`Tracking of Ceotaneous Leishmaniasis by Parasitological, Molecular and Biochemical Analysis

تعقب الليشمانيا الجلدية بالاختبارات التحليلية الطفيلية والجزيئية والكيموحيوية

Sundus Nsaif AL- Hucheimi/ PhD; Department of Microbiology, Faculty of Medicine, University of Kufa-Iraq.

Baqur A. Sultan/ PhD; Department of Microbiology, Faculty of Medicine, University of Kufa-Iraq.

Muhsin A. Al-Dhalimi/MD.; Department of Dermatology, Faculty of Medicine, University of Kufa-Iraq.

Thikra Abdullah Mahmood/ Department of Community Medicine, Faculty of Medicine, University of Kufa-Iraq.

thakraas@gmail .com

الخلاصة

الهدف: تشخيص الأنواع والسلالات والعتر لمسببات الليشمانيا الجلدية لمختلف المناطق الموبوئة

المنهجية: أجريت الدراسة الحالية في خمسة محافظات وغطت سبعة مستشفيات كحقل للدراسة من تشرين الأول/2010 الى كانون الأول/2012، وقد أنجز فحص الترحيل الكهربائي لخلات السليلوز(Cellulose acetate electrophoresis) في معهد (Walter Reed) للبحوث في الولايات المتحدة الأمريكية. الفحص المجهري المباشر تم بثلاثة طرق(السائل المسحوب من القرحة،خزعة الجلد وقطرات الدم المنسابة أثناء الخزعة) . تضمنت الدراسة زراعة العينات على الوسط الزرعي NNNالمحور و RPMI-1640مع مصل العجل الوليد،يتبع بزراعة العينات على الوسط الزرعي Drosophila Schneider. تعرضت هذه الدراسة للتشخيص الدقيق للطفيلي المسبب لليشمانيا الجلدية بواسطة استخدام الفحوصات الجزيئية والكيماحياتية تطرقت تلك الدراسة إلى Isoenzym profil في(20) عينة من مجموع الحالات المرضية التي أظهرت نتائج موجبة للزرع بواسطة الأوساط الزرعية وتم مقارنتها مع السلالات والعتر المصدرية باستخدام الترحيل الكهربائي لخلات السليلوز (Cellulose acetate electrophoresis) لأربعة أنزيمات (glucose phosphateisomerase,leucilphosphate,manose phosphate isomerase and 6phosphogluconate dehydrogenase) .

النتائج :أظهرت نتائج الدراسة ان 90 (71%)من المرضى أعطوا نتائج موجبة عند الفحص تحت المجهر الضوئي. عند زراعة العينات على الأوساط الزرعية فوجد أن 83(65%) من العينات أعطت نتائج موجبةو بينت الدراسة بأن 52(41%) من مجموع الحالات المرضية التي أظهرت نتائج موجبة للزرع بواسطة الاوساط الزرعية درست بواسطة Nested-PCRوبأستخدام نوعين من البادئات لتحديد جنس وسلالة وعترة الطفيلي. أظهرت الدراسة 45(86,5%) من الحالات كانت *L.major* (560 bp) و7(13,5%) كانت *L.tropica* (750 bp).Isoenzymprofile في(20) عينة من مجموع الحالات المرضية التي اظهرت نتائج موجبة للزرع بواسطة الأوساط الزرعية تم مقارنتها مع السلالات والعتر المصدرية بأستخدام الترحيل الكهربائي حيث أظهرت النتائج أن *L.major(LV39)*وجدت في كل الحالات.

الاستنتاج:يعتبر ال (nested-PCR و CAE طرق مثلى في تمييز وتشخيص أجناس الليشمانيا وأنواعها .كانت (*L.major* و*L.tropica* العوامل المسببة لليشمانيا الجلدية في حين *L.major* كانت النوع السائد في منطقة الدراسة.

التوصيات:1-نتيجة لما تم ملاحظته في فترة الدراسة نوصي باستخدام PCR كأساس للتطبيقات المستقبلية.

2 -فتح عدد من المراكز البحثية في القطر لما يسمى بتعقب الطفيلي تستخدم للأغراض الوبائية بالإضافة إلى التشخيص.

3 -إجراء دراسات تجريبية للحصول على لقاح.

Abstract:

Background: In areas of endemicity without sufficient laboratory infrastructure, identification of *Leishmania*parasites is useful for control and preventive plan and very important since species differentiation is instrumental in selection of optimal therapy and treatment regimens.

Aim: The present study was performed to identify the species and strain of *Leishmania*parasiteisolated from different endemic areas .

Materials and Methods: In a consecutive series of 126 patients referred for a suspected CL lesion during October 2010 to December 2012 in five Iraqi provinces, direct smear and culture followed by molecular and biochemical analysis were done using nested-PCR and cellulose acetate electrophoresis.

Results: Direct smears revealed that 90 (71%) patients gave positive results under light microscope. Out of 126 cases,83(65%) gave positive growth in modified NNN medium and RPMI-1640 with fetal bovine serum followed by sub-culture in drosophila Schniders media. Out of 83 postive growth culture,only 52 specimens were studied by nested - PCR .It was found that 45(86.5%) cases in the generation of a 560 bp DNA and 7(13.4%)patients displayed a fragment of 750 bp, corresponding to *L. major* and *L. tropica*, respectively. Cellulose acetate electrophoresis (CAE) was performed by 4 enzyme systems (glucose phosphate isomerase, leucilphosphate, manose phosphate isomerase and 6phosphogluconate dehydrogenase). The results showed that *L.major*(LV39) isolate were in 20 mass cultivated culture

Conclusion: Nested-PCR and cellulose acetate electrophoresis was an ideal method for discrimination of *Leishmania. spp.* and variants. Both *L. major* and *L. tropica* were the causative agents of cutaneous leishmaniasis but *L.major* was the main species in study area **.**

Recommendations: As a result of the observations made during the whole study period, nested- PCR is very suitable method, which can be used as a basis for future applications**.** Opening number of research centres situated in the

country offer DNA applications for so-called “parasite tracking.” Such an application is probably more relevant for epidemiological purposes than for diagnosis. (e.g., in outbreak investigations or tracking drug-resistant parasite strains). After collection in the field, samples can be simply transferred to such a centre for further analysis. More experimental studies for effective vaccine are developed for leishmaniasis.
Keywords: Leishmaniasis, Nested PCR ,

INTRODUCTION:

Leishmaniasis is one of the infectious parasitic diseases transmitted by biting insects (sand flies) with highest incidence in the world [(1)]. World health organization (WHO) has ranked leishmaniasis as one of the six important infectious diseases of the world. Leishmaniasis still constitutes a major public health problem and the burden is increasing. The World Health Organization (WHO) estimated the number of persons at risk to be around 350 million and the number of new cases to be 2357000 per year [(2)] .

Despite the huge number of research conducted on these pathogens in numerous scientific fields since the beginning of the last [(3), (1)] mentioned that the sensitivity of direct microscopy is not high, and tissue culture is not uniformly available and successful [(4)] explain that in molecular methods both kinetoplast DNA and chromosomal DNA can be used as template for identification of *Leishmania* species by PCR. Moreover, molecular method can be carried out in surveys for detection of cases, reservoir hosts and vectors for better understanding of the transmission mechanisms and control of this complex disease although epidemiological and clinical findings are necessary but they are not sufficient for identification of causative agents of CL[(5).]

Cellulose acetate electrophoresis has proven to be a more reliable and precise method to distinguish the parasites at the species and subspecies levels[(6).]

Little is known of the various species of*Leishmania*parasites inIraq. There are no published studies that have characterized Iraqi*Leishmania* isolates in study area by cellulose acetate electrophoresis. Despite considerable progress in cellular and molecular biology and in evolutionary genetics, we are far from understanding how these organisms act in natural populations. They have a complex life cycle and are present in very diverse ecological niches and can infect a wide range of hosts. Furthermore, *Leishmania*spp. can produce a great variety of clinical symptoms in humans. The genetic determinants responsible for this clinical polymorphism are still unknown, making this biological model complex from ecological, genetic and phylogenetic points of view [(7)] . This review explores the advances in knowledge of *Leishmania* genetics in natural populations, epidemiology and pathogenicity with the aid of genetic data [(8)] .

MATERIAL AND METHODS

Study Area and Patients

The study was conducted on 126 patients with lesions clinically suggestive to be cutaneous leishmaniasis from all ages, both sexes whom attended to hospitals from five Iraqi provinces as following: Al-Najaf province (Al-Hakeem and Al-Sadder teaching hospital), Babylon province (Marjan hospital), Al-Qadisya province (AL-Sadder Teaching hospital), Karbala province (Al-Hussain General Hospital) and Kutprovince (Al-Zahraa Teaching and Al-Kerama Hospital) from October , 2011 to December, 2012.

For each case having cutaneous lesions, a questionnaire was completed to record the necessary information such as name, age , sex occupation, residence, duration of the disease, history of travel to an endemic area, rodent finding. On examination, we record the site, number and type of the lesion i.e wet type or dry type. CL patients with a history of travelling to other endemic areas within a period of one year prior to sampling were excluded.

SPECIMENS COLLECTION

Lesions and the adjacent normal-looking skin around them were cleaned and sterilized with ethanol 70%. Sterile saline (0.1 to 0.2 ml) was drawn into a syringe (1-ml, 25-gauge needle), and

the needle was inserted into the nodule or ulcer's margin and rotated gently several times. A small amount of saline was expressed into the tissue, the needle was rotated, and some tissue aspirate and freed tissue were withdrawn. The syringe was removed from the lesion, and some of its contents were expressed into tubes containing culture media [8].

MICROSCOPICAL EXAMINATION

Small quantities of tissue obtained by skin scrapings were smeared on glass slides, air dried and fixed with methanol for a few seconds. Giemsa stain was filtered and diluted 1:20 with phosphate buffer (pH 7.2). After 20 minutes of staining the slides were washed with tap water and air dried. The stained smears were examined under the microscope with a 40 x lens and with a 100 x oil immersion lens. If at least one intra- or extra-cellular amastigote with a distinctive kinetoplast was found the smear was declared positive. When no amastigotes were seen after 15 minutes of inspection, the smear was declared negative. Many of the patient smears were double checked, the observations were in concordance.

CULTURE

The aspirated fluid was discharged into two culture tubes, one containing semisolid RPMI1640 and the other NNN medium (Novy-MacNeal-Nicolle medium) over layed with Schneider's media supplemented with 30% fetal calf serum, 2 mM L-glutamine, 200 µg/ml streptomycin, and 200 U/ml penicillin. The cultures were incubated at 22-25°C.

After a few days one drop of media was examined under the microscope. If promastigotes were found, the cultures were transferred into Schneider's *Drosophila* medium for further culturing. The parasites were cultivated in 5 ml of Schneider's medium for 2-3 days before they were transferred to a larger volume (40 ml). Negative cultures were examined again several times and in case of no growth they were discarded only after 6 weeks.

Sample processing: The sub cultures were inoculated into Schneider's *Drosophila* medium for further culturing that used for cellulose acetate electrophoresis. Samples were kept at −20°C with 70%ethanol untill use, and DNA was extracted from each sample by use of a commercial kit

.DNA EXTRACTION AND PCR AMPLIFICATION OF KDNA

DNA was extracted from promastigotes multiplied in cultivated tubes in order to be used in molecular diagnosis of *Leishmania*species by using the Bioneer Genomic DNA Extraction Kit and done according to the its steps.PCR was performed using 2 pairs of primers of in 2 Steps as the fallowing table.

Table (1): Primers used in this study

KDNA	First step	CSB2XF(forward)	5´-CGAGTACAGAAACTCCCGTTCA-3
		CSB1XR(reverse)	5´-ATTTTTCGCGATTTTCGCAGAACG-3´
	Second step	13Z(forward)	5´ ACTGGGGGTTGGTGTAAAATAG-3´
		LIR(reverse)	5´TCGCAGAACGCCCCT-3´

PCR products were analyzed by1.5 % agarose gel electrophoresis with 5µl of the reaction mixture. Positive and negative controls were included in all tests.Nested-PCR Thermocyclerconditions were

done by using Optimase PCR protocol writer online in Simple PCR Thermocycler (TECH-Belgium).

RESULTS

The study revealed that 90 cases (71.4%) showed positive of the direct smear examination while 36 cases (28.6%) from patients had negative direct smear examination. Culture of cutaneous leishmaniasis revealed that 83 cases (65.9%) gave positive culture while 43 cases (34.1%) from patients get negative culture. Nested-PCR of cutaneous leishmaniasis revealed that 45 cases (86.54%)for nested-PCR (second step) caused by *L-major* while 7 cases were (13.46 %) caused by *L-tropica* .Cellulose acetate electrophoresis of cutaneous leishmaniasis *Isoenzyme analysis.* After electrophoresis and staining, Comparison of isoenzyme patterns of isolates with standardstrains showed that that the isoenzyme profiles of 20 mass cultivated cultures showed that *L.major* (LV39) isolates.

ISOENZYME ELECTROPHORESIS

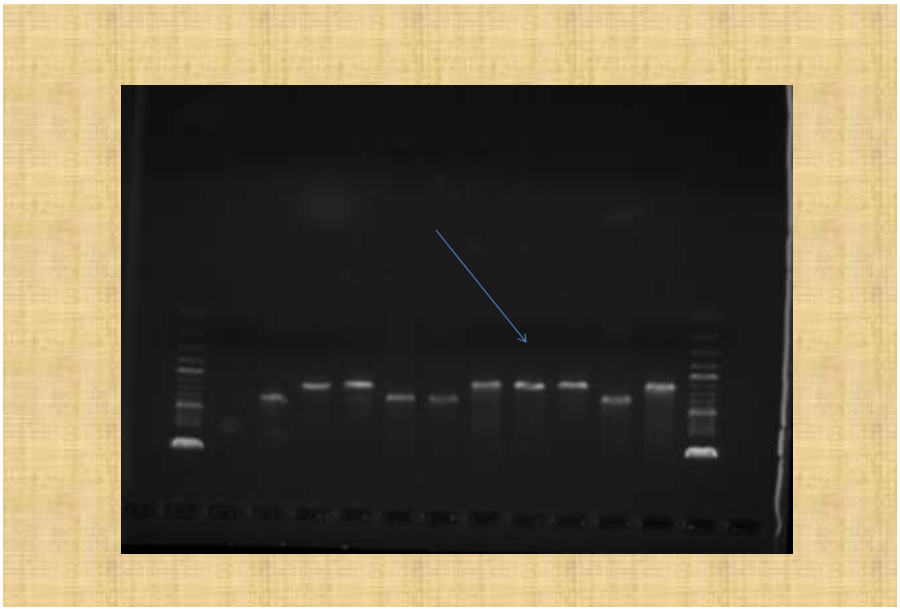

Fig. (1) : Agarose gel electrophoresis of Leishmania isolates in nested-PCR(second step) .Lane 1,13 ,DNA size marker 100 Pb ;Lane 2,negative control ;Lane 3,6,7,11 (560Pb) *L.major*isolates;Lane 4,5,8,9,10,12(750Pb)*L. tropica*isolates

Cellulose acetate were used forisoenzyme analysis of the isolates. Promastigotes were harvested at the end of logarithmic phase by centrifugation at 3000 round/minute at 4°C for 20 min. . Isoenzyme profiles of these isolates were compared with reference strains of *Leishmania spp.* using cellulose acetate electrophoresis and 4 enzyme systems (glucose phosphate isomerase, leucilphosphate, manose phosphate isomerase and 6phosphogluconate dehydrogenase).

STATISTICAL METHODS

Table (2): Cellulose acetate electrophoresis according to sex and age groups of patients.

Age(year)	Sex		Cellulose acetate electrophoresis				Total
			Glucose phosphate isomerase	Leucil phsphate	Mannose phosphate isomerase	6Phosph-ogluconate dehydrogenase	
10 and less	**M**	**1**	A	A	A	B	1
	F	**0**	A	A	A	B	
11-20	**M**	**2**	A	A	A	B	3
	F	**1**	A	A	A	B	
21-30	**M**	**4**	A	A	A	B	6
	F	**2**	A	A	A	B	
31-40	**M**	**3**	A	A	A	B	5
	F	**2**	A	A	A	B	
41 and more	**M**	**3**	A	A	A	B	5
	F	**2**	A	A	A	B	
Total		**20**	A	A	A	B	20

Wilcoxon rank sum test and chi-square were applied to find out the significant difference between the data by (SPSS v.10) under (Windows).

DISCUSSION

Coetaneous leishmaniasis (CL) with two forms of rural and urban types is an endemic disease and regarded as a significant health problem in many parts of Iraqi Provinces with increasing incidence and expanding to new foci. Identification of parasite species and type of disease is very important for treatment of disease as well as for the planning of control program[(9)] .

In a study indicated that the disease is endemic in Iraq and in other neighbouring countries. [(11)] There are estimated 12 million cases worldwide, and about 1.5 million new cases of cutaneous leishmaniasis each year, of which over 90% occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru **.** AlSamarai and AlObaidi Mentioned that the disease is epidemiologically unstable, with large and unpredictable fluctuations in the number of cases. The total incidence rate of cutaneous leishmaniasis in Iraq varies from 2.3 / 100000 to 45.5 / 100000 . [(10)]

Direct methods of identification of *leishmania* by using the direct smear was positive in 90(71.4%) of patients. This result was in agreement with what was reported by [(12, 13)] . These methods have limited sensitivities because they require direct visualization of the parasites and the paucity of parasites within the lesion is a hallmark of lesions with old age. The results were nearly similar to the result reported in Iraq by [(10)], which was 73%.

Vaeznia et al mentioned that the reported positivity rate of *Leishmania spp*. observed by microscopic examination of direct smears for the presence of parasites usually variable in various

studies [14]. The high positivity may be attributed to the fact that three methodes were prepared from each biopsied lesion and this justification come agree with some other studies[15].

Bensoussan et al said that the percent success for microscopic identification of amastigotes in stained preparations varies depending on the number of parasites present and/or the experience of the person examining the slide [4]. There was a relationship between positive direct smear and duration of lesion but there was no statistically relationship between clinical form and positive direct smear rate[16].

The present work, showed a high detection rate of *Leishmania* spp. in the CL lesion aspirate cultures, promastigotes were observed microscopically in the majority of the cultures within one month of sample cultivation.

Cellulose acetate electrophoresis and Nested –PCR could be a valuable tool in selection of optimal therapy and treatment regimens, especially in complex localities where more than one *Leishmania*species is present. This method is also important for strategic planning and future control programs[17] .

*Lesihmania*minicircle Kinetoplast DNA is one of the genetic targets that has been applied and proved to be useful for detection of parasites in clinical specimens and isolate characterization by many researchers [4] . Further studies found that kDNA is more sensitive than other DNA markers such as internal transcribed spacer 1 (ITS1), glucose-6-phosphate (G6P), mini-exon, gp63, for detection and/or identification of both Old and New World CL[18] .

Finding of this study showed that *L. major* was the main species (86.5%) caused ZCL in study areas, However, *L. tropica*is present in low level (13.4%) The predominance of ZCL due to *L. major* has already been confirmed in Iraq by [11] also consistent with **Jarallah,**[19] who indicated that Iraqi isolations of *Leishmania* spp. from cutaneous lesions fell clearly into two groups, one of which *L. major* and the other *L.tropica,*while contrary to those reported in Iraq by [10] who indicated *Leishmania major* is confirmed as the causative agent of CL in this region. **AL-Hamdi** [11] recorded that in Iraq there are 2 main species of the genus *Leishmania*causing the infection: *L. tropica*and *L. major*

The life cycle of L. major involves rodent hosts and the sand fly vector, *P. papatasi*. The transmission cycle of *L. tropica* varies between localities and generally does not require a sylvan reservoir. *Leishmaniatropica* is transmitted anthroponotically in most areas by *P. sergenti* but in other areas with rare or sporadic cases, It is suspected to have a zoonotic transmission cycle with *P. sergenti* transmitting parasites among populations of rock hyraxes and humans [20] . The search for reservoirs (other than man) of ACL has been intensive, but ultimately the inference must be drawn that the animals sofar described are not true reservoirs, but accidental hosts [21] .

This shows that CL foci of *L. major* and *L. tropica*can coexist in the same area, at least in some conditions that remain to be investigated. However, up to now the data indicates that CL caused by *L. major* is still a rural disease, while CL caused by *L. tropica*is an urban disease in Iraq**.** The second round PCR was much more sensitive than the first round. Both first and second primer pairs have been designed to identify all tested *Leishmania* species, therefore it is expected having more positive samples in the second round PCR than the first one. Because of the wide clinical spectrum of CL in Iraq and the appearance of new CL foci, there is a real need for *Leishmania*parasite characterization. So the isoenzymetyping for isolates of *Leishmania*from different geographical areas of Iraq is applied [22]

Electrophoretic techniques yield very precise data on genetic contents of organisms. The large biochemical differences between species makes electrophoretic techniques of great value in describing and identifying members of different subspecies that are morphologically indistinguishable [23] .

The data of CAE show most Iraqi isolates belong to the *L. major,* this confirms previous findings by nested-PCR which reported that *L. major* was the dominant species in study areas. The present data of CAE are different from those deduced by Aljeboori and Evans, [24] who found that

the Iraqi isolations of *Leishmania spp*. from cutaneous lesions fell clearly into two groups, one of which gave isoenzyme patterns identical to those of a marker stock of *L. major*, and the other which gave patterns identical to those given by *L. tropica*. Previously, it had been thought that *L. tropica* alone was responsible for cutaneous leishmaniasis in Iraq. None of the current samples showed to be infected with *L. tropica*. This is because of the samples were chosen randomly and the CL in the study area is most probably zoonotic type.

Gold-standard isoenzyme characterization requires large scale parasite culture without contamination and also it is possible that infections of *Leishmania* parasites will be missed due to disparate growth rates of different parasites in cultures **(25) .** Application of four basic methods used in this study showed a reliable consistency together in identification of *Leishmania*species isolated from different endemic areas. The results showed that *L. major* species are dominant in isolates collected from study area. These data are in agreement with previous epidemiological studies in Iraq**(13; 9**). No difference was observed in analysis of isolates with 4 enzymatic systems. Data show that one pattern with 4 bands belong to *L.major*(LV39).

During transmission, variations have occurred in the original strain of *L. major* and were displayed in the new pattern. In general, no remarkable polymorphism was observed among *L. major* isolates and it seems that in most enzymatic systems a marked degree of homogeneity are seen in 20 *L. major* isolates characterized in this study. The results showed exact consistency with data obtained from other methods used in this study. CL has also been increased in several areas of Iraq during recent decade and there is evidence that this infection is emerging in new foci and during recent years in rural and urban areas. The study area is one of the important geographical regions with different points of views, in particular cultural and economical purposes as well as cross-border movements for different bilateral or multilateral purposes, i.e. trade and business, tourism, exchange visiting of neighbouring countries. Also population growth, immigration of non immune persons to endemic areas, activity of infected sand flies, the presence of rodent burrows and people suffering from active lesions or chronic sores, have provided a suitable environment for the maintenance of the disease . The physicians have only relied on the clinical observation to consider the disease as zoonotic or otherwise. The clinical symptoms are variable and may be confused with other etiological agents.

Parvizi and Ready ,(26) said that one of the major obstacles for the control and understanding of this neglected disease is the detection and identification of *Leishmania* parasites in animal reservoirs. Epidemics may flare up with little warning if vector sandflies are present. Cases due to L. tropica may be persistent and disfiguring, and difficult and expensive to treat also in India Sundar and Rai, (27) mentioned that *Leishmania tropica* causing VL.

CONCLUSION

Nested-PCRand cellulose acetate electrophoresis was an ideal method for discrimination of *Leishmania. spp.* and variants.Both*L. major* and *L. tropica* were the causative agents of cutaneous leishmaniasis but *L.major* was the main species in study area **.**

RECOMMENDATIONS

1-Nested- PCR is very suitable method, which can be used as a basis for future applications**.**
2-Opening number of research centres situated in the country offer DNA applications for so-called "parasite tracking." Such an application is probably more relevant for epidemiological purposes than for diagnosis. (e.g., in outbreak investigations or tracking drug-resistant parasite strains).

After collection in the field, samples can be simply transferred to such a centre for further analysis.

3-More experimental studies for effective vaccine are developed for leishmaniasis.

REFERENCES

1. Pourmohammadi,B.; Motazedian,MH.; Hatam,GR.; Kalantari,M.; Habibi,P.and Sarkari,B.(2010). Comparison of three methods for diagnosis of cutaneous Leishmaniasis. **Iranian J Parasitol**: 5(4)1-8.
2. Ramanathan,R.; Talaat,K.; Fedorko,D.; Mahanty,S. and Nash,T.(2012). A Species-Specific Approach to the Use of Non-Antimony Treatments for Cutaneous Leishmaniasis **Am J Trop Med Hyg**. 84(1): 109 - 117.
3. William, H.; Markle, M. and Khaldoun,m. (2004).Cutaneous Leishmaniasis: Recognition and Treatment. **AmFam Physician**:69(6):1455-1460.
4. Bensoussan,E.; Nasereddin,A.; Jonas,F.; Schnur,L. and Jaffe,J.(2006). Comparison of PCR Assays for Diagnosis of Cutaneous Leishmaniasis.**J ClinMicrobiol**. 44(4): 1435–1439.
5. Mahmoodi, M.; Mohajery, M.; Afshari, J.; Shakeri, M.panah ,M. ;Berenji, F; and , A.(2010).Molecular identification of Leishmaniaspecies causing cutaneous leishmaniasis in Mashhad, Iran. Jundishapur.**JMicrobiol**. 3(4): 195-200.
6. Kumar, V.; Kishore, K.; Palit, A.;Keshari, S.; Sharma, M. and Das, V. (2007). Vectorial efficacy of *Phlebotomusargentipes*in Kala-azar endemic foci of Bihar (India) under natural and artificial conditions.**J Commun Dis**. *33* : 102-9.
7. Bailey, M. and Lockwood, D.(2007). Cutaneous leishmaniasis.Clin Dermatol. 25:203–211.
8. Al-Jawabreh,A.;SchoenianG.; Hamarsheh,O. and Presber,w.(2009). Clinical diagnosis of cutaneous leishmaniasis: A comparison study between standardized graded direct microscopy and ITS1-PCR of Giemsa-stained smears.99(1): 55–61.
9. Qader,A.; Abood,M. And Bakir, T.(2012). Identification of *leishmania* parasites in clinical samples obtained from cutaneous leishmaniasis patients using PCR technique in Iraq. **Iraqi Journal of Science**. 53:457-463.
10. AlSamarai ,A. and AlObaidi,H.(2009). Cutaneous leishmaniasis in Iraq**. J Infect Developing Countries** :3(2):123-129.
11. Al Hamdi,K.;Awad,A. and Moker,H.(2010).Evaluation of intralesional 0.2% ciprofloxacin as a treatment for cutaneous leishmaniasis. **Eastern Mediterranean Health Journal** 16 (1).
12. Rodrigues, E.; Mendonca, M.: Werkhauser, R. ; Coutinho ,E.; Souza ,W.; Albuquerqu, M.; Jardim, M. and Abath, G.(2002): Evaluation of PCR for diagnosis of American cutaneous leishmaniasis in an area of endemicity in northeastern Brazil**. J.Clin .Microbiol**., 40(10): 3572-3576.
13. AL-Hucheimi,S.(2005).Acomportive study of some methods used for cutaneous leishmaniasis.MSc. Thesis,AL-Kufa Univ.
14. Vaeznia, H.;Dalimi, A.;Sadraei, J.and Pirstani, M.(2009). Determination of Leishmania species causing cutaneous leishmaniasis in Mashhad by PCR-RFLP method . Archives of Razi Institute,64, (1) 39-44.
15. Nezhad,H.; Borhani,M.; Norouzi,M. and Merzaie,M.(2012). Cutaneous Leishmaniasis in school children in a border area at southwest of Iran. Sci Parasitol 13(4):153-158.
16. UL- Bari,A. ; Azam,SH. ; Ejaz,A. and Mahmood,T.(2010). Comparion of various cytodiagnostic tests in the rapid diagnosis of cutaneous leishmaniasis. **Pakistan Journal of Pakistan Association of Dermatologists** ; 20: 63-69.
17. Rassi, Y.; Abai, M.; Javadian, E.; Rafizadeh, S.; Imamian, H.; Mohebali, M.; Fateh, M.; Hajjaran, H. and Ismaili, M.(2008). Molecular study on vectors and reservoir hosts of

zoonotic cutaneous leishmaniasis in Central of Iran.Bulletin de la Societe de Pathologie Exotique. 101:425–428.

18. Anders, G.; Eisenberger,C.; Jonas,F. and Greenblatt,C.(2002). Distinguishing *Leishmaniatropica* and *Leishmania major* in the Middle East using the polymerase chain reaction with kinetoplast DNA-specific primers. **Trans. R. Soc. Trop. Med. Hyg**. 96(1):87-92.
19. Jarallah,H.(2011). The effect of Sodium chloride and Cephalexin antibiotic on the growth of*Leishmaniatropica*and *Leishmaniadonovani*promastigote. **Journal of Basrah Researches** ((Sciences)) 37(4) 71-77.
20. Sharifi,F.;kharifi,I.;,Zarean,M.;Parizi,M.;Aflatoonian,M.;Harand,F.;Zahmatkesh,R.; Sundar,S. and Rai,M.(2002).Laboratory Diagnosis of Visceral Leishmaniasis**.Clin Vaccine Immunol** . 9 (5) 951-958.
21. Oshaghi,M.; Rasolian ,M.; Shirzadi ,M.; Mohtarami, F. and Doosti ,s.(2010). First report on isolation of Leishmaniatropica from sandflies of a classical urban Cutaneous leishmaniasis focus in southern Iran. Experimental **Parasitology.**126 .445–450.
22. Al-Shukur, B, S, A (2012). DiGNOSIS OF CUTANEOUS leishmaniasis by PCR Technique and efficiency study of some plant extract in number and viability of of parasite in vitro and Msc Thesis , college of girl .
23. Pratlong, F.; Dereure, J.; Ravel, C.; Lami, P.; Balard, Y.; Serres, G.; Lanotte, G.;Rioux, J. and Dedet, J. (2009). Geographical distribution of cutaneous leishmaniasis. *Parasitol***. Today** 18(7) 156-197.
24. Aljeboori ,T. and Evans, D.(1980). *Leishmania spp*. in Iraq.Electrophoretic isoenzyme patterns. II. Cutaneous leishmaniasis. **Trans R Soc Trop Med Hyg**. 74(2):178-84.
25. Hatam, G.; Riyad, M.; Bichichi, M.; Hejazi, S.; Guessous-Idrissi, N.andArdehali, S.(2005). Isoenzyme characterization of Iranian *Leishmania* isolates from cutaneous leishmaniasis. **Iranian Journal of Science and Technology**.29:
26. Parvizi, P. and Ready,p.(2008). Nested PCRs and sequencing of nuclear ITS-rDNA 400 fragments detect three Leishmania species of gerbils in sandflies from Iranian foci of 401 zoonotic cutaneous leishmaniasis. **Trop Med Int Health** 13:1159-71.
27. Ader,A.; Abood,M. And Bakir, T.(2012). Identification of *leishmania* parasites in clinical samples obtained from cutaneous leishmaniasis patients using PCR technique in Iraq.Iraqi **Journal of Science**. 53:457-463.