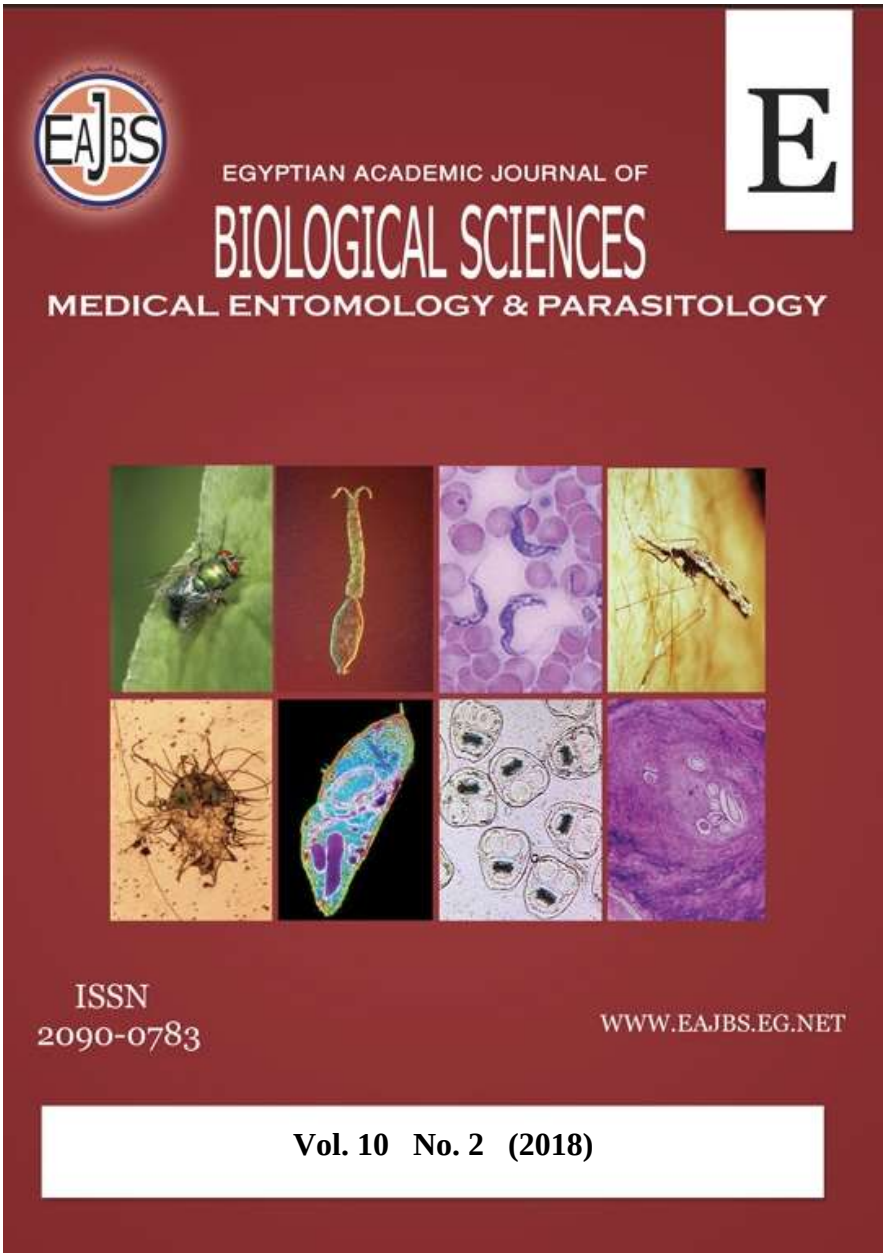

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Detection of Cutaneous Leishmaniasis Based on ITS1 Gene by PCR-RFLP Technique

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ABSTRACT

Background: Cutaneous leishmaniasis is a vector-borne disease transmitted by biting of the sandfly, it is a severe health problem in many countries and endemic in most regions of Iraq.

Objectives: This study was conducted to find the best method for diagnosis of cutaneous leishmaniasis, detect the genotypes of *Leishmania tropica* and *Leishmania major* in Ramadi (Iraq) by PCR-RFLP technique.

Materials &methods: One hundred twenty-two patients 68 were males while the females gender were 54 with age ranged 1-68 years, CL who attended to Department of Dermatology in Al-Ramadi Teaching Hospital and dermatology Private clinics, during the period between November 2017 to April 2018. The Molecular study was carried out to detect the ITS1 gene by (PCR). The restriction fragment length polymorphism (RFLP) was adopted on ITS1-PCR product and after HaeIII digestion at 37Cº for 2 hours.

Results: Laboratory examination of 122 cases showed 62 infection cases in Cutaneous leishmaniasis by using PCR technique and in infection proportion reaches at 51% out of the total number of the cutaneous cases which are similar to leishmaniasis during the months of the study. The demographic study dealt with age, gender, number of lesions and body site of infection, demonstrated that the majority of patients at the age of 1-10 years with percent 28.7%. Also Males (55.7%) had higher infection than females (44.3%), upper limbs had the highest percentage (48%) when compared with other sites of infection, single lesion was documented in 55% of patients, while two lesions were observed in 25% and multiple (3-10) lesions were observed in 20%. Different techniques were used for diagnosis of CL including routine method performed by direct microscopic smear from lesion which showed amastigotes in the macrophage in 50 (41%) positive case. The Molecular study was carried out to detect the ITS1 gene (internal transcribed spacer1) by (PCR). DNA extracted from 122 samples showed 62 (51%) were positive for (ITS1)gene, The restriction fragment length polymorphism (RFLP) was adopted on ITS1-PCR product and after HaeIII digestion at 37Cº for 2 hours obtained two fragments of 60 and 200 bp 42 as *L.tropica*, and two fragments of 140 and 210 bp were identified 20 as *L.major*, genotype techniques were performed for all positive samples.

Conclusion: CL is highly spread with single lesions more than multiple lesions and molecular detection showed that *L.tropica* more common than *L.major*.

INTRODUCTION

Leishmaniasis is a parasitic disease caused by haemoflagellate Leishmania. The disease is widespread and may cause serious health problems in communities throughout the Mediterranean regions and the Middle East, including Iraq (Ashford *et al*., 1992; CDC, 2004).

There are an estimated 12 million cases worldwide, and there are about 1.5 million new cases of cutaneous leishmaniasis each year, of which over 90% occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru (Markle *et al*., 2004). Old World disease primarily is caused by *Leishmania tropica* in urban areas and *Leishmania major* in dry desert areas (CDC, 2004).

There are three main types of leishmaniasis: cutaneous leishmaniasis (CL) caused by *Leishmania tropica, L. mexicana* and *L. major*. Visceral leishmaniasis (VL) caused by *L. donovani* and *L. infantum* and mucocutaneous leishmaniasis (MCL) caused by *L. braziliensis* (Igbineweka *et al.*, 2012). Cutaneous Leishmaniasis is caused by different species of *Leishmania* with a specific inclination of each species to a particular geographical region. (WHO, 2014). It spread by sand fly bites, afflicts hundreds of thousands of the world's poorest people in tropical countries. *Leishmania* spp are intracellular protozoa have a complex digenetic life cycle, requiring a susceptible vertebrate host and a permissive insect vector, which allow their transmission, emphasized the importance of animal reservoirs in facilitating transmission of CL (WHO, 2015).

In Iraq, two species are present: *L tropica*, the agent of anthroponotic cutaneous leishmaniasis (ACL), and *L. major*, the agent of zoonotic cutaneous leishmaniasis (ZCL). Both ACL and ZCL were reported as causative agents of leishmaniasis in Iraq, but ACL is found mainly in suburban areas (WHO, 2003).

Only a minority of infected humans develops the disease: most are infected at a sub-clinical level. These asymptomatic hosts help sustain VL transmission in endemic areas and represent a major challenge for infection control (Singh, 2006).

The Cutaneous leishmaniasis lesions appeared as the sores which can change in size and appearance over time. It may start out as papules (bumps) or nodules (lumps) and may end up as ulcers (like a volcano, with a raised edge and central crater); skin ulcers may be covered by scab or crust. The sores usually are painless but can be painful. Some people have swollen glands near the sores (for example, under the arm, if the sores are on the arm or hand). The lesions of CL in normal infection appeared in the arms, legs, faces and ears, showed solid, dry like volcano area in shape and characterized by erythematous papule, with ulcerative border (CDC, 2012). In such cases, the diagnosis should be confirmed by examination of smears from lesions, culture, and histopathological examination (Singh *et al*., 2003). In developing countries such as Iraq, laboratory equipment and materials such as ELISA test kits or PCR technique materials are not available and dermatologists mostly have to rely on the clinical characteristics of the lesion. Giemsa or Leishman-stained smears obtained from the lesions are a rapid means of diagnosis (Ramírez *et al.*, 2000).

The diagnosis of CL is based on clinical features and laboratory tests, including a direct parasitological examination and/or indirect testing with serology and molecular diagnostics (Singh *et al*., 2003). A universal PCR method targeting the internal transcribed spacer 1 (ITS1) region, which occurs between the genes encoding 18S rRNA and 5.8S rRNA, has proved useful in the direct diagnosis and identification of the Leishmania parasite because this region is highly conserved among species. On the other hand, there are other targeted genes using in the molecular description. The species identification requires additional processing by restriction fragment length polymorphism (RFLP) and DNA sequencing, these last tests are more sensitive but may be applied only in particular centers (Al-Nahhas *et al.*, 2013).

The evidence confirmed increasing in percentage of infection due to a bad situation for hundreds of thousands of people who exposed to the displacing and dived in camps, in addition to the presence of the war

and bad conditions, and presence of swamps near their camps that important for reproduction sand fly (Younis, 2018).

MATERIALS AND METHODS

This study was carried out on Iraqi patients included 122 patients suspected of cutaneous leishmaniasis admitted to Al-Ramadi teaching Hospital and some private clinics during the period from 1st November 2017 to 1st April 2018. Approval for this study was obtained from the Ethical Committee of the University of Anbar.

According to (Eksi *et al*., 2017) with some modification in the method of collecting the aspiration fluid, the lesion sites were cleaned with 70% alcohol. there my a syringe (1ml, 30G*1/2) when intradermal pentostam injection at the periphery was done, we were aspirated the fluids and the blood that oozing from the sites by capillary tubes with anti-coagulants and prepared slide to examine directly by microscopy after staining and the other was saved in screw cap container with the Sterile NaCl solution as dilution fluid and incubated in refrigerator (4^0 c) until DNA extraction.

Microscopic examination conducted on each sample of aspiration prepared the smear by transferring a portion of the sample onto a clean slide. Staining with Giemsa or Leishman's stain solution and examined under a light microscope with a 100_ objective lens. Preparation showing amastigotes is considered to be positive (+ve) for Leishmania spp. and preparation with no amastigotes is considered negative (–ve) for Leishmania spp. All results were recorded (Rahi, 2015; Eksi *et al*., 2017).

The isolation of Leishmania spp. DNA was extracted from the aspiration fluids by using the (DNA extraction for the intracellular organism) Genomic DNA Mini Kit (Geneaid, Taiwan) according to the manufacturer's protocol and stored at -20°C. DNA samples prepared from aspiration blood were quantified by Ultraviolet spectrophotometer (Unico, USA) reading at 260 and 280 nm (Sambrook *et al.,* 1989). All samples were stored at -20°C until use.

All suspension samples examined for DNA extraction which was assayed by PCR amplification process. The specific primers were synthesized from IDT (USA), The forward primer (LITSR) 5-CTGGATCATTTTCCGATG-3 and reverse primer (L5.8S) 5-TGATACCACTTATCG CACTT-3, specific to the ribosomal ITS1 region of Cutaneous leishmaniasis the PCR program, of ITS1 region According to (El Tai *et al*., 2000; Rio de Janeiro, 2009). as the following: Initial Denaturation 95°C for 5 min. Denaturation 95 °C30 sec. Annealing 48°C 30 sec. Extension72 °C 1 min. Final Extension72°C 6 min. for 35 cycles**.**

The PCR products of ITS1 gene were detected and separated by 2% of agarose gel with (2µl of 10mg/ml) ethidium bromide and carried out for one hour with 5volt\cm electrophoresis, followed by detection of the specific bands (350 bp for ITS1) under Ultra Violet Light (Sambrook *et al.,* 1989).

Sample Positive for PCR-products gene was performing of RFLP procedure with endonuclease restriction enzyme HaeIII (Takara Bio Inc, Japan) according to (Eroglu *et al*.,2011; Eksi *et al*.,2017) for restricted of specific sequences for each genotype of cutaneous leishmaniasis For the purpose of knowledge subgenotypes that infected human and enhance the accuracy of diagnosis. As the following steps 10µI of PCR product was placed into Eppendorf tube (1.5 ml), 3 µI from 10X M buffer enzyme, 0.5µI of restriction enzyme,1.5 µI of deionized water were added to all of these components which final volume of reaction mixture was 15µI and the Reaction mixture was incubated at 37°C for three hours. When Incubation period was completed, add 3 µI of 10X Loading Buffer to stop enzyme reaction and apply on agarose gel electrophoresis (2.5% agarose) and visualized with ethidium bromide staining and carried out for observation of restricted bands by restriction enzymes.

According to (Simon 2006) all result was statistically analyzed. Inferential statistics such as Chi-square test by using the SPSS statistical program was used to test

whether or not significant differences between proportion and means exist.

RESULTS

The patients were of different sex, out of 122 specimens, 68 (55.7%) were male while the female gender was 54 (44.3%), the ages were distributed between a year to 68 years where distribution into 7 age groups (Table 1). The total number of specimens that gave positive results of cutaneous leishmaniasis by microscopic examination was 50 (41%) patients. while the specimens that gave positive results by PCR technique for ITS 1 gene was 62 (51%). In addition to that the samples that gave positive result by PCR assay were inserted to nucleic acid restriction enzymes (Hae III) for restricted the ITS1region of the cutaneous leishmaniasis where Hae III was used for restricted the gene ITS1(350 bp) into (210 bp, 140 bp) for *Leishmania major* and (200 bp, 60 bp) for *Leishmania tropica*. There is a statistically significant difference between the rate of infection and the age groups, $P<0.05$. The mean of age were 15.25 with standard deviation $\pm$ 13.4 years.

Table 1: The numbers and percentages of the infections according to the results of microscope diagnosis and PCR assay.

Risk Factors		**Diagnosed samples(Number=122)**			
		Infected patients based on microscopic examination		Infected patients based on PCR assay	
		NO.	%	NO.	%
Total		50	41%	62	51%
Gender	Male	30	24.6%	37	30.5%
	Female	20	16.4%	25	20.5%
Age groups	1-10	30	24.6%	35	28.7%
	11-20	12	10%	10	8.2%
	21-30	5	4%	9	7.4%
	31-40	2	1.6%	5	4 %
	41-50	1	0.8%	2	1.6%
	51-60	0	0%	1	0.8%
	61-70	0	0%	0	0%
Residency	Rural	45	36.9%	54	44.3%
	Urban	5	4.1%	8	6.5%
Body site of infection	Upper limbs	76	48%		
	Legs &feet	45	28.6%		
	Face	33	20.8%		
	Other site	4	2.6%		
Number of lesions	One lesion	67	55%		
	Two lesion	30	25%		
	3-5 lesion	16	13%		
	5-10 lesion	9	7%		
Species of leishmania	**L. tropica**	**42/62**	**67.7%**		
	L. major	**20/62**	**32.3%**		

The infection according to the age groups by using microscopic examination, where the higher rate of infection was in the age group (1-10) was 30 (24.6%), (11-20) was 12(10%), (21-30) years 5(4 %), (31-40) years 2(1.6%), (41-50) years 1(0.8%), (51-60 years) 0(0%) and (61-70) years 0 (0 %) and the rate of infection by using the Conventional PCR assay among different age groups, the higher rate of infection with cutaneous leishmaniasis was in the age group (1 -10) was 35 (28.7%) while the minimum rate of infection was in the age groups (61-70) years 0(0.00%), while the other age groups were (11-20), 10 (8.2 %), (21-30)

years was 9 (7.4%), (31-40) years 5 (4 %), (41-50) was 2 (1.6%) and (51-60), 1(0.8%).

The distribution of cutaneous leishmaniasis infection by using microscopic examination were 30(24.6%) in males while the rate of infection between females were 20(16.4%), also the rate of infection in urban area was 5(4.1%) while in rural area was 45(36.9%), While the rate of infection with leishmaniasis by using PCR assay, this technique was appear the rate of infection in males were 37(30.3%) while the females were 25(20.5%) and the prevalence of cutaneous leishmaniasis in rural areas was 54(44.3%) while in urban areas was 8(6.5%), The rate of infection in male higher than female and in rural area was higher than the urban area.

Different parts of the patient's body were observed with infection of Baghdad boil including face, arms, legs and feet, but number of patients infected in arms (upper limbs) had the highest percentage (48%) when compared to other sites of infection, followed by legs and feet (28.6%), face (20.8%) and other site (shoulder, thorax and neck) (2.6%) ,with highly significant differences (P<0.01).The number of lesions in CL patients ranged between one to 10 in different body parts. One ulcerated lesion was documented in 67 (55%) of patients, while two lesions were observed in 30(25 %) patient, 3-5 ulcerated lesions were found in 16(13%) patient and multiple lesions (5-10) were found in 9 (7%), The result recorded that 41% (50) of direct staining smear from patients lesions have positive and showed the amastigotes in phagocytic cell (macrophage) as neutrophil whereas 59% (72) of smear was negative, with significant differences (P<0.05).

Amplification of the ITS1 region by polymerase chain reaction and PCR products were electrophoresed in 2% agarose gel, the ITS1 PCR determined by the observation of expected bands 350 bp for cutaneous leishmaniasis, showed that out of 122 specimens of cutaneous leishmaniasis 62 (51%) were positive while 60 (49 %) were negative, by using PCR analysis. as shown in Figure 1.

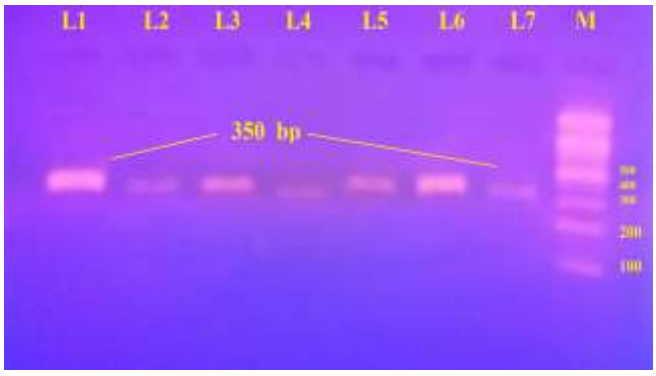

Fig. 1: Amplification of ITS1 DNA from the cutaneous leishmaniasis of the infected human. molecular weight marker (100 bp ladder), Lanes (1,2,3,4,5,6,7) positive samples at 350bp in PCR, Running conditions: Agarose gel (2%), 5 v/cm for 1hrs, stained with ethidium bromide.

The results of restriction amplified ITS1 gene product of Leishmania by the endonuclease Hae III gave two fragments 60 and 200 bp were identified as *L. tropica* and two fragments of 140 and 210 bp as *L. major*. The RFLP analysis gives the same result when incubation at 37^{o}C with three different times (2, 3 and 24) hours (Figure 2).

In the present study, 42 *L. tropica* (dry skin lesion) and 20 *L.major* (wet skin lesion) were diagnosed with RFLP methods.

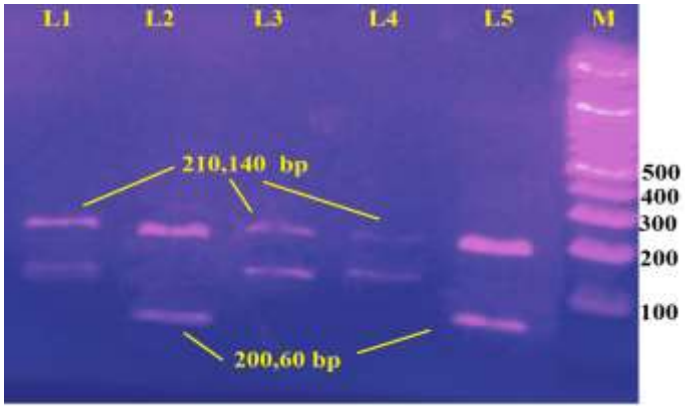

Fig. 2: Gel electrophoresis of PCR- RFLP products of ITS1 of Leishmania isolates using HaeIII restriction enzyme on 2.5% gel agarose, M 100 bp DNA ladder marker, L2, L5 *Leishmania tropica. L1, L3, L4 Leishmania major.*

DISCUSSION

The diagnosis of CL classically relies on microscopic examination. These classical methods require the presence of a relatively high number of viable or morphologically intact parasites; this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low. In contrast, the molecular approach is both sensitive and specific (Laskay *et al*., 1995). In this study, we set up a well-documented, genus-specific PCR to detect *Leishmania* species in clinical cutaneous samples and compared this method with the classical method.

The present study revealed a significantly high prevalence of cutaneous leishmaniasis in the age group between 1-10 years (24.6%) and (28.7%) by using microscopic examination and conventional PCR assay, This result is agreement with other Iraqi studies as (Al-Mayale 2004; Al-Difaie and Jassem 2014) in Al-Qadisiya governorate. Also (Rahi *et al.*, 2014) observed in another study higher infection appeared in the age group 10 years and less. the present result agrees with that in neighboring countries as in Syria (Al-Nahhas and Kaldas 2013), in Jordan (Al-Athamneh *et al*., 2014) decided that age group (2-19) years was also more susceptible to the infection by CL than another age group, in Iran (Mehdi *et al*., 2016) and Turkey (Fahriye *et al.*, 2017).

Whereas (Al-Obaidi *et al.*, 2016) obtained the highest infections in the age group (5-14yr.), While the lowest infection of CL was observed in the age group (>1yr.). also (Al-Warid *et al*., 2017) who confirmed that majority of cases were recorded among age groups 15–45 years old. The reason for the low rate of elderly patients may be related to the fact that they were infected during their early ages and acquired long-term immunity during childhood ,another factor is that older people do not admit to the treatment of CL while they know this disease and disfiguring scars are not as important for them as for youngsters (Akçalı *et al.*, 2007).

Also, this difference could be due to this age playing outdoors for a long time and more exposure to the infected sand flies, many investigators postulated that the decrease in incidence with age was due to development of immunity by previous infections (Al-Samarai and AlObaidi 2009).

The result of present and other studies pointed to this diseases can infect the individuals at any age. Also, the (WHO 2014) reported that people of all ages are at risk for infection if they live or travel where *Leishmania* spp is found.

Depending on genders. The present study revealed the rate of cutaneous leishmaniasis which is higher in males than in females by using microscopic examination and PCR assay. In Iraq (Al-Samarai and AlObaidi 2009) also found that males were (57%) and females were (31.7%). Another study by (Abdulwahab 2013) recorded that the infection in males was 65% than females (35%). In Saudi Arabia (Amin *et al*., 2013) documented that the incidence rate of CL

was higher in males than in females from 2000 to 2010. In Syria (Shanehsaz and Ishkhanian 2013) found that Syrian males are more infected with the parasite than females, In Jordan (Al-Athamneh *et al*., 2014), The same result in Turkey (Mustafa *et al*., 2017), This is probably happened due to the cultural habits of most areas.

Otherwise, the results of the present work appeared to disagree with the other previous Iraqi studies by (Rahi *et al.*, 2014) and (Al-Qadhi *et al*., 2013). These results may be attributed to the fact that males are more exposed to the insect biting more than females due to working outdoors and also due to men are less covering than women then exposed (Al-Samarai *et al*., 2016). Although it is believed that sex hormones may influence the establishment and the course of parasitic diseases, behavioural factors, making male individuals more likely to be exposed to vectors in fields and other transmission environments, are probably equally or more important (Rahi *et al*., 2013). The males are more exposed to the environment where the sand flies present by walking near rivers or swimming beside males work in the farms, while the females mostly staying in the houses (Kharfi *et al*., 2004). Asmaa *et al.,* (2017) found the highest percent of infection related to the geographical site which was near water stream flow all year and abundance of fresh water holes which provide sand flies a suitable environment to complete its life cycle and increase agriculture activities.

The distribution of cutaneous leishmaniasis in rural area was higher than the urban area, this result is agreement with other Iraqi studies as (Rahi *et al*., 2013) and (Al-Samarai *et al*., 2016), in Libya (Sabra *et al*., 2013), in Syria (Al-Nahhas and Kaldas 2013), in Jordan (Al-Athamneh *et al*., 2014), in Iran (Mehdi *et al*., 2016), and in Turkey (Fahriye *et al.,* 2017). However, a different result had been recorded by (AL-Hucheimi 2014; Al-Atabi 2014).

Leishmaniasis usually is more common in rural than in urban areas because there are many factors that play an important role in the presence and distribution of CL in this district, including the presence of animal reservoirs such as rodents, dogs, etc.; the presence of marshes; and the use of clay to build some of the houses in villages that belong to this district area. Furthermore, as an agricultural area, attracts and harbors many kinds of insects; therefore, its population works long hours in the farms where they are more exposed to insects bites (Al-Samarai and Al-Obaidi 2009), but it is found in the outskirts of some cities (Mustafa *et al*., 2017).

Different parts of the patient's body were observed with infection of Baghdad boil including face, arms, legs and feet, but the number of patients infected in arms (upper limbs) had the highest percentage (48%) when compared to other sites of infection.

This result agrees with (Al-Difaie and Jassem 2014) and (Hassan 2017) in Iraq suggested that the highest rate of the lesion on the upper limbs but (Khalifa *et al*., 2004), (Rahi *et al*., 2013) and (Al-Obaidi *et al*., 2016) found the face and hand the highest infection. Also, This result agrees with another study in Iran as (Talari *et al*., 2006 and Hojat *et al.,* 2012).

In general, the presence and distribution of lesions depend on which parts of the body are exposed and on the susceptibility of the host (Al-Samarai and Al-Obaidi 2009). CDC (2014) directed that, to minimize the amount of exposed (uncovered) skin, to the extent that is tolerable in the climate, wear long- sleeved shirts, long pants, and socks; and tuck your shirt into your pants.

The number of lesions in CL patients ranged between one to 10 in different body parts. One ulcerated lesion was documented in 67 (55%) of patients, while two lesions were observed in 30(25%) patient, 3-5 ulcerated lesions were found in 16(13%) patient and multiple lesions (5-10) were found in 9 (7%).

This result agreement with (Al-Mayale 2004) in Al-Qadisiya, (Al-Hucheimi 2005) in Al- Kufa, (Musa 2011) in Baghdad and

(Rahi *et al.*, 2013; Rahi 2015) in Kut city were show the incidence rate of single lesions in CL patients was higher (67.2%) than of the multiple lesions 32.8 % in Iraq, and (Khalifa *et al*., 2004) in Saudi Arabia.

In contrast with (Al-Difaie and Jassem 2014) reported that the incidence rate of multiple lesions in CL patients was higher than of the single lesions, this can be due to the fact that some ulcers do not necessarily lead to the appearance of scars for several possible reasons, i.e. immune system interference or early healing of the ulcers, spontaneously or due to treatment. Also which is in agreement with previous reports indicating more exposure as a result of educational and occupational situations (Lafi *et al.*, 2007).

The direct staining smear considers good first examination to CL, need a small amount of material from the edge of the lesion which stained easily by Giemsa stain (CDC 2011).

The main reason that cells are stained is to enhance visualization of the cell or the cellular components under a microscope, make them easier to see, also it can highlight (Khade-mvatan *et al*., 2012). Staining helps in the identification of the sample by smear colour change without getting into the complete analysis of the sample and easy to observe the morphology, size and shape.

The Giemsa stain provides a better stain intensity, show some details that may be unclear otherwise, especially in cells, but some smears of Giemsa stain gave negative results and the parasite doesn't see or disappeared (Younis *et al*., 2017), that attributed to many reasons, patient take treatment, mistake in time staining, smear thickness and sometimes distortion of the cell wall may occur (Mustafa *et al*., 2017). These classical methods require the presence of a relatively high number of viable or morphologically intact parasites; this may pose a problem particularly in the chronic phase of CL where parasite levels in skin lesions are very low (Laskay *et al*., 1995; Rahi 2015).

Most of the slides that were high scored amastigote numbers as microscopy - positive were also positive by PCR-RFLP. Although the costs for PCR-RFLP diagnosis are higher and its concordance is lower than microscopic examination, but this method can identify Leishmania species without the need for cultivation them (Schönian *et al.*, 2003; Al-Jawabreh *et al.*, 2006).

The ITS1 gene was chosen to detect 18 ribosomal RNA (SSU) RNA in Leishmania, it is found on chromosome 27 and exon 1 in the parasite. This gene consider as an important virulent factor for the parasite to make infection as reported by (Mauricio *et al*., 2004). In the current study, the amplification of ITS1 gene fragment with 350 bp showed clear band when electrophoresis on 2% agarose gel. Belal *et al.*, (2012) established that PCR and Genotyping analysis of *Leishmania* spp. PCR based methods have proven to be highly sensitive and specific compared to the standard methods and are considered exceedingly valuable for diagnosis. Identification of the Leishmania type is important, because different species may require distinct treatment regimens. Furthermore, such data are also valuable in epidemiologic studies where the distribution of *Leishmania* species in human and animal hosts, is a prerequisite for designing appropriate control measures (Maraghi *et al.*, 2013).

The results of restriction amplified ITS1 gene product of Leishmania by the endonuclease Hae III gave 42 *L. tropica* and 20 *L. major* were diagnosed with RFLP methods. These results were confirmed by (WHO 2014) an agreement with many studies, in Iraq (Ali *et al.*, 2015; Hassan 2017), in Afghanistan (Faulde *et al.*, 2008) and Iran (Talari *et al*., 2006) who found the dry lesions more than wet lesions, in contrast with other studies done in Iraq (Al-Difaie and Jassem 2014), Pakistan (Ul Bari and ber Rahman 2006), India by (Sharma and Mahajan 2015) and in America (Amalia *et al*., 2014) reported that dry skin lesions less than wet skin lesion.

The presence of incidence of *L. major* in this and other studies in Iraq may be due to the presence of reservoir animals in large numbers in some areas in Iraq, especially rodents and dogs. Obviously, dense populations of natural hosts of *L. major*, together with abundant vector sand flies are the key elements responsible for the high rate of human infection (CDC 2012). Also, it must know vector sand flies responsible for human infection by *L. topica* only (Craig *et al.*, 2013).

The application of PCR and RFLP benefit to characterize the *Leishmania* species causing cutaneous leishmaniasis in Iraq. two types of *Leishmania* spp., *L .major as* mentioned previously by (Al-Saqur and Al-Obaidi 2013) and *L. tropica* as mentioned previously by (Sharma and Mahajan 2015) and that confirmed another Iraqi study (Rahi *et al*., 2013) and another study in nearby countries such as Saudi Arabia (Amin *et al*., 2013) and Iran (Azizi *et al.*, 2012).

According to the results of this study, we concluded the demographic study investigation revealed that the High infection rate was noticed in the age group of 1 to 10 years. and males were more exposure to infection than females, upper limbs had the highest percentage when compared to other sites of infection, and single lesion appeared in patients more than multiple lesions.

Characterization of *Leishmania* isolates collected from different parts of Al Ramadi city showed that *L. tropica* and *L. major* are the agents of cutaneous leishmaniasis.

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