different culture method used [23,26] .

The use of PCRs has slowly become the preferred way for diagnosing leishmaniasis since conventional parasitological methods are not sufficiently sensitive [15] . From here it became necessary to apply high specificity technology, such as using PCR With the advance of this technology for several years, it has experienced a large number of genetic targets for example (ITS1 region of rRNA genes, rRNA genes, Mini-exon, Kinetoplast DNA minicircles). kDNA had sensitivity up to 98.7% in the diagnosis of leishmaniasis parasite [27]. Many researchers have reported consistent 100% specificity with increasing sensitivity which in overall between 92 and 98 % [17,18, 22]. In the present study, PCR presented 94.7% sensitivity, significantly higher than that of direct microscopy (75.7%) and culture (57.8%) methods alone (*P*=0.001) for diagnosis of cutaneous leishmaniasis. The present study has been detected two types of this parasite first L. major with a molecular weight of 620 bp and L. tropica a molecular weight of 760 bp, the results does not match with Fazaeli *et al.* when studying the types of skin leishmaniasis using the kDNA - PCR in southern Iran, in this study recorded L. major species and unable to determine the presence of L. tropica , suggesting that skin leishmaniasis in endemic areas is Zoonotic Cutaneous Leishmaniasis [18] . The results that carried out by Abdullah *et al*. in Baghdad showed, big similarity with the above result, which showed L. major in two samples at percentage (7.4%) of the total obtained samples [19].However, our findings are corresponding to those of Mahboudi *et al*. and those of Pourmohammadi *et al*. , which was conducted in Iran, there are two types of leishmaniasis have been investigating L. tropica and L. major [28,29] . The PCR appears to be the most sensitive single diagnostic test for each form of leishmaniasis [15] .

However, microscopy and culture improved the sensitivity (75.7%, 57.8%), these are consistent with other reports in different endemic areas of the world [27,28].The values reported by Bensussan *et al.* and Pourmohammadi *et al.* for diagnosis of CL by microscopy and parasite culture were 74.4% ,62.8% and 76.71%,50.9% respectively [15, 29]. Traditional methods (direct, culture) can be time consuming, are limited by access to specialized laboratories and microscopic expertise, and have a reported sensitivity of 50-70% [29,30] .

When the PCR results(94.7%) were compared with the direct microscopy and culture methods, its sensitivity was higher than that of the two other techniques, a significant difference in sensitivity was found, our findings nearly

compatible with results of Pourmohammadi *et al.* who found that the direct microscopic examination and culture, when associated, are not sufficient to diagnose all the CL cases[29] . the PCR assays increased the speed and sensitivity of species specific leishmaniasis diagnosis compared to the conventional techniques [17,27].

In conclusion, the PCR assay indicated a high sensitivity for diagnosis and identification of CL caused by identified species of *Leishmania* parasites including *L. major* and *L. tropica* in the study area. Identification of the parasite in addition to diagnosis of the infection is necessary. In these situations, using a sensitive molecular method such as PCR will be helpful.

References

1- Bañuls, A.L.; Hide, M.; Prugnolle, F.(2007): Leishmania and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Adv Parasitol , 64:1–109.

2- Center of Disease Control and Prevention. (2003). Cutaneous leishmaniasis in U.S. military personal. south west/central Asia, 2003. MMWR Moib. Mortal. Wkly. Rep. **52**: 1009-1012.

3- World Health Organization (2010) : Control of the leishmaniases. Report of a meeting of the WHO experts committee on the control of leishmaniases, Geneva; 22–26 March .

4- Alvar, J.; Velez, I.D.; Bern, C.; Herrero, M.; Desjeux, P.; Cano, J.; Jannin, J.; Den Boer, M.; WHO Leishmaniasis Control Team(2012) : Leishmaniasis worldwide and global estimates of its incidence. PLoS One, 7(5):e35671.

5- Thakur, C. P. (2000). Socio-economics of visceral leishmaniasis in Bihar (India). Trans. R. Soc. Trop. Med. Hyg. **94**: 156-157 .

6- Ben-Ami, R.; Schnur, L.F.; Golan, Y.; Jaffe, C.L.; Mardi, T.; Zeltser, D.(2002) : Cutaneous involvement in a rare case of adult visceral leishmaniasis acquired in Israel. J Infect , 44(3):181–184.

7- Reithinger ,R.; Dujardin, J.C.(2007) : Molecular diagnosis of leishmaniasis: current status and future applications. J Clin Microbiol, 45(1):21–25

8. Magill, A.J.(2005) : Cutaneous leishmaniasis in the returning traveler. Infect Dis Clin North Am , 19(1):241–266.

9- Belhadj, S.; Hicheri-Helali, J.; Kallel, K.; Kaouech ,E.; Abaza, H.; Toumi ,H.; Hammami, M.; Chaabane, T.B.; Chaker, E.(2005) : Place de la culture dans le diagnostic parasitologique des leishmanioses viscérales et cutanées: experience tunisienne. Rev Fr Lab , 369:41–45.

10-Al-Basher ,N. M. (1990) : Axenic amastigote of leishmania parasite .Cultivation and relationship to promastigote and intracellular amastigote .MSc. Thesis, University of Baghdad

11-Azizi, K.; Soltani, A.; Alipour, H.(2012): Molecular detection of Leishmania isolated from cutaneous leishmaniasis patients in Jask County, Hormozgan Province, Southern Iran, 2008. Asian Pac J Trop Med 5(7):514–517.

12-Khademvatan, S.; Saki, J.; Maraghi, S.(2012) : Comparison of traditional methods and PCR for diagnosis of cutaneous leishmaniasis in South-West of Iran. Zahedan J Res Med Sci, 14(8):1–5.

13-El-Beshbishy, H.A.; Al-Ali, K.H.; El-Badry, A.A.(2013) : Molecular characterization of cutaneous leishmaniasis in Al-Madinah Al-Munawarah province, western Saudi Arabia. Int J Infect Dis, 17(5):e334–e338.

14-Azmi ,K.; Nasereddin, A.; Ereqat, S.; Schnur, L.; Schonian, G.; Abdeen. Z.(2011) : Methods incorporating a polymerase chain reaction and restriction Fragment length polymorphism and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis. Diagn Microbiol Infect Dis , 71(2):151–155.

15-Bensoussan, E.; Nasereddin, A.; Jonas, F.; Schnur, L.F. and Jaffe, C.L. (2006) : Comparison of PCR ssays for diagnosis of cutaneous leishmaniasis. J Clin Microbiol 2006, 44(4):1435–1439.

16-Kumar, R.; Bumb, R.A.; Ansari, N.A.; Mehta, R.D. and Salotra, P.(2007) : Cutaneous leishmaniasis caused by Leishmania tropica in Bikaner, India: parasite identification and characterization using molecular and immunologic tools. Am J Trop Med Hyg, 76(5):896–901.

17-Mouttaki ,T.; Morales-Yuste, M.; Merino-Espinosa, Gema.; Chiheb,S.; Fellah,H.; Martin-Sanchez, J. ; Riyad, M.(2014) : Molecular diagnosis of cutaneous leishmaniasis and identification of the causative Leishmania species in Morocco by using three PCR-based assays. Parasites & Vectors, 7:420.

18-Fazaeli, A.; Fouladi, B.; Hashemi-Shahr, SM. and Sharifi, I. (2008). Clinical Features of Cutaneous Leishmaniasis and Direct PCR- Based Identification of Parasite Species in A New Focus in Southeast of Iran. Iranian J Publ Health, 37, 3, pp.44-51.

19-Abdullah, M.Q.; Mushriq, K.A. and Tural, Y.B. (2009). Identification of *Leishmania* parasites in clinical samples obtained from Leishmaniasis patients using PCR technique in Iraq. Iraqi Journal of Science,50(1): 32-36.

20-Rodgers, M.R.; Popper, S.J. and Wirth, F. (1990). Amplification of Kinetoplast DNA asatool in the detection and dianosis of *Leishmania*. Exp. parasitol., 12: 267 – 75.

21- Jarallah, S. A. (2000): Epidemiological of Cutaneous leishmaniasis and visceral leishmaniasis in Iraqi. MSc.Thesis, University of Baghdad . 89 p.

22-Vega-Lopez F.(2003) : Diagnosis of cutaneous leishmaniasis. Curr Opin Infect Dis.16: 97-101.

23-Mobarak , H. M.(2008) : Isolation and Cultivation of Cutaneous Leishmania parasite by using Different Cultures Media. MSc. Thesis,University of kufa

24- Limoncu, M. E.; Balcioglu, I. C.; Yereli, K. and Ozbilgin. Y. (1997): A new experimental in vitro culture medium for cultivation of Leishmania species. J.Clin. Microbiol.

25- Limoncu, M. E; Ozbilgin, A.; Balciogu, I. C. and Ozbel, Y. (2004): Evaluation of three new culture media in cultivation and isolation of Leishmania parasite.

26- Andrea, K .; Bogylel, C. M.; Diego, E.; Jorge, A. and Vanessa, A. (2007): Evaluation of microculture method for isolation of leishmania parasite from Cutaneous leishmania of patient in Peru. J. Clin. Microbiol**.**

27- Saovanee, L.; Suradej, S.; Theerayudh, S.; Tawee, N.; Jeerapun, W. and Mathirut, M. (2007): Identification of *Leishmania* spp. and a report of novel Species in Thailand. Am. J. Trop Med Hyg.76(5):902-5.

28-Mahboudi, F.; Ablhassan, M.; Yaran, M.; Mobtaker, H. and Azizi, M.(2001): Identification and differention of Iranian *Leishmania* species by PCR amplification of kDNA. Scand. J. Infect Dis.33(8):596-8.

29- Pour mohammadi , B. ; Motazedian, M.H. ; Hatam, G.R.; Kalantari, M.; Habibi ,P.and Sarkari, P. (2010) : Comparison of Three Methods for Diagnosis of Cutaneous Leishmaniasis**.** Iraian J Parasitol , 5 ,4: 1-8.

30- Wortmann, G.; Hochberg, L.P.; Arana, B.A.; Rizzo, N.R.; Arana, F.; Ryan, J.R. (2007) : Diagnosis of cutaneous leishmaniasis in Guatemala using a real-time polymerase chain reaction assay and the smartcycler. Am J Trop Med Hyg. , 76(5): 906-908.

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