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Evaluation of the PCR Method for the Diagnosis of Cutaneous Leishmaniasis in AL Hawija District, Kirkuk, Iraq

Husain F. Hassan[1], Moath M. Salman[1], Burhan M. Mohammed[1]

[1]Department of Biology , College of Science , University of Kirkuk, Iraq

Abstract

Males (191) and females (229) with skin lesions were enrolled in this investigation with the highest incidence of the disease in both sexes being in the age group (0-5) years . The clinical features of the lesions were covered by firmly adherent crust and some were edematous. By using classical microscopic examinations, the amastigotes were detectable in 143 cases out of 200 lesion samples. On the other hand , the use of specific PCR system and DNA sequences provided evidence that *Leishmania tropica* is the only etiologic agent of cutaneous leishmaniasis in AL Hawija district, Kirkuk province, Iraq.

***Key words** : Cutaneous leishmaniasis ; Polymerase chain reaction (PCR); Diagnosis, AL Hawija*

Introduction

Cutaneous leishmaniasis (CL) is the most widespread form of anthropozoonotic disease caused by hemoflagellate of the genus Leishmania , wich infect the vertebrate host after a bite by infected sandfly of the genus Lutzomyia in the New World and phlebotomus in the Old World (1). Human cutaneous infections display a clinical spectrum ranging from primary lesions (oriental sore) that often selfheal to sever mutilating mucocutaneous lesions (Chilcero ulcer , espundia) to diffuse cutaneous leshmaniasis (2). In Iraq CL (Baghdad boil) is widely distributed in the central parts of the country with most cases being caused by *L.tropica* (urban , dry CL) or *L.major* (rural, wet CL) (3) . During the past decade polymerase chain reaction (PCR) approaches have been developed as sensitive alternatives (4) to augment existing an early diagnostic methods based largely upon microscopic examination of Giemsa stained smears , culture of lesion biopsies , histopathology and immunological tests (5). The kinetoplast DNA (kDNA) in members of the genus Leishmania represent an ideal PCR target since contains 10000 small minicircular DNAs and have conserved and variable regions that allow differentiation between Leishmania species (6) . In the present study our validation of kDNA amplification for the diagnosis of CL as part of an epidemiological surveys confirmed the high current rate of infection in an area of endemicity in AL hawija district,Kirkuk province,Iraq

Method

Dermal scraping specimens were collected by slitting the border of a skin lesion from 420 patients with clinically suspected CL, who had admitted to ALhawija hospital from August 2018 to July 2019 . the specimens were divided in to three samples . The first sample was used for microscopic smear examination . The tissue aspirate was smeared on to glass slide , fixed with methanol and stained with Giemsa . the second sample was used for in vitro culture wich was performed by inoculation of tissue fragment in Nove-MacNeal – Nicole (NNN medium) and incubated at 26 C . After 5 days , the samples were transferred to fresh medium and observed for at least 6 weeks before they were diagnosed as negative culture . The third sample was processed for PCR .

Extraction of DNA . DNA as purified using the Genomic Prep Cells and Tissue DNA isolation kit (Bioneer,Korea) according to the protocol of the manufacturer. Frozen tissue sample (20 mg) were lysed by incubate 30 µl of lysis buffer (10 mM Tris –HCl , pH 8.0 , 10 mM EDTA , 1 mg / ml of proteinase K) at 65 C for 2 hours with supsequent heating at 100 C for 30 minutes to denature the enzyme . Cell debris and proteins were removed by centrifugation (10000 Xg at 4 C for 10 minutes) and the supernatant fraction containing the DNA used for PCR. Five microliters of the DNA solution was added to the PCR mixture .

Polymerase chain reaction amplification . The PCR amplification was performed using the oligo ptimers forward T2b(5-CGGCTTCGCACCATGCGGTG-3) and reverse B4 (5-ACATCCCTGCCCACATACGC-3) wich were previously reported to specifically amplify the entire 750-bp minicircle kDNA of Leishmania species . The PCR reaction mixture (25µl) containing 10 mM Tris – HCl , 50 mM KCL , 0.1 mg of gelatin per ml , 1.5 mM Mg CL2 , 0.2 mM each deoxynucleoside triphosphate , 25 pmol of each appropriate primer and 2.5 U of Taq DNA Polymearse (Promega,USA) was amplified using Perkin-Elmer thermocycler following thermal profile wich composed 32 cycles (preincubation at 94 C for 5 minutes , denaturation at 94 C for 1 minute , annealing at 60 C for 1 minute , extension at 72 C for 1 minute and extra incubation at for 72 C for 10 minutes). Aliquots (10µl) of amplified PCR mixture were subjected to electrophoresis on 1% agarose gel using X TBE running buffer (0.045 M Tris borate , 1 mM EDTA) and PCR product bands visualized by staining with ethidium bromide (0.4 µg / ml) . Positive control DNA was purified from promastigote of *L.tropica* and from human monocyte and amplified by PCR using the same protocol as described above .

Sequence analysis . PCR was purified using GFX PCR DNA product and Gel band purification Kit (Amersham ,UK) following the manufactures instruction . The purified PCR product was sequenced by Macrogen Corporation Korea . Multiple sequence alignment was done using Clustal W method (Bioedit DNA analysis software)

Result

AL-Hawija district , approximately 70 km from the center of Kirkuk province , was chosen as a site for field studies because it is an area of alluvial plains with scattered irrigation channels moderately cultivated . People of this area mostly poor farmers living in mud houses in small scattered villages and are in close association with domestic animals . Four hundred twenty patients with skin lesions were enrolled in the study ; 191 were males and 229 females . The age distribution of skin lesions (Table 1) showed the highest peak incidence of the disese in both sexes was in the age group (1-5) years . The median duration of the lesions was 6 months with the highest frequency being presented in September (19%) followed by October (15.4%) , November (13%) and December (11.9%) . Seventy nine patients (18.79) presented with multiple lesions (4-7 lesions) . it is interesting to note that one patients was with 19 lesions . The lesions were located primarily on the lower extremity (8.8%) , face (10.7%) or upper extremity (11.9%). Of the 200 lesion samples 143 lesions were positive by Giemsa – stained smear (figure 1) , Yielding a sensitivity of (7 % 5%) and only 12 were culture positive with NNN medium . Total DNA was extracted from 24 lesion samples and assessed by electrophoresis on the agarose gel . The extracted DNA was used as templates in PCR reaction . Molecular characterisation of the isolates revealed amplification of the characteristic 600 bp minicircle band (figure 2) . The PCR of human monocyte DNA samples from healthy control with no previous exposure to CL-endemic area showed amplification of a band of a size (340-bp) wich confirm the fact that the amplicon of 600-bp of lesion isolate being not derived from host DNA (figure 3).The sequencing of the PCR product of 600-bp for each sample after correction and filling the missed nucleotide depending on reverse sequence using chromas Pro , the multiple sequence alignment was done by Clustal W method using Bioedit (DNA analysis program) and compared with previously reported references of Leishmania gentypes was due to *L. major* starins .

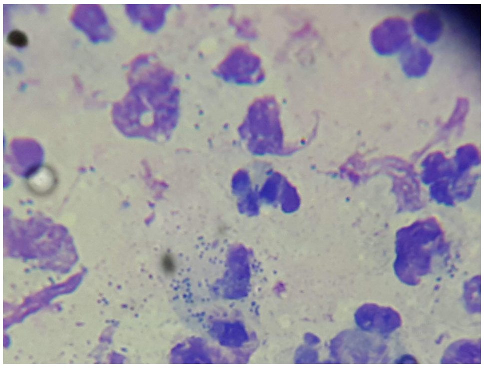


Figure (1) Amastigotes within macrophages . 100X

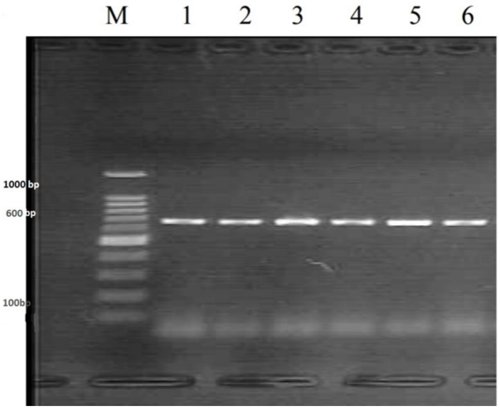


Figure (2) .Representing agarose gel of PCR products obtained from biopsy samples with primers specific for *L.tropica* . Lane M , represent 123 bp DNA Lader ; Lane 1-6 represent positive control .

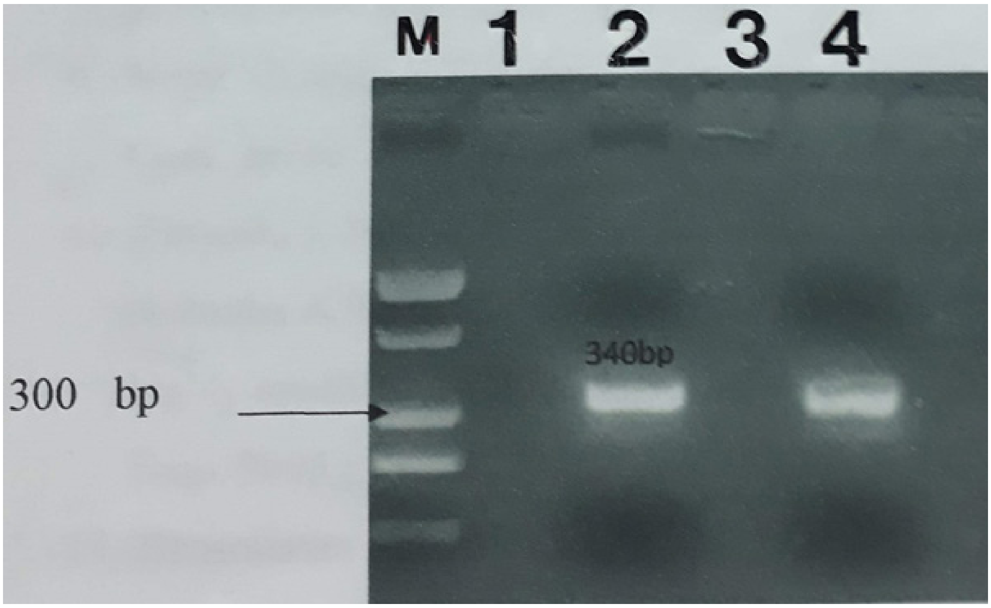
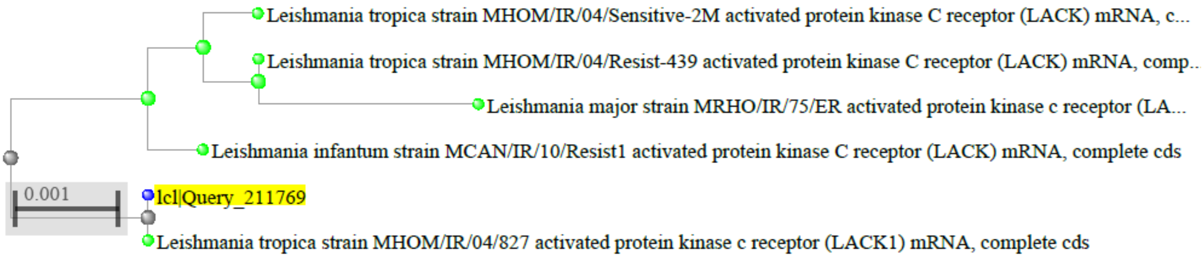


Figure (3) : Representative agarose gel stained with ethidium bromide following PCR amplification of human monocyte DNA by the primers wich amplify a region of the human beta hemoglobin gene , HBBL (forward) 5-GGCAGACTTCTCCTCAGGAGTC-3 and HBBR (reverse) 5-CTTAGACCTCACCCTGTGGAGC-3.M , 100-bp DNA Ladder , Lane 1 , *L. major* amplified DNA ; Lane 2 , Lane 3 and Lane 4 healthy biopsy DNA sample from patients with CL

The phylogentic relationship using the neighboring – joining method by (NCBI) the nucleotide sequence data reported have been submitted to the gen bank data base with accession number of *Leishmania* to show the phylogeny tree



Neighboring-joining tree showing phylogenetic relationship to family tree of some strain of *Leishmania*

Table 1 : Age and sex distribution of 420 cases of cutaneous leishmaniasis recorded in AL-hawija district, Kirkuk provice, Iraq during 2018 – 2019.

Age (year)	Male	% infection	Female	Infection%	Total	Infection%
5-0	57	13.49	67	15.94	124	29.43
10-6	45	10.7	48	11.42	93	22.1
15-11	30	7.1	41	9.7	71	16.9
20-16	22	5.23	29	6.9	51	12.1
25-21	17	4.04	24	5.7	41	9.7
30-26	20	4.76	20	4.76	40	9.5
Total	191	45.4	229	54.5	420	99.75

Discussion

Rural and urban forms of cutaneous leishmaniasis are known to be prevalent in Iraq probably since 3000 - 2000 BC (6) . During the last ten years there have been an outbreak of CL in ALhawija district , Kirkuk province of Iraq . As a result of the second gulf war , the increased human mobility and long distance travel may contribute to the spread of CL in areas where the disease was not endemic . This could possibly make diagnosis and species critical for for the importance of establishing control strategies . Prevaleance of infected female phlebotomine sand flies in urban and suburban areas of Iraq where USA military personal have been stationed could possibly confirm emergence of new Leishmania foci and the coexistence of multiple Leishmania species in the same geographical local (7) . In addition , accurate diagnosis of CL is often difficult because some of the dermal disease have some resemblance to those found in patients with CL . Direct microscopic smear examination of tissue sample for the presence of Leishmania is cheap and simple but rarely successful and require great expertise . Axenic culture of parasites from biopsy specimens or lesion aspirates is sensitive but it is time consuming and is subject to contamination . Therefore , there has been a great need for a more reliable laboratory test for diagnosis of CL in this area . The present study reports the application of PCR as a tool for the identification of Leishmania in lesion aspirates abtained from patients suspected to have CL . In the present study we use PCR primers based on sequences present in World Health Organisation reference strain (8). Although , they also anneal to DNA from strains from ALQadessia district , Iraq (9), this had not formally demonstrated in isolate from regions of endemicity in Kirkuk . Thus typing of Leishmania isolated from patients with CL by PCR yielded results in agreement with thus obtained by typing cultured *Leishmania tropica* (10). The evidence available at present incriminates *L. tropica* as the only causative agent of CL in Alhawija , Kirkuk district of Iraq and this is in agreement with results reported from neighbouring Iran and Turkey . (11) . Thus PCR is more sensitive than conventional methods of diagnosis and is likely to be valuable tools not only for species identification but also for investigation of relationships between causative agents and the clinical manifestation and epidemiology of the disease.

Conclusions

This study showed that PCR technique is the sensitive methods in the diagnosis of leishmaniasis. And from DNA sequencing have found that the main causes of cutaneous leishmaniasis in AL Hawija district is *Leishmania tropica* .

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Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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