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Molecular Detection Of Localized Cutaneous Leishmaniasis (Lcl) Among Iraqi Patients Using Real Time-Pcr

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Abstract

The current work highlights the use of a molecular technique to identify the etiological agent of localized cutaneous Leishmaniasis (Leishmania spp. parasites) in Iraqi patients with clinical manifestations of suspected Leishmaniasis. Twelve participants were recruited for this study between January 2021 and June 2021 at Baghdad hospital's external clinics. According to the manufacturer's instructions, the acquired samples in the form of skin biopsies were directly treated to a DNA isolation technique employing total DNA using the GeneJET Genomic DNA purification kit. Real-time polymerase chain reaction (real-time –PCR) was used to confirm the presence of Leishmania spp. in the twelve samples. Eight of twelve DNA samples (8/12= 66.66%) were positive for the Leishmania suspect pathogen, as determined by positive real-time PCR results. The current findings support the ability of the molecular detection approach used in this work to detect and confirm the presence of Leishmania spp. pathogen in cutaneous Leishmaniasis in a quick, accurate, specific, and sensitive manner.

Keywords: Localized cutaneous leishmaniasis (LCL), Real time-PCR, Leishmania spp., Leishmania specific primer, clinical manifestations

Introduction

Leishmaniasis, a parasitic disease, which is classified as a neglected tropical disease (NTD). Leishmaniasis, a neglected tropical disease (NTD), is a parasitic disease caused by the infection with Leishmaniaspp. parasites. It is considered a tropical and subtropical aliment. The causative agents Leishmania spp. are categorized as intracellular parasites. It was reported that the modeof transmission of leishmaniasis is through the bite of the sand fly Phlebotomus and Lutzomyia in different regions allover the world (i.e., Northern Africa, the Middle East, Europe, Asia, and part of South America). (Vera-Izaguirre et al., 2006). According to WHO (World Health Organization) records, over 20 Leishmania species are the causative agents of leishmaniasis. Additionally, over 90 sandfly species

are reported to be the vehicle of the Leishmania parasites. The WHO website stated that there are 3 main forms of the disease: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneousleishmaniasis (MCL). Cutaneous leishmaniasisis the utmost popular type of leishmaniasis and does cause skin lesions, mostly ulcers, on the unprotectedportions of the body, resulting in generation of life-long scars (Centre for Disease Control and prevention (CDC) records: https://www.cdc.gov/parasites/leishmaniasis/gen_info/faqs.html).

The cutaneous leishmaniasis is further subdivided into two subgroups: localized cutaneous leishmaniasis (LCL) and diffused cutaneous leishmaniasis (DCL) (Torres-Guerrero et al., 2017). At most,the clinical characteristics include a broad spectrum of manifestations of variable severity that mainly rely on the species of Leishmania implicated and the host immune response (Andrade-Narváez et al., 2001). For instance, In Mexico, the utmostdistinctivefeature is the cutaneous-chondral form synonymously named "chiclero's ulcer" (Reithinger et al., 2007).

Approximately 95% of CL patientshappen in the Mediterranean basin, Americas, the Middle East and Central Asia. In 2019 over 87% of new cases with CL happened in 10 countries: Afghanistan, Brazil, Colombia, Iraq, Iran, Pakistan, the Syrian Arab Republic, Tunisia, Algeria, and Libya. It is predicted that between 600 000 to 1 million novel cases happen throughout the world yearly (https://www.who.int/news-room/fact-sheets/detail/leishmaniasis).

According to the wording stated by International Association for Medical Assistance to Travelers (IAMAT) (https://www.iamat.org/country/iraq/risk/leishmaniasis), leishmaniasis is prevalent in Iraq. Cutaneous leishmaniasis (CL) makes up nearly 2/3 of cases whilst and visceral leishmaniasis (VL) does account for 1/3 of cases. The season with high incidence and prevalence of leishmaniasis begins in May. At present, the ailmentremains to do spread in the south region of theQadisiyah province, approximately 130 kilometers south of Baghdad as a consequence of the increase in the Sandfly activity. Hence, the transmission of leishmaniasis illness in Iraq is in a continuous elevation.

The goal of the present study is to monitor the prevalence of LCL cases in Iraq in a small sample size of 12 cases using a molecular approach: real-time PCR using a leishmania specific primer set, in the context of alarm records generated by WHO and CDC regarding the current status of leishmaniasis all over the world and particularly in Iraq.

Materials and methods

Ethical statement

The investigation was approved by the national center for tropical and zoonotic diseases in Baghdad, Iraq. An informed written consent was signed prior to sample collection from the enrolled patients in this study. The written consents were signed directly by the adult participant or the parents in case of children participants.

Study participants and study sites

Twelve patients were enrolled in this study. The study participants included both genders. Moreover, the recruited patients included two categories according to their ages: adults and children. The patients were chosen from those who were accustomed to visit the external clinics in Baghdad hospital.

Clinical manifestations of leishmaniasis

Leishmania imposes a range of cutaneous disease. The spectrum of clinical index might be due to changeability in the virulence of the parasitic etiological agent (and other inherentfeatures).At most, mucosal leishmaniasis (ML) and leishmaniasisrecidivans (LR) are caused by oligoparasitic disease related to a distinct cellular immune response. The center of the spectrum includes localized cutaneous leishmaniasis (LCL), which is the most popular clinical demonstration. At the counterpart end of the spectrum, diffuse cutaneous leishmaniasis (DCL) is driven by polyparasitic disease with a prevalence of parasitized macrophageswithout granulomatous inflammation. This range of clinical presentation and host immune response are like to the clinical presentations in cases suffered from leprosy (Convit et al., 1993).

The clinical manifestations of suspect cutaneous leihsmaniasis were diagnosed and described by the physician. A file record for each patient was built up at first visit of the patient to the external clinic in Baghdad hospital.

Collection of clinical specimens

Biopsy samples from skin (4-mm) were collected from patients with suspected leishmaniasis by the aid of local anesthesia using a sterile scalpel in order to do a split in the utmost inflamed border of the most new lesion. Then the gathered biopsy samples were immersed in 200 µL of transport buffer (10 mMTris-HCl, pH 8.0 and 10 mM EDTA), followed by incubation at 65 ^{o}C for 3 hrs. Storage of samples was performed for 48-72 hrs at 4 ^{o}C.Afterthat, the freezing was done for the samples until being used further.

DNA isolation from biopsy samples

All frozen biopsy samples were thawed and were subjected to DNA isolation protocol using the GeneJET genomic purification kit (Thermo Fisher Scientific Co., USA).

Quality and quantity of isolated total DNA

The isolated total DNA from each biopsy sample was run in 1% agarose gel electrophoresis using DNA submarine unit (Cleaver Co., UK) to assess its quality. After electrophoresis termination, the agarose gel was visualized under UV-transilluminator. On the other hand, the absorbance of the isolated DNA was measured at 260 and 280 nm using Nano-drop Spectrophotometer to assess its quality and quantity. A ratio of > 1.8 of Ab 260/Ab280 indicated good quality of the isolated DNA. The isolated DNA was stored at -20 ^{o}C until being processed.

Real time –PCR usingLeishmania spp. specific primer

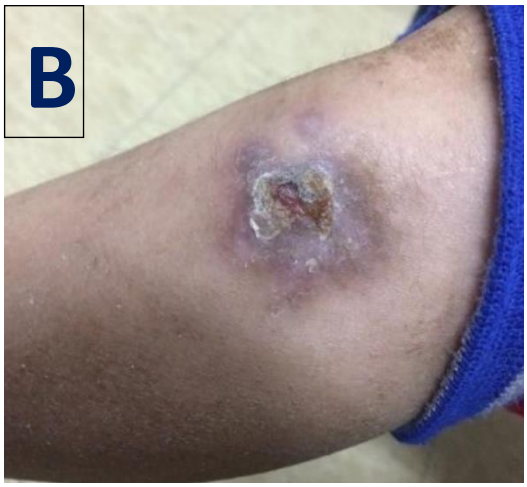
The DNA isolated from biopsy samples was subjected to real time –PCR using the Leishmaniaspp. specific primer set : 13A (5'-GTGGGGGAGGGGCGTTCT) and 13B (3'-TTGACCCCAACCACATTATA) to amplify a partial fragment of the genomic DNA of Lesihmania spp. of 120 bp (Aviles et al., 1999). The PCR mixture contained 50 ng total biopsy DNA, 25 µL SYBR ^{TM} Green PCR Master Mix (Thermo Fisher Scientific Co., USA), 0.3 µM of each forward and reverse primer, and the volume was completed to 50 µL using nuclease free water. Then all reaction mixtures were carried out in Rotor Gene 5-Plex (Qiagen, USA). The real time-PCR conditions were settled to be 94 ^{o}C for 10 min (initial denaturation), 40 cycles (each cycle: 94 ^{o}C for 15 s (for denaturation), 60 ^{o}C for 60 s (annealing, extension, and fluorescence acquisition), and 55-95 ^{o}C hold for melting. After termination of the real time-PCR run, data collected from reaction mixtures was analyzed for evaluating the melting curve and obtaining the Ct value for each reaction. The expected melting temperature of the PCR product (Tm) displayed in the melting curve was spanned from 81-82 ^{o}C. The melting curve with single peak indicated the specificity of the primer and the precision of the data output.

Results

Clinical manifestations of patients with suspect cutaneous leishmaniasis

The clinical manifestations of the suspect cutaneous leishmaniasisdemonstrated by the patients enrolled in this study were represented in Figure 1. A shown, the lesion of suspect cutaneous leishmaniasis was exhibited in different regions: face, elbow, and arm. The clinical manifestation were typical cutaneous leishmaniasis with skin lesions (developed after several weeks or months after infection) and swollen

glands. The lesions, with closed or open sores appearance, changed overtime in size and form. The skin lesions were not painful unless there was a bacterial infection.



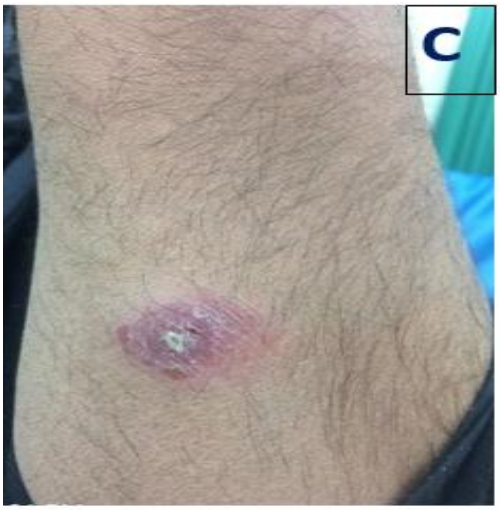


Figure 1: Representative examples of some patients enrolled in this study with cutaneous leishmaniasis.**A**: clinical manifestation of cutaneous leishmaniasis in the face of a child patient. **B**: clinical manifestation of cutaneous leishmaniasis from an adult patient in the elbow region. **C**: clinical manifestation of cutaneous leishmaniasis from an adult patient in the arm.

Molecular detection of Leishmania spp. etiological agent in suspect cutaneous Leishmaniasis DNA biopsy samples

As mentioned previously in the section of materials and methods, the twelve patients with suspect cutaneous leishmaniasis were subjected to real time-PCR experiment using Leishmania spp. specific primer set 13A and 13B. Data revealed that 8 out of 12(8/12= 66.66%) DNA biopsy cutaneousleishmaniasis samples proved to have the etiological agent Leishmania spp. (Figure 2). The amplification curves depicted in Figure 2 reflected different PCR reactions with different Ct values. Real time-PCR with late Ct values indicated low concentration of the Leishmania DNA included in these samples. Conversely, high Ct values in Real time–PCR evidenced high concentration of the Leishmania DNA included in these samples (Table 1). Moreover, the melting curves depicted in Figure 3 revealed a single peak in each Real time PCR that reflected the specificity of the Leishmania specific primer set used in this study. Additionally, all obtained melting curves from all Real time-PCR showed overlapped peaks that would provide additional evidence about the specificity of the used primer set.

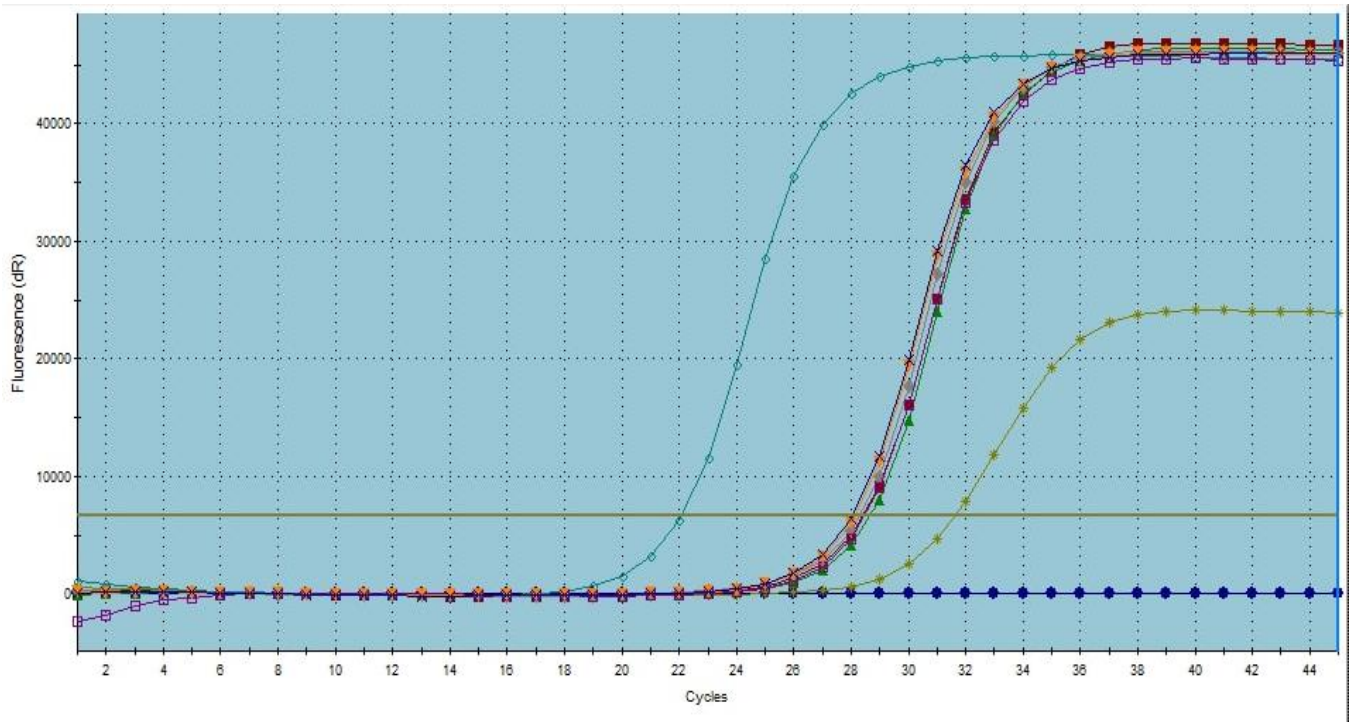


Figure 2: Amplification curves of eight Real time-PCR conducted by 50 DNA biopsy cutaneous Leishmaniasis samples.

Table 1: Ct values of Real time-PCR conducted for 8 DNA biopsy cutaneous leishmaniasis samples.

Sample code	Ct value*
1	28.46
2	28.71
3	28.34
4	31.68
5	22.09
6	25.54
7	28.16
8	28.04

- Ct value: Threshold cycle number at which the first significant luorescence above the background was detected.

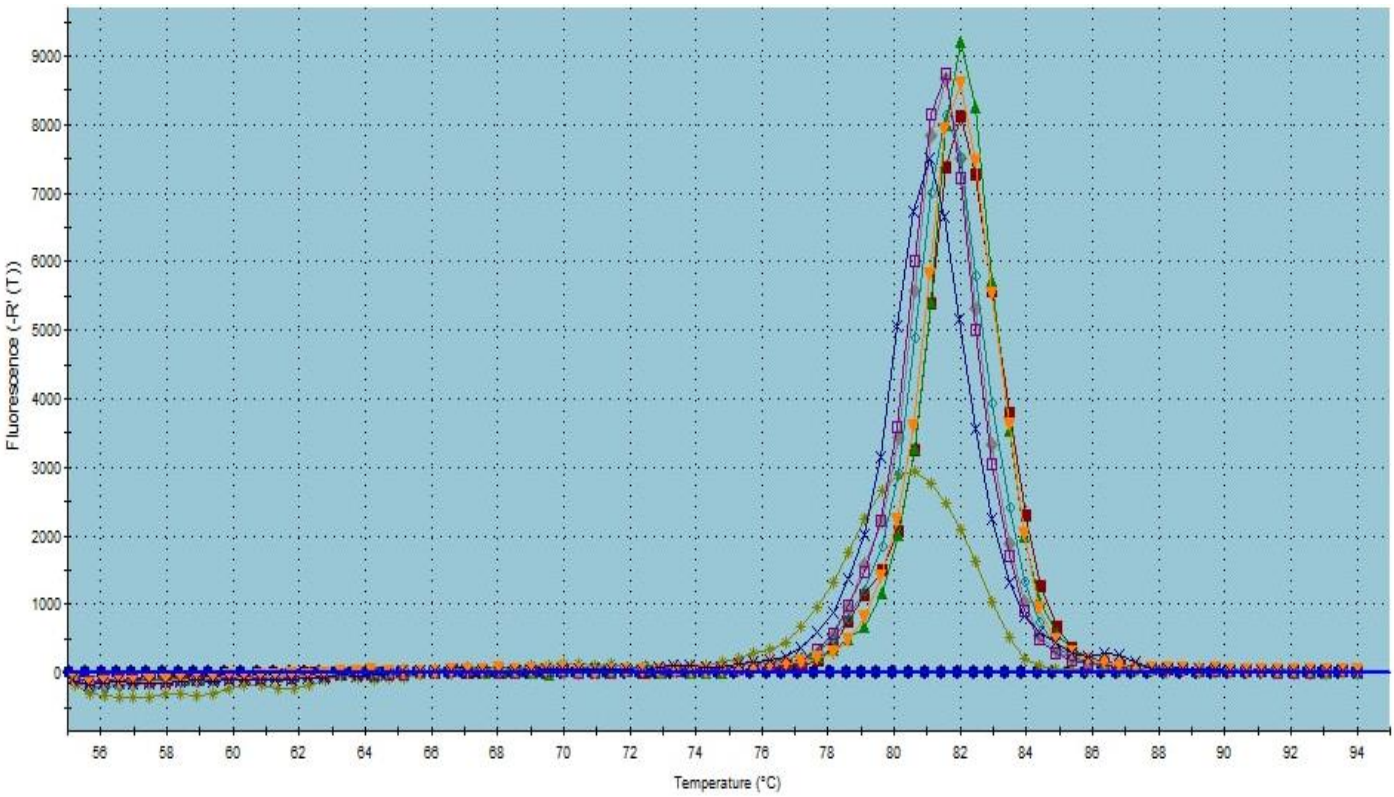


Figure 3: Melting curveshowing overlapped single peaks from eight Real time –PCR conducted with eight DNA biopsy cutaneous leishmaniasis samples.

Discussion

Cutaneous leishmaniasis (CL) is the most popular form of leishmaniasis and has a predestined 1.5 million infections annually worldwide (Desjeux 2004). This disease prevalentlydoes occur in individualsinhabiting in disease-endemic nations neverthelesstoo is a problematic issue in a continuous increasing manner among travelers (Wall et al., 2012). According to WHO records in 20 May 2021 and IAMATrecords, the current status of leishmaniasis in Iraq is sounding an alarm due to elevated numbers of cases of cutaneous leishmaniasis.

Early diagnosis is crucial for haltingrigorousimpairment or even mortality of cases (Schallig and Oskam 2002). The diagnostic protocols employed in the identification of Leishmaniacontagionsby the aid of culturing or serological approaches is somewhat a longduty with diminished specificity (Nicolas et al., 2002). On the contrary, since the invention of the first thermal cycler instrument in 1987, molecular approaches have gained much attention in the context of detection of etiological agents in clinical

specimens (Zarlenga and Higgins 2001). In this context, molecular approaches, basically PCR, have becomevital tools for the diagnosis of infectious diseases (Bastien et al., 2008).

High levels of sensitivity and specificity, quick identification of the parasite, high reliability, reproducibility, and the likelihood of direct application on clinical specimens obtaining results in a matter of a few hours gain numerous advantages of molecular techniques when compared to conventional ones (Paiva-Cavalcanti et al., 2010).

In this study, eight out of twelve samples were proved to harbor the etiological agent of leishmaniasis. The frequency of occurrence of leishmaniasis in the twelve investigated samples was (8/12= 66.66%) as inferred from Real time-PCR experiment. This would underpin the potential, accuracy, efficacy, reproducibility, reliability, rapidness of the Real time-PCR in detection of leishmaniasis pathogens in the clinical specimen especially in LCL cases. In the light of getting rapid diagnosis for cases of LCL, the real time-PCR method is more superior to the conventional methods (i.e., microscopy and immunodiagnostic methods) that necessitate at least three days to get reliable results. Rapid treatment would reduce the complications of LCL.

The real time –PCR methodology has certain advantages over the conventional PCR. Of most paramount importance are recording the data of each cycle online (at the time of the run). Thus, there is no need to carry out an agarose gel post –PCR to evaluate the success of the PCR. Hence, there is a saving in time, facilities and quickness in getting results (Mortarino et al., 2004; Kubista et al., 2006).

Data obtained in this study greatly underpin the indispensable need for performing an confirmatory diagnostic test beside the clinical manifestations. This is attributed to the misleading information that could be derived from the clinical manifestations. Moreover, LCL manifestations could be like those of leprosy. Thus, applying Real time-PCR would be a crucial in the rapid obvious steps towards better and correct diagnosis of LCL cases.

Real-time PCR is superior to traditional PCR as it is quicker, reduced labor-intensive, low level of contamination, and by empolyinh probes the sensitivity and specificity would be improved (Dymond, 2013, Mohammadiha et al., 2013, Yang and Rothman, 2004). Furthermore, by using standard curves real-time PCR can be used for quantification. As a consequence of the high sensitivity, qPCR can be employed in screening of cases and tracing of the asymptomatic carriers. Thereby the gaps in the context of the exploration of leishmaniasiswould be addressed with Leishmania (Francino et al., 2006, Mary et al., 2006, Pourabbas et al., 2013).

There existsnumerousreports of different variants of PCRs targeting Leishmania, by the aid of various molecular targets, nonetheless,the majority of protocols do target a single species (Francino et al., 2006, Srivastava et al., 2011b), a group of closely related species (Harris et al., 1998, Odiwuor et al., 2011), in a non-quantitative manner, Harris et al., 1998, Odiwuor et al., 2011, Srivastava et al., 2011b), and low levels of sensitivity (Wortmann et al., 2005). There are only certain and limited publications addressing the real-time PCR assays that do target all or approximately all of the 20 different Leishmania species existed in humans (Castilho et al., 2008, Tupperwar et al., 2008, Wortmann et al., 2001). Several protocols would target multicopy genes (Bossolasco et al., 2003, Francino et al., 2006, Talmi-Frank et al., 2010) and certainprotocols for multicopy genes also utilizeSYBRGreen (de Monbrison et al., 2007). Multicopy genes are frequentlyfavorable to enhance sensitivity.

Conclusion

Real time – PCR is the gold standard solution for diagnostic purposes of leishmaniasis. It is cost-effective, time saving, labor saving , reproducible, and reliable approach. However, the low percentage of occurrence of LCL among the study participants could be attributed to the sample size. Prospective studies would focus on studying the prevalence of LCL among Iraqi patients in much more sample size.

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