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Molecular study for detection the *Leishmania donovani* in mice tissues

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Abstract

The present study designed to evaluate the efficacy of conventional PCR with small – sub unit18S rRNA gene of the liver and spleen tissues in mice infected with 200 µl of 1.2 x 106 inoculum of Leishmania donovani promastigote after 45 and 60 days of infection. The results show positive results in 30 specimens (75%) of liver and 26 specimen (65%) of spleen of infected mice after these periods. Also the results show the amastigote significantly increased ($p \leq 0.05$) in the number of infected mouse tissue (liver and spleen), large in size of the spleen and significantly increased ($p \leq 0.05$) in the liver and spleen weight of infected mice after 45 and 60 days from inoculum in comparison with non infected mice, also significantly decrease in Hb, RBC, and WBC in comparison with non infected mice after 60 days. This study concluded that the Balb/c mouse model have good indicator for VL resemble to human VL, and conventional PCR with small 18S rRNA gene have high sensitivity for detection Leishmania donovani in the liver and spleen tissues of mice.

Key words: *Leishmania donovani*, clinical signs, PCR.

INTRODUCTION

Visceral leishmaniasis (VL) is a protozoan infectious pathogen transmitted via the insect vector dwell the reticulo-endothelial device[1].Often the disease is deadly where the disease is progressive if left without treatment [2,3]. Is caused by *L. donovani* spp complex depending on geographical zone [4].

Because of the visceral leishmaniasis parasites are dispersed throughout the lymphatic and vascular systems and are hitting other cell in the reticulo-endothelial system as monocytes and macrophages leads to infiltrate the bone marrow, enlarged of spleen and liver (hepatosplenomegaly), sometimes enlarged lymph nodes (lymphadenopathy) [4].

Most studies in Iraq showed numeral clinically significant such as Fever, Splenomegaly, hepatomegaly, Weight loss, Anemia, Cough, Diarrhea, Jaundice, Leukopenia, Abdominal pain, decrease in hemoglobin ratio, white blood cell counts, packed cell volume, increase monocytes, vomiting, bleeding tendency, and lymphoadenopathy as [5,6].

The parasite persists as a chronic infection in the spleen with gradual destruction of spleen architecture [7,8].

Splenic smears have high sensitivity, but are associated with a risk of serious/fatal haemorrhage, while bone marrow smears have low sensitivity and the procedure is painful [9].

Alvar[10] was mentioned that approximately 500,000 people are infected with VL in endemic regions and the disease is accompanied by 20,000 - 40,000 deaths per year.

According to WHO[2] 350 million people are prone to the infection with VL and the disease is endemic in 76 countries in East Africa, South and East Asia and Latin America, above 90 percent of cases located in 6 countries (Baingladesh, India, Eithiopia, Sudan and South Sudan , Brazil), and recently about 300000 new cases are reported yearly.

The available methods for the diagnosis of VL involve several techniques, such as demonstrating the parasite in tissues, *in vitro* culture, animal inoculation, serological and molecular techniques [9,11].

Deborggraeve [12] indicate that PCR alone rather provides a marker for infection than a marker for disease and its role in VL diagnosis in endemic regions.

The PCR assay developed is sensitive enough to detect the 18S rRNA gene in an amount equivalent to a single parasite or less in a one million human cell environment. The high sensitivity of this PCR diagnostic test with relatively non invasive peripheral blood sampling method opens up the possibility of its deployment in field for the routine diagnosis of VL [13].

PCR has been applied successfully in recent years to detect *Leishmania* spp. in cases with any of the clinical manifestations of leishmaniasis. Some PCR methods also allow differentiation between parasite strains, which can facilitate more-efficient treatment [14].

Here with the objective of this study was to evaluate the efficacy of conventional PCR with small - sub unit18S rRNA gene of the liver and spleen tissues in mice infected with *Leishmania donovani*, also to indicate the BALB/c mice as a good model for VL.

METHODOLOGY

Prepare and induction dose of parasite

Pure isolation and diagnosed of *Leishmania donovani* promastigotes were cultured at 26 °C in diphasic media (NNN medium). Subsequently, also promastigotes were cultured in RPMI medium((RPMI)- 1640 Medium powder (Hepes, L. glutamine and sodium pyrovate), 16.4g of it was dissolved in approximately 700 ml of distilled water and then the other component was added (Sodium bicarbonate (4.4%)15 ml, Ampicillin 0.5 ml, Gentamicin 0.25 ml, and Nystatin 0.25 ml, 10% of either fetal calf serum or newborn bovine calf serum, the PH was adjusted to 7, the medium was sterilized by filtration with 0.22 µ Millipore and was stored at – 20ºC) in order to subject to PCR technique as control specimen.

The promastigotes were cultivated in NNN medium at 26±1˚C.Stationary phase promastigote was sub-cultured every 72 hr–96 hr in new media. When the parasite culture equal the growth, stationary phase promastigote was harvested and washed in RPMI medium by centrifuged it at 1500 rpm for ten minute, then supernatant was removed by Pasteur pipettes while the sediment was resuspended in the appropriate size of RPMI medium approximately 5 ml. The number of promastigotes per ml were determined by counting in hemocytometer and the concentration adjusted to 1.2×10^6 cells of parasite.

Promastigotes parasites in 200 µL[15], were inoculated / mice by intrapretonial administrations, then (*L. donovani* –infected BALB /c mice) were maintained in the animal house for 6 - 8 weeks prior to apply the experiment. Liver, spleen, and blood culture were done, and impression smears were done as following :imprints were made on glass microscope slides from the lateral side of one wedge of liver and the butt end of the other after they had been touched on Whatman No.1 filter paper five times. Imprints were also made from the cut spleen surface [16]. Imprints were fixed in methanol for (0.5-1) minute and stained with Giemsa stain for (35) minutes, then washed with water and left to dry [17].

Impression smears"(in terms of Leishman–Donovan units (LDU))were used to calculate the load of amastigote of parasite in

liver and spleen organs using the formula: LDU = [(number of parasites/1000 host nuclei) x organ weight in milligrams (mg)], amastigotes were calculated by optical microscope under oil lens 100 x [16]. Induction of hepato-splenomegaly was assessed using liver and spleen indices" which were "calculated using the formula: Organ index = (organ weight in mg/total body weight in mg) x 100 [18].

Experimental animals

During this study, the mature male mice *(Mus musculus*) of Balb /c strain were used. The animals were purchased from Department of Biology- Faculty of Science/ Thi-Qar University-Iraq. Their aged between 6 to 8 weeks weighing 25-30 g. The all animal was maintained in animal house of the Department of Biology - Faculty of Science / University of Kufa-Iraq, in individual plastic cages, each cage was contained eight "animals. These cages were kept at 21 to 25 °C, under a 12-h dark and light cycle. Animals were given water and a standard pellets feed ad libitum. The procedures adopted in this study were in accordance with [19,20].

The overall number of animals used to be 80. All mice were weighed in animals balance. Animal were anesthetized use a combinations of ketamiane at 24 mg/kg plus xylaziane at 12 mg/kg [21] , and then they were sacrificed . Liver and spleen were weighed by sensitive balance. Blood was within drawn by cardiac punctures by using a 1 ml disposable syringe for determination different blood parameters. About 1 ml of fresh blood was added to each ethylene diamine tetra acetic acid (EDTA) tube. The measurements of the erythrocytes (RBC), hemoglobin (Hb), leukocytes (WBC)were made in an automatic cell counter (AutoHematology analyzer). The results were provided within 5 minutes on the LCD display, printed out on the printer and stored in the resident memory.

Molecular study:

The PCR assay was performed to detect the (18S rRNA) gene for confirming the identification of *L. donovani* according to [12]. The 18S rRNA primer was used in this study was F: 5¢-CGTAGTTGAACTGTGGGCTGTGC-3¢ R:5¢ACTCCCGTGTTTCTTGTTTCTTTGAA-3¢. This primer which was provided as lyophiliized form were dissolved in steriles deionized distill water to gives a final concentrations100 picomole /µl according to recommendation of provider and were kept in a deep freezer to use through practice. The genomic DNA was obtained through the genomic DNA purification kit (FAVORGEN kit).). Gel electrophoresis was used for detection of DNA by UV transilluminator.

Extraction of Genomic DNA from mice tissue and cultured cells (*L. donovani*).

Genomic DNA was extracted from mice tissues using a commercial extraction system (Tissue Genomic DNA extraction mini kit) with Proteinase K (10mg/ml)**.** And the extraction was applied according to the Tissue Protocol was mentioned in this kit. The cultured cells which were grown in RPMI culture media were harvested also according to (Tissue Genomic DNA extraction mini kit), then The Tissue Protocol applied to extract DNA from them.

The reaction mixture was carried out in a volumes of 20µl (5µL Master mix, 5µL DNA template, 2.5µL Forward primers, 2.5µL Reverse primers and 5µ L Deionidied water (d d water) according to information of (Bioneer company/Korea). The PCR program of 18S rRNA primer that apply in the thermocycler was described as following(95 Cº for 15 min Initial denaturation, 45 No.of cycles, 95 Cº for 30 second Denaturation, 60 Cº for 30 second Annealing, 72 Cº for 45 second Extension and finally Final extension 72Cº for 5 min)[12].

Gel electrophoresis was used for the detection of DNA fragments, PCR products which visualized with the aid of ethidium bromide and UV transilluminator and photographed by using digital Camera [22]. The DNA of reference strain *L. donovani* was used as a positive control, and sterile deionized distill water was used as the negative control".

Statistical analysis:- Analysis of data was performed by using Statistical Package for the Social Sciences "SPSS-version 23". The results were expressed as (mean ± standard Deviation). One way analysis of variance "ANOVA" followed by least significant difference "LSD" was used for the statistical comparison between infected mice with different periods and non infected mice.. Statistical significance was accepted at the P≤ 0.05 values [23].

RESULTS

This study, conducted in the laboratories of a faculty of Science / university of Kufa in the period between October 2017 – February 2018. A total of 80 mouse tissues was subjected to PCR amplification to record sensitivity of PCR with 18S rRNA for detecting *L. Donovani* in the liver and spleen mice, this study shows when was quantified load of amastigote parasite in tissue after 45 and 60 days from inoculum the amastigote significantly increased (p≤ 0.05) in the number of infected mice tissues (liver and spleen) (table 1), and the (figure 1) indicates high load of amastigote in spleen, also (figure 2) shows large in size of spleen in comparison with non infected mice. And there were significantly increased (p≤ 0.05) in the liver and spleen weight of infected mice after 45 and 60 days from inoculum where the liver recorded 1.60±0.20 and 1.66±0.11g respectively, and the spleen recorded 0.51±0.24 and 0.55±0.21g respectively in comparison with non infected mice were 1.13±0.11g of the liver and 0.08±0.005of spleen (figure 3 and 4). On the other hand the result, observes a significant reduction (p≤ 0.05) in Hb and RBC and WBC after 60 days of infection were 8.83 ± 1.04, 4.70 ± 3.01 and 1.56 ± 0.72 respectively in comparison with non infected mice were 11.43 ± 0.66, 8.59 ± 0.12 and 5.7 ± 0.43 respectively (figure 5, 6, and 7).

Table 1:- Load of amastigote of *Leishmania donovani* parasite of the liver and spleen after 45 and 60 days of inoculum.

	45 days	60 days
Liver	3985.92 ± 461.24 *	4883.42 ± 708.44 *
Spleen	3127.16 ± 1293.15**	3597.13 ± 1479.52b**

Sig.* p≤ 0.05 refer to significant differences between the periods of load of liver
Sig.** p≤ 0.05 refer to significant differences between the periods of load of spleen

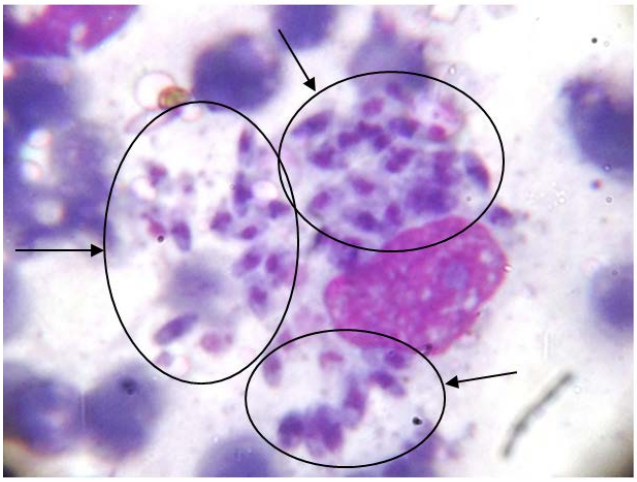

Figure 1:- Large number of amastigote of *Leishmania* parasite in spleen .

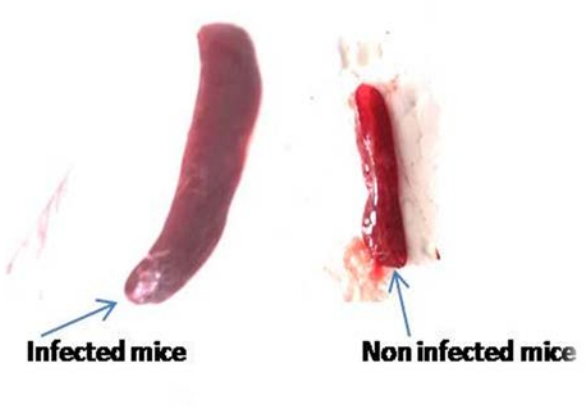

Figure 2:- Massive large in size of spleen in infected mice with *Leishmania donovani* comparison with non infected mice.

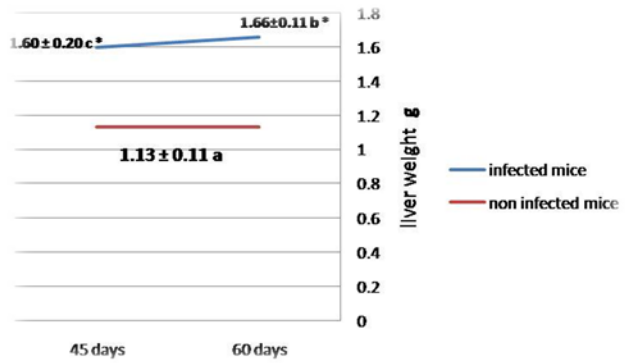

Figure 3:- Liver eight of mice infected with *Leishmania donovani* after 45 and 60 days of inoculum comparing with non infected mice**.**
Sig.* p≤ 0.05 refers to significant differences between the periods.
Sig.[a b c] p≤ 0.05 refers to significant differences between the infected and non infected mice.

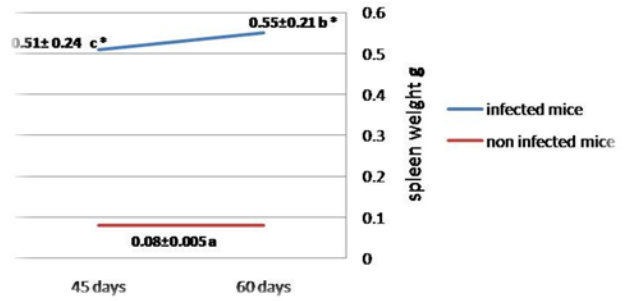

Figure 4:- Spleen weight of mice infected with *Leishmania donovani* after 45 and 60 days of inoculum comparing with non infected mice**.**
Sig.* p≤ 0.05 refers to significant differences between the periods.
Sig.[a b c] p≤ 0.05 refers to significant differences between the infected and non infected mice.

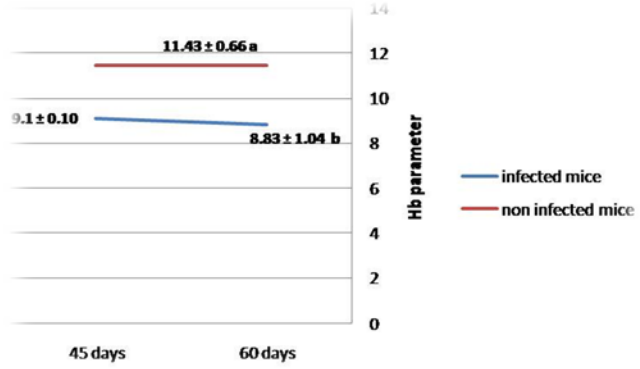

Figure 5:- Hb parameter of mice infected with *Leishmania donovani* after 45 and 60 days of inoculum comparing with non infected mice**.**
Sig.[a b] p≤ 0.05 refers to significant differences between the infected and non infected mice.

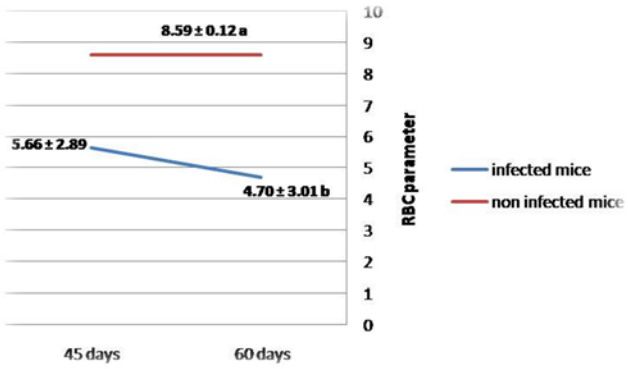

Figure 6:- RBC parameter of mice infected with *Leishmania donovani* after 45 and 60 days of inoculum comparing with non infected mice**.**
Sig.[a b] p≤ 0.05 refers to significant differences between the infected and non infected mice.

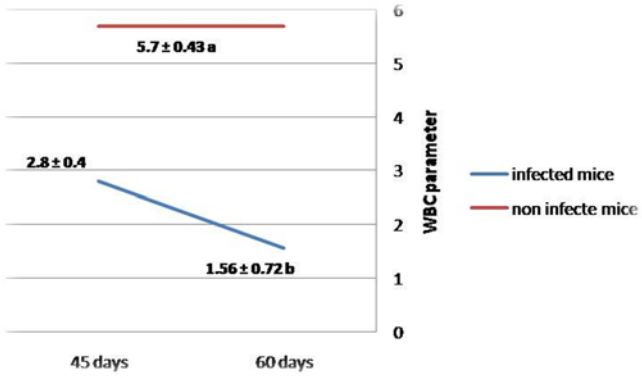

Figure 7:-WBC parameter of mice infected with *Leishmania donovani* after 45 and 60 days of inoculum comparing with non infected mice**.**
Sig.[a b] p≤ 0.05 refers to significant differences between the infected and non infected mice.

Molecular study

The DNA extract with 18S rRNA which amplify at product 115 bp and marker 100 bp were occurring in this study and in which shows positive results in 30 specimens (75%) of liver and 26 specimen (65%) of spleen of infected mice after 45 and 60 days, (table 2) and (figures 5).

Table 2: Positive sample of the liver and spleen for infected mice.

organs / period	liver		Liver		Total		spleen		Spleen		Total	
	45 days		60 days				45 days		60 days			
Total	No/ 20	%	No./20	%	No./40	%	No./20	%	No./20	%	No./40	%
80	16	80	14	70	30	75	11	55	15	75	26	65

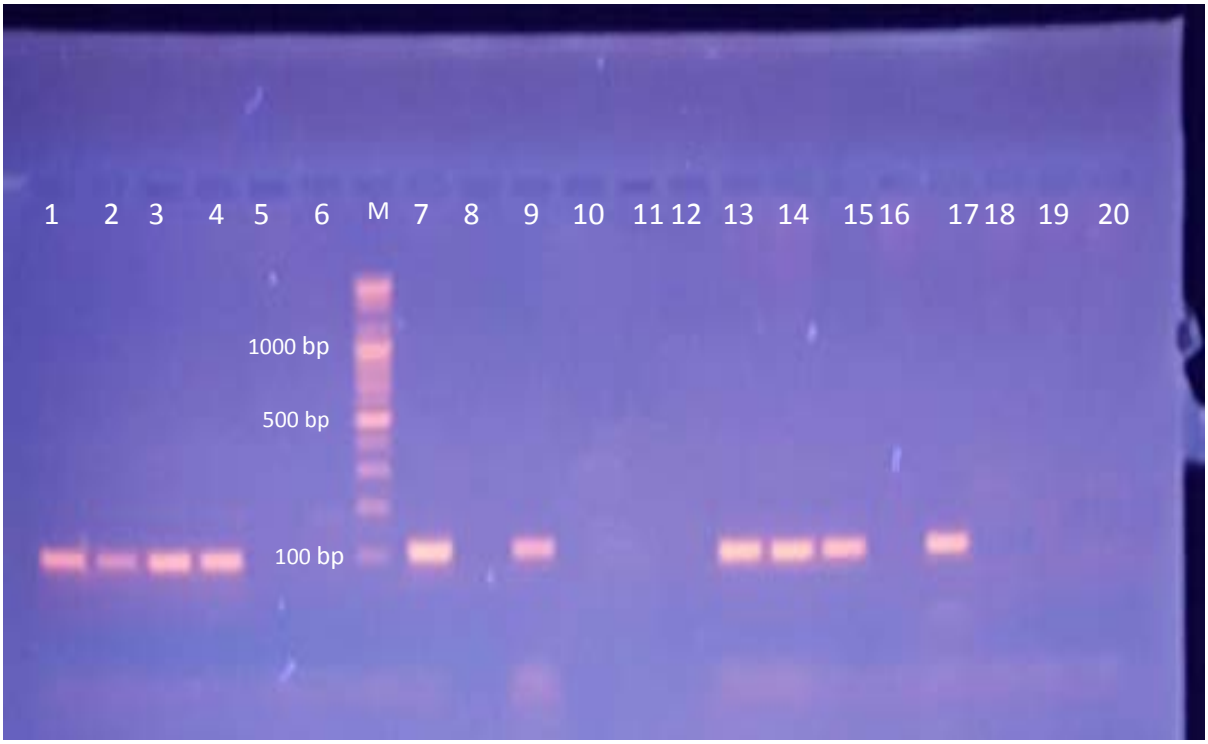

Figure 8:- Ethidium bromide-stained with 1.5% agarose gel for a DNA extract from mice tissues (liver and spleen) amplification with 18S rRNA gene-specific PCR technique at product 115 and marker 100 bp (line M) shows present positive specimen (line 1, 2, 3, and 4 positive spleen specimen), line (7 and 9 positive control) and (line 5, 6, and 8 negative controls) and (line 13, 14, 15and 17 positive liver specimen) and (line 10, 11, 12, 16, 18, 19, 20 negative specimen).

DISCUSSION

Because the reticuloendothelial system is the objective of parasitization the clinical manifestations include hepatomegaly and the spleen becomes much enlarged due to the amastigote is present and proliferate in the macrophage system, especially the liver, spleen, and marrow, this leads to hypertrophy of the macrophage system with resultant troubles in organs which bearing phagocyte, produce blood disorders. Hence, this case is important to hematopathologists [24].

Imprint smears, and other tests were done in infected mice after 45 days and 60 days for injection parasites, also are observed large sized spleen.

Over time infection with *Leishmania donovani* is induced (hepatosplenomegaly) in BALB/c mice. where the liver and the spleen of the infected mice be an much larger than those of noninfected mice [25].

Infective burden were limited by liver and spleen organs [26]. the important in quantifying parasitism is to understand the pathological changes and clinical appearances and that happened through the normal date of experiential VL [27]. In this study, the parasite load was elevated in the liver more than spleen as shown in table (1). This result agrees with [28] when they showed higher parasitism in the liver compared with the spleen during they used using hamster experimentally infected with *L. infantum* via the IC route and with [29] when they showed the amastigote highly increased in liver after 52 days post infection.

This result is in disagreement with [27], where they calculated *L. infantum* parasite load by using real time PCR and demonstrated that the spleen more intensely than liver. Also, it is in disagreement with [30] when they demonstrated *L. infantum* parasite loads high in spleen and low in the liver of dogs. This results show significant elevation ($p \leq 0.05$) of liver and spleen weight between positive control and negative control (figures 1 and 2).

Lafuse [31] observed that the spleen weight increases 20 folds in infected hamster in comparison with non infected hamster after 8 weeks post infection, also [25] showed significant elevation in liver and spleen weight in infected mice compared with non infected mice.

The decline of Hb values was showed in positive mice in the present study with significant difference ($p \leq 0.05$) between positive mice and non infected mice (figure 5), also, there was a reduction of RBC and of WBC between infected mice and uninfected mice (figures 6 and 7). One of the major presenting features in untreated visceral leishmaniasis is anemia [32] .

This result disagreement with [25] they showed no significant difference of Hb and RBC between infected mice after 12 weeks post infection and non infection mice, but they observed severe anemia and decrease in RBC after 24 weeks post infection. Lafuse [31] demonstrated severe anemia and decrease in RBC and WBC in infected hamster after 8 weeks post infection in comparison with non infected hamster.

The most common blood disorder (hematological manifestation) in VL is anaemia. VL can be connected with disseminated intravascular coagulation, pancytopenia, hemophagocytosis, thrombocytopenia, and lycopene [24].

Molecular methods based on PCR or other amplification techniques are increasingly used in both diagnostic and epidemiological studies, and are shown to be more sensitive than the conventional methods for the diagnosis of leishmaniasis[33]. This study was conducted to evaluate the sensitivity and specificity of the 18S rRNA primers in a PCR assay in VL of mice, The subunit ribosomal RNA sequence has been found to be highly sensitive for the confirmation of leishmaniasis from peripheral blood and tissue biopsy specimens [34,35,36]. Several target sequences have been used, including kinetoplastid DNA, rRNA, mini-exon-derived RNA genes, the â-tubulin gene region, glycoprotein 63 (gp63) gene locus and internal transcribed spacer (ITS) regions [37].. many studies were showing that a method based on small subunit gene 18S rRNA gene DNA amplification was the most sensitive as [12,13] study. This result shows high percent in

detection the parasite in the liver and spleen tissues by conventional PCR technique (table 2), Molecular assays have been demonstrated as important diagnostic tools [38]. These can be performed on bone marrow, splenic, lymph node smears, peripheral blood and serum samples [39]. This result agrees with [12,13] when they used small sub unit gene 18S rRNA gene for diagnosis VL patient's peripheral blood and bone marrow. The PCR assay developed is sensitive enough to detect the 18S rRNA gene in an amount equivalent to a single parasite or less in a one million human cell environment. The high sensitivity of this PCR diagnostic test with relatively noninvasive peripheral blood sampling method opens up the possibility of its deployment in field for the routine diagnosis of VL [13].

Nicolas[40] mentioned that a few samples negative by the conventional PCR were positive by the real-time PCR with more sensitive of real-time PCR than conventional PCR.

In conclusion, this study demonstrates that the Balb/c mouse model has good indicator for VL resemble to human VL, and conventional PCR with small 18S rRNA gene have high sensitivity for detection *Leishmania donovani* in the liver and spleen tissues of mice.

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