Viscerotropic Leishmaniasis; Diagnosis and species detection using the polymerase chain reaction with kinetoplast DNA-specific primers in Balb/c mice

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التشخيص وتحديد النوع باستخدام ;اللشمانيا الاحشائية الجلدية تفاعل سلسلة انزيم البلمرة مع بادئ خاص من الحمض النووي للبانية الحركية في الفئران المختبرية

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الخلاصة

تضمنت الدراسة ثلاثة مجاميع من الفئران المختبرية، حقنت المجموعة الاولى(1) مليون بروماستيجوت ثابت الطور والمجموعة الثانية (3) مليون بروماستيجوت ثابت الطور من طفيلي *L. tropica* في راحة القدم الخلفية لهذه الحيوانات وحقنت المجموعة الثالثة محلول الفوسفات بفرسلاين. تهدف الدراسة الى امكانية استعمال الفئران المختبرية كنموذج لدراسة سير المرض في الانسان وكذلك امكانية تشخيص المرض باستعمال تفاعل سلسسة انزيم البلمرة في تشخيص المرض بصورة مبكرة وبحساسية عالية. جمعت نماذج الكبد واستخلص الحمض النووي منها باستخدام طقم استخلاص من شركة بروميكا. استعمل بادئ خاص من الحمض النووي للبانية الحركية في تفاعل سلسسة انزيم البلمرة. تم تشخيص نوع الطفيلي على انه *L. tropica* بالاعتماد على الوزن الجزيئي لناتج تفاعل سلسسة انزيم البلمرة حيث كان الوزن الجزيئي (800) زوج من القواعد النتروجينية وهذا الوزن تنتجه *L. tropica* ومجموعة اللشمانيا الحشوية (*L. donovani*) وبما ان العزلة كانت معزولة من افة جلدية من مريض في العراق حيث ينتشر نوعين فقط اللشمانيا الجلدية احدهما (*L. major*) ووزنها الجزيئي (650) زوج قواعد والاخرى (*L. tropica*) ووزنها الجزيئي (800) زوج قواعد.

استعمل البادئ ذاته في تحديد انتشار الطفيلي الى الاحشاء الداخلية باستعمال الحمض النووي المستخلص من الكبد، ان تفاعل سلسسة انزيم البلمرة كان عال الحساسية واعطى تشخيص مبكر في اليوم (15) بعد الاصابة وخاصة حيوانات المجموعة الثانية. جميع حيوانات المجموعتين باستثناء حيوان واحد من المجموعة الاولى اعطت نتائج موجبة في اليوم (30) بعد الاصابة ولكنها كانت جميعها موجبة في الايام التالية خلال فترة التجربة.

Abstract

The study involves three groups of Balb/c mice, the first and second groups inoculated in the hind footpad (1 x 10^6), (3 x 10^6) stationary phase promastigotes of *L. tropica* respectively, the third group inoculated phosphate buffer saline. The aim of this study was to establish a murine model for human viscerotropic leishmaniasis, and the dissemination of *L. tropica* to visceral organs tissues of BALB/c mice in order to find out the ability to diagnosis the disease by polymerase chain reaction (PCR) as recent application. The samples of liver tissues were collected and DNA was extracted by Promega DNA kit purification.

The polymerase chain reaction (PCR) with kDNA specific primers was used firstly to distinguish the species of parasite which isolate from patient had cutaneous lesion 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. 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.

The same primers were used to detect the dissemination of parasites to visceral organs via extracted DNA from liver tissues of infected animals. PCR application was very high sensitive and early diagnosis in liver tissues as a substrate for PCR reaction, all animals of group (1) which infected with (10^6) promastigotes were negative result at (15) days post-infection, whereas three animals from group (2) which infected with ($3x10^6$) promastigotes showed positive result at the same period. All animals except one from group (1) showed positive results at (30) days post-infection. But at the later sequences days all animals were positive.

Introduction

Leishmaniasis is a disease caused by different species of *Leishmania*, under the kingdom Prostista and phylum Euglenozoa. These flagellated protozoa known as kinetoplastids, include a number of pathogens responsible for serious diseases in humans and other animals (1), (2). Leishmaniasis currently prevalent in (88) countries, infecting (12) million individuals and threatening (350) million people with approximately (2) million new cases per year; (500 000) new cases of (VL); (60 000) deaths occur every year and in the absence of appropriate and timely treatment, most patients die and (l.5) million cutaneous leishmaniasis (CL) (90%) of them in Afghanistan, Algeria, Brazil, the Islamic Republic of Iran, Peru, Saudi Arabia and Sudan) (3). The historical forms of classification of leishmaniasis includes : visceral leishmaniasis (Kala-azar) produced by *Leishmania donovani* (4) ; cutaneous leishmaniasis (Oriental sore) produced by *Leishmania tropica* (5) and mucocutaneous leishmaniasis (tegumentary) form produced by *Leishmania braziliensis* (6). The parasite that causes ulcerative skin lesion in Baghdad diagnosed by (7).The prevalence of visceral leishmaniasis (VL) was reported by (8) in all regions of Iraq except south-east area of Iraq (marshes regions), while (9)

suggested that the visceral leishmaniasis was endemic in Baghdad and adjacent regions.

Viscerotropic leishmaniasis is a comparatively mild form of visceral leishmaniasis caused by *L. tropica*, and is a feature of this disease (10). The first (2) cases of VL caused by *L tropica* in Africa were reported in 1989 (11). Later, in a study of (66) patients in southern Iran diagnosed clinically with VL, (1) of the (66) cases was found to be caused by *L tropica* when splenic and bone marrow aspirates were analyzed (12).This is an oligoparasitic syndrome with nonspecific clinical manifestations caused by the spread of *L tropica* to the reticuloendothelial system. Magill, (13) described the syndrome among (12) US servicemen returning from the Arabian Gulf War in 1991 with nonspecific symptoms including fever, anemia, weight loss, and anorexia, it is notable that none of these originally described cases had concurrent evidence of cutaneous involvement, and subsequent reports have also failed to demonstrate this association. Thus, they were interested in reporting a case of concomitant cutaneous and presumptive viscerotropic . Indeed, *L tropica* is associated with Old World cutaneous disease in urban areas of the Middle East and cities in the Mediterranean area, India, and Pakistan. Francesca, (14) speculate that either strain variants of *L tropica* or specific host immune response to this parasite may explain the dual tropism (dermal and visceral) in patient with viscerotropic syndrome. The rare report of (15) about *L tropica* which induced cutaneous leishmaniasis with presumptive coexisting viscerotropic disease and an unusually prolonged incubation period. Nicolas, (16) suggested that the dissemination into the spleen, as a visceral organ, is associated with a susceptible phenotype, whereas containment of the parasite in the skin and lymph nodes is associated with a resistant phenotype in mice. It is not known whether *L. tropica* disseminates to the visceral organs in BALB/c mice. *Leishmania tropica* remains in the skin and lymph nodes in BALB/c mice and does not disseminate to the spleen (17), but (53) suggested that *L. tropica* disseminates into the spleen. *Leishmania tropica* visceralizes to the spleen and grows there while its growth is controlled in footpad tissues (18). Dissemination of *L. tropica* to visceral organs in BALB/c mice was similar to the growth patterns of this parasite in human viscerotropic leishmaniasis. In contrast to *L. major* infection, however, susceptible BALB/c mice resolve a high level infection with the visceralizing *Leishmania sp*. spontaneously, making them a better model of subclinical infection than of progressive disease. Dissemination of parasites to visceral organs occurred in hamster infected by attenuated *L. tropica* and challenged at day (60) by *L. major*, the parasites disseminate to spleen and liver after (15) days post challenge and resulting in pathological changes (hepatospleenomegaly) in these organs (19).

In molecular biology, the polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence, developed in (1983) by Kary Mullis (20). The method relies on thermal

cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification (21). As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. Amongst the molecular methods used for clinical diagnosis, PCR has been proved to be most sensitive and specific technique, albeit limited to tertiary care hospitals and research laboratories. The specificity of the PCR can be adapted to specific needs by targeting conserved region of the gene (22). Gene amplification through the PCR has several advantages compared to traditional techniques, because of its extremely high sensitivity, rapidity and the ability to be performed with a broad range of clinical specimens (23). Also the detection or identification of the causative agent is possible directly from the clinical specimens.

Several studies have reported that PCR assay could detect parasitaemia a few weeks before the appearance of any clinical signs or symptoms. Martin-Sanchez (24) using PCR-ELISA found (24%) asymptomatic individuals carrying *Leishmania* kDNA in their blood. It was pointed out that these individual could be potential source of transmitting the infections in the community. The authors also found a good correlation between the antibody titers, skin test positivity and PCR positivity. Bone marrow, lymph node aspirates, skin biopsy, skin scrape/exudates and blood samples have been used for PCR in several studies (25). The specificity of PCR on bone marrow aspirates has been reported up to (100%) (26) and sensitivity (80 - 93.3%) as compared to (50 - 60%) sensitivity of smear and culture examination (27). Using PCR methodology, it is no more essential to undergo invasive methods such as bone marrow, splenic punctures, lymph node biopsy, liver biopsy, etc., or collect large volumes of blood samples. Even a few drops of blood on filter paper may be sufficient to diagnosis of visceral leishmaniasis (28), or Filter Paper Lesion Impression in Secondarily Infected Ulcers and Non-ulcerative Lesions of Cutaneous Leishmaniasis (29).

The chronic CL patients are greatest diagnostic challenge and are easily misdiagnosed by clinical criteria because they are often atypical. These patients often have low or no *Leishmania* antibodies, and thus serological tests are not rewarding. In such cases, PCR has been proved to be the most important tool for diagnosis. The sensitivity of PCR in CL has been reported (100%). In a study (30) could confirm the diagnosis in all (50) dogs using PCR. Various types of specimens may be used such as skin biopsies, dermal scrapings from the bottom of the ulcer as well as exudates and syringe-sucked fluid taken from ulcerative lesions. Recently, the sampling method has been further improved using cotton

swab for diagnosis of CL (31). The automization of real-time PCR with comprehensive portable units has made these tools field friendly. The multiplex PCR can be used whenever, double or mixed infections are suspected as in AIDS patients and detect the natural presence of parasites in transport host (32).

Several molecular methods, especially those based on the (PCR), have been developed for the diagnosis and the identification of *Leishmania* species (33). Among them, PCR followed by hybridization using radiolabelled (34) or chemiolumin-escent (35) probes increased the sensitivity in detecting *Leishmania* parasites, but remains unable to identify species, therefore the molecular species identification is still made mainly by iso-enzyme electrophoresis. Spanakos (36) developed a simple, low-cost method for the detection and species differentiation of *Leishmania* directly from clinical samples, for routine use in a Parasitology laboratory. They used samples of peripheral blood, bone marrow and skin lesion material samples, derived from Greek patients with visceral or cutaneous leishmaniasis, and three reference strains. Patrick (37) suggested that quantitative real-time reverse transcriptase PCR (qrtPCR) is not necessarily more sensitive than conventional PCR (cnPCR), but it has many objective advantages over cnPCR, particularly speed, broad dynamic range of target DNA quantitation, and reduction of contamination. Polymerase chain reaction using kDNA should be used for the diagnosis of CL and that an ITS1 PCR can be reliably used for the diagnosis of CL when rapid species identification is needed (38).

The kDNA PCR methods have a higher sensitivity compared with microscopic method. Moreover, PCR could identify the parasite species for specific therapy. Microscopic method had low sensitivity and less value in chronic and atypical CL cases (39).

In Iraq (40) apply observational and descriptive study at outpatient departments of dermatology in the middle Euphrates region of Iraq they examine slit-skin smear and skin biopsy of patients with clinically suspicious CL lesions, the results of PCR examination were positive in (92.5%) when compare with other test like slide-touch skin biopsy method and Histopathologic examination, they conclude the PCR technique is highly specific (100%) and sensitive (92.5%) for the diagnosis of CL.

Material and Methods

Semisolid medium

This medium used to maintenance vitality and viability of the parasite in laboratory and also used to return the parasite from animal tissue, the medium is suitable for multiplication and growth of *Leishmania* promastigotes (41).

Component of 1000 ml

Table (1) components of semisolid media for preparation (1000) ml.

1. NaCl	6.91 gm
2. KCl	0.29 gm
3. $NaHCO_3$	0.10 gm
4. $CaCl_2$ H_2O	0.22 gm
5. D- Glucose	0.77 gm
6. Peptone	1.00 gm
7. Agar agar	4.00 gm
8. beef extract with agar	0.30 gm
9. Gentamycin	500 mg
11. Distilled water	800 ml
12. Rabbit Defibrinated blood	200 ml

All gradients above except blood and antibiotics were solved in distilled water, the pH must be 7.4 and autoclaved at 121 Celsius and pressure one atm. for fifteen minutes then cold to 50 C° and added blood and antibiotics under sterilized conditions, medium divided as 10 ml in each sterilized universal tube (20 ml volume) and incubate at 37C° for 24 hours to ensure free from contamination then store in refrigerator.

RPMI 1640 medium

This media used with 10% fetal calf serum (FCS) to reproduce large number of *leishmania* parasites (42). The direction per liter by dissolve 16.353 grams from media powder and 2 gram sodium bicarbonate in 900 ml of distal water stirring gently until completely solubilized without heating adjust pH to 7.1- 7.3 then adding additional water to 1 liter. Filter sterilizes using a 0.22 micron membrane, aliquot into sterile containers. Antibiotics adding (penicillin 100 IU/ml and gentamycin 500 µg/ml). Medium divided as 5 ml in each sterilized universal tube (20 ml volume) and incubate at 37C° for 24 hours to ensure free from contamination and store it in refrigerator for at least 3 months. When want to use it add 20% filtrated (0.22 micron membrane) FCS.

Sample collection

The parasites isolate in Wassit Province (Al-Zehraa hospital) at 16/10/2010 from child 8 years old with history of cutaneous lesion on face, about 1.5 - 2 cm lesion diameter. The history of infection about four months. The chancre fluid collected by disposable syringe and cultured in semisolid medium, incubate at 26˚C (Celsius) and examine three days later to detect the parasite growth and multiplication. Weekly subcultured the parasites to maintenance.

Preparation doses of parasites

The virulence of *Leishmania tropica* strain was maintained with passage in BALb/c mice. The amastigotes were isolated from the spleen of infected mice and cultured on semisolid medium and subcultured in RPMI 1640 with L-glutamine containing 20% v/v heat inactivated FCS and (penicillin 100 IU/ml, 500 µg/ml gentamycin) incubated at 26˚C (43). Promastigotes in the stationary phase were harvested and washed three times in phosphate buffered saline pH 7.4 (1500 g for 10 min), the pellet containing promastigotes were resuspended in PBS to count by haemocytometer. The parasite concentration was adjusted to 1×10^7 , 3×10^7 promastigotes/ml and 0.1 ml was injected subcutaneously into the hind footpads of group one and group two mice respectively (44).

Counting *Leishmania* parasites

Leishmania promastigotes counting according to (45) by taking 20 ml of cells from the culture and place it in a 1.5ml Eppendorf tube containing 20 ml of 2% formaldehyde mixing thoroughly. Place 10 ml of the fixed cells on the Neubauer haemocytometer and leave at room temperature for 5 minutes to allow the cells settle down. Count 5 squares, multiply the number by 10^5 so the number of cells present in 1 ml of culture.

Study design

Group of (60) Balb/c mice, males, 6~8 weeks- old divided to three subgroup (20 mice), the first and second subgroup inject intradermaly ($1x10^6$) , ($3x10^6$) respectively stationary phase promastigotes of *L. major* in the hind footpad and the third subgroup inject by phosphate buffered saline as control.

Four mice of each subgroup dissected at days (15, 30, 60, 90 and 120) depend on days post-infection, after stupefaction animal using chloroform, blood collected from heart (0.5) ml in anticoagulant (EDTA) tube to isolate genomic DNA for polymerase chain reaction (PCR).

Samples of spleen and liver for PCR

Preserved in deep freeze (-15ºC), used later to DNA purification.

Isolating Genomic DNA from Animal Tissue

The following Protocol was used to isolation DNA from liver tissues using a lit from Promega company.

1. 600µl of Nuclei Lysis Solution was added to a 15ml centrifuge tube, and chilled on ice.
2. The liver tissue Grinded in liquid nitrogen using a mortar and pestle that has been prechilled in liquid nitrogen. After grinding, allowed the liquid nitrogen to evaporate and transferred approximately (10–20mg) of the ground tissue to (600µl) of Nuclei Lysis Solution in a (1.5ml)
microcentrifuge tube.

3. The lysate Incubated at (65°C) for (15–30) minutes.
4. Added (3µl) of RNase Solution to the nuclear lysate and the sample mixed by inverting the tube (2–5) times. the mixture Incubated for (15–30) minutes at (37°C). Cooled the sample at room temperature for (5) minutes before proceeding.
5. Added 200µl of Protein Precipitation to the room temperature sample and vortexed
vigorously at high speed for (20) seconds. Chill sample on ice for (5) minutes.
6. Centrifuged for (4) minutes at (13,000–16,000 × g). The precipitated protein will form a tight white pellet.
7. Carefully removed the supernatant containing the DNA (leaving the protein pellet behind) and transferred it to a clean (1.5ml) micro centrifuge tube containing (600µl) of room temperature isopropanol.
8. Gently mixed the solution by inversion until the white thread-like strands of DNA form a visible mass.
9. Centrifuged for (1) minute at (13,000–16,000 × g) at room temperature. The DNA will be visible as a small white pellet. Carefully decanted the supernatant.
10. Added (600µl) of room temperature (70%) ethanol, and gently invert the tube several times to wash the DNA. Centrifuged for (1) minute at (13,000–16,000 × g) at room temperature.
11. The ethanol carefully aspirated using drawn Pasteur pipette. The DNA pellet was very loose at this point, and care must be used to avoid aspirating the pellet into the pipette.
12. The tube Inverted on cleans absorbent paper, and air-dries the pellet for (10–15) minutes.
13. Added (100µl) of DNA Rehydration Solution, and rehydrated the DNA by incubating the solution overnight at room temperature or at (4°C).
14. Stored the DNA at (2–8°C).

Extraction DNA from culture

The genomic DNA extracted from culture according to (46). 100 µL of Cultures of active promastigotes were transferred to (1.5) mL tube and diluted (1:4) with double distilled sterile water (ddH,O). The cultures were boiled for 5 min to increase cell lysis. (10) µL was used as control positive.

Loading buffer

To prepare (10) ml of loading buffer

- 25mg bromophenol blue
- 25mg xylene cyanol
- 3ml glycerol
- H2O to 10mL

Gel electrophoresis

Fifteen µL of DNA which extracted in previous step mixed with (5) µL loading buffer (1:3) were electrophorised in 2% agarose containing (1µg/ml) ethidium bromide at (70 volt) for (50 minute) visualized with ultraviolet light.

Polymerase Chain Reaction (PCR)

Green Master Mix (GoTaq®) (promega)

It is a premixed ready to use solution containing bacterially derived *Taq* DNA polymerase, dNTPs (400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP) and 3mM $Mgcl_{2.}$ Reaction buffers (pH8.5) at optimal concentration for efficient amplification of DNA templates by PCR. The Green Master Mix contains two dyes (blue and yellow) that allow monitoring of progress during electrophoresis, it have sufficient density for direct loading onto agarose gels. The dye migrates at the same rate as 3-5Kb DNA fragments, and the yellow dye migrates at a rate faster than primers (<50bp), in a 1% agarose gel.

Primer design

It is specific primer used by (46) to Distinguishing *Leishmania tropica* and *Leishmania major* in the Middle East.

The primer designed was based on a published sequence from the *L. maior* kDNA minicircle (47). Primer Uni21 was designed on a sequence within the conserved region (5' GGG GTT GGT GTA AAA TAG GCC 3'). The Lmj4 primer design was based on the variable region of the same *L. major* strain (5' CTAGTTTCC CGC CTCCGAG 3'). The first primer resuspend in (929 µL) of (DDW) to obtain a concentration of (100 µM) (100 picomoles/ µL). The second primer resuspend in (816 µL) of D.D. water to obtain a concentration of (100 µM) (100 picomoles/ µL)

PCR procedure

The PCR was performed according to (46) in a (25 µL) reaction solution consisting (12.5 µL) of green master mixture (Promega), (1.5 µl) (1.5pmoles) of each primer, (1.5 µL) of free nuclease deionized distal water, and 8 µl of each samples as DNA template.

Cycling was performed in a thermocycler (MULTIGENE $_{\text{Labnet}}$) with the following conditions: an initial denaturation step at (5 min at 94 ºC), 36 cycles of denaturation (1 min at 94 ºC), annealing (1 min at 54°C), elongation (1.5 min at 72°C) followed with, finally, an extension step at (10 min at 72°C). For each sample one positive control and one negative control were included.

Gel electrophoresis

Ten µl of the PCR products were separated by electrophoresis on 1.2% agarose containing (1µg/ml) ethidium bromide at (70 volt) for (50 minute), and visualized with ultraviolet light.

DNA Ladder kit

The KAPA Universal Ladder kit was designed for determining the approximate size and and quantity of double strand DNA on agarose gel. It was containing eighteen DNA fragments (in base pairs): 100,150, 200, 300, 400, 500, 600, 800, 1000, 1200, 1600, 2000, 3000, 4000, 5000, 6000, 8000 and 10000. Kit was formulated with DNA loading dye for direct loading on agarose gel. The concentration was (100ng / µl)

Gel analyzer software

This software used to detect molecular weight of DNA that extracted in previous procedures. Gel analyzer developed by scientific adviser Dr. Istvan Lazar, the software version is 2010a, copyright 2010.

Results and discussion

Identification of a Leishmania infection for laboratory clinical diagnosis by culture or serological techniques requires a long time and has poor specificity. With the development of a PCR assay that can be improved for identification of Leishmania species with internal probes or different primers, as has been done for other pathogenic microorganisms (48).

DNA of leishmania promastigotes

Figure (1) shows the high concentration of the extracted genomic DNA which extracted by boiling according to (46). The DNA extracted from leishmania culture and mixed with loading buffer (1:3) then elecrophorised in 2% agarose containing (1µg) ethidium bromide at (70 volt) for (50 minute), , this DNA used as control positive.

Species detection

The species was distinguished by using kinetoplast DNA-specific primer (primer pair Uni21/ Lmj4) that used by (46).

Parasite isolated from patient had CL acquired in Iraq figure (2), 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 (46) suggested 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, figure (3). Mahmoodi, (49) 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.*

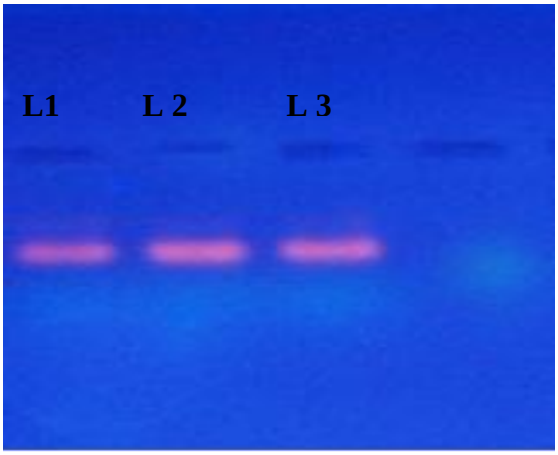

Figure (1) genomic DNA extracted by boiling from leishmania promastigotes in RPMI1640 culture, Lane 1, 2 and 3

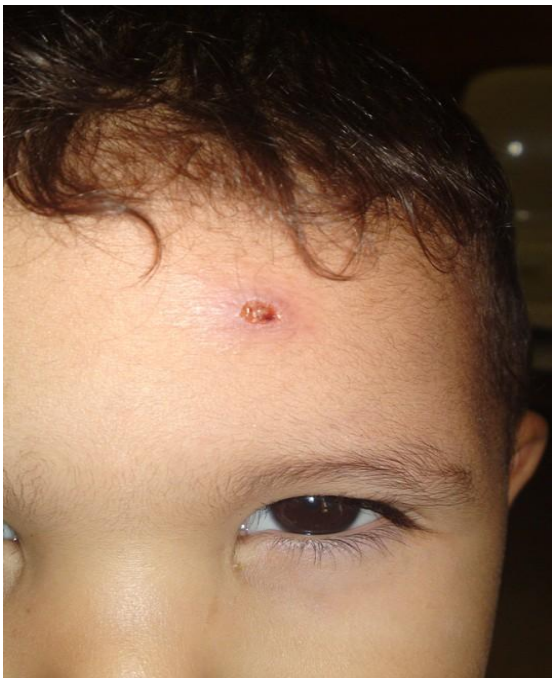

Figure (2) showed cutaneous lesion in child infected with cutaneous leishmaniasis in which parasites isolate

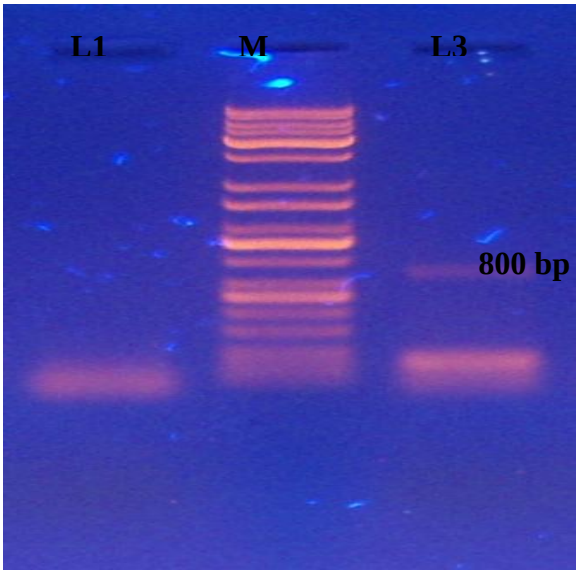

Figure (3) polymerase chain reaction product obtained from (1 µL) of boiling culture using primer pair Uni21/ Lmj4. Lane 1, negative culture; M, DNA size marker (KAPA Universal DNA Ladder); lane 3, leishmania DNA

DNA from liver tissue

Nicolas, (48) developed a highly accurate real-time PCR assay with which to reproducibly detect and quantify the relative *L. major* burden in mouse tissue samples (spleen, liver and bon morrow) using kinetoplast DNA primer, the assay was able to detect as little as (100) bp of L. major DNA per reaction, which is equivalent to (0.1) parasite. Cutaneous leishmaniasis infection involving visceral organs and circulatory blood dissemination in the infected host (50).

Monteiro, (51) used tissue imprints of skin, lymph node, spleen, liver and bone morrow of *L. chagasi*-infected dogs to investigate the presence of *Leishmania* amastigotes by either optical microscopy (OM) or Polymerase chain reaction (PCR) to detection of DNA.

Polymerase chain reaction by using primer pair Uni21/ Lmj4 applied on DNA extracted from liver tissues of infected Balb/c mice groups. Figure (4) showed negative results of group (1) at period of (15) days PI, this indicate no amastigotes in liver tissues at this time in this group, this may be low dose of inoculated parasites in the site of infection. The low dose resulted in only minor pathology but a higher parasite titer in the chronic phase, and it established the host as an efficient long-term reservoir of infection back to vector sand flies (52).

Whereas three animals from four gives positive results in group (2) at the same period, this positive result indicates the presence of amastigotes in the liver tissues at days (15) PI in animals. This result compatible with (18) which reported the *L. tropica*-infected BALB/c mice controls parasite growth at footpad tissues, while the parasite continues to grow in visceral and the parasite disseminate to the visceral organs in *L. tropica*-infected mice increased from (10) to (30) days PI.

The results of PCR of the two groups of infected animals at the period of (30) days PI and later was positive, the bands of DNA appear at the same line of control positive figure (5), the load of parasite in the spleen in *L. tropica*-infected mice continuously increasing at month (4) PI (18). The presence of *L. tropica* amastigotes in the spleen and liver is compatible with previous studies (Centers for Disease Control 1992), (15), (53), (54), (14).

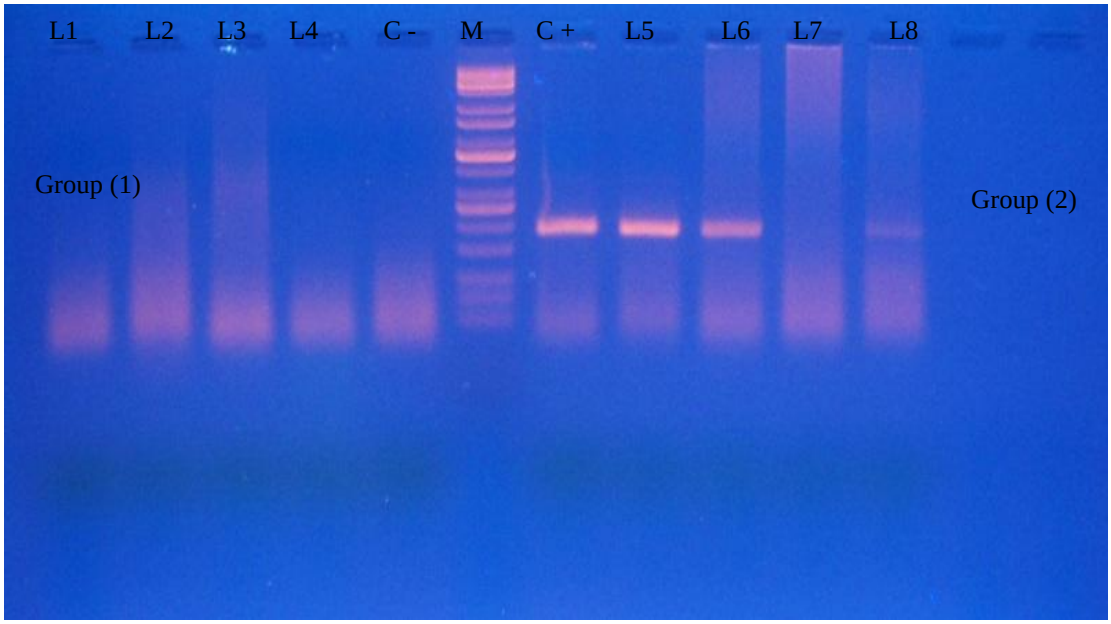

Figure (4) Polymerase chain reaction of DNA extracted from liver of infected lab. animals after (15) days PI using primer pair Uni21/ Lmj4. Lane 1, 2, 3 and 4 represent DNA of group (1). Lane 5, 6, 7 and 8 represent DNA of group (2). (C-) represent DNA from control negative group whereas (C+) is a DNA extracted from promastigotes of culture.

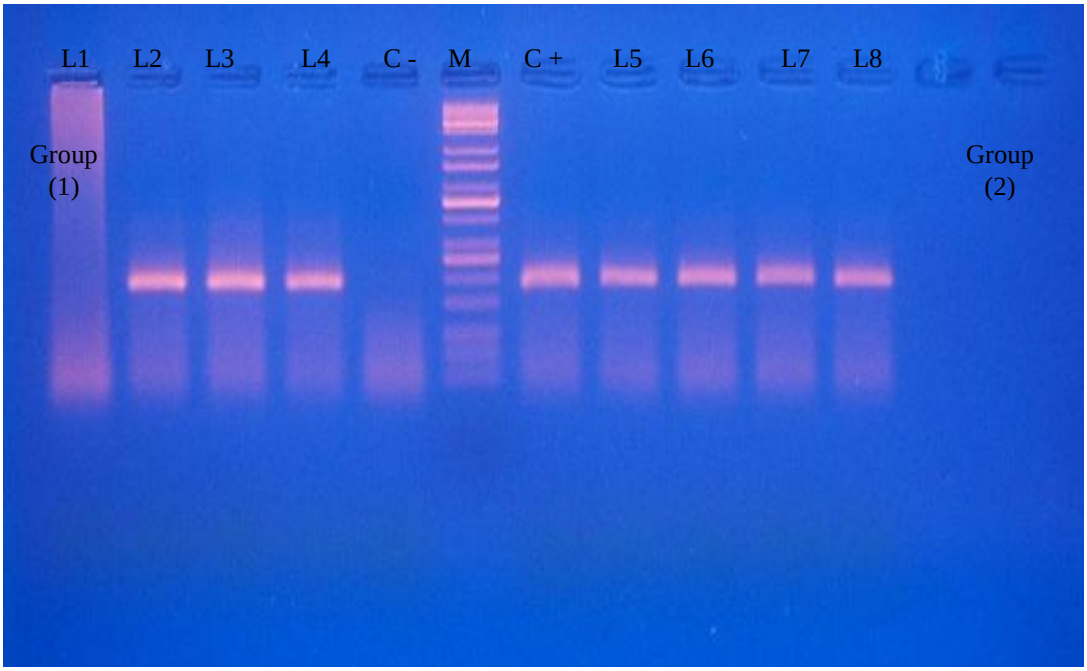

Figure (5) Polymerase chain reaction of DNA extracted from liver of infected lab. animals after (30) days PI using primer pair Uni21/ Lmj4. Lane 1, 2, 3 and 4 represent DNA of group (1). Lane 5, 6, 7 and 8 represent DNA of group (2). (C-) represent DNA from control negative group, (C+) is a DNA extracted from promastigotes of culture.

References

1. Ross, R. (1903).Note on the bodies recently described by Leishman and Donovan. *Brit Med J x*; 2:1261-1262.
2. Roberts, M. (2006). Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. *Br Med Bull* **75–76:115–130.**
3. WHO, (2006). Regional Office for South-East Asia. Report of the 59th session of the Regional Committee of the South-East Asia Region. New Delhi: *World Health Organization*.
4. Laveran and Mesnil (1903). The cytology of Leishmania donovani cited by Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. (1990) Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. *Annls Parasitol hum. Comp*., 65:111-125.
5. Wright, JH. (1903). Protozoa in a case of tropical ulcer ("Delhi sore"). *J Med Res.* Dec;10(3):472-82.
6. Gasper, V. (1911). Sober uma nova especial de Leishmania. *Brazil Medico*., 25:411-412.
7. Wenyon, C. M. (1911). J. trop. Med. Hyg., 14, 103.
8. Rahim, R. F. (1967). Present problems of oriental sore in Iraq. *Bull. Of endemic dis*., Baghdad, 9: 45-58.
9. Nouri, L. and Al-Jeboori, T. (1973). Kala-azar in Iraq; an epidemiological and clinical study. *J. Fact. Med.* Baghdad. 15, pp: 72-85.
10. Hyams, KC, Riddle J, Trump DH, Graham JT. (2001). Endemic infectious diseases and biological warfare during the Gulf War: a decade of analysis and final concerns. *Am J Trop Med Hyg*, 65: 664–670.
11. Mebrahtu, Y.; Lawyer P.; Githure J. (1989). Visceral leishmaniasis unresponsive to pentostam caused by *Leishmania tropica* in Kenya. *Am J Trop Med Hyg*.; 41(3):289-294.
12. Alborzi, A; Rasouli M; Shamsizadeh A.(2006). *Leishmania tropica*-isolated patient with visceral **leishmaniasis in southern** Iran. *Am J Trop Med Hyg*., **74(2):306- 307.**
13. Magill, AJ.; Grogl M.; Gasser RA Jr.; Sun W.; Oster CN.(1993). Visceral infection caused by *Leishmania tropica* in veterans of operation desert storm. *The England Journal of medicine*, 328(19):1383-1387.
14. Weiss, Francesca ; Nicholas Vogenthaler; Carlos Franco-Paredes; Sareeta R. S. Parker (2009). *Leishmania tropica*–Induced Cutaneous and Presumptive Concomitant Viscerotropic Leishmaniasis With Prolonged Incubation. *Arch Dermatol.*145 (9):1023-1026.
15. Magill, AJ.; Grogl M.; Johnson SC.; Gasser RA Jr. (1994). Visceral infection due to *Leishmania tropica* in a veteran of Operation Desert Storm who presented 2 years after leaving Saudi Arabia. *Clin Infect Dis*., 19(4):805-806.
16. Nicolas, L.; Sidjanski S.; Colle JH.; Milon G.(2000). *Leishmania major* reaches distant cutaneous sites where it persists transiently while persisting durably in the

primary dermal site and its draining lymph node: a study with laboratory mice. *Infec Immun*.; 68:6561–6566.
17. Lira, R.; Mendez S.; Carrera L.; Jaffe C.; Neva F.; Sacks D.(1998). *Leishmania tropica*: the identification and purification of metacyclic promastigotes and use in establishing mouse and hamster models of cutaneous and visceral disease. *Exp Parasitol*, 89:331–342.
18. Mahmoudzadeh -Niknam, H.; Simin Sadat Kiaei; and Davood Iravani (2007). Viscerotropic growth pattern of *Leishmania tropica* in BALB/c mice is suggestive of a murine model for human viscerotropic leishmaniasis. *Korean J Parasitol*.; 45(4): 247–253.
19. Al-Tamemy, Abdul Kareem A. R. (1990). Study of the pathological changes and immine response of cutaneous leishmania isolates in laboratory animals; A thesis of Msc. submitted to College of Veterinary Medicicne, Baghdad university.
20. Mullis, K.; Saiki, R.; Scharf, S.; Faloona, F.; Horn, G.; Erlich, H.; Arnheim, N. (1985). "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia". *Science*, 230 (4732): 1350–1354.
21. Bartlett, J. M. S., Stirling D. (2003). A Short History of the Polymerase Chain Reaction. 226. pp. 3–6.
22. Sarman, Singh (2006). New developments in diagnosis of leishmaniasis. *Indian J Med Res* 123, March 2006, pp 311-330.
23. Tavares, C.A.P.; Fernandes A.P.; Melo MN. (2003). Molecular diagnosis of leishmaniasis. *Expert Rev Mol Diagn*., 3: 657-67.
24. Martin-Sanchez J, Pineda JA, Morillas-Marquez F, Garcia-Garcia JA, Acedo C, Macias J.(2004). Detection of Leishmania infantum kinetoplast DNA in peripheral blood from asymptomatic individuals at risk for parenterally transmitted infections: relationship between PCR results and other Leishmania infection markers. *Am J Trop Med Hyg*., 70 : 545-8.
25. Deborggraeve, S.; Thierry L.t.; Diego E.; Gert Van der A.; Margaret M.; Monique W. (2008). A Simplified and Standardized Polymerase Chain Reaction Format for the Diagnosis of Leishmaniasis. *The Journal of Infectious Diseases*, Molecular Dipstick for Leishmaniasis, 198, p: 1-8.
26. Martin-Sanchez, J. Pineda JA, Morillas-Marquez F, Garcia-Garcia JA, Acedo C, Macias J.(2004). Detection of Leishmania infantum kinetoplast DNA in peripheral blood from asymptomatic individuals at risk for parenterally transmitted infections: relationship between polymerase chain reaction results and other Leishmania infection markers. *Am J Trop Med Hyg*., 70 : 545-8.
27. Tavares, C.A.P.; Fernandes A.P.; Melo MN. (2003). Molecular diagnosis of leishmaniasis. *Expert Rev Mol Diagn*., 3: 657-67.
28. da Silva, ES.; Gontijo CM.; Pacheco Rda S.; Brazil RP. (2004). Diagnosis of human visceral leishmaniasis by PCR using blood samples spotted on filter paper. *Genet Mol Res*, 3: 251-7.

29. Boggild, A. K., Ana P. R., Braulio M. V., Nicolas V., Flor C., Jorge A., Donald E. L., and Alejandro Llanos-Cuentas.(2010). Diagnostic Performance of Filter Paper Lesion Impression PCR in Secondarily Infected Ulcers and Non-ulcerative Lesions of Cutaneous Leishmaniasis. *J. Clin. Microbiol*. v1 02457-10.
30. Oliveira, CI.; Bafica A.; Oliveira F.; Favali CB.; Correa T.; Freitas LA. (2003). Clinical utility of polymerase chain reaction-based detection of Leishmania in the diagnosis of American cutaneous leishmaniasis. *Clin Infect Dis*, 37 : p: 149-53.
31. Mimori, T.; Matsumoto T.; Calvopina MH.; Gomez EA.; Saya H.; Katakura K. (2002). Usefulness of sampling with cotton swab for PCR-diagnosis of cutaneous leishmaniasis in the New World. *Acta Trop*; 81 : 197-202.
32. Kato, H.; Eduardo A. Gomez , Abraham G. Cáceres , Hiroshi Uezato، Tatsuyuki Mimori and Yoshihisa Hashiguchi (2010). Molecular Epidemiology for Vector Research on Leishmaniasis. *Int. J. Environ. Res. Public Health*, **7, 814-826.**
33. Andrade, A.S.; Gomes, R.F.; Fernandes, O.; de Melo, M.N. (2001). Use of DNA-based diagnostic methods for human leishmaniasis in Minas Gerais, Brazil. *Acta Trop*., 30 (78), 261–267.
34. Fernandes, O.; Catanho, M.P.; Segura, I.; Labrada, L.A.; Derre, R.; Saravia, N.; Degrave, W. (1999). Minicircle variable region probes for characterization of *Leishmania (Viannia)* species. *J. Parasitol*., 85, 563–568.
35. Bastrenta, B.; Buitrago R.; Vargas F., Le Pont F.; Torrez M.; Flores M.; Mita N.; Breniére S.F. (2002). First evidence of transmission of Leishmania (Viannia) lainsoni in a Sub Andean region of Bolivia. *Acta Trop*. 83, 249–253.
36. Spanakos, G.; Evangelia-Theophano P.; Panagiotis G. M.; Nicholas T.; Alexander F.; Nicholas C. V. (2008). Detection and species identification of Old World Leishmania in clinical samples using a PCR-based method. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 102, 46-53.
37. Patrick, Bastien; Gary W. Procop and Udo Reischl (2008). Quantitative Real-Time PCR Is Not More Sensitive than "Conventional" PCR. *Journal of Clinical Microbiology*, Vol. 46, No. 6, p: 1897-1900.
38. Bensoussan, E., Abedelmajeed N., Flory J., Lione F. S., and Charles L. J. (2006). Comparison of PCR Assays for Diagnosis of Cutaneous Leishmaniasis. *J Clin Microbiol*. 44(4): 1435–1439.
39. Karamian, M.; MH Motazedian; M. Fakhar; K. Pakshir; F. Jowkar; H. Rezanezhad (2008). Atypical presentation of Old-World cutaneous leishmaniasis, diagnosis and species identification by PCR. *Journal of the European Academy of Dermatology and Venereology*, Volume 22 Issue 8, Pages 958 – 962.
40. Al-Hucheimi, S. N.; Baqur A. S.; Muhsin A. Al-Dhalimi (2009). A comparative study of the diagnosis of Old World cutaneous leishmaniasis in Iraq by polymerase chain reaction and microbiologic and histopathologic methods. *International Journal of Dermatology*, Vol. 48, Issue 4, pages 404–408.
41. Adler, S. and Theodor, O. (1972). The behavior of culture of leishmania species Phlebotomus papatasi. *Ann. Trop. Med. Parasitol*., 21: 111- 141.

42. Limoncu, ME.; Ozbilgin A.; Balciog IC.; and Ozbel Y. (2004). Evaluation of three new culture media for the cultivation and isolation of Leishmania parasites. *J. Basic Microbiol*., 44 (2004) 3, 197–202.
43. Badiee, A.; Mahmoud R. Jaafari, Ali Khamesipour (2007). Leishmania major: Immune response in BALB/c mice immunized with stress-inducible protein 1 encapsulated in liposomes. *Experimental Parasitology*. 115, 127–134.
44. Laurenti, M.D.; Orn, A.; Sinhorini, I.L. and Corbett, C.E.P. (2004). The role of complement in the early phase of Leishmania (Leishmania) amazonensis infection in BALB/c mice. Braz. *J. Med. Biol. Res.*, Volume 37(3) 427-434.
45. Mottram laboratory(2008). Protocols for handling working with *Leishmania* species. Working with *Leishmania* for Dummies, p: 4.
46. Anders, Gerlind; Carol L.; Eisenberger, Flory Jonas and Charles L. Greenblatt (2002). Distinguishing *Leishmania major* and *Leishmania major* in the Middle East using the polymerase chain reaction with kinetoplast DNA-specific primers. *Transactions of the Royal Society of Majorl Medicine and Hygiene*, 96, 87-92.
47. Smith, D. F.; Searle, S.; Ready, P. D.; Grimiccia, M. and Ben- Ismael, R. (1989). A kinetoplast DNA probe diagnostic for Leishmania major: Sequence homologies between regions of Leishmania minicircles. *Molecular and Biochemical Parasitolom*., 37, 213- 224.
48. Nicolas, L.; Eric P.; Thierry L. and Geneviève M. (2002). Real-Time PCR for Detection and Quantitation of Leishmania in Mouse Tissues. *J Clin Microbiol*.; 40(5): 1666–1669.
49. Mahmoodi, M. R.; Masoud M.; Jalil T. A.; Mohhamad T. S.; Mohhamad J. Y. P., Fariba B., Abdolmajid F. (2010). Molecular identification of Leishmania species causing cutaneous leishmaniasis in Mashhad, *Iran. J Microbiol*.; 3(4): 195-200.
50. Talmi-Frank, D.; **Charles L. J.; Abedelmajeed N.; Alon W.; Roni K.; Milena S.; Ofer P. and Gad B. (2010).** *Leishmania tropica* **in Rock Hyraxes** (*Procavia capensis*) **in a Focus of Human Cutaneous Leishmaniasis.** *Am J Trop Med Hyg*.; **82(5): 814–818**.
51. Monteiro, H. de Andradea; Alexandre B. R.; Sara L. dos S.; Ângela C. V.; Marcos J. M. and Alvaro J.R. (2006). Use of PCR–RFLP to identify Leishmania species in naturally-infected dogs. *Veterinary Parasitology*, **Volume 140, Issues 3-4, Pages 231-238.**
52. Kimblin, Nicola; Nathan Peters; Alain Debrabant; Nagila Secundino, Jackson Egen, Phillip Lawyer, Michael P. Fay, Shaden Kamhawi, and David Sacks (2008). Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. *Proceedings of the national Academy of Science of the United State of America* , vol. 105 no. 29: 10125-10130.
53. **Mahmoudzadeh-Niknam, H. (2004). Induction of partial protection against** *Leishmania major* **in BALB/c mice by** *Leishmania tropica*. *Scand J Lab Anim Sci*; **31:201–207**.
54. Wilson, E. Mary; Selma M.B. Jeronimo; Richard D. Pearson. (2005) Immunop-athogenesis of infection with the visceralizing Leishmania species. *Microbial Pathogenesis*, 38 147–160.

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