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Biochemical Markers, Trace Elements, and Vitamins in Cutaneous Leishmaniasis

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Abstract

General Background: Cutaneous leishmaniasis is a prevalent parasitic disease in endemic regions and is increasingly recognized as a systemic condition associated with metabolic and nutritional disturbances beyond skin manifestations. **Specific Background:** Alterations in liver function, lipid metabolism, trace elements, and vitamin status have been reported in parasitic infections; however, integrated biochemical and molecular evidence in cutaneous leishmaniasis remains limited. **Knowledge Gap:** There is insufficient empirical data linking molecular identification of *Leishmania* species with comprehensive biochemical, trace element, and vitamin profiles in affected patients. **Aims:** This study aimed to evaluate biochemical markers, trace elements, and vitamins in patients with cutaneous leishmaniasis and to confirm *Leishmania* species using PCR. **Results:** Compared with controls, patients showed significantly elevated liver enzymes (AST, ALT, ALP) and lipid parameters, reduced iron and zinc levels, increased copper levels, and significantly lower vitamin D3 and B12 concentrations. Nested PCR identified *Leishmania tropica* as the predominant species, followed by *L. major*. **Novelty:** The study integrates serological, nutritional, and molecular data, providing a holistic profile of systemic alterations associated with cutaneous leishmaniasis. **Implications:** These findings underscore the importance of biochemical and nutritional monitoring alongside molecular diagnosis to improve clinical management and therapeutic strategies for cutaneous leishmaniasis.

Highlight :

- Cutaneous leishmaniasis was associated with significant elevations in liver enzymes and serum lipid levels compared to controls.
- Marked alterations in trace elements were observed, characterized by reduced iron and zinc levels alongside increased copper concentrations.
- Patients exhibited significantly lower vitamin D3 and B12 levels, indicating a potential link between nutritional status and disease severity.

Keywords : Leishmania, Liver Enzymes, Lipid Profile, Trace Elements, Vitamins

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Introduction

Cutaneous leishmaniasis is a parasitic disease caused by a protozoan belonging to the haemoflagellate genus Leishmania, which is an obligate intracellular parasite of the reticuloendothelial system of the skin (1). It is widespread globally and, according to the World Health Organization, poses a significant health problem for humans, particularly in developing countries(2). Many mammals, including humans, are infected with these parasites, which are transmitted through the bite of the sand fly (3). The genus Phlebotomus is the most important, encompassing species that attack humans and animals, feeding on their blood and thus contributing to the transmission of various diseases, including leishmaniasis (4). Wild and domestic animals are the primary reservoir hosts, although in some cases, humans are the reservoir host, as in the disease caused by Leishmania tropica strains. Other, less common, routes of transmission exist, primarily through blood (5). Cutaneous leishmaniasis, the most common, is caused by L. tropica, leading to scarring skin lesions (6).The disease is characterised by the formation of skin lesions that tend to heal spontaneously within 2-3 months, with an incubation period of 1-4 months. Symptoms begin with the appearance of erythematous papules, which may develop into ulcers as the disease progresses(7).Although the ulcers of cutaneous leishmaniasis are often asymptomatic, a burning sensation is common, reflecting an inflammatory response in the host tissues(8). This disease affects males more than females, and an increase in leishmaniasis cases has been observed in individuals suffering from anaemia. Malnutrition plays a major role in increasing susceptibility to infection, in addition to the increasing incidence resulting from environmental changes and the continued large-scale migration from rural to urban areas (9). Humans become infected when their immune system is weakened and therefore unable to kill the Leishmania parasite (10).Cutaneous leishmaniasis has appeared in Iraq, specifically in the Adhamiya district of Baghdad Governorate, particularly among children aged two to four years, and is locally known as Baghdad boil (11). Highly sensitive and specific molecular techniques based on the detection of parasite DNA in the polymerase chain reaction (PCR) targeting coding and non-coding regions of the Leishmania genome have been successfully applied in recent years to detect Leishmania species. It is a rapid reaction that outperforms traditional methods used to identify the parasite (12)

Materials and Methods

Samples were collected from Tikrit Teaching Hospital from patients with cutaneous leishmaniasis from October 2023 to January 2024 A total of 60 samples were collected from various age groups of both sexes, ranging from 2 to 60 years old. *Leishmania* parasites were isolated from the skin after diagnosis by a specialist physician. The affected area was sterilised with 70% ethanol for three minutes, and 2% lidocaine was injected using an insulin syringe after the scab was removed. The lesion was then incised with a curved scalpel at the edges and center until the tissue material was visible on the blade. The blade was then gently moved across the surface, and the tissue was placed on a glass slide. The sample was fixed to the slide by adding two drops of methanol to the slide and allowing it to dry for 2-5 minutes. It was then stored in a slide holder. Five millilitres of venous blood were withdrawn using a sterile syringe, and two millilitres of blood were placed in test tubes containing an antibody. (EDTA) (ethylene diamin tetra acetic acid) was used for the purpose of measuring molecular tests. The other 3 ml were placed in test tubes free of the anticoagulant in order to obtain serum using a centrifuge at a speed of 3000 rpm for 10 minutes. After that, the serum was withdrawn using a micropipette, and the serum was divided into Eppendorf tubes with a volume of 1.5 ml per tube. The samples were kept frozen at -20°C until the serological and molecular tests were carried out. The tests were carried out in the central laboratories of Tikrit University.

A. Serological test:

Serological tests were measured using a spectrophotometer according to the manufacturer's instructions for the Spectra Liver Function, Lipid and Trace Element Tests. Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure the levels of Vitamin D3 and B12 in the serum of people with cutaneous leishmaniasis and compare them with the uninfected control group using Biolab assays according to the manufacturer's instructions.

B. Molecular Identification:

DNA was extracted from blood samples of each person infected with cutaneous leishmaniasis at the central laboratories of Tikrit University using the Genomic DNA Extraction Kit, according to the manufacturer's instructions and supplied by the German company Sacace. The extracted DNA was detected using a Nanodrop Spectrophotometer, which is used to detect the concentration of nucleic acids (DNA and RNA). The concentration of DNA (ng/µl) is determined, and the purity of the DNA is measured by reading the absorbance at a wavelength between 260 and 280 nm. When the absorbance ratio is between 1.8 and 2, the extracted DNA is pure. The samples were then electrophoresed to evaluate the quality, purity, and quantity of the extracted DNA before it was used in the PCR reaction. Primers specific to the KDNA gene, which are responsible for diagnosing the types of *Leishmania cutaneous* parasite and supplied by (Bioneer, Korea) were used as shown in Table (1) below:

Table (1): primers used in this study with their nucleotide sequence

Target gene	Primer	Sequence primer		Amplification product(bp)
KDNA	CSB2XF Forward	**F**	5'ATT TTT CGC GAT TTT CG C AGA ACG 3'	bp (13)750
	CSB2XR Reverse	**R**	5' CGA GTA GCA GAA ACT CCC GTT C A 3'	
	13Z Forward	**F**	5`ACT GGGG GGT TGG TGT AAA ATA G 3`	bp (13) 560
	LI Reverse	**R**	5`TCG CAG AAC GCC CCT 3`	

F: forward **R:** reverse

Afterwards, nested kinetoplast minicircle DNA-PCR was performed using primers for the KDNA gene, and electrophoresis was performed using

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a 2% agarose gel to read the polymerase chain reaction rate. After the electrophoresis process was completed, the plate was removed from the tank and placed under UV transillumination by exposing it to a wavelength of 260 nm to observe the amplification products. Then, it was photographed, and the molecular weights of the extracted DNA were estimated based on the distance travelled in the gel and compared with the volumetric index of the DNA. The molecular weights of the fragments that resulted from the PCR technique were estimated by comparison with the volumetric index specific to the PCR results (DNA Ladder 1500 bp) (14).

Results and Discussion

A. Serological test:

1.1 The effect of cutaneous leishmaniasis on liver enzymes:

The results of the study examining serum liver enzyme levels in individuals infected with cutaneous leishmaniasis showed a significant increase in aspartate aminotransferase (AST) levels 210.87 ± 119.22 IU/L compared to the uninfected control group 27.23 ± 8.15 IU/L. Similarly, alanine aminotransferase (ALT) levels were also significantly elevated ($p \leq 0.001$) in the serum of individuals infected with cutaneous leishmaniasis 5978.23 ± 2262.69 IU/L compared to the uninfected control group 27.19 ± 8.09 IU/L. A significant increase in alkaline phosphate (ALP) levels ($p \leq 0.001$) was also found in the serum of individuals infected with cutaneous leishmaniasis 585.19 ± 165.47 IU/L. The enzyme (ALP) in the serum of uninfected individuals is 85.11 ± 15.22 IU/L as shown in Table (2).

Table(2): Liver enzyme concentrations of patients with cutaneous leishmaniasis compared to the control group

Liver enzyme	Patients Mean ± S.D N=60	Control Mean ± S.D N= 30
AST	210.87 ± 119.22 a	27.23 ± 8.15 b
ALT	5978.23 ± 2262.69 a	27.19 ± 8.09 b
ALP	585.19 ± 165.47 a	85.11 ± 15.22 b

M: mean **S.D:** standard deviation **N:** Number of samples

***The different letters indicate significant differences at a probability level of ($p \leq 0.001$) between the rates of the different groups**

The results of the current study are consistent with those of (15) in Samarra, which showed elevated liver enzymes in individuals with cutaneous leishmaniasis compared to healthy individuals. They also agree with the findings of (16) regarding elevated AST and ALP levels in their study investigating the impact of diabetes and cutaneous leishmaniasis on hematological, immunological, and biochemical variables. This study involved conducting several tests on leishmaniasis patients without diabetes. The elevated AST enzyme in the blood serum of infected individuals is attributed to its presence in the cytoplasm and mitochondria of liver cells, as well as skeletal muscle cells, cardiac muscle, kidney, and pancreas. Any damage or death to the cells comprising these organs leads to the appearance of this enzyme in the bloodstream within 8 hours. Following the damage, it reaches its peak within 24-36 hours and returns to normal after 4-7 days. The increase in this enzyme is a result of the *Leishmania* parasite invasion, which possesses certain enzymes that break down tissue, leading to the release of this enzyme into the bloodstream and consequently increasing its concentration. (17) This elevation may be attributed to its association with other biological changes occurring within the body, some of which were caused by leishmaniasis infection. Among these changes, zinc deficiency resulting from leishmaniasis may be a cause of the elevated ALT enzyme, given their crucial role in the activity and effectiveness of these enzymes. Zinc deficiency can also lead to non-alcoholic fatty liver disease. The relationship between zinc deficiency and non-alcoholic fatty liver disease is close (18). The elevated ALT enzyme may also be due to some patients from whom samples were taken having a history of infection or to the use of anti-leishmaniasis treatment, which led to an increase in this enzyme. In some studies, the researcher (19) found elevated liver enzymes in his study on the effect of pentavalent antimony compounds used in the treatment of cutaneous leishmaniasis on blood profiles and biochemical variables after treatment of the infection. This resulted in elevated ALT and AST enzymes in the blood serum. ALP is an enzyme found in the bile ducts of the liver and in the intestines, and it is an indicator of liver disease. High levels of ALP can result from bile duct obstruction or damage due to the parasite, and these tests can reveal many details about a range of pathological changes (20).

B. The effect of cutaneous leishmaniasis on lipids

The concentration of both those infected with cutaneous leishmaniasis and the control group was measured, and the results showed an increase in the concentration of cholesterol, triglycerides, and High-density lipoproteins at a significance level of $p \leq 0.001$, which amounted to 647.81 ± 146.30 mg/dl, 532.71 ± 179.70 mg/dl, and 264.90 ± 104.63 mg/dl, respectively, compared to the uninfected control group, which amounted to 115.14 ± 27.15mg/dl, 107.97 ± 13.98 mg/dl, and 36.62 ± 4.80 mg/dl, respectively, as shown in Table No (3).

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Table(3): Lipid concentration levels in patients with cutaneous leishmaniasis compared to the control group

Lipids profile	patients Mean ± S.D N=60	Control Mean ± S.D N= 30
Cholesterol	647.81 ± 146.30 a	115.14 ± 27.15 b
Triglycerides	532.71 ± 179.70 a	107.97 ± 13.98 b
HDL	264.90 ± 104.63 a	36.62 ± 4.80 b

*** The different letters indicate significant differences at a probability level of (p ≤ 0.001) between the rates of the different groups**

The results of the current study are consistent with those of study (15) in Samarra, which showed elevated lipid levels in individuals with cutaneous leishmaniasis compared to healthy individuals. These results also align with findings (21) regarding certain haematological and biochemical parameters in leishmaniasis patients in Dhi Qar, specifically elevated levels of Cholesterol, Triglycerides, and HDL,

reaching 207 mg/dl, 177 mg/dl, and 81 mg/dl, respectively. Cholesterol is a key factor in leishmaniasis infection due to its role in cell membrane dynamics. It is involved in the formation of cell membranes in all tissues of living organisms, and cholesterol metabolism is susceptible to either a decrease or an increase in the disease (22). Lipid and fatty acids are essential for the parasite's survival, as they are the main energy source for completing the parasite's life cycle. *Leishmania* infection leads to changes in some cellular metabolic pathways, and the most affected pathways are the metabolism of fats and fatty acids (glycerolipids, fatty acids, and carbohydrates) as a result of the interaction between the parasite and the host cell's plasma membrane (23).Triglycerides are important because they provide an energy source for the parasite. Triglyceride molecules can accumulate together and are thus stored in adipose tissues, where they are incorporated into lipoprotein molecules along with other components such as cholesterol and phospholipids and are transported in the aqueous medium of the plasma (24). *Leishmania* is an obligate intracellular parasite that depends on the host's fat stores to ensure its survival. It uses fats for energy and thus escapes the immune system. This alters the host's fat metabolism in some way. *Leishmania* uses the host's cholesterol to ensure phagocytosis and avoid an immune response. In addition, the host's lipocytes play key roles in disease development and parasite development within the cell, leading to changes in the patient's serum lipid levels, such as elevated HDL lipids (25).

C. The effect of cutaneous leishmaniasis infection on trace elements levels:

The studied results showed a difference in the level of elements present in the serum of people infected with cutaneous leishmaniasis compared to healthy people. It showed a decrease in the concentration of iron by 31.50 ± 8.01 mg/dl, while its level in healthy people was 102.67 ± 31.10 mg/dl. An increase in the level of copper was observed in infected people, reaching 184.65 ± 21.88 mg/dl, while its level decreased in healthy people to 77.37 ± 17.19 mg/dl. As for zinc, its level decreased in the serum of infected people to 32.97 ± 13.05 mg/dl, compared to healthy people whose zinc level was 105.91 ± 8.51 mg/dl. Through statistical analysis, it was found that there are significant differences at the significance level of p ≤ 0.001 between the results of infected and healthy people, as shown in Table (4).

Table(4): Trace element levels in patients with cutaneous leishmaniasis compared to the control group

trace elements	patients Mean ± S.D N=60	Control Mean ± S.D N= 30
Iron (Fe)	31.50 ± 8.01 a	102.67 ± 31.10 b
Copper (Cu)	184.65 ± 21.88 a	77.37 ± 17.19 b
Zinc (Zn)	32.97 ± 13.05 a	105.91 ± 8.51 b

*** The different letters indicate significant differences at a probability level of (p ≤ 0.001) between the rates of the different groups**

The results of the current study are consistent with those of study (26) in Al-Qadisiyah Governorate, Iraq, which investigated cutaneous

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leishmaniasis, and also with study (27) in India. Both studies observed a decrease in serum iron concentration in patients with cutaneous leishmaniasis compared to the control group. Iron plays a crucial role in the parasite-host interaction, facilitating intracellular replication, increasing parasite numbers, and spreading the parasite. Parasites require nutrients from the host to support their growth and compete with it for nutrient availability (28). Thus, parasites have developed various mechanisms to compete with the host for nutrients. Iron is one of the essential nutrients for both the host and the parasite. The parasite requires iron to cause infection, spread the disease, and resist the host's defences. This triggers the acute phase response of the liver proteins to reduce iron depletion and decrease iron absorption (29). Study (30) elucidated the pathway for iron absorption and digestion by the parasite, demonstrating that Leishmania lacks a stable pathway for iron synthesis and therefore relies on heme in the host's blood. This finding is consistent with study (31), which found a decrease in zinc levels and an increase in iron levels. Copper deficiency was observed in patients with intestinal parasites and cutaneous leishmaniasis in the city of Al-Sharqat. Therefore, (32) emphasized the necessity of administering zinc supplements to patients with skin diseases and cutaneous leishmaniasis as a beneficial treatment due to its antioxidant and immunomodulatory properties, which are associated with effectiveness in treating diseases, including cutaneous leishmaniasis. The study's results were consistent with those of (33)regarding the low zinc levels in individuals with cutaneous leishmaniasis. Studies indicated that the low zinc levels in individuals with cutaneous leishmaniasis are due to the effect of methylathionine, which is synthesised in the liver and other tissues. Metallothionein has a high capacity to bind to zinc atoms in the bloodstream. This binding process is stimulated by the cytokinetic agent Interleukin-1 (IL-1), the levels of which are elevated in individuals infected with the cutaneous leishmaniasis parasite as a result of the immune response. Elevated serum copper levels in infected individuals are linked to ceruloplasmin, a glycoprotein bound to 6-8 copper atoms. Ceruloplasmin binds to a large proportion of the copper in the blood serum, preventing its excretion by the kidneys in urine. This is stimulated by interleukin-1 (IL-1), which is secreted by immune cells to fight infections. Elevated IL-1 levels are attributed to elevated serum copper in infected individuals. Approximately 90% of the copper in the blood is stored as ceruloplasmin. Therefore, elevated copper levels can be observed in many infections that affect this protein (34). Trace elements are essential components of the human body, participating in numerous vital functions such as growth and development. They are also crucial for the immune system and are involved in the synthesis of hundreds of important enzymes (35).This elevation is considered a physiological response and not necessarily indicative of increased copper intake. This often explains the findings in studies that show elevated copper levels in patients with cutaneous leishmaniasis.

D. The effect of cutaneous leishmaniasis infection on vitamin D3 and B12 levels

The results of the current study, as shown in Table (5), indicate a significant decrease at a probability level of $p \leq 0.001$ in the values of vitamin D3 levels in both the infected and control groups. The lowest value was in the group of patients with cutaneous leishmaniasis, reaching 20.82 ± 6.61 ng/ml, which is much lower than the control group, which reached 26.92 ± 8.21 ng/ml. As for the vitamin B12 level, Table (5) showed a decrease in both the infected and control groups at a significance level of $p \leq 0.005$, as it reached 20.36 ± 5.20 pg/ml compared to the control group, which reached 24.99 ± 7.63 pg/ml.

Table(5): Vitamin levels of patients with cutaneous leishmaniasis compared to the control group

Vitamins	**patients** **Mean ± S.D** **N=60**	**Control** **Mean ± S.D** **N= 30**
Vitamin D3	20.82 ± 6.61 a	26.92 ± 8.21 b
Vitamin B12	20.36 ± 5.20 a	± 7.6324.99 b

***Different letters indicate significant differences at the probability level ($p \leq 0.001$ and $p \leq 0.005$) between the rates of the different sums**

The study's findings are consistent with those of (36), which showed that patients with cutaneous leishmaniasis, especially those who did not receive vitamin D3 supplements, had significantly lower vitamin D3 levels compared to healthy individuals. Vitamin D3 is a fat-soluble vitamin that is formed when the skin is exposed to ultraviolet (UV) radiation (37). Approximately 90% of the body's required vitamin D is supplied by UV exposure, while the remaining 10% is obtained from food (38). Therefore, patients may suffer from limited sun exposure in addition to malabsorption or impaired metabolism of vitamin D3, especially if they have co-existing conditions that reduce the conversion or fixation of the vitamin in the body. These findings are also consistent with those of (39), who found a significant decrease in serum vitamin B12 levels in patients infected with the parasite compared to the control group. Vitamin B12 deficiency is associated with increased disease severity or prolonged healing of skin lesions in patients with cutaneous leishmaniasis. This highlights the role of nutritional factors in influencing the immune response and the course of parasitic infection, as patients often live in impoverished areas, making them more susceptible to the disease. Prone to malnutrition and therefore low intake of foods rich in vitamin B12, such as dairy products, eggs and meat (40). These results indicate that monitoring and correcting deficiencies in these vitamins is part of the strategy to help treat cutaneous leishmaniasis, especially in chronic or widespread cases.

E. Molecular diagnosis of the parasite *Leishmania* spp. using PCR technology

The current study showed that the infection rate of cutaneous *Leishmania* species by molecular examination was 80% after examining 20 randomly selected serum samples, all of which were positive in clinical examination. The molecular examination confirmed that 16 samples carried one of the species of *Leishmania tropica* and *Leishmania major*, and 4 samples had negative results in the molecular examination. The proportions of the recorded species are varied among them, as follows:

1-L. tropica :

This type recorded the highest infection rate by molecular testing, with 10 samples, representing 50% of the total samples tested. Figure (1) shows

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the PCR results for the positive samples using KDNA gene.

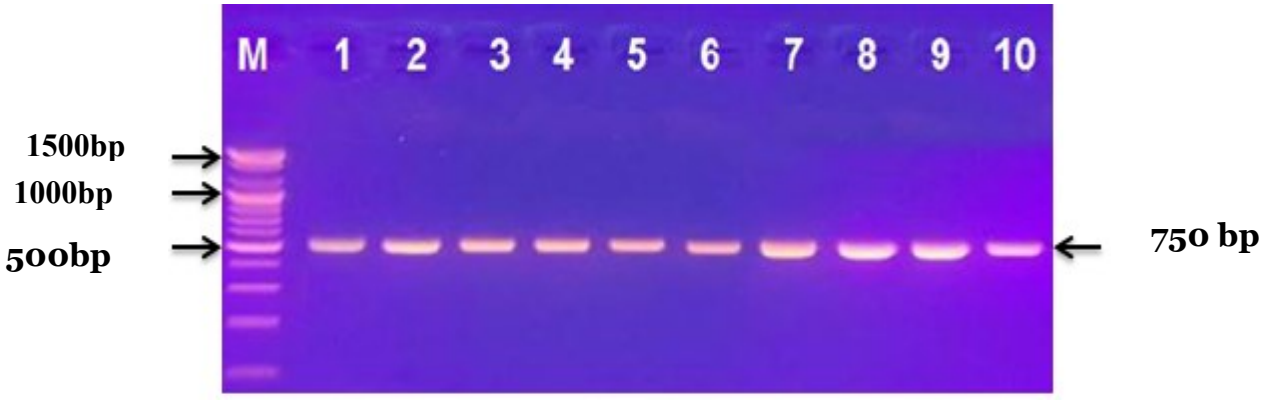

Figure (1): Electrophoresis with agarose gel at a concentration of (120V/45) Min %2 for the PCR products of the KDNA gene of the *L. tropica* parasite, where M represents the volumetric index, while the numbers from (1-10) represent some of the genes that tested positive for the KDNA gene and the bp750 result

2- *L. major* :

This type recorded an infection rate of 6, representing 30% of the total samples examined by molecular testing. Figure (2) shows the PCR result for samples positive by the KDNA gene.

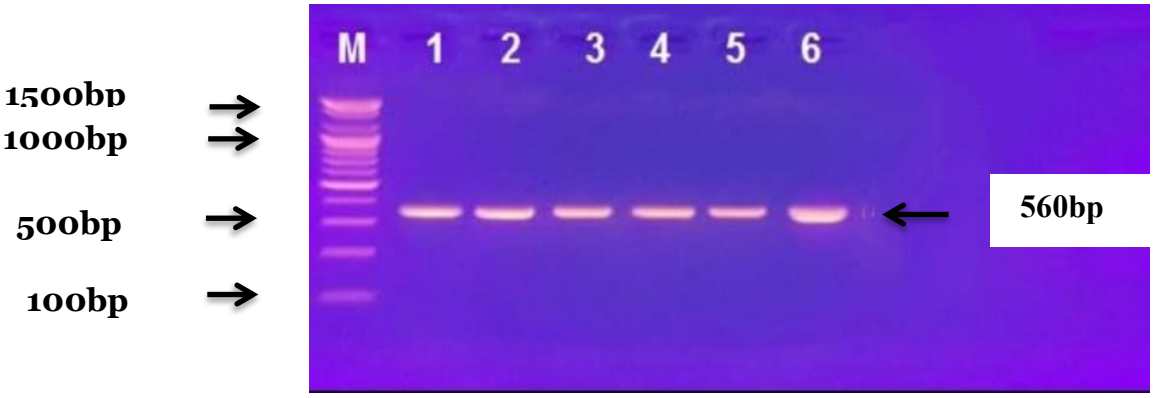

Figure (2): Electrophoresis with agarose gel at a concentration of (120V/45 min) 2% for the PCR products of the KDNA gene of the *L. major* parasite, where M represents the volumetric index, while the numbers from (6-1) represent some genes that tested positive for the KDNA gene and the bp560 product

The results of the current study are consistent with those of study (41), showing the presence of *L. tropica* in 57.5% and *L. major* in 23.75% of the samples. The results of the current study are also similar to those of study (42) in Tikrit, where 48% of the samples were found to be infected with *L. tropica* and 10% with *L. major*. The infection rates recorded are close to those of the current study, which may be due to similarities in social environment, cultural levels, experimental conditions, and opportunities to obtain *Leishmania* genotypes.

It did not agree with (43), where the results showed the presence of *L. tropica* in 60% and *L. major* in 20% of the samples, and it did not agree with (44) in Iran, where the results showed the presence of *L. tropica* in 12% and *L. major* in 65.8% of the samples. The reason for the difference in the results recorded in the current study compared to previous studies is attributed to the difference in the geographical distribution of the parasite, the use of pure isolates of the parasite grown on culture media or gene banks, the number of diagnosed samples, the method of diagnosis, the testing technique and its sensitivity, the duration of the study, the age groups of infected people, as well as the method of taking and isolating samples, the amount of DNA obtained from the parasite, its purity and concentration, the extraction method used, the PCR and electrophoresis programmes, and the experimental conditions. All of these factors can explain the reasons for the difference in what the various studies concluded.

Conclusion

1.Elevated liver enzyme and lipid levels were observed in patients with cutaneous leishmaniasis compared to the control group.

2.Trace element levels were significantly affected, either increased or decreased, in patients with cutaneous leishmaniasis compared to healthy individuals.

3.Vitamin D3 and B12 levels were decreased in both groups, with significantly lower values in patients with the disease compared to the control group.

4.Nested PCR results showed that 16 out of 20 randomly selected samples carried two Leishmania species (L. tropica and L. major), while 4 samples had negative results in molecular testing.

5-Statistical Analysis:

All study results were subjected to statistical analysis using IBM-SPSS version 27. Significant differences were determined at the probability levels of $p \leq 0.001$and $p \leq 0.005$ using the Welch t-test to identify significant differences between individuals with cutaneous leishmaniasis and

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the control group..

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