ISSN: 0067-2904
GIF: 0.851

Using PCR for detection of cutaneous leishmaniasis in Baghdad

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Abstract

Cutaneous Leishmaniasis (CL) is an endemic disease and one of the major health problems in Iraq. *Leishmania tropica* is known as the causative agent of Cutaneous Leishmaniasis in Baghdad.The classical serological methods of diagnosing leishmaniasis is a poor sensitivity especially for the sub genus and time consuming Here we have investigated two primer pairs, one specific for *Leishmania* as genus and the primer specific for the species of *L. tropica* to be detected by polymerase chain reaction (PCR).Samples were collected from (AL-karama Teaching Hospital) and whole genomic DNA was extracted from axenic promastigotes.The extracted DNA was amplified by PCRwith two KDNA primer pairs, for genus specific (13A/13B) and (Lmj4/Uni21) to identify the specific species of cutaneous leishmaniasis. Amplified PCR products then run on gel electrophoresis and two visible bands of the two primers were seen, 120 and 800 bp, respectively. Our results indicate that the PCR technique is sensitive and specific for the detection and differentiation of cutaneous leishmaniasis agents and can be recommended to applied in hospitals and research centers.

Keywords: PCR, characterization, cutaneous leishmaniasis.

استخدام تفاعل البلمرة المتسلسل للكشف عن الإصابة باللشمانيا الجلدية في بغداد

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الخلاصة:

داء اللشمانيات الجلدي (CL) من الامراض المتوطنة ويشكل احدى المشاكل الصحية الرئيسية في العراق. *Leishmania tropica* هي من المسببات المسؤلة عن اللشمانيا الجلدية في بغداد .الطرق المصلية التقليدية في تشخيص داء اللشمانيات هي طرق قليلة الحساسية خاصة بالنسبة للاجناس الفرعية اضافة الى أنها مستهلكة للوقت . في هذه الدراسة قمنا بفحص بادئين اثنين احدهما خاص بجنس اللشمانيا والاخر خاص بنوع *L . tropica* للكشف عنه بطريقة تفاعل البلمرة المتسلسل (PCR). تم في هذه الدراسة جمع العينات من مستشفى الكرامة التعليمي في بغداد ، اذ تم استخلاص الحامض النووي الدنا من الطور السوطي للطفيلي وبعدها خضع للتضخيم باستخدام بادئين خاصين للبانية الحركية بواسطة تفاعل السلسلة المتبلمر. (13A/13B) خاص بجنس اللشمانيا و(Lmj4/Uni21) خاص لتشخيص انواع اللشمانيا الجلدية. أظهرت نتائج تضخيم تفاعل البلمرة المنتج بواسطة الترحيل الكهربائي حزمتين مرئيتين للبادئين (120، 800) زوج نيتروجيني على التوالي، النتائج اثبتت ان الPCR طريقة حساسة ودقيقة للكشف والتفريق بين انواع اللشمانيا الجلدية لذلك نوصى باستخدامها في المستشفيات ومراكز البحوث.

Introduction

Leishmaniasis is avector-borne disease caused by protozoa of the genus *Leishmania*.The disease is transmitted via sand flies of the genus *Phlebotomus* in the old world (Europe, Asia and Africa) and *Lutzomyia* the new world (Latin America)[1].There are over 14 species of *Leishmania* which may

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cause up to three different clinical syndrome [2, 3]. Leishmaniasis can manifest itself in different forms depending upon the infecting species of *Leishmania*,the disease could emerge as cutaneous, muco-cutaneous, or visceral leishmaniasis [4].Cutaneous leishmaniasis (CL) manifests as ulcerated skin lesions or muco- cutaneous leishmaniasis (MCL) affects the mucous membranes of the nose, mouth, throat and can lead to partial or total destruction of the associated membranes, and visceral leishmaniasis (VL) which is a systemic, potentially lethal disease caused by parasites of the *Leishmania donovanii* complex [5,6]. In Iraq, leishmaniasis is an endemic disease especially CL and its widely spread in the central parts of the country.The incidence of the disease in Iraq was found to increase in months of September and October and reached the rate maximum in January and February. The incidence declines from March and reaches its lowest point in July and August [7]**.** There are an estimated 1.2 million cases of CL and 400,000 cases of VL reported annually worldwide [8]. Cutaneous leishmaniasis CL is the most common and least fatal form of the disease, identified by ulcerative skin lesions, and it was caused by *Leishmania major, L. tropica, L. aethiopica, L. mexicana, L. braziliensis, L. guyanensis, L. panamensis, L. peruviana, and L. amazonensis*. Almost two-thirds cases of CL were reported from six countries: Afghanistan, Algeria, Brazil, Colombia, Iran, and the Syrian Arab Republic [9]. In the past year, there were 1600 cases of cutaneous leishmaniasis and 4 cases of visceral leishmaniasis diagnosed in American soldiers deployed to Iraq, Kuwait, and Afghanistan [10].Diagnosing of leishmaniasis is based on endemicity, clinical symptoms, and laboratory test results.Often diseases of different etiology but similar symptoms like schistosomiasis and malaria are present in the same endemic region as leishmaniasis; therefore, careful differential diagnosis is very critical for epidemiological surveys and therapeutic purposes [11]. Differentiation between the *Leishmania* species is an issue because there are overlapping and dynamic geographic regions of risk and different susceptibilities to treatment [12, 13]. Thus, a method of diagnosis that is sensitive enough to detect low levels of the parasite in asymptomatic or early symptomatic infection and can distinguish between the different *Leishmania* species could be of tremendous utility in regions of endemicity and nonendemicity [14].A molecular approach for the diagnosis of leishmaniasis, based on the detection of *Leishmania* DNA has two goals: detection of *Leishmania,* similar to other parasitological methods and identification of the *Leishmania* species which is not achieved by other methods, except when cultured promastigotes are analyzed using *Leishmania* species-specific monoclonal antibodies [15]. Thus, in this study we have investigated two primer pairs to identify the genus and species of cutaneous leishmaniasis in one of the major hospitals in Baghdad where patients with CL usually visit for diagnosis and treatment.

Materials and Methods

Leishmania parasite isolation:

Samples from patients suspected with cutaneous leishmaniasis in Al-Karama teaching hospital, were collected during the period of Nov – Dec (2014) for molecular identification of these isolates by polymerase chain reaction (PCR) using species-specific primers and a sample taken from hand lesion a needle aspirate was obtained by using 2ml syringe with 20 gauge needle containing 0.2-0.3 ml normal saline.The lesion of CL was sterilized with 70% alcohol to eliminate bacteria and remove the crust and debris, then 0.2 ml of normal saline was injected to the base near the border of the lesion. The injected solution was re-aspirated without taking the needle out of the skin. This aspirate was used in direct smear by smearing on drop of the aspirated material on a clean slide. The slides were examined microscopically under 40X lens. These samples were cultured in NNN media for retrieval of the procyclic promastigotes of the parasite before they were transferred to M199 culture media for maintenance.

DNA extraction:

DNA extraction of procyclic promastigotes of the isolates was extracted by using QIAamp DNA MiniKit(50)No.51304 QIAGEN(Germany). According to the manufacturer's instructions. The concentration and purity of the extracted DNA was measured by the Nanodrop spectrophotometer. Nanodrop is highly sensitive and directly provides concentration of DNA at A260/A280 ratio, and A260/A230 ratio then, the DNA was stored at −20°C until PCR amplification.

Preparation of primers: the primer pairs used in this study were purchased from Alpha DNA (Canada) in lyophilized form and was dissolved in TBE buffer to give a final concentration of 100 pmol/ µl for each, as instructed by the company and stored in a freezer until use.

PCR amplification of *leishmania* genus-specific primer 13A/13B according to Mouttaki *et al* [16]: The Primers (13A /13B) of the kDNA minicircles forward (GTGGGGGAG GGGCGTTCT) reverse (ATTTTCCACCACCCCCAGTT) was amplified by PCR, this sequence is conserved in all minicircle classes of all *Leishmania* species [16]. According to the manufacturer's GoTaq® Green master mix, Promega (USA) protocol: The reactions mix was prepared and each working tube was up to volume of 25 µl containing (12.5 µl ,1X) PCR MIX, (1 µl, 1 µM) of each primers, and (5 µl, 25 ng) of the DNA template and final concentration with nuclease free H_2O. The PCR amplification was run in a DNA thermocycler (eppendorf) using of 94°C for 4 min for initial denaturation, followed by 30 cycles, each consisting of 94°C for 1 min, 60°C annealing temperature for 1 min, 72°C for 1 min and final extension 72°C for 10 min (30 cycles). PCR produces were hold 4°C for 10 min. PCR products were detected by electrophoresis in 1.5 % agarose gel at 100 V in the presence of ethidium bromide 0.5 µg/ml gel solution.

PCR amplification of *leishmania* genus-specific primer (Lmj4/Uni21) primers according to Mouttaki *et al* [16]:

This primer pair was used for differentiation between the two forms of cutaneous leishamaniasis, *L.tropica* and *L.major* by PCR, Forward (CTAGTTTCCCGCCTCCGAG)and Reverse (GGGGTTGGTGTAAAATAGGCC). Mouttaki *et al* [16].According to the manufacturer's procedure, the reaction was set up in a total volume of 25 µl containing 12.5 µl 1x PCR mix, (1 µl, 1 µM) of each primers, and (5 µl, 25 ng) of the DNA template and final concentration with nuclease free H_2O. The PCR amplification was run in a thermocycler (eppendorf) using of 94°C for 4 min for initial denaturation, followed by 35 cycles, each consisting of 94°C for 1 min, 55°C for 1 min annealing temperature, 72°C for 1 min and final extension 72°C for 10 min and was hold at 4°C for 10 min. PCR products were detected by electrophoresis in 1.5 % agarose at 100 V in the presence of ethidium bromide 0.5 µg/ml gel solution.

Results and Discussion:

In this study, random samples of patients suffering from skin ulcers and suspected for infection with cutaneous leishmaniasis were diagnosed by PCR and specifically identified as *L. tropcia* infection, by using genus-specific and species-specific primer pairs for this purpose. The extracted DNA of the six samples was first run on agarose gel to confirm the presence of DNA before PCR amplification run, Figure-1.

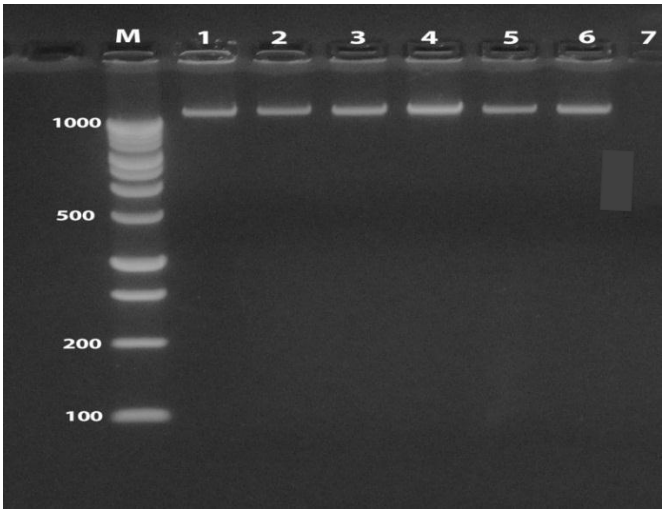

Figure 1- Genomic DNA extracted from promastigotes. M= 100 bp ladder. lane (1-6) DNA of promastigotes.

PCR amplification of *Leishmania* genus-specific primer 13A/13B :

All the six samples showed ~120 bp of the primer pair 13A/13B as in Figure-2.The primer pairs 13A/13B showed higher efficiency to diagnose the genus of *Leishmnaia*, as a previous study showed that this primer pair is being able to detect an equivalent of 0.1 promastigote cells per PCR tube. This may be explained in part by the higher copy number of the kDNA target (approximately 10,000 copies). Furthermore, the smaller size of the 13A/13B PCR products 120 bp [17]. A previous study found that the kDNA 13A/13B PCR is a highly sensitive diagnostic and thus seems a valuable tool for

the diagnosis of CL, however, it is not able to differentiate between the *Leishmania* species and can be considered as control positive to all *Leishmania* species. All samples in this study showed gel bands of 120 bp and have been confirmed that all isolated samples are of *Leishmania* genus,[16].

Another study [18] ninety two Giemsa-stained smears of lesions from suspected cases of cutaneous leishmaniasism DNA was isolated and used for PCR-based diagnosis of *Leishmania* infection. Each smear had been examined under a light microscope at 1000X and scored for amastigote numbers, all the microscopy-positive slides were also positive by 13A/13B amplification PCR and four of the 14 smears that were negative by microscopy (although of lesions that were clinically consistent with leishmaniasis) were also PCR-positive. Similar work in Shiraz Iran, used the same primer for diagnosis which amplify the DNA from a wide range of *Leishmania* spp including *L. major* and *L. infantum*, to amplify the 120-bp conserved region of the *Leishmania* kinetoplast minicircle [19,20].Karamian *et al*.[21] found that fifty-one patients clinically suspected of CL with living in the endemic regions of Fars province, Iran, after microscopic examination extraction of DNA was performed on their smears and analyzed by two specific PCR assays for diagnosis and species identification. A typical forms of CL were evaluated among PCR positive patients PCR results were positive in 37 out of 51 cases (72.5%), among whom microscopic examination revealed *Leishmania* amastigotes in only 3 (5.9%). These results showed that kDNA PCR methods have a higher sensitivity compared with microscopic method,also PCR could identify the parasite species for specific therapy. Microscopic method had low sensitivity and less value in chronic and atypical CL cases. It used primers to diagnosis cutenous leishmaniasis to amplify the 120-bp conserved region of the *Leishmania* kinetoplast minicircle. it consider control for all *leishmania* species [22].

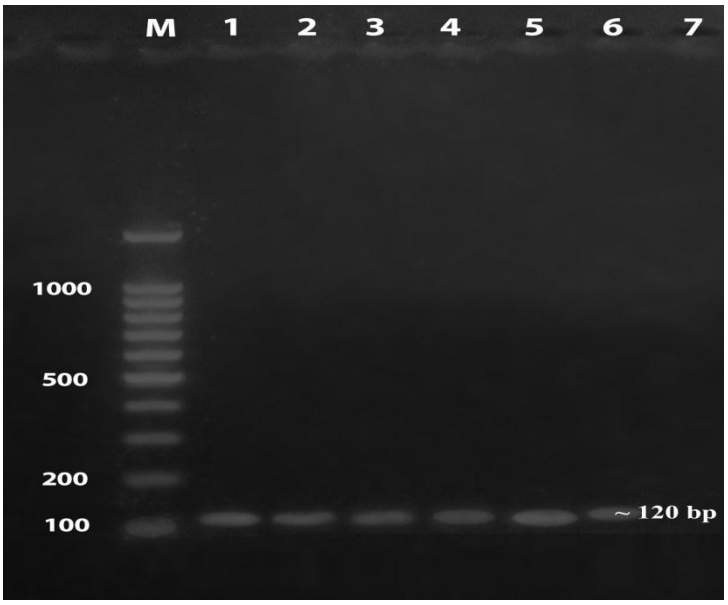

Figure 2- 1.5% Agarose gel electrophoresis of 13A/13B PCR products. M=100bp ladder lane (1-6) *Leishmania tropica*, lane (7) control negative.

PCR amplification of species specific primer pair (Lmj4/Uni21);

The second primer pair used in this study was Lmj4/Uni21, which allows the differentiation between *L. tropica* and *L. major* as the gel electrophoresis result was considered positive for *L. tropica* when a band of the expected size was obtained equal to 800 bp for Lmj4/Uni21 Figure-3.

Andres *et al*. [23] found that samples obtained from skin lesions of patients with suspected CL used kDNA primer pair Lmj4/Uni21 was used for differentiation between *L. major* and *L. tropica* in the Middle East . The primer designed was based on a published sequence from the *L. major* kDNA minicircle PCR products showed consistent species specific differences in size *L. major* (650 bp) could be clearly differentiated from all the other 'Old World' species *L. tropica, L. donovani and L. infantum* yielded the same size of band (800 bp), whereas that from *L. aethiopica* was slightly larger (850 bp) [24].

Similar work done by [25] in which ninety-eight patients of suspected of CL were recorded between November 2004 and April 2006 in India, used (Uni21/Lmj4) primers and the results showed

that it was suitable for amplification and identification of parasite DNA of *Leishmania tropica*. [23] They were used similar primer pair for diagnosis and species identification in a large number of patients with CL as positive controls PCR with all the culture isolates 14 gave a consistent pattern of *L. tropica* (800 bp), clearly differentiated from *L. major* (650 bp).The kDNA PCR was the most sensitive diagnostic assay and was established as a valuable tool in the diagnosis of CL in India. The test may be used as a new standard for the detection of parasite in patients suspected of CL with negative microscopic examination and or culture results.

In another study [26]. The species was distinguished also by using kinetoplast DNA-specific primer Parasite isolated from patient had CL acquired in Iraq , where only *L.major* and *L. tropica* were expected to be the causative agents. The size difference between the PCR products of *L. major* and *L. tropica* allowed differential diagnosis Anders [23] suggested that the smaller product (650 bp) could be identified as derived from *L. major* whereas the larger product (800 bp) was due either to *L. tropica* or to a member of the *L. donovani* complex which yielded the same size of band, similar work done by [27] they were used PCR technique based on kDNA specific primer to differentiate the species of parasites that causes cutaneous leishmaniasis in different region of Iran, they suggested the presence of (620) bp fragment indicated *L. major* and (800) bp indicated *L. tropica.*

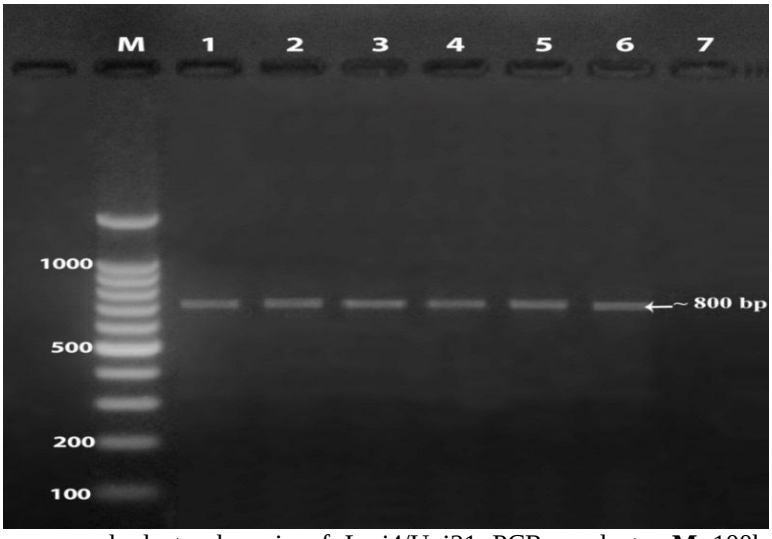

Figure 3- 1.5% Agarose gel electrophoresis of Lmj4/Uni21 PCR products. **M** 100bp ladder **lane (1-6)** *leishmania tropica*, **lane(7)** control negative.

This study is one of the first attempts to identify *Leishmania* species using molecular methods in Baghdad. In this study showed that PCR has a specifically compared to classical method and used PCR technique based on kDNA specific primer to differentiate the species of parasites that causes cutaneous leishmaniasis,The results of this study confirmed that all the isolated samples are cutaneous and specifically diagnosed as *L. tropica* by a sensitive molecular technique which is PCR and is highly recommended to use the outcomes of this study in the diagnosis of *Leishmania tropica* in Iraq.

References

1. Dostalova, A and Volf, P. **2012**. *Leishmania* development in sand flies: parasite-vector interactions overview. *Parasit.Vectors.*5, p: 276.
2. Herwaldt, B.L. **1999**. Leishmaniasis.*Lancet.*, 354, pp: 1191–1199.
3. Barratt, J. L., Harkness, J., Marriott, D, Ellis, J.T. and Stark, D. **2010**. Importance of nonenteric protozoan infections in immunocompromised people. *Clin.Microbiol. Rev.*, 23, pp: 795–836.
4. Murray**,** H. W, Berman, J. D, Davies, C. R and Saravia, N.G. **2005**. Advances in leishmaniasis.*Lancet.*, 366, pp: 1561–1577.
5. Alvar, J., Yactayo, S. and Bern, C. **2006**. Leishmaniasis and poverty. *Trends. Parasitol.*, 22, pp: 552–557.
6. Bart, A., Van Thiel, P. P., de Vries, H. J., Hodiamont, C. J. and VanGool, T. **2013.** Imported leishmaniasis in the Netherlands from 2005 to 2012: epidemiology, diagnostic techniques and sequence-based species typing from 195 patients. *Euro.Surveill* ., 18, pp: 20544**.**
7. Rahim, G.F. and Tatar, I.H**. 1966.** Oriental sore in Iraq. *Bull.Endem.Dis. Baghdad.*, 8, pp:29-54.

8. Alvar, J., Velez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den-Boer, M. and WHO Leishmaniasis Control Team. **2012.** Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*., 7, p: e35671.

9. Reithinger, R., Dujardin, J. C., Louzir, H., Pirmez, C., Alexander, B. and Brooker, S. **2007**. Cutaneous leishmaniasis. *Lancet.Infect.Dis.*, 7, pp: 581–596.

10. Centers for Disease Control and Prevention. **2004**. Update: cutaneous leishmaniasis in US military personnel—southwest/central Asia. *Morb.Mortal Wkly*, 53, pp:264–265.

11. Reithinger, R. and Dujardin, J.C**. 2007**. Molecular diagnosis of leishmaniasis: current status and future applications. *J.Clin.Microbiol.*, 45, pp: 21–25.

12. Berman, J. **2005**. Clinical status of agents being developed for leishmaniasis. Expert.Opin. *Invest. Drugs*, 14, pp:1337–1346.

13. Croft, S. L., Sundar, S. and Fairlamb, A. H. **2006**. Drug resistance in leishmaniasis. *Clin. Microbiol. Rev.*, 19, pp:111–126.

14. Magill, A., Grogl, J. M., Gasser, R., Wellington, S. and Oster, C. N. **1993.** Visceral infection caused by Leishmania tropica in veterans of Operation Desert Storm. *N. Engl. J. Med.*, 328, pp:1383–1387.

15. Grimaldi, G. and McMahon-Pratt, D. **1996**. Monoclonal antibodies for the identificationof New World *Leishmania* species. *Mem. Inst. Oswaldo.Cruz.*, 91, pp:37–42.

16. Mouttaki, T., Morales-Yuste, M., Merino-Espinosa, G., Chiheb, S., Fellah, H., Martin-Sanchez, J. and Riyad, M. **2014**. Molecular diagnosis of cutaneous leishmaniasis and identification of the causative Leishmania species in Morocco by using three PCR-based assays. *Parasit. Vectors*. 7, p:420.

17. Lachaud, L., Marchergui-Hammami, S., Chabbert, E., Dereure, J., Dedet, J.P. and Bastien, P. **2002**. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. *J. Clin .Microbiol*., 40(1), pp:210–215.

18. Motazedian, H., Karamian, M., Noyes, H. A. and Ardehali, S. **2002**. DNA extraction and amplification of *Leishmania* from archived, Giemsa-stained slides, for the diagnosis of cutaneous leishmaniasis by PCR. *Ann .Trop.Med.Parasitol.*, 96, pp: 32–34.

19. Rodgers, M. R., Popper, S. J. and Wirth, D. F. **1990**. Amplication of kinetoplast DNA as a tool in the, detection and diagnosis of *Leishmania*. *Exp.Parasitol.*, 71, pp:267–275.

20. Laskay, T., Miko, T. L., Negesse, Y., Solbach, W., Rollingho, V. M. and Frommel, D. **1995**. Detection of cutaneous *Leishmania* infection in paraYn-embedded skin biopsies using the polymerase chain reaction. *Trans. R. Soc. Trop. Med. Hyg.*, 89, pp:273–275.

21. Karamian, M., Motazedian, H., Fakhar, M., Pakshir, K., Jowkar, F. and Rezanezhad, H. **2008**. Atypical presentation of Old-World cutaneous leishmaniasis, diagnosis and species identification by PCR. *J.E.A.D.V.*, 22, pp:958 – 962.

22. Reale, S, Maxia, L, Vitale, F, Glorioso, N.S, Caracappa, S and Vesco, G. **1999**. Detection of *Leishmania infantum* in dogs by PCR with lymph node aspirates and blood. *J Clin Microbiol* . 37: 2931–2935.

23. Anders, G., Eisenberger, C. L., Jonas, F. and Greenblatt, C. L. **2002**. Distinguishing *Leishmania tropica* and *Leishmania major* in the Middle East using the polymerase chain reaction with kinetoplast DNA-specific primers. *Trans.R.Soc.Trop.Med Hyg*., 96, pp: 87-92.

24. Smith, D. F., Searle, S., Ready, P.D., Gramiccia, M. and Ben-Ismail, R. **1989**. A kinetoplast DNA probes diagnostic for *Leishmania major:* Sequence homologies between regions of *Leishmania* mini circles. *Mol Biochem Parasitol*., 37, pp: 213–224.

25. Kumar, R., Bumb, R. A., Ansari, N. A., Mehta, R. D. and Salotra, P. **2007**. Cutaneous Leshmaniasis caused by *Leshmania tropica* in bikaner. Indi, parasite identification and characterization using molecular and immunologic tools. *Am. J. Trop. Med. Hyg.*,76(5), pp:896–901.

26. Altamemy, A.A., Kawan, M.H. and Al-Saqur, I.M. **2012**. Viscerotropic Leishmaniasis, Diagnosis and species detection using the polymerase chain reaction with kinetoplast DNA-specific primers in Balb/c mice. *W.J.S.M.* (1), pp:32-48.

27. Mahmoodi, M. R., Masoud, M., Jalil, T. A., Mohhamad, T. S., Mohhamad, J.Y.P., Fariba, B. and Abdolmajid, F. **2010**. Molecular identification of *Leishmania* species causing cutaneous leishmaniasis in Mashhad, *Iran. J Microbiol*., 3(4), pp: 195-200.