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IDENTIFICATION OF *LEISHMANIA* SPECIES CAUSING CUTANEOUS LEISHMANIASIS USING REAL-TIME PCR IN IRAQ

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ABSTRACT

A total of 60 suspected cutaneous leishmaniasis (CL) cases were diagnosed during the period from December, 2013 to February, 2014 with females representing 67% of the cases. The incidence rate with CL was 88.3% by using Real-Time PCR. Fourty -two (70%) of isolates from different patients were typed as *L. major* and 11 (18.3%) of isolates were typed as *L.tropica* while 7 (11.7%) of cases were gave negative results. The present investigation revealed that the highest number of patients 24 (40%) was in age group (10 and less) year. Clinically, Our results showed that 26(43%) of CL patients were had single lesion and 34(57%) had multiple lesions, most of them 45(75%) in arm. The highest incidence of disease 39(65%) was observed in rural areas, and the lowest incidence rate 21(35%) was in urban areas. The statistical analyses were carried out with Minitab version.

INTRODUCTION

Leishmaniasis is a major vector-borne disease caused by obligate intramacrophage of the genus *Leishmania* (Zavitsanou *et al*., 2008). Leishmaniasis is still one of the world's most neglected tropical diseases, affecting largely the poorest of the poor, mainly in developing countries; 350million people are considered at risk of contracting leishmaniasis and some 2 million new cases occur yearly in 88 countries (WHO, 2010; Siqueira-Neto *et al*., 2012). There are several different forms of leishmaniasis. The most common form is cutaneous leishmaniasis (CL), which causes skin sores. Visceral leishmaniasis (VL), which affects some of the body's internal organs, (most commonly the spleen, liver and bone marrow) is the most serious of the infections (Siqueira-Neto *et al*., 2012). Over the last decade, several studies have shown PCR to be both highly specific and more sensitive than the classical methods for the diagnosis of leishmaniasis. PCR is more suitable for diagnosis, as it can be performed in any biological sample, including skin tissue, blood and bone marrow. Additionally, it is always recommended to use more than one diagnostic test (Singh, 2006; Guizani, 2004). BothVL and CL have been reported in Iraq caused by *Leishmania donovani*, *Leishmania major* and *Leishmania tropica* respectively (WHO, 2003).

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The cases of cutaneous leishmaniasis caused by *L. tropica* mostly occur in the suburbs of big cities (Baghdad, Mosul) among large conglomerations of people where the sanitary conditions are unsatisfactory. Incidences caused by *L. major* are much more common; they appear primarily in rural areas, especially in the northern and southern provinces of the country (Korzeniewski, 2005; Abdulsadah A. Rahi, 2013). The present study aimed to identify the species of *Leishmania* by Real-time PCR in Iraq.

MATERIALS AND METHODS

Samples collection

Sixty patients with clinically diagnosed cutaneous leishmaniasis from both sexes and different ages were included in this study. They were attended to Al-Karamah Teaching Hospital in Kut, Iraq during the period from December, 2013 to February, 2014. Samples are taken from the skin leishmanial lesion, and kept into two tubes; one stored in freeze at -20 °C for Real-time PCR and the second tube for direct smear. After the smears dried completely, they were fixed with 100% methanol, allowed to dry again, and stained with Geimsa stain for microscopic examination for presence of amastigotes (Robinson *et al*., 2002).

Real-time PCR

Genomic DNA was extracted from skin lesions and aspirates by using AccuPrep®Genomic DNA extraction kit (Bioneer.

Korea) and done according to company instruction. The extracted genomic DNA was checked by using Nanodrop spectrophotometer (Thermo, USA), and measured the purity of DNA through reading the absorbance at (260/280 nm). The Real-Time PCR primers that used in this study were design by using the complete sequence of *L. major* and *L. tropica* kinetoblast DNA genome (GenBank: Z32845.1), (GenBank: Z32843.1), using NCBI Gene-Bank data base and provided by (Bioneer company, Korea) as following table:

Primer		Sequence	PCR product size
L. major	F	TCGCGTGTTCTGACTTTTGC	95bp
	R	ACTCAAGTCCCGTCCATCAAC	
L. tropica	F	AGGCTGTTTTTGGGCTTGAC	90bp

qPCR Thermocycler conditions was designed for each primer *L. major* or *L. tropica* according to primer annealing temperature and qPCR Syber green kit instructions. qPCR data analysis was performed by calculation the threshold cycle number (CT value) that presented the positive amplification gene in Real-time cycle number.

Statistical Analysis

The suitable statistical method was used in order to analyze and assess the results by using T-test in Minitab version (Meyer Ruth and David D. Krueger, 2004). The comparison of significant (P-value) in any test were: S= Significant difference (P<0.05), HS= Highly Significant difference (P<0.01), and NS= Non Significant difference (P>0.05).

Table 1. Distribution of CL cases in relation of Age, Gender and Residence

Age (year)	Gender	Residence		Subtotal	Total
		Urban	Rural		
10 and less	M	2	7	9	19
	F	0	10	10	
11-20	M	2	1	3	10
	F	2	5	7	
21-30	M	2	0	2	11
	F	4	5	9	
31-40	M	1	1	2	8
	F	2	4	6	
41 and more	M	1	1	2	6
	F	2	2	4	
Total	M	8	10	54	
	F	10	26		
%	-	(18) 33%	(36) 67%	100%	

M = Male, F= female T=-4.31 P=0.140

Table 2. Types of CL infection in according to the Age and Gender

Age (year)	Gender	Type of CL infection		Subtotal	Total
		Dry	Wet		
10 and less	M	4	5	9	19
	F	3	7	10	
11-20	M	1	2	3	10
	F	4	3	7	
21-30	M	1	1	2	11
	F	1	8	9	
31-40	M	1	1	2	8
	F	4	2	6	
41 and more	M	1	1	2	6
	F	1	3	4	
Total	M	8	10	54	
	F	13	23		
%	-	(21) 39%	(33) 61%	100%	

M = Male, F= female T=-3.38 P=0.090

RESULTS

A sixty patients were detected for *Leishmania* amastigotes using Giemsa-stain smeared, out of which, 35 (58.3%) were gave positive by microscopic observation and 53 (88.3%) by Real-Time PCR; 42 (70%) of samples were typed as *L. major* and 11 (18.3%) were typed as *L.tropica* while 7 (11.7%) of cases were gave negative results.

Real-Time PCR

1- Leishmania major

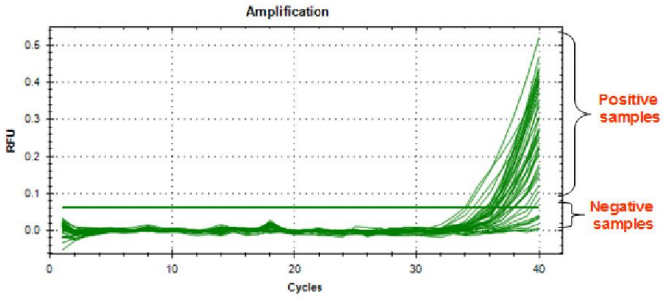

Figure 1. Real-Time PCR amplification plot of Kinetoplast DNA genome of *Leishmania major* in positive and negative samples

2- *Leishmania tropica*

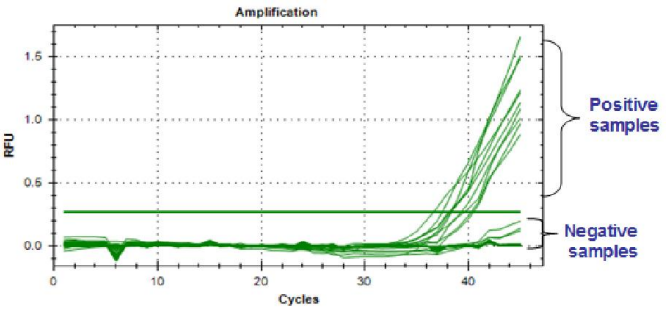

Figure 2. Real-Time PCR amplification plot of Kinetoplast DNA genome of *Leishmania tropica* in positive and negative samples

DISCUSSION

Over the past several years, Real-time PCR (RT-PCR) has become the leading tool for the detection and quantification of DNA or RNA. Using these techniques, you can achieve precise detection that is accurate within a two-fold range, and adynamic range of 6 to 8 orders of magnitude (VanGuilder *et al*., 2008). Our finding showed that CL was significantly associated with illiteracy and farmers as an occupation which is usually more common in rural population (67.4 %). Similar findings were reported in previous studies (Sabra *et al*., 2013; Abdellatif *et al*., 2012 and Ranjan *et al*., 2005). There was a strong tendency for cases to be more prevalent, significantly in female than in male (P<0.05), but there is no clear explanation for such a gender distribution. It might be due to behavioural and individual risk factors (Mirzaei *et al*., 2011). Also, the high prevalence of CL in female might be explained by the fact that female in this group are more exposed to insect bites than male in the same group because most farm workers were female in rural areas. This finding is consistent with those found by others (Korzeniewski, 2005; Rahman *et al*., 2009). On the contrary of other studies that found the higher incidence of infection among male than female (Stewart and

Brieger, 2009; Rastogi and Nirwan, 2007). Moreover, the highest proportion of infection (48.9%) was recorded in 11-20 years age group, and the lowest (20.7 %) was in the 1-10 years age group, which is in agreement with previous reports indicating more exposure as a result of educational and occupational situations (Abdullah *et al*., 2009; Alimoradi *et al*., 2009; Sharma *et al*., 2005). Ulcerative wet type lesions were present in 61%, while the nodule dry type lesions were present in 39%. These observations are in agreement with those reported from Iraq (Korzeniewski, 2005), Iran (Talari *et al*., 2006), Colombia (Ramírez *et al*., 2000), Pakistan (Ul Bari A1 *et al*., 2006), and Afghanistan (Faulde *et al*., 2008). In the current study, the ulcers were observed among all age groups.

The T- test showed non-statistical significant differences in the prevalence of the CL ulcers of all age groups (T= - 4.31, -3.38, P= 0.140, 0.090) by sex and type of infection, respectively. The sensitivity of direct microscopy is not high, and is not uniformly available and successful, while RT-PCR is a sensitive test for the detection of low amounts of DNA in tissues (Sharifeh *et al*., 2012). The results of our study is similar or close to the results of similar studies (Foulet *et al*., 2007; Wortmann *et al*., 2007; Wortmann *et al*., 2004). Moreover, they showed that comparing with other methods, RT-PCR was a rapid test (Van der *et al*., 2008; Wortmann *et al*., 2005). In addition to the diagnosis will direct manner swab be more prone to error and that is due to several reasons, including the method of taking the swab, staining, installation, number of parasites present and/or the experience of the person examining the slide and the time consumption (Bensoussan *et al*., 2006). The present study showed that *L.major* represented 42(70%) and *L.tropica* was 11(18%). These results are consistent with the findings of other studies in Iraq (Korzeniewski, 2005; AL-Hucheimi, 2005) and other countries (Amro *et al*., 2012; Mirzaei and Sharifi, 2011). These results are inconsistent with the findings of other studies (Maraghi *et al*., 2007; Soukkarieh *et al*., 2012).

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