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DETECTION OF sHLA-G PROTEINS IN THE SERA OF PATIENTS INFECTED WITH VISCERAL LEISHMANIASIS

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Abstract

Visceral leishmaniasis (VL) or kala-azar is one of the worlds most neglected tropical diseases in mortality and fourth in morbidity, rK39 dipstick was used to diagnose the suspected infected patients as cheapest simple technique which can differentiate recent from chronic infection, for disease out-coming, naïve T-lymphocyte cells should be differentiated into pathogen-specific immunity responses, such as T-helper 1(Th-1) or (Th-2). HLA-G is a special protein defined as non-classical HLA class I molecule can suppress the immune system through prevention of T-cell function by foul all T-cell mechanisms. So, this study aimed to detect and evaluate the level of sHLA-G in the sera of patients infected with VL. The results showed that there was a highly significant increase in its level in patient's sera and this level impressed by different clinical parameters such as age, sex, and symptoms.

Key words : Visceral leishmaniasis. VL diagnosis, rK39, MHCG1, sHLA-G.

Introduction

Visceral leishmaniasis (VL), classified as a Neglected Tropical Climate Disease (NTD) (WHO, 2017). Characterized by acute, sub-acute or chronic, disease (Pelissari *et al.,* 2011). VL caused by the infective stage (promastigote) of obligate intracellular parasite belonging to genus *Leishmania*, as *Leishmania donovvani* in human and *Leishmania infantum(chagasi)* in both human and dogs (Bankoti and Stager, 2012). Bone marrow, spleen and liver are the main organs for *Leishmania*'s establishment. The incubation period ranged from (2 week-18 months), associated with scandalous inflammatory in viscera swelling through (2-8) months after infection (Ready, 2014). The clinical signs include hepato-splenomegaly, long-term and low-grade fever, anemia, leucopenia, hyper gammaglobulinemia, cachexia, weight loss, (Karunaweera and Ferreira, 2018). The up-regulation in HLA-G expressed clearly in a various pathological condition such as visceral Leishmaniasis, cancers, viral infection, organ transplantation, and autoimmune and inflammatory diseases (Gonzalez *et al.,* 2012). HLA-G known as a non-classical HLA class I protein molecule, belonging from HLA family. Among extra villous cytotrophoblasts, cornea, thymic medulla and pancreatic islets, HLA-G proteins are resident (Kovats *et al.,* 1990; Cirulli *et al.,* 2006; Ferreira *et al.,* 2017; Ribeyre *et al.,* 2018). There are two forms of HLA-G proteins: Membrane-bond HLA-G (HLA-G1, HLA-G2, HLA-G3 and HLA-G4) and soluble isoforms, (HLA-G5, HLA-G6 and HLA-G7) as a result of alternative splicing in the primary mRNA. However, proteolytic cleavage of cell surface HLA-G1 by metalloproteinases (MMPs) such as MMP-2 results in another soluble isoform called shedding HLA-G1 (Rizzo *et al.,* 2013). The differential in HLA-G forms related to presence or absence in one or more (α1, α2 and α3) domains beside to β_2m covalently binding (Yan, 2011; Carosella *et al.,* 2015). (ILT2)/LILRB1, ILT4/LILRB2, KIR2DL4, CD8 and CD160 are receptors located on immune cells surfaces, by which HLA-G conjugated and immune-suppressive functions achieving (Fainardi *et al.,* 2011; Lin and Yan, 2019). ILT2/LILRB1 is present on all monocytes and B lymphocytes, and on subsets of dendritic cells (DCs), myeloid derived suppressive cells (MDSCs), natural-killer (NK) cells and T- cells. ILT4/LILRB2 is expressed on DCs and monocytes, neutrophils and MDSCs (Baudhuin

et al., 2013; Kostlin *et al.,* 2017). In several recent studies showed that, the ILT4 could be recognizing both associated with β_2m and free HLA-G heavy chains, whereas ILT2 could only recognize HLA-G associated with β_2m and for this singularity ILT4 is binding with HLA-G in high affinity than ILT2 (Lin and Yan, 2018). HLA-G/receptors (particularly ILT2 and ILT4) mechanisms were summarized by hindering the proliferation, differentiation, cytotoxicity, cytokine secretion and chemotaxis of immune cells, induction of regulatory cells and MDSCs or M2 type macrophages (Morandi *et al.,* 2014; Lee *et al.,* 2015). This study was aimed to compare the levels of soluble HLA-G molecules and its receptor LILRB4 in Iraqi visceral leishmaniasis patients with healthy peoples to understand the creation of immune-suppressive micro-environments by studying the following parameters:

1. Detection of the presence of the immunosuppressive molecules sHLA-G in Iraqi visceral leishmaniasis patients.
2. Seeking of the expression of one of HLA-G receptors (LILRB4) on immune cells in the sera of patients.
3. Evaluation of the immunosuppressive property of sHLA-G molecules by determination of the level of IL-12 in the sera of patients.

Materials and Methods

Blood sample collection

Five ml of venous blood were drawn from forty-seven children samples from different Iraqi hospitals were suspected they might be infected with VL infection. The blood was placed in a sterilized plain tube and left to stand for 30 minutes at room temperature to clot and then centrifuged for ten minutes at 3000 rpm for serum collection. The aspirated sera were stored in a deep freeze into sterile tubes until used. All the forty- seven samples succumbed to diagnostic tests with rK39 and MHCG1 tests.

Leishmania IgG/IgM Rapid Test Cassette (Dipstick test)

Immunochromatography or rK39 kit was used for detection and differentiation of IgG /IgM to subspecies of Visceral *Leishmania*. The procedure was done according to the direction of manufacture (Weifang Kanghua Biotech). However, the assay procedure was achieved according to the test's guidance and all of the sensitivity, specificity and predictive values calculated by Trevethan, (2017) method. Human MHCG (Major Histocompatibility Complex Class I G) ELISA kit. The test used in detection of human HLA-G proteins. The procedure was done according to the direction of manufacture (My biosource), MHCG kit.

Results and Discussion

This study included forty- seven children samples that suspected of having VL and twenty apparently healthy children as a control group. All samples were tested by *Leishmania* IgG/IgM rapid test cassette (immunochromatography) for simultaneous detection and differentiation of IgG and IgM to the subspecies *Leishmania donovani*. The results showed that out of 47 patients only two patients revealed –ve result by this test and none of healthy samples showed +ve result. However, all the positive results 45/47 were IgG positive, no patient with IgM appeared by this study. Which means all patients were chronic infected with VL (Table 1). All 45 children proved having visceral leishmaniasis, their ages ranged between three months to ten years old and the infection rate was non-significant ($P > 0.05$) between children, 26 of them were males while 19 females. The percentage of male (57.77%) was high significantly ($P \leq 0.05$) than females (42.22%). Beside a high significant ($P \leq 0.01$) increase was showed at late stages (55.55%) than patients at initial stages of infection (44.44%) (Table 2).

This study found rK39 test distinct with simple technique, easy handling, fast results observation, safety using and special for *Leishmania donovani* identification and identify recent from chronic infection. Despite the

Table 2: Percentage distribution of patient's samples according to different clinical parameters.

No. of case			Total No.%
Age	Less than year	21(46.66%)	45 (100)
	More than year	24 (53.33%)	
Sex	Female	19 (42.22%)	45 (100)
	Male	26 (57.77%)	
Symptoms	Initial stage	20 (44.44%)	45 (100)
	Late stage	25 (55.55%)	

Table 1: The percentages of IgG/IgM rapid test for the total patient samples.

Subject samples	Total No.	Positive		Negative
		IgM	IgG	
Suspected patients	47	.....	45(95.74%)	2(4.255%)
Control apparently health	20	.....	.....	20(100%)

low sensitivity expression in a compare with Freire *et al.,* (2018). The present study found that, the results of rK39 recorded 81.39% sensitivity and 91.6% specificity. In addition to 94.5% positive predictive value and 73.33% negative predictive (Table 3).

Table 3: The values of (SN, SP, PPV, NPV) of *Leishmania* IgG/IgM rapid test.

Sensitivity (SN)	81.39%
Specificity (SP)	91.6%
Positive predictive value (PPV)	94.5%
Negative predictive value (NPV)	73.33%

These data were disagreement with Freire *et al.,* (2018) results who showed the sensitivity of rK39 was 92.8% to 100% and 96.0% to 100% for specificity in India and from 36.8% to 92% respectively in Brazil and East Africa. The major differences between these data and the current results may be related to epidemiology spread conditions, patients immunity response, manufactory brands currency and type of *Leishmania* species where L. *infantum* used in this study instead of L. *donovani* as in others studies.

In addition to comparison with rKE16 that used by (AL-Kayyt and AL-Qadhi, 2014; Bangert *et al.,* 2018) the sensitivity was (100%) for rKE16 and (92%) for rK39. This study proffered that rKE16 was better one in survey study than rK39 in VL diagnosis only, because it could not distinguish between recent from chronic infection.

However, this study showed highly significant increase in children with ages more than one year (53.33%) than less ages (46.66%) suggested that the young children, who are above one year gave influence to VL infection and the high exposure beginning from one year old.

The above data gave an agreement with Jervis *et al.,* (2017) and Chapmen *et al.,* (2018) results, which explained that the correlation occurred between ages and infection were found the patients who ranged from (5-20 years) had a high exposure to the bite of infected sand flies in a comparison with younger.

Besides, the results agreed with Terefe *et al.,* (2015) who suggested that 94% states recorded in individual with 15 years, 3.3% in children younger than 5 years, 19.2% with children ranged between 5-14 years and 8.6% in elder. However, Perry *et al.,* (2013) estimated 0–10 ages individual to be at highest risk and 11–20 ages at lowest risk of VL.

The different point in this study that was disagreed with above researches, was the infected children with ages above one year had impaction to VL disease instead of below ages and the highly exposure begin from one year instead of 5 years. This study reached to reckon that the simplest clarification that the risk of infection given increase expression with age up to 1–20 instead of 5-20 years while decreased in infants. The main reasons must be taken into consideration are immunity development, nutrition, the individual sleeping behavior and host kinetics.

Moreover, this study reached to the high percentage of infection was recorded in males (57.77%) more than in females (42.22%). This point was agreement with many other studies as Rodríguez *et al.,* (2018) who showed that the VL infection more common in males than females in spite of similar exposure occurred. Cloots *et al.,* (2020) results reached to the males VL cases in India were significantly more frequently reported by the health services than female VL cases.

The main differences between both genders may be related to several reasons as both males and females had a similar number of genes and the only differing in those encoded by the sex chromosomes. In addition to, this study hypothec that the sex hormones might be have influence roles in immune system and altering pathogenesis of leishmaniasis has been established, for instant: Zhang *et al.,* (2001) suggested the testosterone aid in increasing the uptake of L. *donovani* parasites by macrophages, elevated of infection rates and infection levels of these cells.

Lezama-Davila *et al.,* (2007 and 2008) found the exogenous estrogen supporting to increase leishmanicidal activity in macrophages in both male and female mice. Other hypothesis claims the sex-differences are absent in children before the age of puberty (Jervis *et al.,* 2017)

However, another reasons may be depending on human activities, for example: the males activities outdoor increased the chance to exposure to the bite of sand-flies more than in females especially in agrarian, farm and other open environments as playing outdoor, sleeping in the farm at night and through agriculture working.

In addition to, the most of VL patients recorded in this study were at late stages (55.55%) than initial (44.44%). That is mean the patients were in chronic stage and terrible infection.

VL infection has a different phase. The first one known by early or initial phase who characterize with variable duration and early symptoms. In this stage the patient suffered from many swings in the body such as, intermittent fever, malaise and shivering after special period. The firstly symptoms altered to be concluded splenomegaly, in some patients can observe hepatomegaly

associated or not with splenomegaly and that absence in auto-immunodeficiency as AIDS patients (Rodrigues *et al.,* 2016).

Another sign is a hyperplasia of the reticuloendothelial system this sign may be accompanied with wasting and pale appearance of the mucous membranes, mononuclear phagocytes appeared heavily parasitized in many organs, atrophy in white pulp of spleen, polyclonal hyper gammaglobulinemia accompanied with abundance of plasma cell in spleen, failure in bone marrow function, Immune complexes associated with nephritis, proteinuria, microscopic hematuria, Anemia, thrombocytopenia and neutropenia (Rodrigues *et al.,* 2016; Jayakumar *et al.,* 2019).

Another phase called with late or advanced, involved thrombocytopenia, severe mucosal hemorrhage, Jaundice, and ascites (accumulation of fluid in abdominal cavity) also occur (Osman *et al.,* 2001; Rodrigues *et al.,* 2016).

Further less, The current study showed that, all the 45 children confirmed with VL infection gave a highly significant increase (Pd" 0.01) in the level of sHLA-G (17.951 ± 7.78 ng/ml) in comparison to control (0.177± 0.12ng/ml) (Table 4).

This result was agreed with previous result by Pistoia *et al.,* (2007) who found higher significant increase in sHLA-G in a serum from patients affected by a variety of disorders in comparison to controlgroup (Fig. 1).

This finding suggest that sHLA-G level may predict VL presence in suspected patients and have therefore prognostic value. However, many studied with large number of patients are needed to obtain conclusive evidence in support of this possibility.

However, an up-regulation of HLA-G expression has been reported in this study based on different pointers. According to age, the young children with the ages above one year showed non-significant (P> 0.05) increased (18.865± 7.39 ng/ml) in sHLA-G level in comparison to the below one year old of patients (16.906 ± 8.27 ng/ml).

While according to sex, the results found that males scored significant increase (19.64 ± 7.40 ng/ml) in comparison to females (15.221± 7.64 ng/ml). This result was disagreed with previous result by Rudstein-Svetlicky *et al*., (2007), who showed that sHLA-G was influenced by several variables. Among them is the gender of donors, since the level of sHLA-G is higher in women than men.

Such difference may related to age group difference between the current and previous study when all patients in this study included small ages which they not exposed to accumulation of epigenetic environment that affect HLA-G gene polymorphism or hormonal variation due to age.

Whilst late stage of disease patients showed a highly significant increase (23.685 ± 3.82ng /ml)in sHLA-G level in comparison to patients with initial stage (10.784 ± 4.97 ng/ml).

Table 4: The mean level of sHLA-G protein in the sera of VL patients in comparison to healthy control group.

Samples	VL patients	Control
No.	45	20
Level ng/ml	17.951± 7.78	0.177 ± 0.12

**(P≤ 0.01)-HS

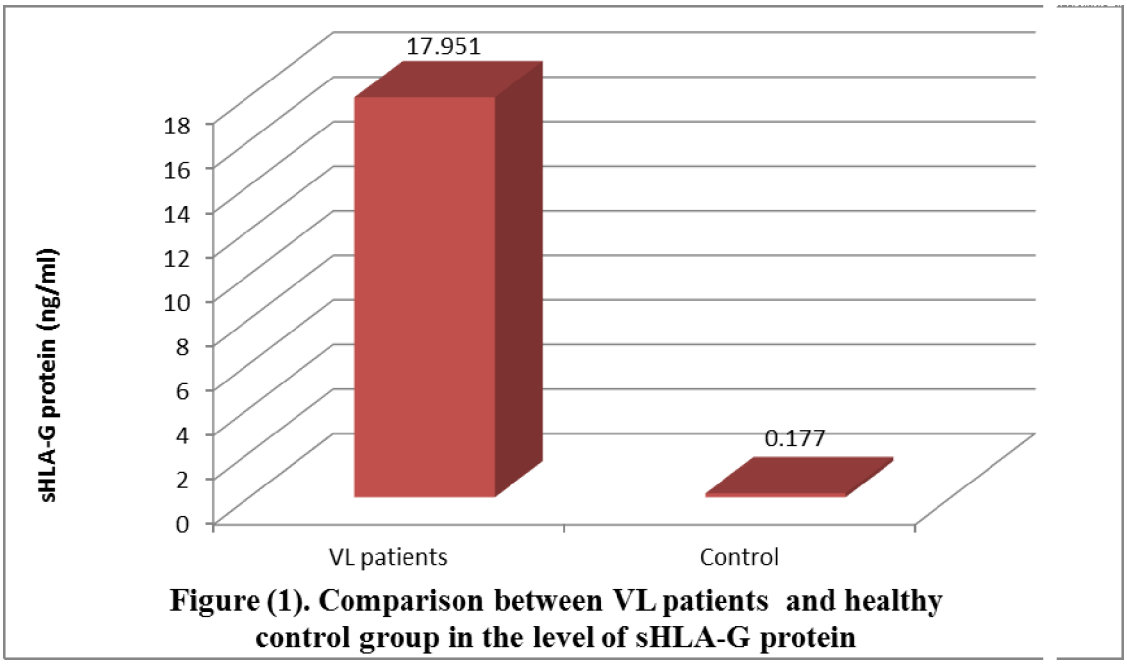

Figure (1). Comparison between VL patients and healthy control group in the level of sHLA-G protein

Table 5: The estimation of sHLA-G levels in patient's sera according to different clinical parameters.

No. of case		Meanz SD of HLA-G (ng/ml)	Total No.%	t- test (p- value)
Age	Less than year	16.906 ± 8.27	45 (100)	4.708(0.406) NS
	More than year	18.86 ± 7.39		
Sex	Female	15.221 ± 7.64	45 (100)	4.569 (0.0430)*
	Male	19.94 ± 7.40		
Symptoms	Initial stage	10.784 ± 4.97	45 (100)	2.641 (0.0001)**
	Late stage	23.685 ± 3.82		

* ($P \leq 0.05$)-S, ** ($P \leq 0.01$)-HS.

This result came in line with previous result by Morandi *et al.,* (2007) who showed the serum levels of sHLA-G were significantly higher in patients who developed a local or disseminated neuroblastoma (NB) than in those who remained in remission (Table 5).

This study found that, some discrepancy noticed in two parameters, gender and symptom results. Little variance observed in sHLA-G level in boys more than girls. Furthermore, noticed changes in sHLA-G level were observed and that depended on infection stages and immunity activation. In late stage of infection could be recognized wide difference in sHLA-G level with immunity regression in a comparison with initial stages of infection.

On the other hand, slight dissimilarity showed in sHLA-G level between children above one year and in the less. It is worth attention that, this study suggested that sHLA-G level in each individual may be little or no impacted with the age but in contrast with the sex. This point shared with many other studies as Carlini *et al.,* (2013) and Jeong *et al.,* (2014) results who suggested that the sHLA-G level had a negative relationship with different parameters as patient's age, gender and also location. In spite of the agreement in ages upset but this study differed in the sex argument.

Also, suggestion that the up-regulation in sHLA-G expression due to increase in immunity cytokines releasing. The immune regulation to *Leishmania* depended on the type of T-lymphocyte and special cytokines as IFN-^{3}, IL-12, 1L-10, TNF-β etc.

In VL infection, HLA-G can suppress the immune system through, prevention of T cell function by foul all of T-cell mechanisms as proliferation, cytotoxicity, apoptosis and regulatory T cells. Also, the differentiation, proliferation mechanisms and cytokine production in B lymphocytes (Morandi *et al.,* 2016).

Prevention of proliferation and cytotoxicity of peripheral blood NK cells stimulated of two mechanisms as proliferation and releasing of pro-angiogenic factors in blood and uterine NK cells. By down-regulating of chemokine receptors appearance on T, B and NK cells surfaces inhibits can suppress the chemotaxis of different of these cells populations, inhibition of phagocytosis process by impaired the production of reactive oxygen species in innate cells (Alvar *et al.,* 2020).

HLA-G proteins can be achieved their immunomodulatory functions through conjugate occurred between their receptors and other receptors expressed on immune cells surface. As LILRB1 (ILT2/CD85j), LILRB2 (ILT4/CD85d), and KIR2DL4 (CD158d) (Alegre *et al.,* 2014).

Keep in mind, that the expression of these receptors can be subjected to HLA-G without any costimulatory requirement as " autonomous" from any immune response (Lemaoult *et al.,* 2005) despite some inflammatory cytokines may be managed HLA-G expression (Ullah *et al.,* 2019).

From this study we conclude that the rK39 test is better than rKE16 in *Leishmania donovani* identification because it identifies recent from chronic infection, but it has a low sensitivity in L.*infantum* diagnostic.

Besides, visceral *Leishmania*species followedseveral ways to life insurance inside the hosts, and the immune-suppressive conditions were done with the aid of soluble HLA-G it appeared with the upregulation of this protein through the infection. So, these molecules may have verminous role in parasites passing from immune controlling.

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