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Assessment of *miRNA-99a-5p, LOC100294145*, and Interleukin-27 Levels Before and After Pentostam Treatment for *Cutaneous Leishmaniasis* in Children

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ABSTRACT

Background: In patients with *cutaneous leishmaniasis*, MicroRNAs modulate immune response pathways both before and after treatment.

Objective: The study objective to evaluate the level of IL27 and relative gene expression of *miR-99a-5p* and gene expression *LOC100294145*pre and post treatment with Pentostam.

Materials and Methods: A total of 60 volunteers participated in this study; 30 had *cutaneous leishmaniasis* and were examined both pre and post therapy, while the remaining 30 individuals as a control group. ELISA was used to measure the amount of IL27, and qRT-PCR was used to quantify the gene expression of *miR-99a-5p* and *LOC100294145*.

Results: According to the current study's findings, the control group's relative The control group's *miR-99a-5* expression was substantially greater than that of the patient's two groups (pre, p=0.001 and post, p=0.001, respectively). Comparing the control group to the patient groups both before and after therapy, the level of IL27 increased significantly (pre, p=0.001 and post, p=0.004, respectively). The differences in gene expression levels of *LOC100294145* between study groups were not statistically significant. The slight change after treatment may highlight the possibility that this gene has an immunological role in the response to treatment.

Conclusion : The study results indicate that IL-27 and *miR-99a-5p* are effective diagnostic indicators for *cutaneous leishmaniasis*, as they showed significant differences between patients and the control group, while they were not significantly affected by treatment. The *LOC100294145-LncRNA* gene did not show significant differences, warranting further studies to determine its potential role in the immune response to the disease.

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INTRODUCTION

Leishmaniasis is a vector-borne disease caused by obligate intracellular parasites of the genus Leishmania (Trypanosomatida: Trypanosomatidae). According to recent research, leishmaniasis is endemic in 89 countries, with an estimated 1.3 million new cases annually, resulting in 20,000 to 40,000 deaths each year (2)

The life cycle of Leishmania is complex, i.e., indirect, and is exposed to various environmental and intracellular factors. These organisms are classified as biparental parasites, indicating that their life cycle involves the passage through two distinct hosts and culminates in the completion of two distinct phenotypic stages. The initial stage is known as the Amastigote, which is present in the leishmanial form within the mammalian host. The subsequent stage is referred to as the Promastigote, also designated as Leptomonas, within the sand fly host (3).

The healing of LCL lesions or their transformation into chronic lesions is contingent upon the initial immune response (4). The development and outcome of Leishmania infection are influenced by vector, parasite, and host factors. The inability of vertebrate hosts to control the infection appears to be related to two main factors: the ability of some Leishmania strains to resist the microbicidal effect of active macrophages and the breakdown of cellular immune responses that protect the host (5

IL-27 plays an important role in the innate and adaptive immune responses. These responses stimulate the production of TH1 cells, which protect humans from early-stage leishmaniasis infection. IL-27 also regulates the inflammatory response of Th17 cells (6). IL-27 is a cytokine that exerts various effects on innate and adaptive immune cells. IL-27 is produced by innate cells, macrophages, and dendritic cells during the early stage of leishmaniasis infection. It contributes to protection against major leishmaniasis infection but inhibits the protective Th1 response against donovaniasis, Amazonian leishmaniasis, and Brazilian leishmaniasis. This indicates IL-27's dual function. During the late stage of infection, IL-27 mediates pathogenic immune responses and tissue damage (7) .

Sequencing mRNA molecules from infected cells and determining the expression levels of different genes has revolutionized disease diagnosis. This allows us to identify changes in the quantity and type of biomolecules within cells and understand the molecular basis of clinical changes. Many studies indicate that overactivation of the immune system plays an important role in causing leishmaniasis(8) , but proteins involved in maintaining gene expression that regulate the immune response of infected individuals have rarely been studied.

One approach to studying leishmaniasis involves microRNAs (miRNAs), a class of small non-coding RNAs that suppress gene expression in most cases. miRNAs play a role in the complex interaction between the host and pathogens, either as part of the host's immune response to neutralize infection or as a molecular strategy used by the pathogen to modify host pathways to its own advantage (9) .

The microRNA gene *miR-99a-5p* acts as a tumor suppressor by inhibiting the proliferation, migration, and invasion of cells. It has been found to be dysregulated in many tumors (10, 11). Specifically,

breast cancer tissue was found to consistently have lower levels of *miR-99a-5p* (12, 13, 14, 15). One study (16) showed a significant decrease in the level of *miR-99a-5p* in breast cancer tissue compared to healthy tissue, and higher levels of *miR-99a-5p* were found in patients with breast cancer than in healthy individuals. These results suggest the possibility of using *miR-99a-5p* expression levels as a diagnostic biomarker for breast cancer.

Another study (17) showed that *miR-99a-5p* inhibits the release of TNF-α and IFN-γ, which promotes MTB growth in macrophages. Therefore, downregulation of *miR-99a-5p* after MTB infection may play a protective role in innate immunity against MTB.

LOC100294145, a long non-coding RNA (lncRNA), is associated with dermatitis and participates in regulating multiple genes and signaling pathways. This RNA is activated by the p53 gene, and its dysregulation has been demonstrated in Alzheimer's disease patients with dermatitis (18). Long non-coding RNAs (lncRNAs) are emerging as regulators of mammalian immune responses during host interactions with pathogens (19, 20). They are a heterogeneous class of non-coding RNA typically longer than 200 base pairs(21) . The tissue-specific expression patterns of most lncRNA genes suggest that their expression is finely regulated during development and disease progression. Dysregulated lncRNA signatures have also been found to play a role in the pathogenesis of multiple diseases (23, 22). Studies (24, 25, and 26) have shown that lncRNAs may play an important role in the pathogenesis and progression of leishmaniasis caused by different Leishmania species

MATERIALS AND METHODS

Study Design and Sample Collection

This study was conducted on patients diagnosed with cutaneous leishmaniasis by a dermatologist and before they started treatment. The study took place at the dermatology unit of Baquba General Teaching Hospital and private clinics in Kirkuk Governorate (Hawija district, Shmit village) from October 1, 2024, to February 20, 2025. The study included 90 samples divided into three groups: 30 samples from individuals before treatment, 30 samples from individuals after treatment, and 30 healthy individuals (control group) ranging in age from two to 12 years.

Five milliliters of venous blood was drawn from each infected individual (before and during treatment) and from the control group using a 5-milliliter syringe. Each blood sample was divided into two parts. Three milliliters of whole blood were placed in a 10-milliliter plain tube and left at 37°C for 30 to 15 minutes. Then, it was placed in a centrifuge at 2,500 rpm for 10 minutes. The serum was withdrawn using a micropipette(27) and placed in clean Eppendorf tubes, which were sealed with cellophane tape to prevent contamination and evaporation. The serum was stored at -20°C until immunological tests were performed using the ELISA technique.

The second part of the blood was placed in an Eppendorf tube containing 800 µL of triazole at a ratio of 3:1. It was then mixed for one minute, incubated for 10 minutes at room temperature, and stored at -20°C until molecular tests were performed.

Immunological tests:

These tests were performed at the Central Laboratory of the Faculty of Science at the University of Tikrit in Iraq. The concentrations and activity of the studied biomarkers were determined using the sandwich ELISA technique. The reaction resulted in the formation of a yellow compound, and the intensity of this color was measured using a spectrophotometer at a wavelength of 450 nm according to the manufacturer's instructions

Molecular Tests:

Total RNA was extracted from the blood samples used in the study to estimate the gene expression level of microRNA (miRNA) 99a-5p. Real-time polymerase chain reaction (RT-qPCR) was performed using the Transzol Up Plus RNA Kit (TransGen Biotech) according to the manufacturer's instructions.

Gene expression analysis was performed using two-step RT-qPCR, which included:

•Conversion of single-stranded RNA to complementary DNA (cDNA) using the Tinzyme cDNA synthesis kit.

•Use of the GAPDH gene as a housekeeping gene to calibrate gene expression results.

Probes for detecting miR-99a-5p and LNC145 expression were designed based on sequences approved by the NCBI database by Prof. Dr. Ahmed Abdul Jabbar Suleiman Antar from Anbar University. All primers were lyophilized and supplied by Macrogen in South Korea and used uniformly in all samples.

The primer sequences used were:

Gene primer: forward 5′-AACACGCAACCCGTAGATCC-3′ and reverse 5′-CAGTGCAGGGTCCGAGGT-3′

LNC145 gene primer: forward 5′-GAAGGAGCCAGGGCTAGAG-3′ and reverse 5′-TGGAGAGTCTGCGTTTTTCC-3′ The reverse primer was 5′-TGGAGAGTCTGCGTTTTTCC-3′. The GAPDH gene primer sequence was 5′-GGCTGTATTCCCCTCCATCG-3′ (forward) and 5′-CCAGTTGGTAACAATGCCATGT-3′ (revers) .

Statistical analysis:

The data were analyzed using GraphPrism software. Then, the arithmetic mean ± standard deviation was tested using a Student's t-test. A p-value of less than 0.05 was considered statistically significant. We used ROC analysis to determine normal gene and immune marker levels as a clinical diagnostic feature to differentiate between patients and healthy individuals. ROC curves were used if the Youden index determined areas below the acceptable cutoff value. We also calculated sensitivity, specificity, negative predictive value, and positive predictive value, a method based on the Leffak method (also known as the broad method) for analyzing quantitative polymerase chain reaction (qPCR) data. This method allows for the relative quantification of gene expression levels by comparing the target gene expression to a reference gene across different samples.

RESULTS

Cutaneous leishmaniasis is a global public health and social problem, particularly in Iraq and other developing countries. Several cases have appeared in our country in recent years.

The results in Table No. (1) show the IL-27 levels in the study samples. There was no statistically significant difference between the pre-treatment group (223.6 ± 50.29) and the post-treatment group (242.3 ± 51.97) ($p = 0.316$), indicating that treatment did not clearly change IL-27 levels. However, a highly significant difference was found between the pre-treatment group and the control group ($p < 0.001$), as well as between the post-treatment group and the control group ($p < 0.001$.)

We also performed a ROC analysis to evaluate the diagnostic efficiency of interleukin 27 in distinguishing between patients and healthy individuals. Curve (2) shows the results of the sensitivity analysis, which reached 91.67%, indicating a high ability to detect positive cases. The specificity reached 73.33%, and the probability value reached $p < 0.0001$, indicating strong statistical significance. A ROC curve analysis was performed to evaluate IL-27's efficacy as a biomarker for distinguishing pathological cases from the comparison group. Figure 3 shows that the sensitivity was 91.67%, reflecting a high ability to detect positive cases. ROC curve analysis was performed to evaluate IL-27's efficacy as a biomarker for distinguishing pathological cases from the comparison group. Figure 3 shows that the sensitivity was 91.67%, reflecting a high ability to detect positive cases. Meanwhile, the specificity was 73.33%, indicating an acceptable ability to exclude negative cases. The p-value was less than 0.0001, confirming the analytical model's strong statistical significance.

Table (2) shows that the expression level of the *miR-99a-5p* gene was significantly lower in patients before treatment (1.988 ± 0.4621) than in the control group (0.5733 ± 0.1047), with statistical significance of $p < 0.001$. After treatment, the results were also lower than those of the control group, at 2.656 ± 0.5733. There was no statistically significant difference between the two groups before or after treatment ($P = 0.457$). However, a clear difference remained between the post-treatment group and the control group ($P < 0.001$) . The ROC curve for the *miR-99a-5p* gene indicates its effectiveness as a diagnostic tool for distinguishing patients before treatment from the control group. It has a sensitivity of 70%, a specificity of 86.67%, and a high statistical significance with a P-value < 0.0001.

Also, the ROC results in curve No. (6) were used to evaluate the ability of ROC in the miR-99a-5p gene to distinguish between the post-treatment group and the control group, where the sensitivity reached 56.67% and high specificity 86.67% and a statistical difference P-value 0.0005. This indicates that the gene after treatment may reflect a state of recovery or improvement in gene expression associated with the disease.

Table 3 shows the gene expression levels of *LOC100294145 (lncRNA*) in the different study groups. The average gene expression level in the patient group before treatment was 2.182 ± 0.6149. After treatment, it decreased slightly to 2.155 ± 0.5353. There was no statistically significant difference between the two values ($P = 0.693$).

The slight increase after treatment may indicate a relative improvement in the body's response to treatment and may reflect the role of treatment in reducing inflammation or modifying the immune response associated with *cutaneous leishmaniasis*.

When comparing the pre-treatment patient group to the control group (healthy individuals), the mean gene expression level in the control group was 2.386 ± 0.5658. This difference was also not significant (P = 0.357). A non-significant difference was also observed when comparing the post-treatment group with the control group (P = 0.78) .

DISCUSSION

Patients with *cutaneous leishmaniasis* exhibit a robust type I immune response to Leishmania antigens, characterized by elevated levels of TNF-beta and IFN-beta. Interleukin-27 (IL-27) is a cytokine that affects innate and adaptive immune cells in multiple ways. It is primarily expressed in macrophages and dendritic cells during the initial stage of Leishmania infection, contributing to the prevention of cutaneous leishmaniasis caused by *L. major*. However, IL-27 impedes the protective Th1 response against infections caused by *L. donovani*, *L. infantum*, *L. amazonensis*, and *L. braziliensis*, demonstrating its dual function. In the late stage of infection, IL-27 limits harmful immune reactions and tissue damage (7) .

Additional factors that may play a role in promoting pathophysiology include the patient's genetic background, the effect of the vector, the site of infection, comorbidities, the presence or absence of secondary bacterial infection, and the species or strain of the parasite. These factors may lead to a more pronounced early immune response after natural infection (28,29) .

This study is consistent with the findings of studies (6) and (30), which indicate reduced levels of IL-23 and IL-27 produced by macrophages isolated from peripheral blood mononuclear cell cultures of patients with different types of *cutaneous leishmaniasis* lesions. These patients may be either recovered or still infected. The studies also found that IL-27 is associated with the development of CL infection. *Cutaneous leishmaniasis* after treatment and control group.

MicroRNA-99a-5p (miR-99a-5p) is a genetic molecule involved in regulating immune responses and modulating host interactions with the parasite during leishmaniasis infection. It regulates cytokines in the immune response to *cutaneous leishmaniasis* and can regulate the expression of pro-inflammatory cytokines, which are essential for the immune response to leishmaniasis and may affect the course and outcome of the infection. More Specifically, it may affect the activation, differentiation, and polarization of immune cells, such as macrophages and dendritic cells, that are essential for controlling infection (9). It also affects macrophage polarization, potentially promoting polarization toward the M2 phenotype, which is associated with an anti-inflammatory response that promotes parasite survival (31). Interactions between microRNAs (miRNAs) and cytokines in macrophages can promote either parasite elimination or persistence, highlighting the dual role of miRNAs in regulating immunity (32).

Taken together, these findings suggest that *miR-99a-5p* plays a role in the immune response to Leishmania infection, the causative agent of *cutaneous leishmaniasis*, by regulating inflammatory responses through the modulation of immune cell signaling pathways, particularly those of macrophages and T cells.

Although the differences in LOC100294145 gene expression levels between the study groups were not statistically significant, the slight change after treatment suggests that this gene may play a role in the immune response to treatment. Some studies suggest a relationship between long non-coding RNA (LncRNA) genes and the regulation of the immune response and inflammation. This highlights the need for future studies to determine the exact mechanism behind this change and its role in *cutaneous leishmaniasis* infection. study by (33) also examined the response of human skin to *tropical leishmaniasis* infection. The key genes identified in this study warrant further evaluation as potential targets for developing more effective CL treatments and preventive measures caused by *tropical leishmaniasis*.

The slight change in *LOC100294145* expression after treatment may indicate an immune role for this gene in how the body responds to treatment. Some research has shown a relationship between LncRNA genes and the regulation of the immune response and inflammation. This calls for future studies to understand the specific mechanisms behind this change and its role in *cutaneous leishmaniasis*.

CONCLUSION

The results of the study indicate that IL-27 and *miR-99a-5p* are effective diagnostic markers for *cutaneous leishmaniasis*. They showed clear, significant differences between patients and the control group and were not significantly affected by treatment. However, the *LOC100294145-LncRNA* gene did not show significant differences, suggesting the need for further research to determine its potential role in the immune response to the disease.

Conflict of interest: None.

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TABLES

Table (1): Shows the levels of interleukin 27 among the study groups.

IL27	Before T	After T	Control
Number of values	30	30	30
Minimum	138.0	167.9	253.6
25% Percentile	186.2	201.3	282.2
Median	217.0	231.3	326.4
75% Percentile	278.6	292.0	366.6
Maximum	307.2	374.6	399.1
Range	169.2	206.7	145.5
Mean	223.6	242.3	324.7
Std. Deviation	50.29	51.97	47.30
Std. Error of Mean	9.182	9.488	8.636
Lower 95% CI of mean	204.8	222.9	307.0
Upper 95% CI of mean	242.3	261.7	342.3
Coefficient of variation	22.50%	21.44%	14.57%

Table (2): Shows the levels of Relative *miR-99a-5* pexpression among the study groups.

Relative pexpression *miR-99a-5*	Before T	After T	Control
Number of values	30	30	30
Minimum	1.220	1.440	1.490
25% Percentile	1.643	1.797	2.281
Median	1.901	2.044	2.787
75% Percentile	2.275	2.603	3.105
Maximum	3.178	3.106	3.601
Range	1.958	1.665	2.111
Mean	1.988	2.143	2.656
Std. Deviation	0.4621	0.4535	0.5733
Std. Error of Mean	0.08437	0.08280	0.1047
Lower 95% CI of mean	1.816	1.973	2.442
Upper 95% CI of mean	2.161	2.312	2.870
Coefficient of variation	23.24%	21.16%	21.6%

Table (3): Shows the levels of LOC100294145-LncRNA among the study groups.

LOC100294145-LncRNA	Befor T	After T	Control
Number of values	30	30	30
Minimum	0.9594	0.9751	1.000
25% Percentile	1.788	1.742	2.016
Median	2.333	2.063	2.400
75% Percentile	2.623	2.339	2.763
Maximum	3.123	3.130	3.467
Range	2.163	2.155	2.467
Mean	2.182	2.061	2.386
Std. Deviation	0.6149	0.5353	0.5658
Std. Error of Mean	0.1123	0.09773	0.1033
Lower 95% CI of mean	1.952	1.861	2.174
Upper 95% CI of mean	2.411	2.261	2.597
Coefficient of variation	28.19%	25.97%	23.72%

FIGURES

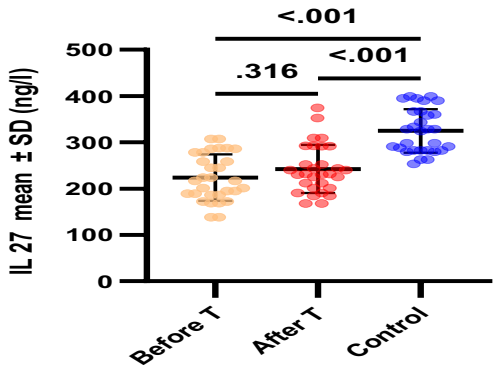

Figure (1): Shows the measurement of IL-27 among the study groups.

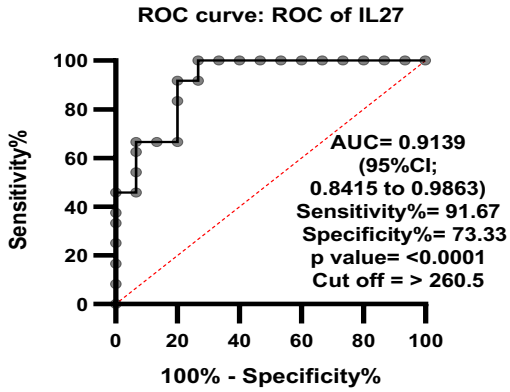

Figure (2): Shows the ROC analysis evaluating the diagnostic efficiency of IL-27 in distinguishing *cutaneous leishmaniasis* patients before treatment from the control group.

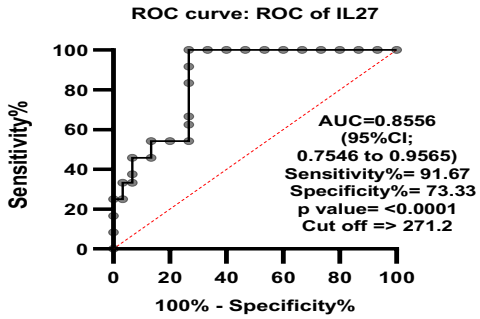

Figure (3): Shows the ROC analysis evaluating the diagnostic efficiency of IL-27 in distinguishing patients with *cutaneous leishmaniasis* after treatment from the control group

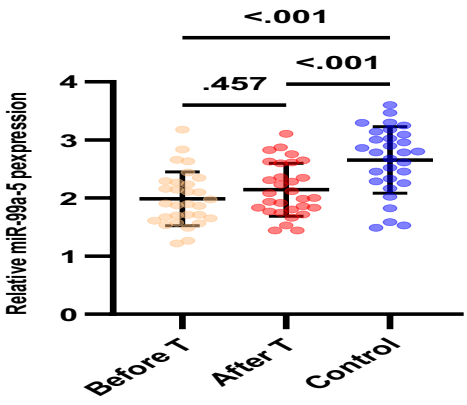

Figure (4): Shows the level of the *miR-99a-5p* gene in the blood of the study groups.

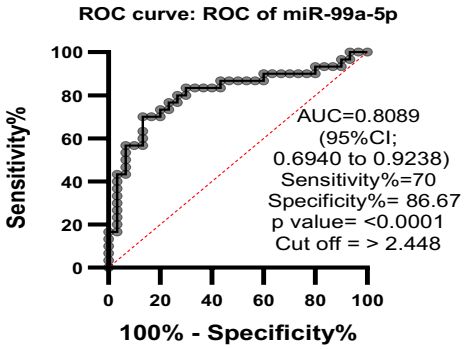

Figure (5): Shows the ROC curve of *miR-99a-5p* gene expression level between patients with *cutaneous leishmaniasis* before treatment and the control group.

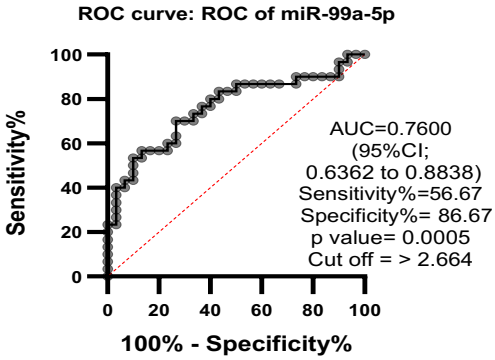

Figure (6): Shows the ROC curve of the gene expression level of *miR-99a-5p* in patients with *cutaneous leishmaniasis* after treatment compared to the control group.

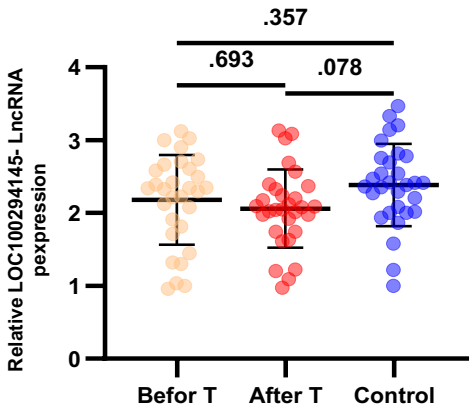

Figure (7): Shows the relative gene expression of LOC100294145-LncRNA in the study groups.