IDENTIFICATION OF *Leishmania* PARASITES IN CLINICAL SAMPLES OBTAINED FROM CUTANEOUS LEISHMANIASIS PATIENTS USING PCR TECHNIQUE IN IRAQ

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Abstract

Cutaneous Leishmaniasis (CL) is an endemic disease and one of the major health problems in Iraq. *Leishmania major* is confirmed as the causative agent of CL in this region. In Al- Qadessia province alone, the recorded total cases of CL were 618 from 2005-2007. We aimed to determine the effectiveness of a polymerase chain reaction (PCR) technique for identification and differentiation of the *Leishmania* parasite in clinical samples. The lesion exudates were collected from 27 suspected patients (12 males and 15 females) and used for smear slide preparations, culture on NNN medium then DNA isolation. The isolated DNA was amplified by PCR, including primers selected on repetitive kDNA for identification of a *Leishmania* subgenus, and the amplified DNA electrophoreses was done. The PCR results showed that only *L. major* exists. Our results indicate that the PCR technique is sensitive and specific for the detection and differentiation of agents of CL in this area.

التعرف على طفيليات اللشمانيا في العينات السريرية المستحصلة مِنْ المرضى المصابين بالطور الجلدي بإستخدام تقنيةَ سلسلة تفاعل الانزيم المتبلمر في العراق

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الخلاصة

يعتبر داء اللشمانيات النوع الجلدي من الامراض الطفيلية المتوطنة في العراق فهو يشكل مشكلة صحية حقيقية في هذا البلد. ففي محافظة القادسية لوحدها سجلت 618 حالة اصابة للفترة الواقعة بين 2005 – 2007. تهدف هذه الدراسة الى تحديد فعالية استخدام تقنية سسلة تفاعل الانزيم المتبلمر PCR في الكشف والتفريق بين انواع طفيلي اللشمانيا في العينات السريرية. جمعت عينات من افات الطور الجلدي من 27 مريض (12 ذكر و15 أنثى) مصاب بالطور الجلدي . حيث كان مدى عُمرهم 12–60 سنةَ. العينات اجريت عليها الفحوصات المختبرية من تحضير المسحات الزجاجية والزرع المختبري على الوسط الزرعي الثلاثي (NNN). تم استخلاص الدنا من العزلات المعزولة وبعد تضخيمه بأستخدام بادئات خاصة مصممة للجين المشفر للدنا المسمى kDNA الخاص بعترات طفلي اللشمانيا. تم تمرير نواتج الدنا المضخم في جهاز الترحيل الكهربائي للدنا حيث ان النتيجة اظهرت بأن الطفيلي نوع L. major هو النوع الوحيد الذي تم التحري

عنه. النتيجة اكدت بأن تقنية سلسلة تفاعل الانزيم المتبلمر هي طريقة حساسة ودقيقة للتحري والتفريق بين انواع طفيلي اللشمانيا.

Introduction

Leishmaniasis is one of the 8 priority targets of the WHO and almost 1500000 new cases of the disease occur annually (1). Cutaneous leishmaniasis (CL) caused by *Leishmania major*, *L. tropica* and visceral leishmaniasis (VL) caused by *L. infantum,* are major health problems in Iraq (2). The majority of VL cases are reported from south of Iraq and CL is also endemic in different parts of Iraq (2). As the leishmanial signs and symptoms can be varied, characterization of *Leishmania* species is crucial for correct diagnosis and prognosis of the disease as well as for making decisions regarding treatment and control measures (3, 4).Traditionally, *Leishmania* parasites are directly detected by microscopic examination of clinical specimens. However all *Leishmania* species are very similar and their species identification is not possible morphologically (4). Currently isoenzyme analysis is a gold standard for differentiation of *Leishmania* species but this technique is demanding, laborious and usually requires prior cultivation *in vitro* (4,5). Frequently *Leishmania* species are identified based on their geographical distribution and on clinical manifestations of the resulting disease. However, geographical origin is an inadequate criterion in non-endemic areas, as well as endemic regions where multiple species of *Leishmania* may co-exist (6, 7). Identification of the infected *Leishmania* species based on clinical signs and symptoms can be problematic because several species cause both visceral and cutaneous involvement (8). With the advent of the PCR technology, several PCR based assays such as the ssu rRNA gene (9), repetitive sequences (10), the gp63 gene locus (11), kinetoplast minicircle sequences (12, 13), mini-exon gene sequences (7) for *Leishmania* species differentiation, were developed. While all these different approaches provide a multitude of valid taxonomic characters for differentiation, most of the time it's necessary to multiply parasite in culture before using them (15). Culture techniques require a sophisticated laboratory set up, time-consuming and increase risk of contamination (14). Thus, in this study; we aimed to use PCR method for the direct identification of *Leishmania* species on clinical samples.

Materials and Methods

Skin lesions exudates, dermal scrapings, and cutaneous biopsies were taken from 27 patients with suspected CL who had consulted Al-Khadmyia teaching hospital / Dermatology and venereal disease department from March (2005-2006). Patients evaluation including history and physical examination by the consultant medical staff at the hospital. Both conventional parasitological examination and molecular diagnosis were applied to each sample. Parasitological diagnosis consisted of microscopic examination (magnification, X1,000) of smears for amastigotes after Giemsa staining and *in vitro* culture in Nove-MacNeal-Nicolle (NNN) medium as previously described (17). When promastigotes were obtained in culture, for molecular diagnosis, DNA extraction was performed with DNA mini kits (Bioneer AccuPrep®, Korea), according to the manufacturer's recommendations. PCR amplification was carried out with the *Leishmania* kinetoplast DNA (kDNA) primers forward T2 (5′-CGGCTTCGCACCATGCGGTG-3′) and reverse B4 (5′-ACATCCCTGCCCACA-TACGC-3′) according to the manufacturer's recommendations. The reactions were done in a total volume of 25 µl containing 20 µl 1x PCR MIX, 0.3 µl of *Taq*-DNA polymerase, and 5 µl of the DNA was added to the mixture. The PCR amplification was done in a DNA thermocycler (eppendorf) using 1 cycle of 95°C for 180 sec, 63°C for 30 sec, and 72°C for 60 sec. Followed by 35 cycles of 93°C for 40 sec, 63°C for 40 sec, and 72°C for 60 sec. The PCR was done using CinnaGen *Leishmania* species PCR Determination and Detection Kit (All reagents were purchased from CinnaGen® Inc, Iran). Each experiment included a positive and negative control. The presence of amplification products was confirmed with 2% agarose gel electrophoresis analysis, and visualized by ethidium bromide staining (0.5 µg/ml).

Results and Discussion

The diagnosis of CL is traditionally based on microscopic demonstration of amastigote forms in tissue biopsies or smears. However, this method usually presents low sensitivity, and in atypical forms, CL may be overlooked because

of similarity to other dermal diseases. Thus, it is necessary to apply specific diagnostic methods as PCR (16). In the present study, we investigated the benefits of PCR as a tool for the detection of *Leishmania* parasites in lesion aspirate specimens obtained from patients with CL.

All patients enrolled in this study had characteristic signs and symptoms of CL, from small erythematous papules to nodules and ulcerative lesions. Patients ages varied from 12 to 60 years (age mean =32 years). They were 44% males and 56% females (Table 1). DNA purified from patients samples yielded an amplification product of the expected size (620 bp) which confirm the presence of *L. major* DNA. Band patterns from the samples were compared with standard positive control of test kit and identified *L. major* in 2 samples (7.4%) (Figure 1). No contamination or inhibition was detected.

In the present study, we used PCR with primers from the conserved region of *Leishmania* minicircles (19). This approach is very useful for diagnosing CL and can provide a better understanding of the epidemiology of VL and CL in endemic areas (20, 21). Campino *et al*., (19) used PCR to detect *Leishmania* DNA in peripheral blood obtained from HIV-positive patients with co-infection, but the species of *Leishmania* involved was not identified (22). In another study conducted in Columbia district/U.S.A when *L. major* identified using genus-specific PCR assay in 21 (95%) of 22 military personnel deployed during 2002-2003 to three countries (Afghanistan, Iraq, and Kuwait). Surveillance for infected female *Phlebotomine* sand flies, the vectors of leishmanial parasites, has been conducted in and near urban and preurban areas of Iraq where U.S military personnel have been stationed. Analysis indicated that the most common genus were *Phlebotomus papatasi* and *P. sergenti*. The overall infection rate in the sand flies was 1.4%,

Table 1: Patient data and results

Patient no.	Age	Gender	Direct examination	In vitro culture	PCR result	Molecular identification
1	14	F	-	-	-	ND
2	45	M	+	-	-	ND
3	30	F	-	-	-	ND
4	23	F	-	-	-	ND
5	22	M	+	-	-	ND
6	34	M	+	+	+	*L. major*
7	36	F	-	-	-	ND
8	55	M	-	-	-	ND
9	12	M	-	-	-	ND
10	23	F	+	-	-	ND
11	17	F	-	-	-	ND
12	31	F	-	-	-	ND
13	50	F	-	-	-	ND
14	52	F	-	-	-	ND
15	60	M	-	-	-	ND
16	56	M	-	-	-	ND
17	18	F	-	-	-	ND
18	20	F	-	-	-	ND
19	33	M	-	-	-	ND
20	24	F	+	-	-	ND
21	29	F	-	-	-	ND
22	47	M	-	-	-	ND
23	15	F	-	-	-	ND
24	23	F	-	-	-	ND
25	28	M	+	+	+	*L. major*
26	35	M	-	-	-	ND
27	32	M	-	-	-	ND

M, male; F, female; +, positive; -, negative; ND, not determined.

the infection rates for sand flies collected were as follows: 2.3% for Tikrit, 1.6% for AL-Nasiriyah, 0.08% for Baghdad, and 0.06% for Balad (23). These findings have led us to propose guidelines for the diagnosis of CL that use PCR as the principal means of parasitological confirmation of cases.

Recent studies document the emergence of new Leishmania foci and the coexistence of multiple Leishmania species in the same geographic locale, including much of northern Africa (18, 24). We recommend that treatment protocols, particularly in areas of coexistence, be predicated on diagnosis of not only the clinical form CL versus but additionally the disease causing species.

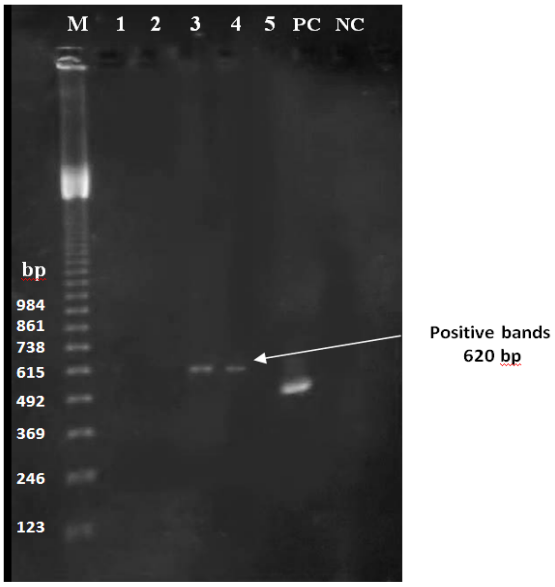

Figure 1. PCR reaction for diagnosis of Leishmania parasite of samples of lesion exudates of patients with cutaneous leishmaniasis, Electrophoresis was carried out in 2% agarose at 8V/cm for 45 minutes and stained with Ethidium bromide. Lane M, represent 123-bp DNA Ladder; lanes (3, 4) represent L. major amplified DNA; lane PC, represent positive control; Lane NC represent negative control.

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