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Using of Species-Specific Primers for Molecular Diagnosis of *in Vitro* Promastigotes of *Leishmania donovani*

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Abstract:

Visceral Leishmaniasis (VL) is a disseminated protozoan infection caused by *Leishmania donovani* parasites which affects almost half a million persons annually. Classical diagnosis methods of VL still not very sensitive and time consuming. In this study, we reported the success of polymerase chain reaction (PCR) method to identify *L. donovani* based on kinetoplast deoxyribonucleic acid (kDNA) for the diagnosis of the parasite using *in vitro* promastigote cultures. LdI species - specific primer was used to identify *L. donovani* and the result showed a single band of about ~600bp. It can be recommended that this primer is to be used for detection the visceral *L. donovani*.

Keywords: *L. donovani*, PCR, specific primers.

استخدام باديء متخصص للتشخيص الجزيئي لطيفلي الشمانيا الاحشائية للاطوار المسوطة خارج الجسم الحي

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الخلاصة

داء الليشمانيا الحشوي (VL) هو اصابة بابتدائيات منتشرة تسببها طفيلي *Leishmania donovani* الذي يصيب ما يقرب من نصف مليون شخص سنويا. طرق التشخيص التقليدية للشمانيا الحشوية لا تزال غير حساسة للغاية وتستغرق وقتا طويلا. في هذه الدراسة، تم استخدام طريقة تفاعل البلمرة المتسلسل (PCR) لتحديد *Leishmania donovani* على أساس حمض النووي الرايبونوكليوتيد منقوص الأكسجين (kDNA) لتشخيص الطفيلي باستخدام الاطوار المسوطة المزروعة. وقد استخدم باديء LdI للكشف عن *L. donovani* وأظهرت النتيجة وجود حزمة واحدة ~600bp وبذلك يمكن استخدام هذا الباديء للكشف عن الشمانيا الحشوية *L. donovani*.

Introduction

Leishmaniasis is a neglected disease which affects 12 million people in nearly 90 countries presenting a worldwide public-health problem [1]. More than 90% of reported cases are located in 11 countries, India, Bangladesh, Nepal, Sudan, Brazil, Bolivia, Peru, Afghanistan, Iran, Saudi Arabia, Syria and Iraq [2]. The protozoan *Leishmania* is obliged intracellularly and cause a wide spectrum of clinical syndromes: visceral leishmaniasis (kala azar), cutaneous leishmaniasis and mucocutaneous leishmaniasis (espundia) [3]. Leishmaniasis is transmitted by the bite of phlebotomine female sand flies of the genera *Phlebotomus* and *Lutzomyia*, in the old and new worlds, respectively [4].

L. donovani attacks the reticuloendothelial system and the symptoms are characterized by fever, hepatosplenomegaly, leukopenia, progressive weakness and emaciation, which can result in death if

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left untreated. Children are at greater risk than adults of developing VL in endemic areas [5]. Leishmaniasis is endemic in Iraq, where both forms of the disease, cutaneous and visceral, are found. Iraq, with a population of nearly 32 million, where 23% are living below the national poverty line, has seen much strife and struggle in the past 25 years. Maximum numbers of cases of Leishmaniasis were reported in these early years of war and population displacement; in 1992, the number peaked at 45.5 cases per 100,000 of population [6].

Current parasitological diagnostic tests, including microscopic examination and *in vitro* culturing, offer limited sensitivity with respect to the direct detection of *Leishmania*, as well as time consuming. In addition, parasite-specific antibody tests, such as the immunofluorescent-antibody test (IFAT), direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA), although widely used to diagnose infection, employ crude antigens derived from whole parasite extracts and lack the appropriate sensitivity and specificity required for accurate serodiagnosis [7].

Reliable laboratory methods become mandatory for accurate diagnosis, especially in immunocompromised patients such as those infected with HIV [8]. Several authors consider the detection of parasite DNA in biological samples as alternative for leishmaniasis diagnosis [9]. The standardization of molecular biology such as amplification of species-specific genomic regions by the polymerase chain reaction (PCR) is of great importance, since that will support the diagnosis of the disease and the identification of *Leishmania* species, in cases that the serological and parasitological tests do not elucidate the diagnosis. This technique has the advantage of replicating the genome of the agent from the minimum quantity of circulating DNA in biological samples [10]. Several PCR assays for the combined detection and differentiation of parasites exist, including species-specific PCR, single-strand conformation polymorphism (SSCP) analysis, and restriction fragment length polymorphism (RFLP) analysis [11]. The objective of this study was using of species – specific oligonucleotides for molecular detection of Visceral Leishmaniasis (VL), *Leishmania donovani*, of an Iraqi isolate previously diagnosed with VL based on classical methods, such as, clinical features and serological tests.

Materials and Methods:

Isolation of the parasite that used in this study:

Leishmania donovani (MHOM/IQ/2005/MRU15) isolate, from a patient clinically diagnosed with VL, was kindly provided from Medical Research Unit, College of Medicine, AL-Nahrain University.

Parasite culture: Procyclic promastigotes was cultured in M199 media supplemented with 10% FCS and incubated at 26°C. After three days, the culture was examined under light microscope to ensure the growth of parasites and the absence of any contamination.

DNA extraction:

DNA extraction of healthy cultures of procyclic promastigotes of *L. donovani* was made using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA was kept at -20°C.

Oligonucleotide sequence: The *L. donovani* kinetoplast mini-circle sequence (accession no. Y11401) was purchased from Alpha DNA. The two primers sequence used were:

5-AAATCGGCTCCGAGGCGGGAAAC-3 and 5-GGTACACTCTATCAGTAGCAC-3 together designated the LdI primers. The LdI primers amplify a fragment of approximately 600 bp, which can be seen on gel after PCR amplification.

PCR amplification: DNA from cultured parasites was amplified using the LdI primers as described above, GoTaq[®] Green Master Mix, Promega (USA), was used for this PCR amplification according to the manufacturer's protocol. The PCR amplification mixture which used for detection of LdI gene includes GoTaq[®] Green Master Mix, 2X (12.5 µl), 3 µl of 25 ng DNA template, 1 µl (1 Mm) of each forward and reversed primers and 7.5 µl of nuclease free water to complete the amplification mixture to 24µl. Amplification was performed in a thermal cycler (Eppendorf[®]) programmed for 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min, preceded by an initial denaturation of 2 min at 94°C. Final extension was for 3 min at 72°C. Products were analyzed by electrophoresis of 1.5% agarose gel containing Ethidium bromide (0.5 mg/ml) in TBE buffer and photographed under UV illumination.

Results and Discussion:

In the present study, we used PCR with specific primers from the conserved region of *Leishmania* minicircles [12]. This approach is very useful for diagnosing VL and can provide a better understanding of the epidemiology of visceral and cutaneous leishmaniasis (CL) in endemic areas [13,14].

The DNA was isolated from samples to be amplified with species-specific primers for the diagnosis of *Leishmania donovani* strain isolate from MHOM/ IQ/2005/MRU15 of Iraqi origin. Whole genomic DNA was first isolated from promastigote cultures and the isolated DNA was electrophoresed in 1% agarose gel to determine the presence of DNA, Figure-1.

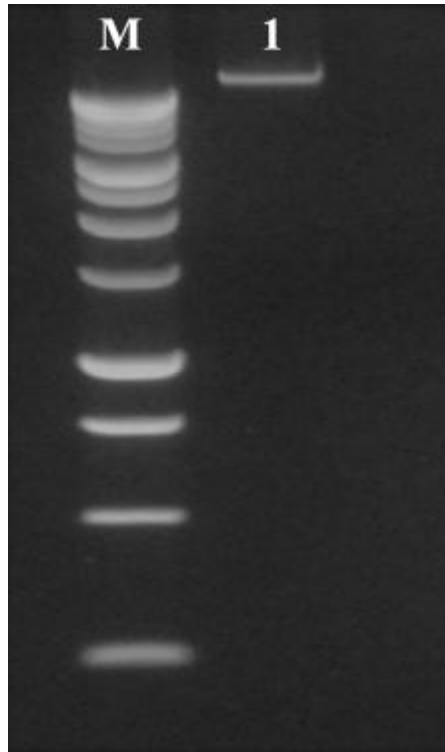


Figure 1- 1% Agarose gel electrophoresis of extracted DNA of *Leishmania donovani*, M-100bp Ladder, Lane (1) extracted DNA.

VL diagnosis may be carried out by direct parasitological tests that can produce false-negative results, either due to the low number of *Leishmania* spp. organisms in clinical samples (bone marrow and lymph nodes) or because morphological identification is complex. Moreover, such methods are invasive. Conventional serological techniques are limited by cross-reactivity with other parasitic diseases and because several technical procedures have not been standardized [15]. However, serological methods are still widely employed in epidemiological surveys.

On the other hand, Molecular diagnosis exploiting PCR combines several advantages, it is minimally invasive, has a high sensitivity and specificity, is capable of identifying relapses and reinfections in treated VL patients, and can provide species identification [16,17]. Many different PCR assays have targeted conserved and variable regions of kDNA minicircles [18-22], genomic DNA, splice leader mini-exon (SLME) [23], telomeric repeats [24], rRNA gene [25] and gp63 [26]. The amplification of LdI primer pair was used to identify *Leishmania donovani*, kDNA of ~ 600 bp was observed for our isolate, and no non-specific side product or artifacts appeared on the gel, Figure-2. We have found that the PCR using kDNA primers LdI is useful in diagnosis of *Leishmania donovani*.

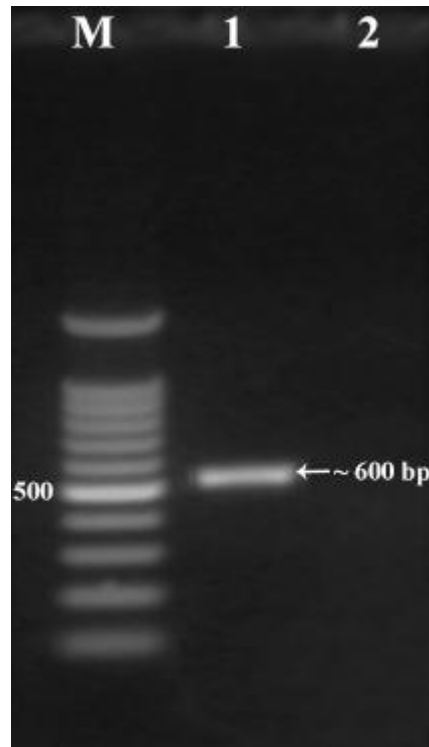


Figure 2- Agarose gel electrophoresis of PCR product of ~ 600 bp of LdI, M-100bp Ladder, Lane (1) DNA template (25 ng), and Lane (2) negative control sample (No DNA template).

The assay could detect as little as 1 fg of parasite DNA from Indian strains of *L. donovani*, an amount that represents the equivalent of approximately 0.1 parasite [27]. The primers were found to be species-specific for *L. donovani*, as DNA from other *Leishmania* species examined (*L. major* and *L. tropica*) was not amplified [19]. In earlier studies for diagnosis of VL due to *L. donovani*, the sensitivity of PCR for blood samples has been found to be in the range of 45 to 94% based on smaller sample sizes ranging from 17 to 42 [28].

Detection of *L. donovani* bodies in skin lesions by microscopy gives a positive result in only about 58% of cases, as parasites are scanty [29]. Early recognition and treatment of Post-kala-azar dermal leishmaniasis (PKDL) would contribute significantly to the control of KA, as patients with PKDL constitute a reservoir for the *Leishmania* parasite [30].

According to previous study by [19], PCR provided a highly sensitive method by LdI primers for diagnosis of PKDL. The sensitivity of the assay was 93.8% for PKDL, which is significantly higher than that reported (82.7%) in a study with 32 patients with PKDL in Sudan [31]. Another study used LdI primers pair approved 100% specificity of this amplification, as all of the control tissues examined 32 leprosy lesions and 19 dermal samples from healthy regions of skin of patients with PKDL were negative [19].

In another study by [32] Blood samples from 10 parasitologically confirmed patients with VL were examined by loop-mediated isothermal amplification (LAMP), conventional PCR, and nested PCR. Eight of 10 samples showed positive results with the LAMP and nested PCR. Seven of the eight samples with positive results also showed positive results with the conventional PCR by using LdI primers.

In conclusion, conventional PCR with LdI primer pair can be a developed method recommended for central laboratories to diagnose patients infected with Iraqi strain of *L. donovani*, in comparison with classical diagnosis methods.

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