Role of Evaluation of some cytokines in Diagnosis of PediatricVisceral Leishmaniasis in Mid- Euphrates area

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Abstract

Visceral leishmaniasis (VL) is commonly known as Kala-azar. It is a disease caused by species of *Leishmaniadonovani*. Leishmaniasis is still one of the world's most neglected diseases, affecting largely the developing countries. VL is widely spread in different parts of Iraq, which is regarded as an endemic place, especially in the middle and south parts, the main reasons are due to adaptation of the vector sand fly in these areas.

This study was carried out during the period from January 2012 to March 2013. Five hundred and eighty seven children aged 1- 60 months were admitted to paediatric ward of Pediatric and Maternal Hospitals of Al- Najaf, Babil, Karbala, and Al-Qadisiyyah provinces with confirmed or clinically suspected visceral leishmaniasis were included; most of them had clinical manifestation of fever, hepatosplenomegaly, weight loss, Leukopenia, and anemia. The diagnosis was under the supervision of paediatrician from each hospital.

This study was evaluated the effectiveness of Enzyme-linked immunosorbent assay (to detection of the concentration of two cytokines, INF- γ and IL-12/p40)

Then it was assessed the efficacy of these diagnostic techniques for detection the early stage of infection by determining the sensitivity and specificity for test .ELISA, the concentration of INF- γ and IL-12p40 were highly significant increase in 46.84% of patients and both the sensitivity and specificity of ELISA tests were 100%.

Index Terms—Visceral leishmaniasis ,Kala-azar,*Leishmaniadonovani*,*Pediatric Visceral*,ELISA, INF- γ and IL-12/p40.

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INTRODUCTION

VisceralLeishmaniasis (VL) is also known as Kala-azar. It is a vector-borneparasitic disease which is nearly always fatal if it is left untreated. Protozoa ofthe leishmania spp. causes an obligate intramacrophage infection. The clinical syndrome is characterized by fever, weight loss, splenomegaly, lymphadenopathy and hepatomegaly. VL is one of the world's most neglected diseases, largely affecting the poorest people, mainly in developing countries [1]According to the World Health Organization (WHO), 350 million people are considered at risk of Leishmania infection around the world. Currently an estimated 12 million people are infected and about 2 million new infections occur each year, half a million being VL [2], [3] .Iraq is a well-known area for endemicity with the Kala-azar disease [4], which is a long-lasting disease since 1954[5]. Then factors such as population movement and the destruction of health and interruption of control methods, like insecticide spraying on early diagnosis and treatment of positive cases contributed to the outbreak of leishmaniasis in Iraq[6], [7] .

In human leishmaniasis, immunity is predominantly mediated by T lymphocytes, which play a major role in generating specific and memory T-cell responses to intracellular parasitic infections and these have been extensively characterized in Leishmania infection. Th1 and Th2 cells can be distinguished by the cytokines they secrete: Th1 cells secrete activators of cell-mediated immunity such as IFN-γ, while Th2 cells secrete cytokines such as IL-10, which represent the main macrophage-deactivating cytokine[8] .

Infected macrophages and dendritic cells (DCs) play important role in the production of IL-12 leading to activation of natural Killer (NK) cells, the production of IFN-γ and the subsequent Th1 activation [9] .

The early activation of NK-cell is also induced by chemokines (IP-10, MCP-1 and lymphotactin) [9] .

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Prajeeth and colleagues (2011) [10] found that NK cells activated by IL-12 and IL-18 stimulated macrophages to kill intracellular Leishmania in a cell contact-independent but gamma interferon, tumor necrosis factor, and inducible nitric oxide synthase-dependent manner .

Activated NK cells have been shown to be cytolytic for Leishmania-infected macrophages, but NK cell-derived IFN-γ plays a more prominent role in host defense by activating macrophages to kill the intracellular parasite through the generation of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI). The importance of NK cells in the control of intracellular parasites by inducing IFN-γ production has been well studied. NK cells purified from unexposed human PBMCs (peripheral blood mononuclear cells) proliferate and secrete IFN-γ in response to Leishmania antigen [11] .

ELISA is the best choice for the development of a rapid and reliable diagnostic method, because it is more practical, easy to standardize and suitable for mass screening. Specificity and sensitivity of the ELISA based immunoassay strictly depends on antigen quality and can be improved by use of recombinant technology, which drives the expression and purification of diagnostically relevant proteins in large amounts [12] .Human visceral leishmaniasis (VL) is routinely diagnosed by detecting IgG that specifically binds to Leishmania antigens [13] .

Recently, many studies had suggested that the Cytokine levels in the sera of patients can be used as markers in epidemiological studies conducted in endemic areas to investigate VL [14].

1. Materials and Methods

This study was carried out, during the period from January 2012 to March 2013, in the laboratories of Pediatric and Maternal Hospitals of Al-Najaf, Babil, Karbala, and Al-Qadisiyyah provinces.

2.2 Study Groups

Patients Group: including 587 children aged 1-60 months, admitted to pediatric ward of Pediatric and Maternal Hospitals of Al- Najaf, Babil, Karbala, and Al-Qadisiyyah provinces. Most cases had clinical manifestation of fever, hepatospleenomegally, weight loss,leukopenia, and anemia. Cough and vomiting were frequently reported accompanying symptoms. Thediagnosis was under thesupervision of pediatrician from each hospital.

The Bone marrow aspiration was carried out for just 38 cases under the supervision of pediatricians at private hospitals in each province. Microscopic examination method applied and detected the Leishmaniadonovani in their Pathological Lab, and then the results of aspirated bone marrow were sent. Only 34 cases were positive of Kala-azar by the demonstration of the parasite -the amastigotes- from direct smear of bone marrow, while 4 cases were negative.

Control group: consists from 20 healthy children all were with no history of parasitic infection, and without clinical manifestation of any disease.

2.1Collection of Samples

Blood samples: two ml of venous blood was collected from clinically suspected patients and control, was allowed to clot at room temperature then centrifuged at 3000 rpm for 5 minutes the serum was harvested into clean dry screw-capped tubes used for ELISA tests.

Determination of cytokines:

Interferon-γ(IFN-γ) and interleukin-12/p40 (IL-12/p40). Serum levels of IFN- γ and IL-12 were measured by means ofenzyme immunoassay using ELISA kits .It is a product of Cusabio Com., Wuhan Huamei Biotech, Inc. (Wuhan, Hubei, China).

2.3Assay procedure

1.100µl of Standard, Blank, or Sample was added per well. Wells were covered with the adhesive strip, and then incubated for 2 hours at 37°C .

2. The liquid of each well was removed, without washing .

3. 100µl of Biotin-antibody working solution was added to each well, and then incubated for 1 hour at 37°C .

4. Each well was aspirated and washed. The process was repeated three times for a total of three washes. Wash: each well was filled with Wash Buffer (200µl) using autowasher, and it let stand for 2 minutes. After the last wash, the plate

was inverted and blotted against clean paper towels .

5. 100µl of HRP-avidin working solution was added to each well. The microtiter plate was covered with a new adhesive strip and then incubated for 1 hour at 37°C .

6. The aspiration and wash was repeated five times as step 4 .

7. 90µl of TMB Substrate was added to each well, and then incubated for15-30 minutesat37°C .

8. 50µl of Stop Solution was added to each well, the plate was gently tapped to ensure thorough mixing .

9. The optical density of each well was determined within 5 minutes, using a microplate reader set to 450 nm .

10. The result were calculated by using (Graphpad prism version 5.0u) computer software to determine each point by estimate the relationship between the optical density (O.D) and the samples concentration (pg/ml) by using the standard curve.

2.4STATISTICAL ANALYSIS OF THE RESULTS

The data were represented as a mean ± SD (standard deviation).Student t-test was used for comparison between two groups. In all cases a P-value < 0.05 was considered to be significant.

Sensitivity, specificity and accuracy were calculated according to Lalkhen and McCluskey (2008) [15] (which described in terms of TP: true positive, TN: true negative, FN false positive: and FP: false positive) as the following:

Sensitivity = TP/ (TP + FN) ×100
Specificity = TN/ (TN + FP) ×100
Accuracy = (TN + TP) / (TN+TP+FN+FP) ×100

2. RESULTS

The concentration cytokines in the patients group suspected of having the disease and control group are shown in table 1 The concentration of INF- γ and IL-

12p40 were highly significant increase (P < 0.05) in 275 (46.84%) patients

Table 1

The Results of the Enzyme Linked Immunosorbent Assay (ELISA):

Cytokines(Pg/ml)	Healthy controls (n=20) (Mean±SD)	Disease Suspects (n=275) (Mean±SD)
INF- γ	13.75±7.8	37±6.5*
IL-12p40	29±5.3	84±12*

*P < 0.05 significant

Evaluation of The results of the EnzymeLinked Immunosorbent Assay (ELISA):

All cases that positive after performing bone marrow aspirations were positive by ELISA, so, both the sensitivity and specificity of ELISA tests were 100 % (Table 2)

Table 2

Accuracy of ELISA for Diagnosis of VL.

Test		Bone marrow aspirations		Total
		Positive	Negative	
ELISA	Positive	34 True positive	0 False positive	34
	Negative	0 False negative	4 True negative	4
Total		34	4	38

Sensitivity = 34/34 x100 = 100%

Specificity = 4/4 x 100 = 100%

Accuracy rate = (34+4) /38 x 100 = 100 %

3. DISCUSSION

In the present study the concentration of INF- γ and IL-12p40 were highly significant increase in 46.84% of patients, and both the sensitivity and specificity of ELISA tests were 100 %.

In Iraq a study was carried out to investigate some cytokines (INF- γ, IL-10, TNF-

α) on blood samplesof Iraqi children with visceral leishmaniasis (VL)using ELISAkits. The results of cytokine showed that there were significant increases in the levels of all the investigated cytokines (INF- γ, IL-10, TNF- α) in the sera of patients with VL during active disease in comparison with the control group[16] .

Many studies had proved that The plasma concentrations of most of the analysed cytokines (IFN-γ, TNF-α, IL-2, IL-12, IL-4 and IL-10) were higher in the subjects with active VL relative to asymptomatic individuals, demonstrating an exacerbation of the immune response in these patients ([14] ,[17], [18] , [19],[20].

A significant reduction in these cytokines, particularly a decline in IFN-γ, which has been indicated to be a reliable marker of the cured disease, has been observed in patients after treatment of VL [20] , [21], [22] .

Costa and colleagues (2012) [14]concluded that the production of Th1 and Th2 cytokines is increased in patients with VL during the active disease. Cured subjects have a cytokine profile similar to that seen in subjects with asymptomatic infection, including basal levels of IFN-γ.
They had proved IL-12, together with IFN-γ, play an important role in the immune response and contribute to parasite death.

These findings show that cytokine levels should be evaluated in combination with the clinical and epidemiological characteristics of VL.

In this study the specificity and sensitivity of this method was considerably high, for symptomatic leishmaniasis, and it was considered important for epidemiologic studies. The most important advantage of these methods is that they are non-invasive.

4. REFERENCES

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