Epidemiological And Molecular Characteristics Of Cutaneous Leishmaniasis In Al-Diwanyiah hospital

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Abstract:
Aims of study: The current study aimed to epidemiological identification and molecular detection of Cutaneous Leishmaniasis in Diwanyiah province by using the nested PCR method.
Material and methods: The present study include (145) patient came to the dermatology lab. of Al-Diwanyiah hospital with suspected cutaneous leishmaniasis at period from (September 2016 to the April 2017). Epidemiological finding including study of age, sex and periods of infection, than, (50) suspected patient samples out of (145) patients samples undergo to performed Nested PCR technique, the frozen skin lesion samples were exposed to genomic DNA extraction step and Nested PCR was carried out by using specific primer for Kinetoplast DNA (kDNA) for detection and identification cutaneous Leishmania species, such as L. major and L. tropica.
Results: Our study shows infection of CL in the male(67) (46.2%) and the female(78) (53.8%) and it found Age (1-12month)(5) (3.5%) ; (1-10year):(42)(28.9%) ; (10-20year):(46%) (31%); (20-30 year): (19)(13.4%) ; (30-40 year):(14%) (9.85%) ; in more than 40 year(19) (13.4%); .Also the highest infection rate of CL were appeared during December 2016 (30.5 %), while the lowest were during April 2017 (2%).The nested PCR was shown specific identification of cutaneous leishmaniosis. Where, the results shown (38) positive samples out of 50 samples at (78%) as cutaneous leishmaniosis. 28/38 positive at (73.7%) as *L.major* and 10 /38 positive at (26.3%) as *L. tropica.* The nested PCR amplification was 560bp product size for *L.major* and 750bp product size for *L. tropica* on Agarose gel electrophoresis.
Conclusion: Cutaneous Leishmaniasis infected both sex , all age and more prevalence in young patients with high infection rate during December. Both L. *major and L. tropica* were the causative agents of cutaneous leishmaniasis but *L.major* was the main species in study area.
Key words: Epidemiological , Molecular, Cutaneous Leishmaniasis , Al-Diwanyiah.

Introduction:

Cutaneous Leishmaniasis are a parasitic disease that caused by protozoa of the genus *Leishmania* . Two important *Leishmania* species are responsible to causes cutaneous leishmaniasis are L. *major and L. tropica* and the disease is endemic in 88 country (1). These protozoa infected humans through bite sandflies vector called (*Phlebotomine*) (2). Cutaneous Leishmaniasis caused by *L. major* and *L. tropica* was previously recorded occurs in Iraq as called (Baghdad sore) WHO which recorded the Cutaneous Leishmaniasis as a major public health problem in all regions of Eastern Mediterranean (3,4). Thus WHO and Eastern Mediterranean Region Organization are established health care programs as strategic plan to control on Cutaneous Leishmaniasis infection (5). The infective stage of Leishmania (Promastigotes) are infected the human skin by the bite of a sand fly then invades human leukocyte macrophages and complete intracellular replication. Skin raised and red lesion was develops in site of the insect bite then ulcerates and may be

include secondary bacterial infection (6). Cutaneous Leishmaniasis skin lesions are sometime spontaneously heal in some Leishmania species then reappear as satellite skin lesions around original infection site or along lymphatic drainage (7). The primary skin lesion representing local dissemination reaction of antigenic products of parasite (8). The laboratory diagnostic methods of Cutaneous Leishmaniasis infection are generally depend on clinical and epidemiological confirmation and detection of parasite. So far , no single laboratory method has been accepted as a gold standard for diagnosis Cutaneous Leishmaniasis infection (9). Tissue sampling using dental broach and stained with Giemsa stain although is a simple technique but always need personal experience in order to find the parasite in the smear. As the parasites are located in the smear in loci and not in uniform pattern and the positivity rate from (54.33 – 71.6 %)(10). Still tissue sampling by dental broach is useful for culturing procedure. Biopsies and staining with H & E stains is also a useful to see the LD bodies and to detect the parasite with high rate. Culturing of the parasite is very specific & sensitive technique but it is a time consuming and the positivity rate from (52.9 – 80%)(11,12). Molecular diagnostic techniques that used nested PCR to amplify target DNA of parasite from host tissues. These molecular techniques are powerful tool to direct detect of *Leishmania* species in clinical samples and provided highly sensitive and specific when compared with traditional methods(13. 14). So that current study aimed to epidemiological identification and molecular detection of Cutaneous Leishmaniasis in Diwanyiah province by using the nested PCR method.

Materials and Methods:

Samples collections:

The present study include (145) patient came to the dermatology lab. of Al-Diwanyiah hospital with suspected Cutaneous Leishmaniasis at period from (September 2016 to the April 2017). Then (50) suspect patient skin lesion were collected in sterile containers under aseptic conditions and transported as soon as possible to laboratory and stored in -20°C refrigerator until use for DNA extraction.

DNA extraction:

The frozen skin lesion samples were subjected to DNA extraction by using commercial DNA extraction kit (Tissue-DNA Kit. Geneaid-Biotech Ltd. USA) and according to kid provided sheet instructions with using Proteinase K (10 mg/ml).**then extracted** DNA was checked by nanodrop spectrum. at 260 and 280nm, and then store at deep freezer until used in Nested PCR method.

Nested PCR:

Nested PCR was carried out by using specific primer for Kinetoplast DNA (kDNA) in genus Leishmania that include External primers and Internal primers were using to amplify 750bp PCR product for tropica and 560bp PCR product L. major (17). These primers were provided by (Bioneer company . Korea). As following table:

Primer	Sequence (5'-3')		Amplicon
External primers	CSB2XF	CGA GTA GCA GAA ACT CCC GTT CA	750bp
	CSB1XR	ATT TTT CGC GAT TTT CGC AGA ACG	
Internal primers	13Z	ACT GGG GGT TGG TGT AAA ATA G	560bp
	LiR	TCG CAG AAC GCC CCT	

Nested PCR master mix preparation: the mix was prepared using (Accu-Power®PCR-PreMix-Kit) master mix reagent and done depend on company instructions as following :

First PCR Run:

First run PCR master mix	Volume
Genomic DNA template	2.5µL
CSB2XF Forward primer (10pmol)	1.25µL
CSB2XF Reverse primer (10pmol)	1.25µL
Free nuclease water	20µL
Total volume	25µL

The PCR matermix placed in PCR premix tubes provided by kit that contain other PCR components Then, all the PCR tubes transferred into vortex for 2minutes. Then transferred into PCR thermocycler (Biorad, Bioneer. Korea).

PCR thermocycler conditions:

First PCR step	Temp.	Time	Repeat cycle
Initial Denaturation	94 °C	5 minute	1
Denaturation	94 °C	30 sec	30
Annealing	55 °C	30 sec	
Extension	72 °C	1 minute	
Final Extension	72°C	5 minute	1
Hold	4°C	forever	

Second Nested PCR Run:

First run PCR master mix	Volume
First PCR product template	1µL
13Z Forward primer (10pmol)	1.25µL
LiR Reverse primer (10pmol)	1.25µL
Free nuclease water	21.5µL
Total volume	25µL

Also, the PCR tubes transferred into vortex for 2 minutes. Then transferred into PCR thermocycler (Biorad, Bioneer. Korea).

PCR thermocycler conditions:

Second PCR step	Temp.	Time	Repeat cycle
Initial Denaturation	94 °C	5 minute	1
Denaturation	94 °C	30 sec	30
Annealing	55 °C	30 sec	
Extension	72 °C	1 minute	
Final Extension	72°C	5 minute	1
Hold	4°C	forever	

PCR product analysis: The Nested PCR products were checked in electrophoresis in a 1% agarose gel and 1X TBE buffer, then stained by ethidium bromide, and investigation under UV-transilluminator.

Statistical analysis :

A computerized program ,the statistical package for social sciences (SPSS) was used to calculated the statistics analysis .The statistical analysis of data had done by chi square with the confidence limit was accepted at 95% ($p>0.05$)(15).

Results:

Epidemiological study results:

1- Prevalence of Cutaneous Leishmaniasis by sex and age:

Our study shows infection of CL in the male(67) (46.2%) and the female(78) (53.8%) and it found Age (1-12month)(5)(3.5%) ; (1-10year):(42)(28.9%) ; (10-20year):(46) (31%); (20-30 year): (19)(13.4%) ; (30-40 year):(14) (9.85%) ; in more than 40 year(19) (13.4%); figure (3) and (4).

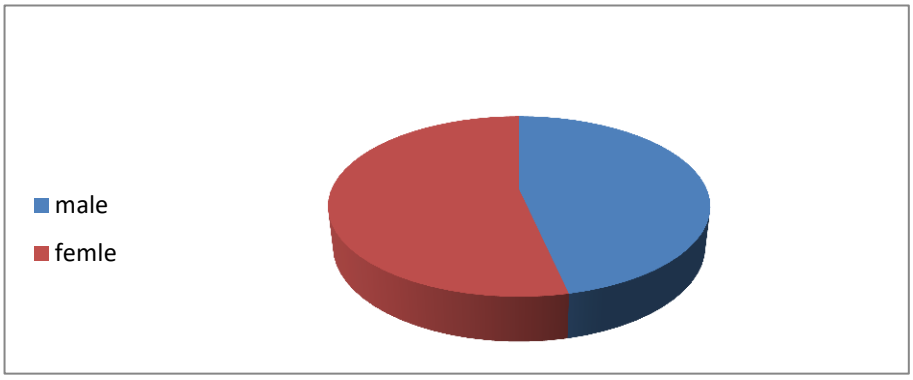

Figure (1): Chart included the prevalence of Cutaneous Leishmaniasis According to the sex

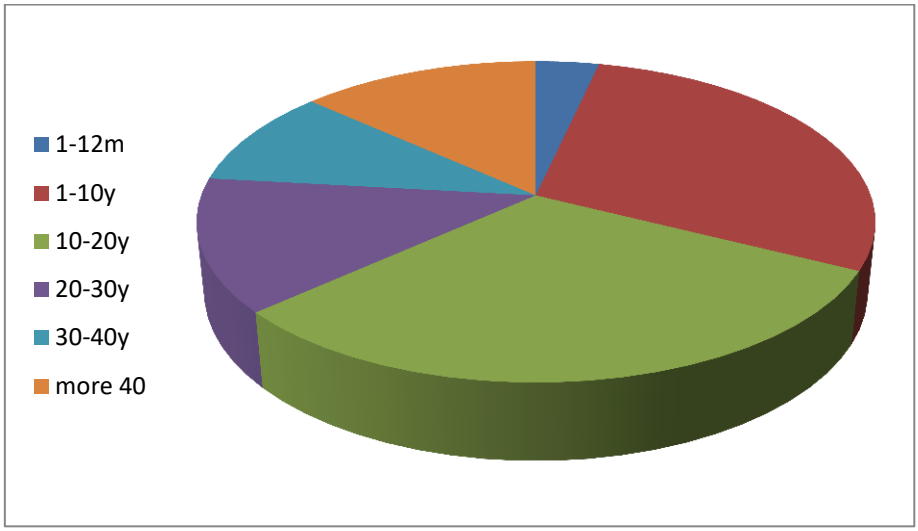

Figure (2): Chart included the prevalence of Cutaneous Leishmaniasis According to the age

2- Monthly distribution Rates of Cutaneous Leishmaniasis Infection:

In our study the highest infection rate of CL were appeared during December 2016 (30.5 %), while the lowest were during April 2017 (2%) (Figure 5).

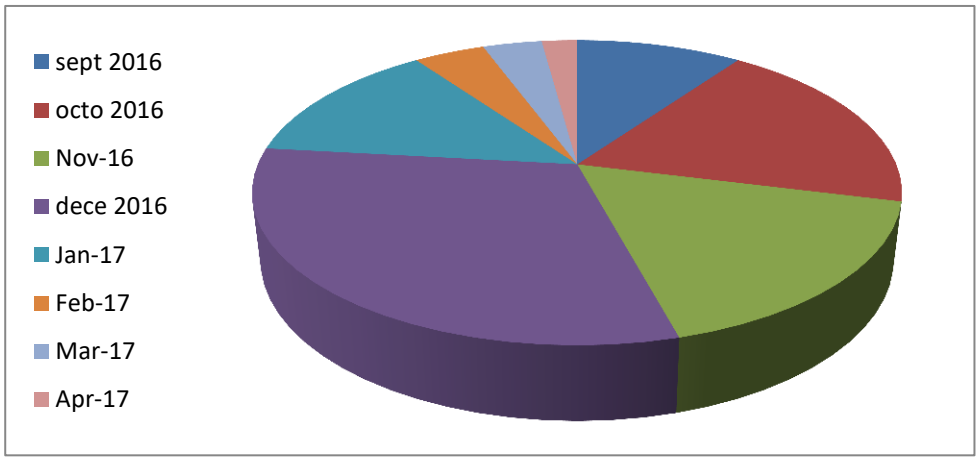

Figure (3): Chart included Monthly distribution of Leishmaniasis

3-Molecular identification results:

The nested PCR was shown specific identification of cutaneous leishmania. Where, the results shown (38) positive samples out of 50 samples at (78%) as cutaneous leishmaniosis. 28/38 positive at (73.7%) as *Leishmania major* and 10 /38 positive at (26.3%) as *Leishmania tropica.* The nested PCR amplification was 560bp product size for *L.major* and 750bp product size for *L. tropica* on Agarose gel electrophoresis as in following figure (1).

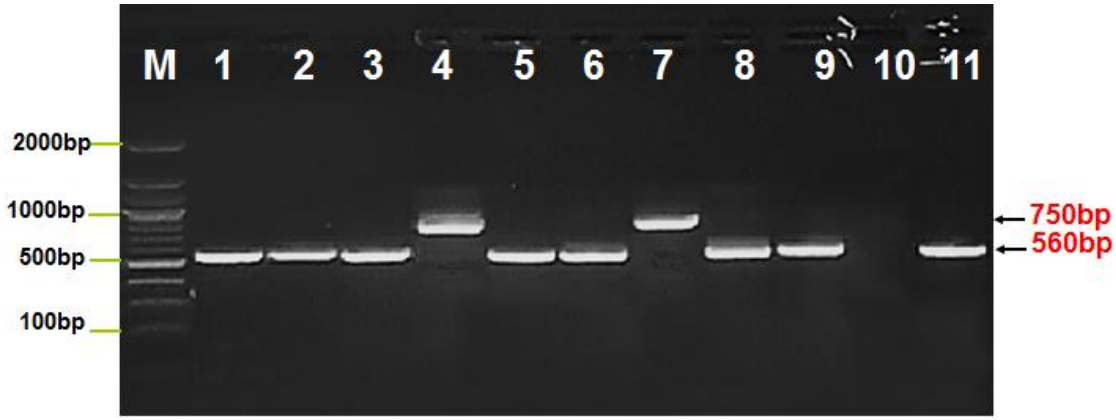

Figure (4): Agarose electrophoresis explain that the analysis of Nested PCR kDNA product in Leishmania positive isolates. Where M: marker (2000-100bp), lane (1, 2, 3, 5, 6, 8, 9 and 11) positive *L. major* at (560bp) PCR product size and lane (4 and 7) positive *L. tropica* at (750bp) PCR product size, whereas Lane (10) negative samples.

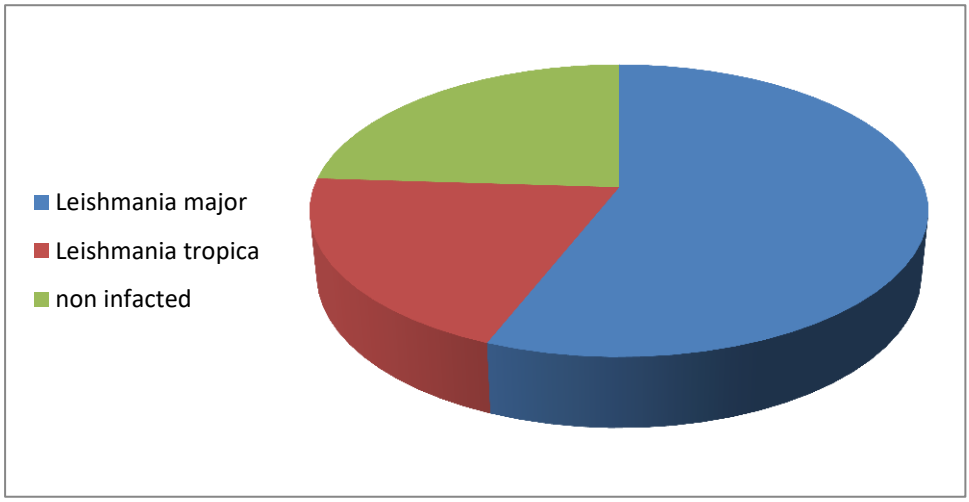

Figure (5): Chart included the prevalence of infection rates in *L.tropica* and *L. major* in patients

Discussion:

In our study used molecular methods (Nested PCR technique) to detection CL that agreement with some researchers that they use same the technique like (16), (17). The PCR has more specific and sensitive by capability of detect low levels of parasite from the peripheral blood (18). The Nested PCR technique was capable to detection and differentiation of *Leishmania major* and *Leishmania tropica* that used in our study, and it highly sensitive to detect the parasite in clinical samples from Iraqi patients with CL. The technique could detect as little as fragment of parasite DNA equivalent of approximately 1 parasite DNAs of Cutaneous Leishmaniasis with high sensitivity rates (19).

According to our results total infection of CL was (78%) that close and similar to (20) where (74.14%) were determined to be positive for CL in Sri Lanka by use PCR methods, also similar to (21) in Brazil

where recorded (71.3%), while (22) found (39.6%) as a percentage of total CL in Iran, that considered less than our results . (23) In Iraq and (24) in Ethiopia recorded percentage more than our results by using PCR, it was (91.66%) and (95%) respectively.

CL infection depended on many reasons, the living standard, way of living, the presence of reservoir or domestic animals, and above all the viability of infected sand fly due to suitable environmental conditions (25). The peak number of cases was in patients with age (10-30 year) This agreement with the results (26) they found the highest incidence in the (20-29) year group this may due to many young are working on the farms ,the rural areas provided suitable conditions for the vector and reservoir, like rodent and dogs.

High and low in the incidence of CL cases depend on the presence of number of reservoir animals and vectors that spread in the around. Obviously, dense populations of natural hosts of *L. major* and *L.tropica* together with sand flies are the key that responsible for the great rates of human infection(23).

The results of this study indicate that reported CL among female greater than men in prevalence of leishmaniasis spp. These results are consistent with the results of studies in (27),(28). But they are inconsistent with the results of studies in (29) and (30) they found the infection in males more than females.

The infection was more common in female as compared to male and this may be due to the ecological condition of the areas .

It is assumed that this difference between two groups of studies because the CL cases has come from different cultural behavior in the study area, where some societies the men do agriculture works while some other the women do that (31).

The monthly distribution of cases showed that the highest cases registered during winter mainly December due to the growth and propagation of vector and declined in April, these results are consistent with other previous studies done in Iraq, the peak incidence of cases was during December and January (32),(33), and in other countries (34).

The data of studies show most Iraqi isolates belong to the L. major, this confirms previous findings by nested-PCR which reported that L. major was the dominant species in study areas. The present data are different from those deduced by (35) who found that the Iraqi Leishmania isolations from skin lesion divided into two group, one of which gave isoenzyme pattern similar to those of a marker stock of Leishmania major, and the other which gave pattern similar to those given by Leishmania tropica. Previously, it had been thought that Leishmania tropica alone was responsible for Cutaneous Leishmaniasis in Iraq. None of the current samples showed to be infected with L. tropica. This is because of the samples were chosen randomly and the Cutaneous Leishmaniasis in the study area is most probably zoonotic type. (36) said that one of the main problems for the understand and control of this neglected disease is the detection and identification of Leishmania parasites in animal reservoirs. Epidemics may flare up with little warning if vector sandflies are present. Significant changes in the epidemiology of the disease in relating to its geographic location, climate, and temperature also depend on the rate of distribution of vectors, reservoir variety, more importantly, parasite identification at the species level(37).

Conclusion:

Conclusion: Cutaneous Leishmaniasis infected both sex , all age and more prevalence in young patients with high infection rate during December. Both L. *major and L. tropica* were the causative agents of cutaneous leishmaniasis but *L.major* was the main species in study area.

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