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IDENTIFICATION OF *Leishmania tropica* and *Leishmania major* BY REAL-TIME PCR USING ITS1 and KDNA GENES IN BAGHDAD

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ABSTRACT

Cutaneous leishmaniasis (CL) is caused by *Leishmania* spp, this disease is a problem in Iraq. The purpose aims of the study is using a Qualitative - Real-time PCR (Q-R.T) to diagnosis compared with traditional methods to investigation of Leishmania species in Baghdad. **Total 75** suspected patient with CL from both sex was enrolled in the present work study during the period between March / 2016 to March / 2017 who attended, AL-Yarmook, AL-Karamaa Al-Kadhimiya, Al-Kindi, Teaching Hospitals in Baghdad, all the demographic feature of the diseases, were recorded. The direct Microscopic examination was carried out by direct smears using Giemsa stain, aspiration fluid from lesions were cultured on Novy–MacNeal–Nicolle (NNN) medium and used R T-PCR for detection of internal transcribed spacer1 (ITS1) and Kinetopast (KDNA) genes. The results revealed that **43%** of cases were positive by Microscopic examination while **16%** by culture and **91%** by R.T- PCR positive for kDNA gene , **60%** for (ITS1) gene , it was found , **62%** were typed as *L. tropica* and **38%** of isolates were typed as *L.major* . Highly Significant (**P<0.01**) correlation was observed between diagnosis methods.

Keywords*: Leishmania tropica,Leishmania major* ,Q-Real-Time PCR, KDNA gene ,ITS1 gene.

INTRODUCTION

Cutaneous leishmaniasis (CL) a vector-borne parasitic disease caused by *L. major* and *L. tropica* which found in most countries of the Eastern Mediterranean region. Some countries of the region have stable endemic foci of zoonotic and anthroponotic CL (WHO, 2012). The diagnostic methods available at present are mostly based on clinical and epidemiological evidence and parasite detection.

The polymerase chain reaction (PCR) to amplify parasite DNA from host tissues. PCR-based methods, as a powerful tool to detect *Leishmania spp.* directly in clinical samples as well as for parasite characterization, have proven to be sensitive and specific compared with routine methods (Jutta *et al*., 2003) and faster results in one or two working days (Foulet *et al*., 2007) The new method Qualitative real-time PCR (Q-R.T) can process a sample in less than an hour, to differentiate single nucleotide mutation within a target DNA sequence (Wortmann *et al*., 2005). This assay has very important to identification of the *Leishmania* species, which subsequently offers the possibility for targeted treatment strategies (Castilho *et al*., 2008).

MATERIALS AND METHODS

Patients: Seventy-five patients with CL were enrolled in the present work during the period between March 2016 to March 2017 who attended, AL-Yarmook, AL-Karamaa Al-Kadhimiya, Al-Kindi, Teaching Hospitals in Baghdad. All the demographic feature of the diseases was recorded.

Diagnosis of Cutaneous Leishmaniasis:

Direct Microscopic examination: A small amount of material from skin lesions after cleaned by 70% ethanol was carried out by direct smears and using Giemsa stain to show amastigotes inside macrophages.

Culture: Aspiration fluid from lesions for cultureing in Novy–MacNeal–Nicolle (NNN) media and smear was taken from the culture on this media with Giemsa stain, promastigotes were showed with active motile

Molecular: Biopsies from lesions were processed of CL were prepared by Filter paper for DNA Extraction.

DNA Extraction: From 75 patients to DNA extraction by using Kit (Qiagen, Hilden, Germany) the extracted DNA were stored at −20°C for further process.

Primers

Name of primer	Sequence of primer
ITS1-(L ITSR) Forward	5 – CTGGATCATTTTC C GATG- 3
ITS1-(ITS1R-TR1-newly) Reverse	5-GAAGCCAAGTCAT CCATCGC- 3
kDNA(Forward)*L.majo*	5-TCGCGTGTTCTGAC TTTTGC-3
kDNA(Forward *L.tropica*	5-AGGTGTTTTTGGGC TTGAC-3
kDNA- (Reverse)	5-ACTCAAGTCCCGTC CATCAAC-3

Detection of KDNA gene by Real –Time PCR: Primers of *L.tropica* and *L.major* were used by (BenAbda *et al*., 2011) for amplifying the variable region of the Leishmania (Nasser *et al*., 2014). Twenty (20) microlitre *r*ection containing Master

mix (Bioneer, Korea), 10 pmol primers and DNA 2*m*L. PCR protocol was: initial denaturation at 95ºC for 5min by 40 cycles of 95ºC for 30 sec, 60ºC for 40 sec and 72ºC for 40 sec and extenstion at 70ºC for 5min. PCR product identity by confirmed with melting curve analysis (Talmi-Frank *et al*., 2010).

Detection of ITS1 gene by Real-Time PCR: For investigation of ITS1 by Real-Time PCR was perf ormed on all 75 extracted DNA samples. Amplification reaction was performed in volume 20µl have PCR Master mix (Bioneer, Koera), primer 10pmol and DNA 5*m*L, nucleus free water as control negative. Reaction were amplified in thermo cycler; initial denaturation at 95ºC for 5min by 35 cycles of 94ºCfor 30 sec ,48ºC for 40 sec, 72ºC for 1min, final extension 72ºCfor 10min.

Statistical Analysis: Chi-square test was used to significant compare between percentage in this study (SAS, 2012).

RESULTS AND DISCUSSION

Leishmania spp the productivity and the potential isolation of (amastigotes) in NNN media were assessed during the present study for evaluating the efficiency in the diagnosis of different life stages of the parasite. The results of microscopic examination, cultivation and Q-Real-Time PCR of the specimens from the patients having suspected CL lesions (Table 1).

The results revealed that 43% (32/75) of cases were positive by the Microscopic examination), whereas only 16% (12/75) by culture. In molecular method by Real-Time PCR it was found 91% (68/75) for kDNA gene; 61% (26/68) were typed as *L.tropica* (figure 1) and 38% (26/68) were typed as *L.major* (figure 2) while 60%(45/68) of R.T PCR positive for ITS1 gene [figure 3]. Highly Significant (P<0.01) correlation was observed between diagnosis methods.

Table 1: Comparative detection of *Leishmania* in direct microscopy, culture and RT- PCR for (KDNA, ITS1) genes.

Assay	Positive No.	%	Negative No.	%	Total	χ^2: P-value
Microscopic examination	43	57	32	43	75	5.032 * (0.039)
Culture	12	16	63	84	75	13.406 ** (0.001)
RT-PCR KDNA	68	91	7	9	75	14.215 ** (0.001)
RT- PCR-ITS1	45	60	30	40	75	8.250 ** (0.0047)

** (P<0.01)-Highly significant

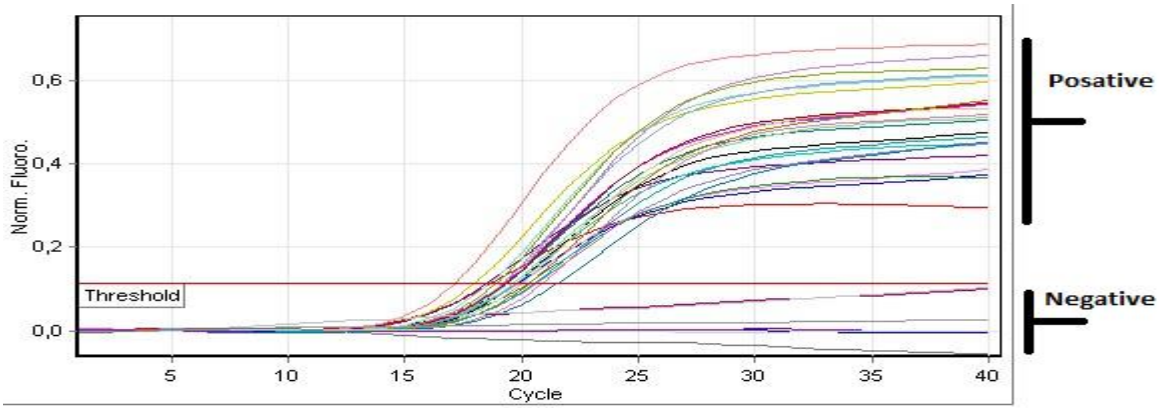

Figure 1: Amplification of Kinetoplast DNA genome of *L.tropica* in positive , negative samples by Q- Real-Time PCR

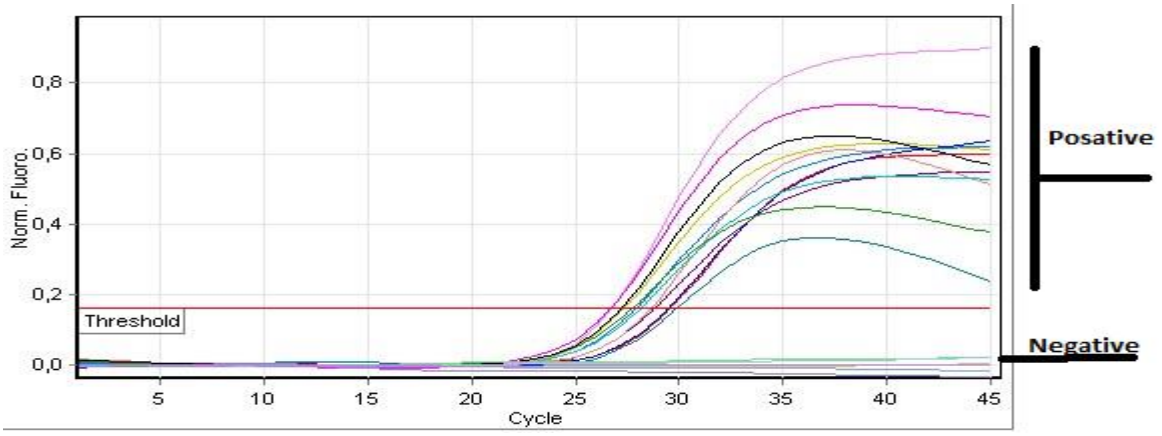

Figure 2: Amplification of Kinetoplast DNA genome of

L. major in positive and negative samples by Real-Time PCR

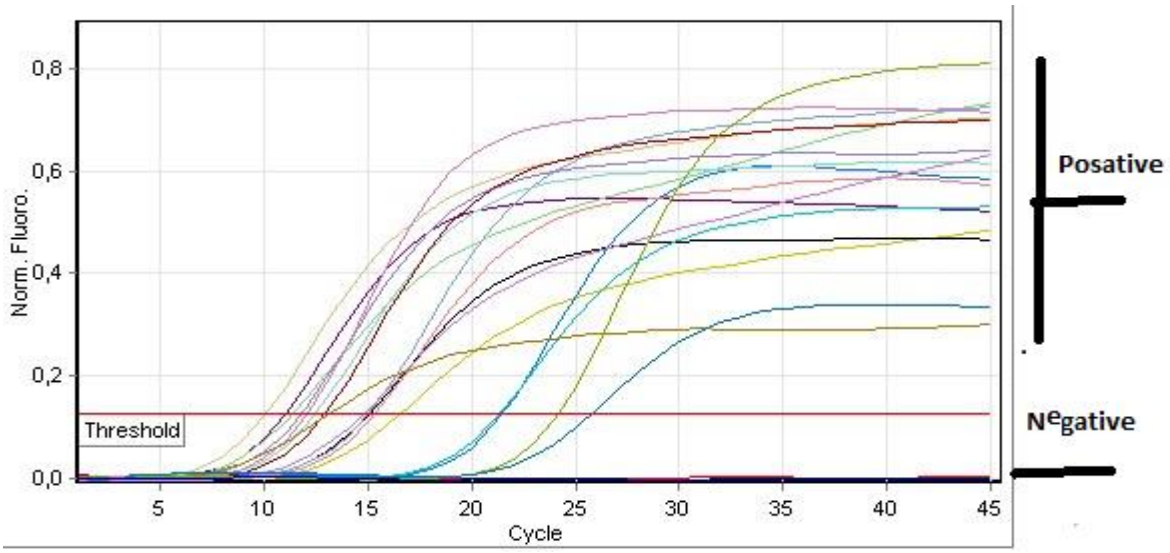

Figure 3. Amplification of **ITS1** gene for *Leishmania tropica and L. major* in positive and negative samples by Real-Time PCR

Cutaneous leishmaniasis is disease is widespread and may cause serious health problems in communities throughout the Mediterranean regions and the Middle East, including Iraq (Connolly, 2016). Many factors that play important role in the presence and distribution of Baghdad boil in this district, the presence of animal reservoirs such as rodents, dogs, the presence of marshes; and the use of clay to build some of the houses in villages, its population are more exposed to insect's bites (AlSamarai and AlObaidi, 2009).

This study emphasized diagnosis and species identification of CL in sample of Iraqi patients in some Baghdad hospitals by traditional and molecular methods using Real –Time PCR and it was found that *L. major* 38% while *L. tropica* 61% etiological agent for the infection. In current study the results showed that the R.T-PCR was the best methods for identification 91% for K DNA gene and 60% for ITS1 gene, the R.T- PCR for K DNA gene was recognized as the most reliable method in the diagnosis of CL (CDC, 2011).

The gene (k DNA) has 10000 copies its suitable for diagnostic purposes (Janaina *et al*., 2016). Results of figure 1 and 2 showed the two types of Leishmania spp. in Iraq by using R.T.- PCR assay, it was appear that *L.tropica* 61% further *L. major* 38% agreement with (Mustafa *et al*., 2017) and this in agreement with other Iraqi study (Abdulsadah, 2014). The ITS 1 gene is important in molecular diagnosis because of having diverse sequences even among genus and different species of one genus. Thus, the results can help to identify polymorphisms (Fahriye *et al*., 2017).

The present study showed the Q- R.T- PCR was more specific technique for diagnosis of cutaneous leishmaniasis with (95%) specificity; this result agreed with Marfurt *et al*., (2013) and (Markle and Makhoul, 2014). The SYBR green dye used in this study binds to double-str-and DNA, results indicate in Q-R.T-PCR the amplification was registered as a Ct value (cycle threshold), The lower Ct value indicates the presence of higher copies of the target and vice versa. In terms of gene expression, high Ct values indicate low DNA concentration of parasites and low gene expression and low Ct value indicates a high DNA concentration of parasites and high gene express-ion (Nolan *et al*., 2006).

CONCLUSION

The Q- Real-time PCR for diagnosis and identification of Leishmania spp. in Iraq, procedure extremely rapid with results and more samples can be processed at one time, higher sensitivity decreased contamination risk, and specific compared with routine methods and *L. tropica* more infection in Iraq than *L. major*

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