Molecular Typing of Two Suspected Cutaneous Leishmaniasis Isolates in Baghdad

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Received 26/11/2019, Accepted 24/2/2020, Published Online First 6/12/2020, Published 1/3/2021

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Abstract:

Leishmaniasis is a group of parasitic diseases caused by Leishmania spp., an endemic infectious agent in developing countries, including Iraq. Diagnosis of cutaneous lesion by stained smears, serology or histopathology are inaccurate and unable to detect the species of Leishmania. Here, two molecular typing methods were examined to identify the promastigotes of suspected cutaneous leishmaniasis samples, on a species level. The first was species-specific B6-PCR and the second was ITS1-PCR followed by restriction fragment length polymorphism (RFLP) using restriction enzyme HaeIII. DNA was extracted from in vitro promastigote culture followed by amplification of kDNA by B6 or amplification and digestion of LITSR/L5.8S. PCR produced bands of ~359 bp and ~450 bp for B6 and ITS1, respectively. Digestion of ITS1 by RFLP revealed two distinct bands of ~150 bp and ~300 bp size. The results revealed that the two isolates belong to cutaneous Leishmaniasis, specifically Leishmania tropica. In conclusion, the confirmation of the studied methods will improve rapid and accurate diagnosis of Leishmania species of the most prevalent Iraqi strain of cutaneous leishmaniasis, L. tropica.

Key words: B6-PCR, Cutaneous leishmaniasis, Promastigotes, TS-1-PCR-RFLP.

Introduction:

Leishmaniasis is one of the neglected diseases caused by infection with protozoan parasites belong to a member of Leishmania species (1). Cutaneous leishmaniasis is caused by numerous species of Leishmania and are able to cause human leishmaniasis counting at least twenty-one species and subspecies (2). Leishmaniasis is prevalent in tropical and subtropical countries; the disease affect about twelve million people with three hundred and fifty million people at infection risk and an estimated yearly incidence of 2 million cases (3). In Iraq, the cutaneous form is predominant and endemic; the main types responsible of ulcer formation are the anthropopnotic L. tropica and zoonotic L. major (4). Visceral leishmaniasis is found but in less incidence than cutaneous, the confirmed species of visceral leishmaniasis are L. donovani and L. infantum (5). Total reported cases of leishmaniasis infections from 2008 to 2015 in Iraq were 17001 as mean of 2.9-10.5 per 100,000 individuals were officially reported by WHO, the highest occurrence cases were reported in 2015 (4000 cases) (6). Furthermore, regional crisis resulted in outbreaks of cutaneous leishmaniasis in areas with no history of leishmaniasis and the vector-born threat spread from endemic to non-endemic areas (7). Recent official data have declared the increasing incidence of cutaneous leishmaniasis in Baghdad and other governorates, such as Al-Khalis district/ Diyala provenance with more than 50 cases of Baghdad boil were recorded in the first two weeks of 2020 (8).

Epidemiologically, the diagnosis of cutaneous leishmaniasis, in Baghdad hospitals and suburban endemic areas generally relies on clinical presentation, microscopic examination in stained smears, rapid agglutination test and parasite culture due to the lack of developed diagnostic tools, which in most cases, cannot identify the causative species (9,10,11). Furthermore, the skin lesion appearance is similar by different species of Leishmania and sometimes it is problematic to be characterized from other skin infections (12).
In recent years, the polymerase chain reaction and molecular tests have developed as advanced sensitive tools rather than classical diagnosis, although, the molecular-based assays demonstrate variable outcomes depending on the target sequence determined (13, 14). Different *Leishmania* species were submitted to several molecular targets which have been evaluated for PCR application including minicircle kinetoplast DNA which is abundant in many copies per parasite cell (15,16), the minixeon gene (17), the gp63 (18), and the internal transcribed spacer (ITS-1) (19). However, few molecular and phylogenetic studies were conducted in Iraq for genotyping the local *Leishmania* strains. Moreover, new surveys confirmed the transition of foreign cutaneous *Leishmania* strains from the northern borders, in which several cases were identified as Iranian strain of *L. major* for the first time in Garmian, southeast of Kurdistan, North of Iraq; this indicates the entry of sand flies that carry this specific strain from Iran (20).

In the present study, local clinical samples were investigated by two species-specific diagnostic assays including B6-PCR amplification and ITS1-PCR-RFLP for cutaneous leishmaniasis molecular identification of parasite species as described by Altamemy (21) Kermanjani et al. (22) and Schönian et al. (23). The studied methods demonstrated rapid and sensitive assays for typing of Iraqi isolates of *Leishmania* on a species level.

Materials and Methods:

**Collections and cultivation of isolates:**

Two suspected cutaneous leishmaniasis samples were kindly provided at the Department of Biology, College of Science, University of Baghdad. The samples were previously isolated from skin ulcers of two patients attended Al-Karama Teaching hospitals in Baghdad and confirmed as cutaneous leishmaniasis by clinical presentation according to the dermatologist. Procyclic promastigotes were cultured in M199 medium supplemented with 10% heated inactivated foetal bovine serum and 1% Penicillin/Streptomycin then incubated at 26°C for 3 days to maintain log-phase harvest culture (24,25).

**DNA extraction:**

DNA was extracted from log-phase of axenic promastigote culture using QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer’s protocol. Extracted DNA was stored at -20°C until polymerase chain reaction amplification.

**Species-specific primer B6:**

Primer pair B6 (alpha DNA, Canada) was used for detection of kDNA by PCR, as a described by (26), which is specific for *L. tropica*. B6-Forward- (GCTCTGCCACGACACACAG) and B6-Reverse- (CGGTGCCTGCCAAGTA). Amplification reaction was carried out in 25 μl using GoTaq® Green Master Mix (Promega, USA). Thermal Cycler program was: 1 cycle of initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 minutes.

**ITS1-PCR-RFLP:**

- **Genus-specific PCR primer:**

  In order to detect *Leishmania* as a genus, the ITS1 region was amplified by LITSR/L5.8S (alpha DNA, Canada) according to (23), ITS1-forward (CTGGATCATTTTCCGATG) and ITS1-Reverse (TGATACCCTTATCAGCCTT). Amplification reactions were carried out in 25 μl using GoTaq® Green Master Mix (Promega, USA) according to the manufacturer’s protocol. Amplification was accomplished in a thermal cycler (Eppendorf®) programmed as following: 1 cycle of an initial denaturation for 2 minute at 94°C followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 63°C and 55°C for B6 and ITS1 respectively for 1 minute, and extension at 72°C for 2 minute. At the end 1 cycle of final extension for 3 minutes at 72°C.

- **Restriction enzyme digestion:**

  The amplicons of ITS1 were digested with *HaeIII* enzyme (Promega, USA) according to manufacturer’s protocol, 1μl (10U) of enzyme was added to 20 μl (100 ng) of PCR products and incubated at 37°C for 2 hours, according to the manufacturer’s protocol and (27).

**Results and discussion:**

**Species-specific B6-PCR:** This amplification of B6 primer pair was used to detect *Leishmania* subgenus (species) of *L. tropica* in other parts of the world, k-DNA of ~359 bp was observed for both Iraqi isolates as shown in Fig.1.
Figure 1. Detection of cutaneous *Leishmania* isolates from culture promastigotes on 1% agarose gel electrophoresis of B6-F/B6-R, M: 100bp ladder, lane 1: first isolate, lane 2: second isolate, lane 3: negative control.

**PCR RFLP of ITS1**: Results of PCR amplification using the primer pair LITSR/L5.8S, both isolates produced a band of ~450 bp, which confirmed the presence of *Leishmania* as a genus (Fig.2a). Subsequently digestion of LITSR/L5.8S PCR products with *Hae*III for *Leishmania* species identification, agarose gel electrophoresis yielded two distinct bands of ~300 bp and ~150 bp (Fig.2b).

![Figure 2](image)

(a) (b)

Figure 2. Detection of *Leishmania tropica* from promastigotes culture on 1% agarose gel electrophoresis of (a) LITSR/L5.8S primer pair, (b) Digesting of ITS1 with *Hae*III restriction enzyme profile of two *Leishmania* isolates. M: 100bp ladder, lane-1: first isolate, lane-2: second isolate, lane-3: negative control.

The development of sensitive molecular test for *Leishmania* species molecular identification in endemic areas is significant because of the existence of several *Leishmania* species with similar clinical presentation and geographical distribution (10). Furthermore, correct diagnosis is vital, in order to prevent the progress of human leishmaniasis and to select the appropriate treatment (28). Species identification of leishmaniasis agent in Iraq is still challenging, because the country contains many areas with different environmental and ecological status; furthermore, the presence of multiple *Leishmania* sub-types of cutaneous and visceral forms including *L. tropica, L. major, L. infantum* and *L. donovani* (29,30).

Primer pair B6-plan represents an important step towards fast, affordable, and reliable diagnostics of *L. tropica*, since it is based on direct PCR amplification of a species-specific DNA fragment and can distinguish this species from other causative agents of old and new cutaneous leishmaniasis agents (26). A similar study proved that B6 specific primer verified its sensitivity to amplify the 359 bp of *L. tropica* isolates originating from diverse geographical areas with nil PCR results of 12 other cutaneous species of old and new leishmaniasis (31).

A worldwide PCR technique targeting the internal transcribed spacer 1 region was designated for identification of various clinical infections of leishmaniasis and verified the ability of parasite identification on a genus level (32). A previous study used the same primer pair to detect *Leishmania* in different biological materials of human with leishmaniasis and proved that this primer is highly species-specific and sensitive detecting around 0.2 parasites per sample (23). In this study, RFLP of the ITS1-PCR product revealed that the isolates belonged to *Leishmania tropica* because this species contains only one restriction site for *Hae*III enzyme, which is (GG/CC), so only two bands appeared with distinct *L. tropica* standard strain base pair sizes, ~300 and ~150 bp. In contrast, *L. major* has difference sizes (~203 bp and ~132 bp). Furthermore, in case of visceral leishmaniasis *L. donovani* and *L. infantum*, both have two restriction sites, therefore, three bands should appear with different sizes (164, 75, and 54 bp) or (184, 72, and 55 bp) respectively (33).

ITS1 PCR/RFLP also can be used in a variety of sample type including direct species identification using Giemsa-stained smears or other samples without prior parasite culturing, microscopic analysis or other techniques, yet, RFLP analysis should be carefully organized to avoid cross-contamination (13, 34, 35). This method has previously been reported for species-specific identification of Old World species and New World leishmaniasis (23, 34, 36). The main feature of ITS1 digestion is that species characterization can
be accomplished by digesting the PCR product with just one restriction enzyme and this one-step typing can be used to detect the sub-genus of medically important isolates (36). Moreover, recent laboratory assessment proved that PCR-RFLP is more applicable with less complexity than multi-locus enzyme electrophoresis and less expensive from the time consuming Isoenzyme technique; it is also more accurate and sensitive than routine microscopic or Giemsa staining diagnostic methods (37, 38). Similar applications of RFLP-PCR assay is recommended for other Leishmania isolates from all parts of Iraqi provinces, for the detection of another local Leishmania species. This is to be examined for diagnosis, prevalent and epidemiology purposes.

**Conclusion:**
This is one of very first trials of molecular typing of Leishmania in Iraq. B6-PCR and/or LITSR/L5.8S – PCR in combination with RFLP technique using Haell III enzyme is a rapid and accurate method using only one primer pair for the detection and identification of Iraqi strain of cutaneous leishmaniasis, specifically L. tropica. It is recommended for further application of the above techniques on other isolates from different provinces in the country.

**Authors’ declaration:**
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

**References:**
التنميط الجزيئي لاثنين من عزلات اللهشمانيا الجلدية المشتبه بهما في بغداد

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الخلاصة:
يعتبر داء اللهشمانيا من الأمراض الطفيلية التي يسببها طفيلي اللهشمانيا، وهو مرض معدي متوطن في العراق. التشخيص بالطرق التقليدية غير دقيق وغير قادر على تشخيص طفيلي اللهشمانيا على مستوى النوع. في هذه الدراسة تم التشخيص بطريقتين جزيئيتين بتنميط الجزيئي للإطوار المسوطة لداء اللهشمانيا الجلدي، على مستوى النوع. الأول كان تفاعل البلمرة المتسلسل (PCR) للبادئ B6 والثاني ITS1-PCR متبوعًا بتعدد الأشكال المقيدة لطول الجزء باستخدام إنزيم التقييد HaeIII. تم استخلاص الحمض النووي من الأطوار الولفقية الخارجية المقوية باستخدام زوج البادئ B6 و ITS1-RFLP باستخدام زوج البادئ B6 و ITS1-RFLP. نواتج تفاعل البلمرة ITS1-PCR كانت 359bp و 450bp و ITS1-RFLP كانت 150bp و 300bp. هذه النتائج أثبتت أن العزلتين تعودان إلى داء اللهشمانيا الجلدي، تحديدا النوع الاستوائي. نوصي بالطريقتين الجزيئيتين للفحص المباشر للعينات المأخوذة من قرحة الجلد للمرضى الذين يشتبه في إصابتهم بداء اللهشمانيا الجلدي للتشخيص الحساس والسريع لأكثر السلالات العراقية انتشارًا من داء اللهشمانيا الجلدي وهي Leishmania tropica.

الكلمات المفتاحية: داء اللهشمانيا الجلدي، B6-PCR، ITS1-PCR، RFLP، Promastigotes، TS-1-PCR.