

Immunology study and the Assessment of parameters in difference Age, Sex of stage resistance infection leishmaniasis in Dayla

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ABSTRACT: The Leishmania parasite belonged to trypanosomatidae family,. Cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are endemic in Iraq as one of the neglected tropical diseases. The current drugs which are used as antileishmanial treatment characterized by enormous side effects including their toxicity to human, long term treatment, liver problems, hugely cost. Therefore, there is a necessity to find an alternative treatment marked as low cost, more specific against parasite's stages, Tumor necrosis factor-alpha (TNF- α), a cytokine that generated by the innate immune response to CL infection, can influence disease clearance in the human host. The effect of this pro-inflammatory cytokine in CL ulcer development during the infection is not well established. The current study was conducted on 88 individual of both sexes, they were from Diyala province and the capital Baghdad, during the period from October 2020 to February 2021.

All suspected cutaneous or visceral patients involved in this study were prediagnosed in the laboratory before processing Tumor Necrosis Factor-alpha (TNF- α) and Adenosine Deaminase Enzyme (ADA) by ELISA colorimetric detection. Total number of confirmed leishmaniasis patients were (n=57) cutaneous patients and (n=16) visceral patients, collected from different hospitals in Diyala province and Baghdad. Positive CL or VL samples were confirmed by microscopic Giemsa staining, In this study, the level of circulating tumor necrosis factor alpha and adenosine deaminase enzyme levels were detected in CL patients of newly diagnosed infection and in patients undergoing different doses of treatment, 2nd and 3rd Pentostam trail-treatment, in addition, VL patients of newly diagnosed infection with no treatment; in comparison to control subjects. Regardless the type of leishmaniasis and according to age, the results showed that TNF- α concentration was higher in patients less than 15 years old, in comparison with other age group over 15 years (15-60) years old, which was 1169.899 ng/ml and 961.206 ng/ml respectively. While according to sex, the higher concentration of TNF- α was recorded in female rather than male, 1030.243 ng/ml and 996.458 ng/ml Regarding ADA enzyme, the highest enzyme activity also recorded in the age group of 1-14 years old, which was 4.749 nanomol/minute/ μ g, in comparison of patients between 15-60 years old, which was 3.706 nanomol/minute/ μ g. While according to sex, the maximum enzyme activity was seen in male rather than female (4.296, 4.483) nanomol/minute/ μ g, respectively. The perceived rise of TNF- α and ADA serum levels may give insights into this pro-inflammatory cytokine as a biomarker in prognosis and tracking disease progression.

Keywords: Leishmania cutaneous Leishmania visceral, ADA, TNF- α

1. INTRODUCTION

Leishmaniasis are category of neglected tropical diseases resulting from the genus of Leishmania parasite[1]. All genus of Leishmania are obligating intracellular, infect macrophage cells of the mammalian hosts [2]. It's transmitted to humans through a bite of a female of sand fly vector, Phlebotomus (with old-world leishmaniasis), and Lutzomya (with new world leishmaniasis) [3]. All Leishmania parasites have two main life stages in their life cycle, and need two hosts to complete its life cycle, the first one the invertebrate stage. The second one is the vertebrate host (human), who has an intracellular, non-motile or non-flagellated form called amastigote stage which invade the macrophages of the vertebrate host [4]. World Health Organization (WHO) considers leishmaniasis as one of the nine most important infectious tropical and subtropical diseases that occurring in all continents[5]. Leishmaniasis regards one of the worldwide disease affecting more than 89 countries including: Asia, Africa, southern Europe (old world), and Latin

America (new world), particularly those countries that suffer from poverty in social and economic conditions. According to estimates about 12 million people are affected by this disease, in addition, about 2 million of new cases and an average of 60,000 deaths were recorded annually [6]. More than 20 known species of *Leishmania* can infect humans and can cause various clinical symptoms; the most three known clinical manifestations are Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL), Visceral Leishmaniasis (VL) (kala-azar or black fever). The difference in the clinical form dependent on several factors: species of *Leishmania*, the type of vector that transmits the *Leishmania*, and a person's immune status [7,8]. The most endemic *Leishmania* species in Iraq are VL and CL; *L. tropica* and *L. major* are the causative agents of the CL and they spread in all country except three provinces in the north of the country. While, VL which represent the most serious form of leishmaniasis is widely distributed among children under five years (90 %), most cases appear in central and some southeastern provinces in Iraq, and it caused by *L. donovani* and *L. infantum*, [9,10]. Currently, no certified vaccines against leishmaniasis in humans are used. As a result, to some of difficulties related to the vector control, and other factors, all of which are contributed together to increase disease cases that lead to the failure of controlling the spread of the disease [11]. The first medicines that used for leishmaniasis treatment are antimonials which include Pentostam (sodium stibogluconate) and Glucantime (meglumine antimoniate). However, various adverse effects are resulted from using these drugs including their toxicity to human, prolonged treatment, increased parasite resistance, leukopenia, liver problems, cardiotoxicity and cardiac arrhythmia [12]. TNF- α is secreted by macrophages and lymphocytes, it is considered a crucial mediator of many inflammatory reactions and contains a major role in innate response in pathogenesis in relevancy human diseases, to provide synthase stimulation oxygenated products and nitric oxide, components important for killing of *Leishmania*, via encounter *Leishmania* invasion, synergistically with other cytokines including IFN- γ [13,14]. Some macrophage-secreted cytokines are considered indirect biomarkers for leishmaniasis infection, including IFN- γ , TNF- α and IL-10 [15]. IL-10 was first described as a Th2 specific cytokine with the power to inhibit cytokine synthesis, this activity largely reflects indirect actions on antigen presenting cells instead of direct effects on Th1 cells [16].

Previous studies have identified a central role for IL-10 in susceptibility immunopathology, and parasite persistence, however, IL-10 inhibits cytokine production and cytotoxic activity of macrophages [17]. Gene expression of TNF- α has proved its correlation with the lesion size, furthermore, the serum concentration of TNF- α has strikingly raised in muco-cutaneous leishmaniasis [18]. In addition, similar studies demonstrated that TNF- α was found to be increased during infection and decreased during treatment to its healthy level [19]. Adenosine Deaminase enzyme or Adenosine Aminohydrolase (ADA) has also been considered as a biomarker in some infectious and hereditary diseases, such as Tuberculosis (TB) and squamous cell carcinoma in terms of clinical confirmation and diagnostic prognosis [20,21]

Similar studies showed that ADA level is increased or decreased during VL which, in turn, reveals better understanding of pathology and physiology of the internal systemic infection [22]. Growing evidences that ADA is increased in serum and lymphocytes in cutaneous leishmaniasis infection via its role in cell-mediated immunity [23]

IFN- γ levels in supernatants of *Leishmania* antigen-stimulated lymphocyte cells were measured before and after antimonial therapy to see whether the release of these cytokines might be employed as therapeutic response markers [24].

This study was aimed. to investigate the serum level of ADA in patients of CL in newly infected patients and patients undertaking Pentostam treatment in different stages of intake and the possible consideration of ADA as a biomarker in cutaneous leishmaniasis disease. The results of this study highlighted a new concept of investigating immunological molecules, such as ADA enzyme, as possible biomarkers for cutaneous or visceral leishmaniasis prognosis and development.

1.2 Materials and methods:

Patient's collection: The participants in this study had cutaneous leishmaniasis assembled between October 2020 to February 2021 from Baqubah General Hospital, Diyala provenance north of Baghdad. All 73 patients were suffering from cutaneous ulcer/s and were diagnosed by the resident dermatologist depending on clinical manifestations and laboratory diagnosis through Giemsa stain preparation of suspected ulcers; amastigotes were screened under light microscope 100x oil immersion. as shown in table1. as shown in figure1



Figure1: people patients both female and male of different Age infection with multiple cutaneous leishmaniasis lesions

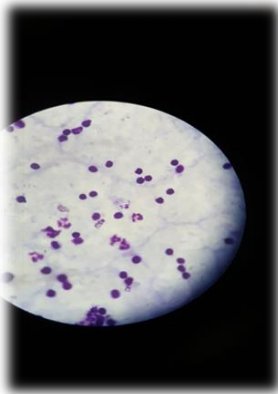


Figure2: Macrophage showing intra-cellular amastigotes from ulcer swab, light microscope 100X.

Table (1):The number, sex and type (cutaneous or visceral) of all collected samples.

Type/ Sex	1-14 years old	15-50 years old	Over 50 years old	Total
Cutaneous/ male	22	9	2	33
Cutaneous/ Female	19	4	1	24
Visceral/ male	9	0	0	9
Visceral/ Female	7	0	0	7
Total				73

2.2 Blood samples:

Samples of 5 ml venous blood from each patient were collected in a gel tube; in addition, personal information was documented including: age, sex, the number of lesions, and the location of the lesions of family, duration of infection and number of Pentostam treatment. Blood samples were centrifuged and the serum was divided into an Eppendorf tube each containing at least 500 µl of pure serum and stored at -20°C for later investigation [26].

Experimental design:

OF the total 73 patients, the experimental groups were divided according to Pentostam treatment, as the following:

Group-1: 22 CL patients of a new infection, no Pentostam treatment.

Group-2: 23 CL patients with 2nd trial-treatment of Pentostam.

Group-3: 12 CL patients with 3rd trial-treatment of Pentostam.

Group-4: 4: 16 VL patients of a new infection, no Pentostam treatment

In addition, 15 blood samples were taken from healthy people in the area

Adenosine Deaminase (ADA) Activity Assay Kit (Colorimetric)

This Adenosine Deaminase (ADA) Activity were ordered from Abcam® Company, USA and stored at -20°C. ADA activity is an assay where inosine formed from the breakdown of adenosine is detected via a multi-step reaction, resulting in the formation of an intermediate that reacts with the ADA convertor and developer to generate uric acid that can be easily quantified at OD293 nm. The kit measures total activity of Adenosine Deaminase with limit of quantification of 1 mU recombinant Adenosine Deaminase.

2.3 TNF-α Human ELISA

*Sandwich Enzyme Linked Immunosorbent:

Sandwich ELISA laboratory kit was ordered from Abcam® Company, USA and stored provided at -20°C

Human TNF alpha ELISA kit (Enzyme-Linked Immunosorbent Assay) is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TNF alpha in serum plasma and cell culture supernatants.

1.3 Results and Discussion:

Adenosine deaminase enzyme activity and tumor necrosis factor alpha result:

The distribution of Patients infected with Cutaneous and visceral

The total number of patients with Leishmaniasis included in this study were 73. The recorded cases were more in male than female. The most frequent age ratio in all studied groups was revealed in the age (1-14 and 15-50 years). The higher cases were males (42) patients, while females were represented (31) of patients. Males and females shared a similar frequency in the age groups without significant differences between them, they were 31(42.47%), 9 (12.33%) and 26 (35.61%), 4 (5.48%) in age groups (1-14) and (15-50) of males and females respectively, as shown in the Table2.

Statistical analysis proved that there were no significant ($P \leq 0.01$) differences between genders of the studied groups.

Table2: Distribution of patient samples according to sex and age groups

Age groups (year)	Male No (%)	Female No (%)	P-value
1-14	31 (42.47%)	26 (35.61%)	0.279 NS
14-50	9 (12.33%)	4 (5.48%)	0.091 NS
More than 50	2 (2.74%)	1 (1.37%)	0.633 NS
Total	42	31	
P-value	0.0001 **	0.0001 **	---
** ($P \leq 0.01$).			

The distribution of studied groups according to some parameters:

Age ratio:

All CL and VL studied cases were distributed in (42) male infections and (31) female infections, the percentage distribution used of subjects in the present study according to the age groups is shown in table (3-3). The most frequent age ratio was found in the age 1-14 years which represent 57 (78.08 %) of all studied patients. The age group 14-50 years represent 13(17.81%), while patients age who enrolled in this study ranged from 1 to over-50 years reveled 3 (4.11 %) as in table 3.

The statistical analysis showed that there were significant ($P \leq 0.01$) differences

between the age groups.

Table 3: Distribution of sample study according to Age groups

Age groups (year)	No	Percentage (%)
1-14	57	78.08 %
14-50	13	17.81 %
More than 50	3	4.11 %
Total	73	100%
P-value	---	0.0001 **
** (P<0.01).		

Gender ratio:

In the current study, all VL and CL patients were distributed into male and female groups. The relative distribution of each group according to the gender is shown in table 4. The majority of studied subjects for the two groups were males which accounted 42 (57.53 %) of patients and the rest ratio were females which represent 31(42.47 %) of infected patients.

Statistical analysis proved that there were statistically non-significant ($P \leq 0.01$) differences between genders of the studied groups.

Table 4: Distribution of sample study according to Sex

Sex	No	Percentage (%)
Male	42	57.53 %
Female	31	42.47 %
Total	73	100%
P-value	---	0.197
NS: Non-Significant.		

To validate the results of the present study, the development and progression of leishmaniasis has been subjected to some risk factors that are related to age, gender, immunological status of patients[27].

With respect to age, most of patients of present study (about 90%) were children equal or less two to 5 years and this in agreement with most studies that suggested a critical role of age in the development of VL, and children are actually more susceptible to the disease when they are at a younger age, and especially they are malnourished [28]. Previous studies also reached malnutrition and age in VL epidemiology may have been confounded by transmission rate, different mechanisms of acquiring immunity that progresses with age might have its effect. Therefore, adult immunity to *Leishmania* can develop quite rapidly, while children may develop inefficiently against the parasite [29].

The current study revealed that the highest rate of infection in cutaneous leishmaniasis was observed in the age group 1-14 years, with statistically significant differences from other ages. In the age groups of (over-50) years, the similarities and differences with other studies showed that cutaneous leishmaniasis affects all ages without exception [30]. This study coincide with other Iraqi studies as [31].who confirmed that majority of cases were recorded among age groups 15–45 years old also similar results were obtained by [32]. who found cases were recorded from areas

endemic provinces with moderate annual rainfalls and a high rural population and same age rang, other studies agree with that in neighboring countries as in Syria[33].Iran and Turkey.

The results of the statistical analysis showed that disease mostly affects patients of (15–50 years) of age and generally effects extremities, most patients have a single lesion [34]. The study also agreed that the frequency of cutaneous leishmaniasis in people between the ages of (15-50 years) and the outdoor young individuals participate in more activities than other age groups and thus are more likely to be exposed to the disease. The lower rate of cutaneous leishmaniasis in the elderly may be linked to the fact that they were infected during at a young age and developed long-term immunity while growing up [35]. In terms of sex, the majority of researchers have found that both male and female are susceptible to infection, the study on the onest of the vector different slightly, with some indicating that instances peaked after January and February and then declined dramatically in February the bite of a female sandfly that is a little insect vector that is 2-3 mm long and work as a vital vector of leishmaniasis over the world with its identified species is well known as the most company way of transmission of the parasite to people[36].

The statistical examination of the association between leishmaniasis infection and the sex of the host revealed that females were more infected than males [37]. This results confirmed by who has showed that cutaneous Leishmaniasis in females which recorded more infections than males while [38].found infections in males were more than in female .found the largest percent of infection was associated to the geographical site which was near water stream flow all year and abundant of fresh water holes providing sand flies with a perfect environment to complete their life cycle and increase agriculture activities .Men were shown to be the more susceptible to infection than females, in the study with a rate that might be linked to males being more exposed to infected vectors than females [39,40].Males who work or sleep in the open and in rooftops areas with minimal body covered are more vulnerable than others according to the study [41].In addition, that females have more intense cellular and humoral immune response than males; therefore, they were considered to be more resistant to certain infections, especially intracellular parasites [42].

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