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DETECTION OF *LEISHMANIA TROPICA* USING NESTED PCR AND MEASURE SOME BLOOD PARAMETERS IN THI-QAR PROVINCE, IRAQ: EARLY DERMAL LESIONS

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ABSTRACT

The study includes three local locations, Al-Hussein Teaching, Suq Al-Shyokh General and Al-Shatrah General Hospitals in province for the period from December 2018 to September 2019. The samples were collected from 80 patients suffering from cutaneous *leishmaniasis* (CL). Nested-PCR was used to amplify kinetoplast minicircle fragments DNA (*kDNA*). Also, 3ml of the venous blood was collected before they take a drug, then putted in EDTA tube for order complete blood count (CBC) test. Electrophoresis results for 80 samples of kinetoplast minicircle fragments DNA gene were amplified by Nested PCR, discovered 65 positives for cutaneous *leishmaniasis* and Furthermore, the results were recorded 46 (57.5%) positive samples of *L. tropica* at 750bp. In present study, there are not significant differences of erythrocytes count and hemoglobin concentration by compared between patients and control, whereas platelet blood count showed a significant increase. On the other hand, the statistical analysis findings were showed significant differences by compared between leukocytes count and WBC differential (neutrophils, lymphocytes, eosinophils and basophils) of patients group and control specimen, except of monocytes which showed an insignificant difference at compared between patients group and control.

KEYWORDS: *Leishmania tropica*, blood parameters, CL, cutaneous *leishmaniasis*, Nested PCR.

INTRODUCTION

Leishmaniasis is a third the most important vector-borne diseases, after malaria and lymphatic filariasis. It is caused by a protozoan intracellular obligatory parasite of the genus *Leishmania*, and which they cause human and animal diseases, transmitted by the bite of sand flies (Soong, 2009). *Leishmaniasis* is one of the endemic diseases and important public health problems, which spread in a wide global region such as: Asia, Africa, Europe and the Americas. The infection is difficult to control, cause deformities and fatalities and their capacity for cause epidemics. However, approximately 20 *Leishmania* spp. can transmit human infection from among the 53 that they are reported (Santos-de-Souza *et al*., 2019). CL is the comprises nearly 70–75% from the total global cases. Currently, there is no vaccine available against *leishmaniasis* infections. Control of CL is difficult due the variety of *Leishmania* spp., reservoir hosts and biological vectors (Aflatoonian *et al*., 2019). CL is often leaves disfiguring scars, especially on visible body sites, causing also social, psychological, and economic problems (Bilgic-Temel *et al*., 2019). Generally, CL diagnosis depends on clinical cases and laboratorial examinations as parasitological, serological and molecular tests (Eksi *et al*., 2017). Numerous studies have referred that a sensitivity of the parasitological identification increases from direct smear to culture to PCR technique (Olliaro *et al*., 2013). It is a necessary to distinguish between *Leishmania* species for identification of clinically different manifestations of the disease (visceral, cutaneous or muco-cutaneous) in order to the correct diagnosis, administration of the appropriate treatment and control of the disease (Mesa *et al*., 2020). Nested-PCR technique is consider a suitable method, has high sensitivity and accurate identification of *Leishmania* parasites in clinical samples (Al-Tamemy and Al-Qurashi, 2017; Akhoundi *et al*., 2017; Ramezany *et al*., 2018). Moreover, kDNA gene has shown highly sensitivity and specificity in detecting *Leishmania* (Naseri *et al*., 2016). However, routine CBC parameters, such as white blood cell count (WBC), lymphocyte, neutrophil, red blood cell (RBC), platelet counts, and others, gave an idea around the evaluation of systemic inflammations, the diagnosis and a stages of the disease treatment (Sula and Tekin 2015).

MATERIALS AND METHODS

Study area and pateints

The study includes three locations Al-Hussein Teaching, Suq Al-Shyokh General and Al-Shatrah General Hospitals in Thi-Qar province/ South of Iraq for the period from December 2018 to September 2019. The

hospitals have received patients which suffering from cutaneous diseases. All medical information was taken from infected patients. The samples were collected from lesion fluid of 80 patients by CL, both genders, different ages, various residence places in the province and from single and multiple lesions. Skin infection lesion was injected by normal saline (0.2 ml) in lesion edge, then draw back with bloody stained fluid for get parasite, after that the fluid was putted in plain tube. Also, 3ml of the venous blood was collected before they take a drug, then putted in EDTA tube for order complete blood count (CBC) test in advisory laboratory.

Genomic DNA extraction

From lesion fluid, DNA was extracted using gSYAN DNA extraction kit Geneaid according to protocol of produced company (Geneaid/ Taiwan). DNA concentration was checked with Nanodropspectrophotometer and then stored at -20˚C until used in PCR amplification.

Nested PCR amplification

The kDNA *Leishmania* isolates was amplified with Nested-PCR, with some modification of PCR according to (Izadi *et al*., 2016), it was included two steps. Target DNA undergone the first run using external primers: CSB2XF (ATT TTT CGC GAT TTT CGC AGA ACG) and CSB1XR (CGA GTA GCA GAA ACT CCC GTT CA), then first run product undergone second run using internal specific primers:13Z (ACT GGG GGT TGG TGT AAA ATA G) and LiR (TCG CAG AAC GCC CCT). PCR-master mix was prepared by (AccuPower® PCR PreMix kit. Bioneer, Korea). The Nested PCR primers were provided by Macrogen Company, Korea. Nested PCR master mix prepared 5µL of DNA, 1µL of each external primer and 13 µL of PCR water and placed in standard PCR tubes. PCR reaction thermal condition included an initial denaturation at 95°C for 5 minutes followed by 30 cycles at 95˚C for 30 seconds., 55˚C for 30 secondes. and 72˚C for 1minute and finally final extension 72˚C for 5minutes. A nested PCR master mix of second run included 3µL from first run product, 1µL of each internal primer and 15 µL of PCR water and placed in standard PCR tubes with thermal conditions. The PCR products passed electrophoresis with 1% agarose gel with 3µL from ethidium bromide. 10µl of PCR product was added into each comb well and 5 µl of (100bp ladder) inside each well. The electric current was adjusted at 100 volts and 80 mA for 1 hr. PCR products were visualized with an ultraviolet transilluminator.

Hematological examination

Hematological samples of patients with L. tropica were done complete blood count (CBC) including (total and differential WBCs counts, RBC, HGB) using auto hematology analyzer that automatically, measures and prints the results.

Statistical Analysis

In the study, method of (t) test was used in order to analyzed the data with SPSS statistical software V.17. Significant level was at $P < 0.05$.

RESULTS AND DISCUSSION

Electrophoresis results for 80 samples of kinetoplast minicircle fragments DNA gene were amplified by Nested PCR, discovered 65 positives for cutaneous *leishmaniasis* and Furthermore, the results were recorded 46 (57.5%) positive samples of *L. tropica* at 750bp.

In short, high abundance of kDNA minicircles in each kinetoplast make an ideal target for diagnosis of *Leishmania* parasites (Abdolmajid *et al.*, 2015; Kocher *et al.*, 2017; Ramezany *et al.*, 2018). Koltas *et al.* (2016) mentioned that kDNA fragments were the most sensitive from routine and molecular diagnosis of leishmaniasis. However, it is a necessary to identification and detection of *L. major* and *L. tropica* infections, because the treatment is administered with different protocols (Salloum *et al.*, 2016). In any case, *L. major* is may be high prevalence in the northern and central provinces of Iraq, while *L. tropica* is more diffused in southern provinces. Generally, both zoonotic cutaneous *leishmaniasis* (ZCL) and anthroponotic cutaneous *leishmaniasis* (ACL) are endemic in Syria, Iraq, Saudi Arabia Kingdom, and Iran (Alkulaibi *et al*., 2019).

This finding is close to Mezher (2017) recorded appearance of *L. tropica* with 69.53%, while *L. major* was 22.58% in Al-Muthanna province. Also, Saroufim *et al.* (2014) diagnosed *L. tropica* in 85%, while *L. major* in 15% out of 948 cutaneous lesion of Syrian refugee persons, which living in Lebanon. Abdolmajid *et al.* (2015) showed 66 (55%) of positive cases; included 46 *L. tropica* and 20 *L. major* in Khorasan‑Razavi province, Iran. Sharifi *et al.* (2015) mentioned that 95.5% of CL samples were identified as *L. tropica* and 4.5% was *L. major* in Kerman province, Iran. Mohammadiha *et al.* (2017) identified in Khorasan‑Razavi province/Iran, *L. tropica* in 61(65%), while *L. major* in 33 (35%) of CL cases. Ramezany *et al.* (2018) showed that *L. tropica* represented 88.5% and *L. major* 11.5% of positive samples in Kerman province/south-eastern Iran.

The result was inconsistent with Jafer *et al.* (2015) recorded appearance of *L. major* in 94(75.2%) and *L. tropica* in 31(24.8%) of CL cases in kerbala province. Al-Hassani (2016) conducted molecular examination of 52 (94.5%) positive samples in Eastern Al-Hamzah district, AlQadisiya province and observed 49 (89.1%) samples were infected by *L. major* and 3 (5.45%) were infected by *L. tropica*. Al-Tamemy and Al-Qurashi (2017) in Wasit province, recorded appearance of *L. major* and *L. tropica* were 89.7%, 10.2% respectively. Also, Amro *et al.* (2012) conducted a molecular epidemiological study in Libya, *L. major* and *L. tropica* showed by 75.9%, 24.1% respectively.

In present study, there are not significant differences of erythrocytes count (RBCs 10^6/µL) and hemoglobin concentration (HGB g/dL) at P>0.05 by compared between patients group and control specimen, whereas platelet blood count (PLT 10^3/µL) showed a significant increase at probability level P<0.05 as shown in Table (1).

On the other hand, the statistical analysis findings were showed significant differences at P<0.05 by compared between leukocytes count (WBCs 10^3/µL) and WBC differential of patients group and control specimen, except of Monocytes which showed an insignificant difference at compared between patients group and control specimen.

Table (1): Some blood physiological parameters of patients group and control specimen.

Parameter	Patient (N=46), Mean±SD	Healthy(N=25), Mean±SD	t. Value	P. Value
RBC 10^6/µL	5.02 ± 0.54	5.04 ± 0.53	-0.18	0.850
HGB g/dL	14.12 ± 1.38	14.02 ± 1.29	0.30	0.333
PLT 10^3/µL	293.22 ± 86.82	247.76 ± 61.04	2.34	0.02*
WBC 10^3/µL	6.49 ± 1.24	8.03 ± 1.27	-5.22	0.00*
NEU 10^3/µL	3.45 ± 0.94	4.70 ± 1.17	-4.99	0.00*
LYM 10^3/µL	2.22 ± 0.53	2.56 ± 0.53	-2.58	0.012*
MONO 10^3/µL	0.57 ± 0.24	0.51 ± 0.16	1.21	0.229
ESO 10^3/µL	0.15 ± 0.10	0.22 ± 0.17	-2.36	0.021*
BASO 10^3/µL	0.05 ± 0.03	0.13 ± 0.11	-2.96	0.026*

df = 69 , *P.value ≤ 0.05 Significant

In present study does not observed significant differences of erythrocytes count and hemoglobin concentration compared between patients group before treatment and control specimen, whereas platelet blood count showed a significant increase. The reason may be the infection remains on external skin surface and does not transmits to the viscera such as liver and spleen, also this parasite infects phagocytic cells, while platelets are raised in the event damage or infection of the skin surface.

Platelets are important for tissue repair after an occurrence of the infection. They are attached to the vessel wall at sites of the infection (Mezger *et al*., 2019). Furthermore, platelets interact with viruses, bacteria, protozoa and fungi which appear anti-microbial functions. For example, after *Leishmania* infection, platelets adhere to *Leishmania* parasites, this is a key mechanism believed to enhance phagocytosis process (Ali *et al.*, 2016). Platelets play an essential role in clot formation. Recent studies show that platelets are also related in infection, inflammation, host response and cancer. They congregate to gather to secrete adhesion molecules in the damaged site and adhere to white blood cells. Generally, they release chemo massages such as cytokines which are targeting lymphocytes, monocytes and neutrophils to inflammation site (Sonmez and Sonmez 2017).

The results of this study are somewhat close to finding of Abdulghani *et al.* (2014) have not recorded a significant change of HGB between CL patients and control sample and observed an insignificant increase in platelet count. AL-Hoot *et al.* (2017) did not observed a significant different of RBC count between CL patients and control sample, whereas HGB concentration recorded a significant decrease in patients. Hassan *et al.* (2017) recorded a significant increase of platelets in CL patients compared with control, did not found significant change of RBCs, while HGB concentration decreased.

There is a significant decrease in WBCs count and WBC differential of patients comparted with control specimen, except of monocytes which not showed a significant difference.

Axiomatically, *Leishmania* parasites infect phagocytic cells such as macrophages and neutrophils. Also, virulence factors act as an inhibition or modification of host cell signaling pathways which inhibit lymphocytes production and/ or other immune cells (Gupta *et al*., 2013). In early stage, they infect and exploit neutrophils. Although neutrophil is mainly engulfing *Leishmania spp*. via the first hours for infection, promastigote does not differentiate to amastigote within neutrophil, but in macrophages (Menezes *et al.*, 2016). In *L. major* has been document a similar scenario of other microbe intracellular infections which apoptotic neutrophil is used as a Trojan horse in order inactive macrophage, allowing a silent entry of *Leishmania* parasites (Tasew *et al*., 2016). Sousa *et al.* (2014) observed an accumulation of neutrophils about 6-24 hrs. after infection, then neutrophils reduced after 72 hrs. Neutrophils decrease resulted to fast lesion development and increased *Leishmania* parasites via the first week from BALB/c mice infection. Further, the lymphocytes number is reduced as result to increased lymphocyte apoptosis in chronic infection (Sula and Tekin 2015). On the other hand, eosinophils are store a wide spectrum of cytokines in the granules, which effect the immune response to certain pathway cascades, also they have variety mechanisms activate phagocytic cells and reactive oxygen species (ROS) (Beat, 2018).

Presence of parasites are usually inducing inflammation by cascade of cellular signals that leads to recruitment of innate immune cells, such as neutrophils, macrophages, eosinophils, and dendritic cells which engulf parasites and/or produce cytokines and other materials that activate immune responses. Resulting responses may be protect and eliminate *Leishmania* parasites, or ineffective to result to chronic inflammation (Slapničková *et al*., 2016). However, several studies referred to enormous recruitment of inflammatory cells such as neutrophils, macrophages and eosinophils was appeared at the site of the infection and it is relative with lesion development (Abu Musa *et al*., 2019). Macrophages have various functions such as phagocytosis, release of cytokines, response to microbial molecules, cell's recruitment to infected places (Kiessling *et al.*, 2017). Singh *et al.* (2018) observed an increased monocytes in patients with cutaneous *leishmaniasis*.

The results are consistent with Sula and Tekin (2015) that have recorded a significant decrease for each of total WBC count, neutrophils, lymphocytes and eosinophils of patients infected by CL comparted with control. Oleiwi *et al.* (2019) recorded a significant decrease of total WBCs in patients of CL. These results are inconsistent with Abdulghani *et al.* (2014) that have recorded a significant increase of WBCs count in patients of CL comparted with control specimen. Al-Rasheed *et al.* (2015) observed HB decrease and WBCs increase in patients of CL compared with control. AL-Hoot *et al.* (2017) showed increase of WBCs, lymphocytes, monocytes and neutrophils for patients of CL.

CONCLUSION

There is a very little data about the identification of *Leishmania* species responsible of human CL. The findings have showed that most cases of cutaneous lesions have been infected with *L. tropica*, that is the prevalent species. Also, there are significant differences of some blood parameter and other do not recorded any differences. However, nested-PCR is a suitable method for direct diagnosis of *Leishmania* at species level.

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