
# Antibody and Cytokine Levels in Visceral Leishmaniasis Patients with Varied Parasitaemia Before, During and After Treatment in Patients Admitted to Arba Minch Hospital, Southern Ethiopia, 2017

By:

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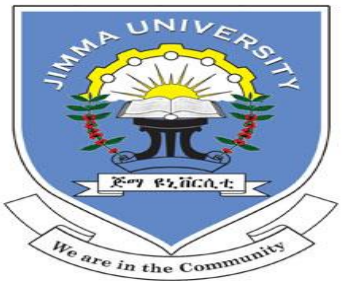


Jimma University

Institute of Health

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## I. Abstract

**Background:** *Visceral Leishmaniasis (VL) is a disseminated protozoan infection caused by Leishmania donovani parasites which affects almost half a million persons annually. Most of these are from the Indian sub-continent, East Africa and Brazil. In this study, we aimed to determine the levels of antibodies and cytokines in visceral leishmaniasis patients at the time of diagnosis, during treatment at different time points and after treatment so as to examine associations with parasitaemia and clinical states (prognosis) of patients.*

**Methods:** *A prospective study enrolling a total of 48 active Visceral Leishmaniasis (VL) patients were evaluated before treatment, during treatment at different time point and follow-up after treatment up-to three month to determine their serum cytokine concentration, antibody levels, splenic aspirate, laboratory and clinical parameters.*

**Results:** *Statistically significant differences in the absolute counts of WBC, Platelet and measurement of Hemoglobin (Hb) were observed before treatment at day 0 compared to during treatment at day 7, 14, 18, 18(EOT) and 30(EOT) days ($P \leq 0.001$). Measurement of serum cytokine showed that VL patients had elevated levels of circulating IL-10, IFN-γ, and TGFB regardless of microscopic parasite load and levels of antibody. The observed highly elevated level of IL-10 cytokine in serum was sharply decreased after start of treatment within seven days. High level of anti-leishmania antibodies was present in all VL patients regardless of spleen aspirate microscopic parasite grade before treatment and at different time during treatment. However; a significant decrease of antibody level observed at 120 days post treatment with median O.D. values of 1.47. IL-2 serum levels were below detection limit in VL patients.*

**Conclusions:** *In conclusion, the present results suggest that cytokines can be used as markers of disease in epidemiological studies to distinguish between the different clinical forms of VL. In addition, measuring circulating cytokines concentration could be used as criteria for cure in combination with other clinical evaluations and their usefulness should be confirmed in investigations conducted in other endemic areas.*



*Key words: Visceral Leishmaniasis, L. donovani, Kala-azar, ELISA, Cytokine, Arba Minch*

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| | |
|---|---|
| **AIDS** | Acquired Immuno Deficiency Syndrome |
| **BMA** | Bone marrow aspirate |
| **CL** | Cutaneous Leishmaniasis |
| **CRS** | Composite Reference Standard |
| **DNDi** | Drugs for Neglected Diseases initiative |
| **ELISA** | Enzyme-Linked Immunossorbent Assay |
| **EOT** | End of treatment |
| **GCLP** | Good Clinical Laboratory Practice |
| **GCP** | Good Clinical Practice |
| **HIV** | Human Immunodeficiency Virus |
| **IgG** | Immunoglobulin G |
| **IgM** | Immunoglobulin M |
| **IL-2** | Interleukin-2 |
| **IL-10** | Interleukin-10 |
| **IFN-γ** | Interferon-γ |
| **LRTC** | Leishmaniasis Research and Treatment Centre |
| **MCM** | Micro-Culture Method |
| **MOH** | Ministry Of Health |
| **NNN** | McNeal, Novy & Nicolle |
| **PBMCs** | Peripheral Blood Mononuclear Cells |
| **PICT** | Provider-Initiated Counseling and Testing |
| **PKDL** | Post-Kala-azar Dermal Leishmaniasis |
| **SPA** | Spleen aspirate |
| **SSG** | Sodium Stibogluconate |
| **TGFβ** | Tumor Growth Factor β |
| **Th** | T-helper cells |
| **TOC** | Test of cure |
| **VCT** | Voluntary Counseling and Testing |
| **VL** | Visceral Leishmaniasis |

# CHAPTER ONE: INTRODUCTION

## 1.1. Background

Species of the genus Leishmania that comes under subkingdom Protozoa, order Kinetoplastida, and family Trypanosomatidae, are the causative agents of leishmaniasis or leishmaniosis (1,2). Leishmaniasis is a parasitic infection caused by a range of *Leishmania* parasites harbored by a wide range of vectors and reservoirs distributed in all inhabited continents(3). The disease affects the poorest populations in 88 countries, 66 are in the Old World, 22 in the New World and of which 72 in the developing countries. There are an estimated 12 million cases worldwide; 1.5 to 2 million new cases occur every year(4), and about 350 million people are considered to be at risk(5,6). Even though leishmaniasis is now considered as one of the most neglected diseases, there is a clear and alarming increase in the number of cases in several regions.

These diseases are usually transmitted between vertebrate hosts by the bite of infected blood sucking female phlebotomine sand flies (Diptera, Psychodidae)(1) and are caused by over 20 species of *Leishmania*(7), which are obligatory intracellular parasites surviving within phagolysosomes of host macrophages. The parasite load in peripheral blood is generally so high that transmission among intravenous drug users by use of shared syringes has also been demonstrated(8). Leishmaniasis consists of four main clinical syndromes: Cutaneous Leishmaniasis(CL); Muco-Cutaneous Leishmaniasis (MCL; also known as espundia); visceral leishmaniasis (VL; also called kala-azar); and Post-Kala-azar Dermal Leishmaniasis (PKDL)(9).

Visceral leishmaniasis is a life-threatening parasitic infection that is endemic in 62 countries worldwide, including the Mediterranean region(10). More than 90% of global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil(4). VL is the second largest cause of parasitic death after malaria and is responsible for an important public health problem with almost 500,000 new cases and more than 50,000 deaths per year (4,11). There are two types of VL, which differ in their transmission characteristics: zoonotic VL is transmitted from animal to human and anthroponotic VL is transmitted from human to human. In the former, humans are occasional hosts and animals, mainly dogs, are the reservoir of the parasite. Zoonotic VL is found in areas of *Leishmania infantum* transmission and is found mainly in the Mediterranean basin and Latin America whereas anthroponotic VL is found in areas of *Leishmania donovani* transmission and is prevalent in the Indian subcontinent with estimated 300,000 cases/year; and in East Africa, primarily in Sudan, South Sudan and Ethiopia, with an

estimated 30,000–57,000 cases/year. The east Africa region is the second in disease burden next to the Indian subcontinent (4,9,11,12).

In Ethiopia, the first case of VL was documented in 1942 in the lower Omo plains, the Southwestern part of the country(13). The disease is prevalent mostly in lowland, arid areas, and the parasite involved is mainly *L. donovani*, with an estimated 4,500 to 5,000 new cases every year, and the population at risk is more than 3.2 million(1,14). The most important foci are found in northwestern and southwestern parts of the country. However, the ecology, and the vectors responsible for parasite transmission and epidemiology of VL differ between these regions. Northwestern Ethiopia (NW) accounts for 60% of the VL cases, and the majority of the HIV - VL co-infections, the main endemic region being the Metema - Humera plains nears the Sudanese border. *Phlebotomus orientalis* is the indicated vector responsible for transmission in this region(15). In southwestern Ethiopia (SW), VL foci are mainly located in the Omo River plains, Segen river plains and Woito Valleys, and near the border with Kenya(16). These regions include savannah forest, and *P. martini* and *P. celiae* have been implicated as vectors(17). Disease in Southern Ethiopia appears to be sporadic and stable occurring most frequently among children or young adults(16).

According to the Federal Ministry Of Health (FMOH)(18), the annual burden of VL in Ethiopia is estimated to be between 4,500 and 5,000 cases. Unfortunately, this data is unlikely to give a true reflection of the field condition, as the health facilities where this disease is diagnosed are not well equipped to identify VL cases. According to the risk map of VL in Ethiopia(19), the estimated total population is 3.2 million. This estimate is based on the national population census of 2007, and the proportion of the country's landmass considered suitable for VL transmission; which is around 375,633 $km^2$ -33% in northeastern, northwestern, western, and southeastern parts of the country. Given the increasing mobility of people, the wide geographic distribution of sandflies, and the high HIV-VL co-infection, there is an impending potential for VL to spread to the vast and highly populated areas of the country. The resurgence of leishmaniasis, its emergence in newer geographical areas and in newer hosts, besides changing the clinical profile of infected patients, has put forward newer challenges in the areas of diagnosis, treatment, and disease control.

The absence of antigen specific responses is thought to underlie the disease progression. Paradoxically the acute phase of VL is associated with elevated expression of IFN-γ mRNA in

lesional tissue, such as the spleen and bone marrow, as well as increased circulating levels of multiple pro-inflammatory cytokines and chemokine's, including IL-12, IFN-γ and TNF-a(20,21). These results imply that the failure to respond to Leishmania antigen stimulation observed in VL patients is not due to a defect in the ability to mount protective Th1 responses per se, but rather to induction of suppressive factors, e.g. IL-10, resulting in unresponsiveness of infected macrophages to activation signals(22). IFN-γ neutralization promotes parasite growth in active VL cases ex-vivo. These findings demonstrate that the elevated levels of IFN-γ in patients with active VL serve to limit parasite replication and suggest that therapeutic administration of IFN-γ may still hold potential(21).

The diagnosis of VL is complex because its clinical features are alike other commonly occurring diseases, such as trypanosomiasis, malaria, typhoid, schistosomiasis and tuberculosis; many of these diseases can be present along with VL (in cases of co-infection); and can confuse the clinical picture at the time of initial diagnosis. Sequestration of the parasites in the spleen, bone marrow, or lymph nodes further complicates this issue(9,23). As the clinical presentation of VL lacks specificity, confirmatory tests are required to decide which patients should be treated. Such tests should be highly sensitive (>95%) as VL is a fatal condition, but also highly specific because the current drugs of choice used to treat VL mostly antimonials, have high cost and significant toxicity(7,24). Ideally, a test should be able to make the distinction between acute disease and asymptomatic infection, because none of the drugs currently available is safe enough to treat asymptomatic infections. Moreover, such tests should be simple and affordable(25,26). Although major advances have been made in the diagnosis of leishmaniasis during the last decade, there is no specific method that can be adopted as the gold standard for detection and diagnosis of *Leishmania* infections.

The clinical presentation of VL follows with an incubation period that generally lasts between 2 and 6 months, VL patients present symptoms and signs of persistent systemic infection (including fever, fatigue, weakness, loss of appetite and weight loss) and parasitic invasion of the blood and reticulo-endothelial system (that is, the general phagocytic system**)**, such as enlarged lymph nodes, spleen and liver. Fever is usually associated with rigor and chills and can be intermittent. Fatigue and weakness are worsened by anaemia, which is caused by the persistent inflammatory state, hyper-splenism (the peripheral destruction of erythrocytes in the enlarged spleen) and sometimes by bleeding(9).

VL symptoms often persist for several weeks to months before patients either seek medical care or die from bacterial co-infections, massive bleeding or severe anaemia(7,9). Untreated VL patients act as reservoirs for parasites and therefore contribute to disease transmission in anthroponotic VL areas. Early case-finding and treatment is therefore considered an essential component of VL control(27). Patient management, screening of asymptomatic infections, surveillance including verification of elimination, and epidemiological studies are some of the areas where diagnostic tests play a major role. Consequently, Reliable laboratory and new methods become mandatory for accurate diagnosis that promotes early identification (detection) of the disease followed by rapid and adequate therapy.

The diagnosis of VL is traditionally based on direct demonstration of *Leishmania* parasites by microscopic examination, serologic testing, and in vitro isolation(23). Direct microscopic examination of spleen aspirates is considered the gold standard for VL diagnosis(28), but both the aspiration procedure and the reading of slides require a high level of expertise that makes it unsuitable for generalized field use. Lymph node and bone marrow aspirates are alternative procedures but lack sensitivity(28). Serological tests have been developed to replace parasitological diagnosis in the field. Accepted limits of *Leishmania* serologic testing are reduced sensitivity in patients with severe underlying immunosuppression, the absence of discriminating power between active or past infections, and, consequently, lack of value in monitoring the parasitological response to specific therapy. Finally, in vitro parasite isolation still remains the reference method to unequivocally identify the parasite, but it is expensive, cumbersome, and generally unhelpful in clinical practice because of the long duration of time (days to weeks) required to obtain a definitive result(23). However an urgent need exists for better diagnostic tests for VL in East Africa.

The outcome of the typical symptomatic clinical form of VL is critically influenced by the immune response developed by the host wherein the systemic infection, with spread of the parasites to the spleen, liver, lymph nodes, bone marrow and other organs, is accompanied by a high titre of circulating antibodies and a depression of Type 1 T-cell mediated immunity, with decreasing production of IFN-γ and IL-12 and a marked up-regulation of IL-4 and IL-10 (29). In addition, IL-10 protects against the side effects of an exaggerated inflammatory response, playing an important role in the regulation of the inflammatory response; all Th cells share the important task of controlling over exuberant immune responses by means of IL-10 production(30,31).

VL is becoming a growing public health threat; the spatial distribution and burden of VL is up surging year after year(32). VL–HIV co-infection is rising in north Ethiopia, and it poses a new and difficult challenge to VL control effort. VL–HIV co-infection is characterized by a number of complexities, including challenging diagnosis, increased drug toxicity, and poor treatment response. Hence, VL is developing both on a spatial and temporal basis in Ethiopia. New diagnostic tools are needed for the confirmation of VL disease and to establish test of cure in treated VL patients. Clinicians do not have the tools to distinguish re-infection from relapse in cases of recurrence, and control programs do not have validated assays for the surveillance of drug resistance in parasites. To decrease the VL burden in Ethiopia, community efforts should be supplemented with effective treatment programs to ensure access to appropriate and affordable diagnosis and case management. In addition to the VL confirmation, the available diagnostic markers should also serve as a test-of-cure after therapy, a marker for asymptomatic infections and for detecting drug resistance(33). Furthermore, in the context of the VL elimination initiative, it would be desirable to have better markers of leishmanial infection at the population level. Considering its recent challenge in diagnosis, this research would try to propose a way forward on comparison of parasitological and immunological methods for VL control programs.

In this study, we aimed to determine the levels of antibodies and cytokines in visceral leishmaniasis patients at the time of diagnosis, during treatment at different time points and after treatment so as to examine associations with parasitaemia and clinical states (prognosis) of patients.

## 1.2. Statement of the problem

As the clinical presentation of VL lacks specificity, confirmatory tests are required to decide which patients should be treated. Such tests should be highly sensitive (>95%) as VL is a fatal condition, but also highly specific because the current drugs of choice used to treat VL mostly antimonials, have high cost and significant toxicity(7,24). Ideally, a test should be able to make the distinction between acute disease and asymptomatic infection, Moreover, such tests should be simple and affordable(25,26). Although major advances have been made in the diagnosis of leishmaniasis during the last decade, there is no specific method that can be adopted as the gold standard for detection and diagnosis of *Leishmania* infections. In addition to the search for newer diagnostic tools, coordinated efforts are now required at the international level to standardize and move the available assays from the laboratory to the field.

The development of diagnostics to guide treatment is the first step to achieve the goal of VL elimination. In patients with typical clinical presentation and a positive serology, parasitological confirmation is advised as the finding and quantification of parasites help in assessing response to treatment. In patients with positive serology but atypical clinical disease, parasitological diagnosis is recommended. The sensitivity of microscopic examination of bone marrow or lymph node examination is sub-optimal; however, a negative result does not rule out a leishmaniasis infection(34). On the other hand, none of the common methods routinely used for the parasitological diagnosis of visceral leishmaniasis is satisfactory and specific serology is unreliable for immunocompromised patients.

Leishmania specific cellular immune response seems to play a fundamental role in the control of infection. T-helper response patterns determine outcome of Leishmania infections in that Th1 responses enhance cell-mediated immunity leading to limiting parasite growth, control of infection and resistance to reinfection whereas Th2 responses lead to a dominantly humoral response and to exacerbation of the infection(35). Th1 unresponsiveness is characteristic of active visceral leishmaniasis(36). The increase in inflammatory cytokines could be associated with the physio-pathological alterations seen during the active disease, whereas the production of IL-10 has been suggested to be important for the survival and persistence of the parasite inside macrophages(37). High antibody titers and immune complex formation in VL may contribute to the elevated IL-10 levels observed and participate in the progressive decline in the immune status of VL patients(38).

# CHAPTER TWO: LITERATURE REVIEW

The diagnosis of Visceral Leishmaniasis is based on a combination of clinical symptoms, epidemiological, and laboratory approaches such as parasitological detection, immunological tests and molecular techniques(2,39). Various published works and reports documented different kinds of diagnostic techniques associated with Visceral Leishmaniasis. Some of the available literatures on accuracies of diagnostic techniques of VL are reviewed as follows.

### 2.1.1. Composite Reference Standards

In endemic areas, clinical symptoms of VL do not appear in all infected individuals, and a fraction of the infected population remain asymptomatic(40). The seroprevalence in healthy populations varies from <10% in low to moderate endemic areas to >30% in high-transmission foci or in household contacts. In Latin America, where VL is zoonotic, rK39-ICT is widely used for diagnosis of VL in humans, and has clear advantages over the Indirect Fluorescent Antibody Test (IFAT) or ELISA based tests(41). The DAT has shown similar diagnostic performance but is not as user-friendly as the rK39(41). A study from Sudan compared the diagnostic performance of rK39 and DAT for VL both qualitatively and semiquantitatively leading to a recommendation on the combined application of these two tests for optimizing diagnosis and simultaneously assessing the magnitude of immune response to *L. donovani* infection(42). Qualitatively both rK39 and DAT demonstrated comparable reliability for VL detection (sensitivity = 96% and specificity = 98.7% or 99.3%)(42).

In selected villages in Libo Kemkem and Fogera districts (Amhara State, Ethiopia), they conducted a study to assess the usefulness of different approaches to estimate the asymptomatic infection rate. Of 605 participants, the rK39 Immunochromatographic test was able to detect asymptomatic infection in 1.5% (9 of 605), direct agglutination test in 5.3% (32 of 605), and leishmanin skin test in 5.6% (33 of 589); the combined use of serologic methods and leishmanin skin test enabled detecting asymptomatic infection in 10.1% (61 of 605). They conclude that the best option to detect asymptomatic infection in this new visceral leishmaniasis–endemic focus is the combined use of the direct agglutination test and the leishmanin skin test(43).

### 2.1.2. Parasite detection

#### 2.1.2.1. Direct Microscopy

The most suitable diagnostic method for leishmaniasis is detection of the amastigote form of the parasite by microscopic examination of tissue aspirates(44). Although the specificity is high, the sensitivity of microscopy varies depending on the tissue used, being higher for spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates(45). However, spleen and liver aspiration can be complicated by life threatening hemorrhages in ~0.1% of individuals, which is also painful and unpleasant for the patients and therefore requires considerable technical expertise, as well as facilities for nursing surveillance, blood transfusion and surgery. Moreover, the accuracy of microscopic examination is influenced by the ability of the laboratory technician and the quality of the reagents used(4,7,25).

#### 2.1.2.2. Parasite culture

Parasite culture method from bone marrow or splenic aspirates is simple, cheap and relatively sensitive, (sensitivity for SA culture in an NNN tube is around 97 to 100%, whereas the sensitivities for bone marrow and lymph node aspirates are estimated at 70-80% and 60-70%, respectively) and facilitates the diagnosis but suffers from its vulnerability to contamination. In addition, the culture method of the parasite is usually time-consuming, which makes it not an ideal method for field use and therefore remains restricted to referral labs. Advancements in the culture method include the development of a more sensitive micro-culture method (MCM) for isolation of Leishmania(46). Another modification in this method involves the use of buffy coat and peripheral blood mononuclear cells (PBMCs) isolated from patient blood that improves the sensitivity of the culture based VL diagnosis(47). The detection of parasites in the blood or organs by culture or by using molecular techniques such as PCR is more sensitive than microscopic examination but these techniques remain restricted to referral hospitals and research centers, despite efforts to simplify them(7,11,26).

#### 2.1.2.3. Antibody-detection tests

Immunological tests are based upon the detection of antileishmanial antibodies and leishmanial antigens, which are useful in both individual diagnosis and epidemiological surveys. To avoid the problems described for parasite identification in tissue smears, several noninvasive diagnostic tests that detect specific antileishmanial antibodies have been developed, but sometimes accompanied by major limitations. First, though serum antibody levels decrease after successful

treatment, they remain detectable up to several years after cure(48). Therefore, VL relapse cannot be diagnosed by serology. Second, a significant proportion of healthy individuals living in endemic areas with no history of VL are positive for anti-leishmanial antibodies owing to asymptomatic infections(24,49).

Antibody-based tests must therefore always be used in combination with a standardized clinical case definition for VL diagnosis. Current serologic tests, such as rk39, direct agglutination test (DAT), immunofluorescence antibody tests (IFAT) and enzyme-linked Immunossorbent assay (ELISA), use crude antigen preparations and are limited in terms of both specificity and assay reproducibility.

#### 2.1.2.4. Indirect Immunoenzyme based ELISA

ELISA is one of the most sensitive tests for the serodiagnosis of VL. The test is useful for laboratory analysis or field applications and to screen large numbers of samples. The recombinant product of K39 (rK39) has been proven to be very sensitive and specific antigen to be used in ELISA for the serodiagnosis of VL in different endemic foci. For example, the excretory, secretory and metabolic antigens released by *L. donovani* promastigotes into a protein-free medium used for serodiagnosis of VL showed 100 % specificity and sensitivity(34). The recombinant antigen, rK39 is specific for antibodies against VL caused by *L. donovani* complex members. It is highly sensitive and predictive for onset of acute disease(50). Although the sensitivity of the test is high, it is entirely influenced by the antigen used in the test(34). In contrast, rK39 does not show detectable antibodies in cutaneous or mucocutaneous leishmaniasis(51). The antibody titre to rK39 ELISA has a good correlation to the efficacy of chemotherapy in visceral leishmaniasis; during the period in which the disease is active, the antibody level is very low (51,52). rK39 based ELISA has been used as a prognostic test for monitoring VL patients undergoing drug therapy and is also useful in predicting clinical relapse(23). rK39 based ELISA has been shown to have high predictive value for detecting VL in immunocompromised patients with VL-HIV co-infection (53). ELISA using rK39 detects asymptomatic infection earlier than the DAT(54). However, Due to the requirement of skilled personnel, sophisticated equipment, and electricity, using ELISA for diagnosing VL is not usual in the endemic areas(26). In addition, rK39-ELISA has a high predictive value for detecting VL in immunocompromised patients, like those with AIDS patients(53). However, there are also reports from Sudan and other countries where assays based on this antigen have much lower

sensitivity and specificity. For example, in Sudan, 7 % of parasitologically proven cases were missed by the rK39 ELISA(55).

Studies by Saha et al (56) have evaluated the utility of leishmanial membrane antigen specific Immunoglobulin(Ig) Isotypes and IgG subclasses for the specific diagnosis of PKDL. Most of the PKDL patient's exhibited elevated levels of antileishmanial IgG, IgM, IgA, and IgG subclass (IgG1, IgG2, and IgG3) antibodies, on the other hand, the absence of IgE and low levels of IgG4 were documented. The sensitivity and specificity of the IgG ELISA using membrane proteins for the diagnosis of PKDL was 100% and 96.7% respectively, higher than the specificities of ELISAs with promastigote and amastigote antigen extracts or rK39(56).

**2.1.2.5. Cytokine Profile (IFN-γ, TGFB1, IL-10 and IL-2)**

The factors determining the development or not of visceral leishmaniasis (VL) have not been completely identified, but a Leishmania specific cellular immune response seems to play a fundamental role in the final control of infection. Cytokines can be used as markers in epidemiological studies conducted in endemic areas to distinguish between different clinical forms of VL(57).

Studies by Hailu et al., (20) shows a follow-up study of individuals infected with *Leishmania donovani* was undertaken in an area of south-western Ethiopia endemic for visceral leishmaniasis (VL). Plasma levels of IFN-γ, IL-12p40, IL-18, IL-15, IP-10, and Mig were markedly elevated in symptomatic VL patients (n=70) compared with individuals with asymptomatic Leishmania infections (n=39), malaria patients (n=13), and healthy controls from the endemic area (n=12). A significant decrease of IFN-γ and all mediators was observed after treatment (n=33) of VL patients(20,36). Plasma concentrations of IFN-γ and IL-10 were elevated in VL(36). levels of the serum cytokines, IL-10, IL-12, and IFN-γ , were higher in patients than in family members and control individuals(58). These data show that increased plasma levels of IFN-γ, as well as the mediators involved in the production and the activity of this cytokine, are characteristic of active VL in humans, and may play an important Immuno-pathogenic role.

Studies by Pradyot et al (59) revealed a significant positive correlation between parasite load and plasma as well as antigen specific levels of IL-10 and TGFβ, in addition, they demonstrates IL-10 and TGFβ in antigen-stimulated PBMCs from culture supernatants, with parasite loads of VL patients, supporting their roles in disease pathology. Verma et al., (2010) establish that the high parasite load in VL is strongly correlated with a high level of IL-10, implicating IL-10 as a marker

of disease severity(60). TGFβ has been shown to impart down-regulatory effects on macrophages and its blockade could restrict parasite progression in these cells(61).

Caldas et al., high levels of IL-10 and IFN-γ observed during active VL are sharply decreased in most patients by 30 days post treatment and IFN-γ are almost vanished 120 days after treatment(62). Kumar et al(21) shows in treated ex-vivo Splenic Aspirate cultures; IL-10 levels in the Splenic Aspirate supernatants were not affected by neutralization of IFN-γ, suggesting that the inhibitory effect of IL-10 on parasite killing does not completely abolish the parasite-controlling effects of endogenous IFN-γ.

## 2.2. Significance of the study

Accurate diagnosis of visceral leishmaniasis (VL) still remains a problem for clinicians. Though the gold standard still remains to be the demonstration of parasites, it has several disadvantages. Antibody based diagnosis, like rK39 strip test, is being widely used in the endemic countries despite their inherent disadvantage of being positive in significant proportion of healthy individuals. The main limitations and problems in diagnosis of VL are; the invasive sampling techniques, the detection of asymptomatic *Leishmania* in infected patients, and absence of a reliable test-of-cure. Although molecular diagnostic tests are considered more expensive than conventional diagnostic techniques, the ease with which molecular tests can now be performed and the rapid results generated enable timely diagnosis, resulting in overall savings. Cost savings may be realized as rapid diagnoses prevent invasive diagnostic procedures limiting unnecessary or potentially toxic treatments. They also shorten hospital stays, replace labor-intensive conventional (e.g. cell culture) methods, and/or shorten hands-on laboratory technologist time.

To understand how the immune response generated during different treatment stages of active VL before treatment, during treatment and post treatment in the host is impacted by the presence of different cytokines resulting in clinical pathogenesis and disease, we have studied the relation between different immunological markers, including antibody levels in VL patients. This will help in prescribing accurate treatment, improving quality of the care of the patient and to reduce the morbidity and mortality rates. We chose to work with peripheral blood serum sample, as this type of sampling is noninvasive, straightforward, and easy to repeat. In this context, we planned to identify possible immunological biomarkers that could be correlated with patients' clinical and parasitological presentation as well as the response patterns to treatment in visceral leishmaniasis patients of south western Ethiopia. The test panels includes; Cytokine concentration (IL-10, IFN-Γ, TGFB andIL-2), *Leishmania donovani* amastigote visualization by direct microscopic examination of spleen or bone marrow aspirate sample; antibody tests by Indirect Immunoenzyme based ELISA.

## 2.3. Conceptual frame work

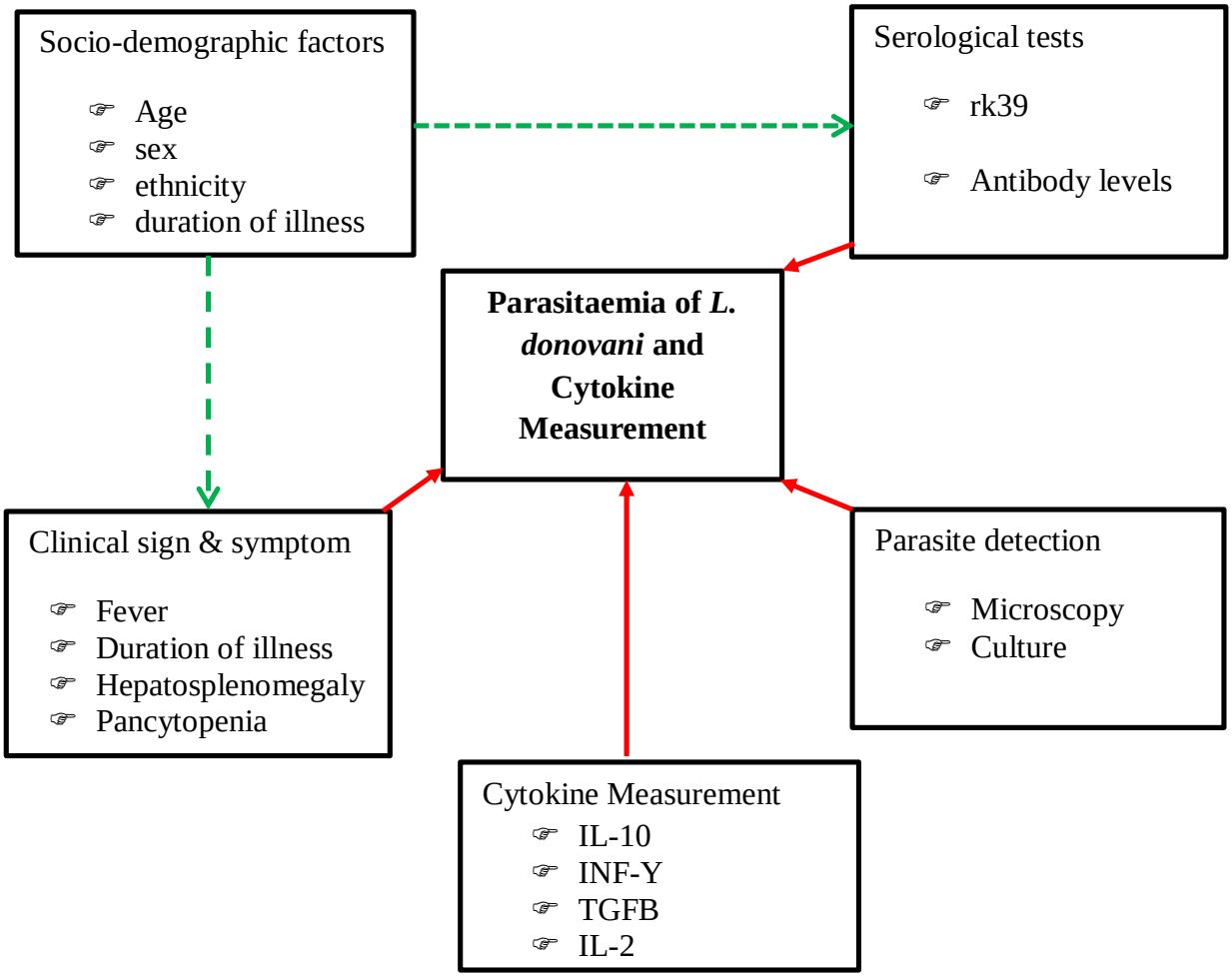



**Figure 1: Conceptual frame work**

**Indicators**

- - -> The broken line indicates that there is association between the factors; however, it is not the interest of this study to see these associations

——> The activated line indicates that there is association between the factors, and it is the interest of this study to examine these associations

Figure 1: Conceptual frame work for this study developed based on laboratory diagnosis of Visceral Leishmaniasis (VL).

# CHAPTER THREE: OBJECTIVES

## 3.1. General objectives

- To evaluate the diagnostic value of serum cytokine levels in visceral leishmaniasis patients and to compare with standard parasitological diagnosis by using antibody levels as measured by recombinant antibody based ELISA and serum cytokine levels before treatment, during treatment at different stages and as a test of cure (TOC) after treatment.

## 3.2. Specific Objectives

1. To compare the conventional parasitological diagnostic methods and serum cytokine levels in the diagnosis of VL at day 0 and as a TOC at EOT in patients defined as true cases of VL by composite reference standard (CRS) diagnostic criteria.

2. To determine the serum cytokine levels involved in the immune response of individuals with the clinical form of VL in order to establish a correlation between the clinical-evaluative behavior of the disease and cytokine production.

3. To compare antibody levels determined by tests to the recombinant k39 antigen with serum cytokine levels of VL patients at different stages of diagnosis and treatment.

4. To compare conventional parasitological diagnostic methods and rk39-specific antibody levels in the diagnosis of VL at day 0 and as a TOC at EOT in patients defined as true cases of VL by CRS diagnostic criteria.

# CHAPTER FOUR: METHODS AND MATERIALS

## 4.1. Study area

The study was conducted at Arba Minch General Hospital in Gamo Gofa zone, Southern Nations, Nationalities' and Peoples' Region (SNNPR) ; Southern Ethiopia. Arba Minch town is the administrative and trading center of the Gamo Gofa zone, located at 505 km from Addis Ababa and 275 km south west of Awassa. The total area of the town was estimated about 1095 hectares and it lied at an altitude of 1300 meters above sea level; its average temperature is 29°C and the average annual rainfall is 900 mm.

Arba Minch hospital is a general hospital originally built to house 50 beds, later expanded to above 300 beds and serving a population of approximately two million. The Hospital provided general outpatient service, surgical & obstetric emergency services, general medical and pediatric in-patient services(63). There was a specialized Centre for leishmaniasis treatment and research in the Hospital that is supported by Drugs for Neglected Diseases initiative (DNDi) and Lead by Professor Asrat Hailu. The LRTC was an established clinical trial site adhering to Good Clinical Practice (GCP) and Good Clinical Laboratory Practice (GCLP) standards. The Centre had been its own laboratory where hematological and clinical chemistry were done routinely for patients with leishmaniasis. Patients presented to the Centre either directly by themselves or were referred from other Health services after screening by rk39 rapid test.

## 4.2. Study design and Period

A descriptive cross-sectional study was conducted among VL patients admitted to the Leishmaniasis Research and Treatment Centre (LRTC) of Arba Minch General Hospital (AMGH) from 09/Sep/2015 to 05/May/2017.

## 4.3. Population

### 4.3.1. Source population

All patients who were present at the study site either directly by themselves or were referred from other Health services.

### 4.3.2. Study population

The study population comprised of VL cases diagnosed at AMGH based on composite diagnostic criteria (clinical or serological diagnosis combined with favorable therapeutic response or

parasitology by smear and/or culture) during the study period. VL diagnosis as such was regarded as those diagnosed by Composite Reference Standard (CRS). Based on CRS, the definitive diagnosis was by either of the following: 1) presence of definitive clinical sign and symptoms as well as positive antibody test with rk39 rapid test) combined with a favorable response to treatment; and 2) parasitological confirmation by smear or culture. Cases of VL diagnosed as such was further assessed for enrollment eligibility, and asked to consent and must meet all the inclusion criteria, and must not have any of the exclusion criteria listed below.

### 4.3.3. Reference standard

Several published VL diagnostic accuracy studies suffer from reference test bias. Researchers comparing a new test to a reference standard with high specificity but low sensitivity, such as bone marrow or lymph node smears, will underestimate the true specificity of the new test. This kind of sub-optimal reference standard misses many true VL cases; such cases might test positive with the new test, e.g. qPCR. Moreover, lymph-node-positive VL patients probably comprise only a sub-set of all VL patients in a given region, and this might again bias the sensitivity estimates. All of the tissue aspirate assays have another inherent problem: they cannot be applied indiscriminately to healthy controls, which complicates the ascertainment of control status(24).

The demonstration of parasite amastigotes in smear or culture of splenic aspirates should be used as the reference standard in VL diagnostic accuracy studies, if the procedure can be carried out safely. Given flawless technical execution, it will be both specific (~100%) and sensitive (>95%). Some Centre's use sequential lymph node and/or bone marrow aspirates; followed by splenic aspiration if the other aspirates are negative. This has the advantage of limiting the number of splenic aspirations while maintaining high sensitivity and specificity.

In cases where splenic aspiration cannot be used, researchers can opt to use either a composite reference standard (CRS) as mentioned above or latent class analysis (LCA)(64). Both involve the use of several diagnostic tests as comparators for the test under evaluation, the former being an empirical definition of disease status and the latter a mathematical approach based on the probability of disease given the observed test pattern. Notwithstanding their inadequate specificity for acute disease, serological tests for VL can be included in the panels of tests used in CRS or LCA, but cannot be considered as a reference standard for stand-alone use. In the past, response to specific VL treatment was used to confirm that a diagnosis was correct, as antimonials have a very narrow spectrum(24).

## 4.4. Inclusion and Exclusion Criteria

### 4.4.1. Inclusion criteria

Patients must meet all the following criteria to be considered eligible for inclusion in the study:

- Male or female ≥ 5 years of age;
- Had been in an area thought to have VL transmission;
- Had a history of persistent fever (2 weeks or more);
- Had splenomegaly;
- Positive result either with rk39 rapid test or DAT titres of 1:800;
- Is willing to provide signed informed consent as an individual or via his/her lawful guardian (for those under 18).

### 4.4.2. Exclusion criteria

Patients who were met any of the following criteria were excluded from the study ("study ineligible"):

- Clinical acute malaria episode during diagnosis proven by parasitological examination of blood films;
- Pregnant patient;

## 4.5. Sample size determination and Sampling technique

### 4.5.1. Sample size

The sample size was determined according to the expense and number of VL patients and non-probability sampling methods (purposive sampling technique) was used to enroll a total of 48 VL patients' from the study site.

### 4.5.2. Sampling Technique

Purposive sampling technique was used, in which all study participants that was admitted for VL treatment and fulfill the inclusion criteria were enrolled consecutively. Samples were collected before treatment at day0, during treatment at day7, 14, and 18, end of treatment (EOT) at day 18 or 30 and post-treatment after 120 days of follow up.

## 4.6. Study variables

### 4.6.1. Dependent Variables

- Cytokine concentration levels (IL-10, IFN-Γ,TGFB1 and IL-2)
- Parasitological tests (Microscopy and culture);
- Antibody levles of ELISA OD values

### 4.6.2. Independent variables

- Socio-demographic factors such as age, sex, ethnicity, duration of illness, etc.
- Fever for two weeks or more;
- Splenomegaly;
- Positive result with rk39 rapid test; and
- Laboratory parameters
- Stage of treatment response and outcomes

### 4.6.3. Operational definition

- **Visceral leishmaniasis (kala azar) –** is a parasitic disease caused by *L. donovani* following the bite of female phlebotomine sand fly with clinical features of fever, spleen and/or liver enlargement and pancytopenia among others and confirmed by serologic (DAT) and/or spleen or bone marrow aspirate.
- **Presumptive Visceral leishmaniasis (kala azar) -**is a parasitic disease caused by *L. donovani* with clinical features of fever, spleen and/or liver enlargement and pancytopenia among others and confirmed by serologic test either by rk39 rapid test and/or DAT titre.
- **Composite Reference Standard (CRS) -** an empirical definition of disease status by the use of several diagnostic tests as comparators for the test under evaluation.
- **Successful treatment/ recovery/cure** – discharge after receipt of at least 25 drug doses (including if indicated, the additional doses specified after a test of cure specimen was positive for parasites) and no admission within a year of follow up.
- **Relapse** – discharge after receipt of at least 25 drug doses (including if indicated, the additional doses specified after a test of cure specimen was positive for parasites.) and became positive after declared cured.
- **Splenic aspiration**- is a procedure done by an experienced clinician under favorable condition in an attempt to demonstrate Leishmania amastigots in spleen tissue.

- **Kala azar** – Another commonly used name for visceral leishmaniasis.
- **Defaulter** – One who withdraws by himself/ herself any time before 25 drug doses is received and test of cure (T-O-C) is done.

## 4.7. Methods of data collection

### 4.7.1. Diagnosis and consenting procedures

Eligible clinical cases were duly informed about the objectives and procedures of the study. In the event he/she agreed to participate, a consent form was signed, and the patient was enrolled in the study. Informed written consent was taken from the parents or guardians of patients under 18 years of age. Impartial witnesses were able to sign for the patient in case the patient was unable to read or write. In addition, if the child was able to provide assent, his/her assent was taken as well.

For the purposes of this study, a Study Enrollment Log was maintained at enrollment/testing area. Study patients who signed informed consent were a subset of patients triaged according to routine standard of care and found eligible for the study. Information on triaged patients who didn't meet study eligibility was documented according to standard clinic practice, for example a clinic registry.

For each enrolled patient, a Case Report Form (CRF) that records pertinent demographic, clinical data and treatment given was completed, as well as results for CBC labs, parasitology, rk39 tests and HIV rapid test results. All patient-related data was handled on the basis of confidentiality.

Once a VL diagnosis was made, patients were received a combination drug of Paromomycin (750mg) Sulphate(250mg) (15mg(PM)/kg) and Sodium Stibogluconate (SSG) (20mg/kg with max. 0f 850mg/kg) for 17 days. Supportive care such as blood transfusion and treatment of undercurrent infections were given.

In patients suspected to had VL, clinical sign and symptoms suggestive of VL (abdominal swelling with or without left upper quadrant pain, hepatosplenomegaly, lymphadenopathy, pancytopenia, anemia, and fever of at least 2 weeks duration), and rk39 RDT test was observed.

Only those VL patients who fulfill the above criteria were included in this study. As a routine procedure, all aspirates for microscopy were simultaneously inoculated into NNN media in an

attempt to grow parasites in vitro. After inoculation, the culture tubes were incubated at 22-28°C and transported to Addis Ababa University LRTC for reading the results and for further sub-culturing.

## 4.8. Laboratory investigations

Prior to study consent and enrollment, presumptive VL diagnosis was made based on the WHO clinical case definition: prolonged fever (at least 2 weeks), weight loss and splenomegaly in a patient from an endemic area or with a travel history. In patients who meet those case definition, rK39 dipstick test (InBios International, Inc.Seattle, WA, USA) were performed as part of standard of care "triage" to rule out suspected VL patients and to assess eligibility for enrollment. Since the late 1990s, ample evidence has been generated that a combination of the WHO clinical case definition for VL and a positive antibody test is an adequate and safe basis for the decision to treat(65).

As part of further standard of care testing, all VL suspects who were enrolled in the study was had Red Blood Cell, WBC, Hemoglobin and Platelet counts systematically done as well as direct microscopic examination of splenic or bone marrow aspirates and culture of parasites from these aspirates. The classical confirmatory test for VL by microscopic detection of the amastigote stage of the parasite in tissue aspirates (spleen, bone marrow, and lymph node) was presently a routine procedure in AMGH, which creates an opportunity to gather data on microscopy and culture of aspirates.

Other diagnostic tests requested by the clinician in charge of the patient were not to be denied and should be reported in the CRF. That includes testing for HIV either by Voluntary Counseling and Testing (VCT) or by Provider-Initiated Counseling and Testing (PICT) and Chemistry test results. All results were recorded in the appropriate CRF.

In addition, the laboratory tests outlined below (4.9.1, 4.9.2, 4.9.3, 4.9.4, 4.9.5) were performed according to study SOPs, as described in the Study-Specific Procedures (SSP) and the study's manual of operations. Staff was trained in the execution of these laboratory tests prior to the initiation of this study.

### 4.8.1. Microscopy

Direct, semi-quantitative microscopic examination of 5 minute methanol-fixed, 5 minute Jenner-stained and 15 minute Giemsa/Leishman-staining of two good-quality smears were prepared

from splenic or bone marrow aspirates. In preparations, after staining with Giemsa or Leishman stain, amastigotes were oval with nucleus and kinetoplast. The cytoplasm appears to be pale blue, with a relatively large nucleus that stains red and the kinetoplast was deep red or violet rod-like body(44). Splenic or bone marrow smears were read in a standardized way according to the SOP in the SSP. Parasites were graded on a scale from 0 to 6+ parasites per 1000 fields as viewed with a 100x oil-immersion lens(6) (see Table 1).

**Table 1: Grading of parasite smears**

| NUMBER OF AMASTIGOTES PER 1000 FIELDS | Grade |
|---|---|
| Greater than 100,000 | 6+ |
| 10,001 – 100,000 | 5+ |
| 1,001 – 10,000 | 4+ |
| 101 – 1,000 | 3+ |
| 11 – 100 | 2+ |
| 1 – 10 | 1+ |
| 0 | 0 |

### 4.8.2. Culture

Materials from splenic or bone marrow aspirate (BMA) was immediately placed in culture tubes containing biphasic medium McNeal, Novy & Nicolle (NNN). The cultures was incubated at 22-28°C and after one week, one drop of media was examined by inverted microscope (400+magnifications) for the presence or absence of promastigote of *Leishmania* species; and positive samples were sub-cultured for other investigations. Negative cultures were kept for three to four weeks with weekly regular examination before being declared negative and discarded.

### 4.8.3. Indirect Immunoenzyme based ELISA

The ELISA method is based upon the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. Antibody levels in patients' serum were measured using an Indirect Enzyme Linked Immunosorbent Assay (ELISA) reagents (Vircell, Granada, Spain) to test IgG+IgM antibodies against *Leishmania donovani* in human serum, 96 tests; Performed according to manufacturer's instructions. Briefly, add 100 µl of serum dilution solution to all wells were added and 5 µl of each sample, 5 µl of positive control, 5 µl of cut off serum (cut off

serum in duplicate) and 5 µl of negative control into the corresponding wells were added and incubated at 37±1ºC for 45 min. After extensive wash with $EL_X$ 800 Bioelisa Washer (biokit, Highland Park, USA) to remove unbound antibodies 100 µl of conjugate solutions added into each well; sealed and incubated at 37±1ºC for 30 min, 100 µl of substrate solution added and incubated at room temperature for 20 min, after adding 50 µl of stopping solution the absorbance of antigen-antibody complex was read with $EL_X$ 800 Bioelisa Reader (biokit, Highland Park, USA) at 450/630 nm within 1 hour of stopping. Positive and negative sera were run in each plate to standardize the readings and the plate to plate and day to day variations and for validation of the assay and kit. OD values were converted to an "index" of numbers such that they could be given in full integer semi-quantitative numbers that could be translated to dichotomized qualitative values by using the formula:

*AI (Antibody Index) = (Sample OD/Cutt-off OD)*10.*

### 4.8.4. Cytokine Profile (IL-10, IFN-γ, TGFB1 and IL-2)

Cytokine levels in patients' samples were estimated using Enzyme Linked Immunosorbent Assay (ELISA) reagents (affymetrix, eBioscience Inc., San Diego, CA, USA) for IL-10, IFN-γ & IL-2 and (affymetrix, eBioscience Inc., Vienna, Austria) for TGFB; performed according to manufacturer's instructions. Briefly, 100µL/well of capture antibody in 1X Coating buffer coated in corning costar 9018 (or Nunc Maxisorp®) with specific capture antibodies against one of the four cytokines (IFN-γ, TGFB1, IL-10, and IL-2) was incubated overnight at $2\text{-}8^0$. Standards, patient serum samples and quality controls for each cytokine were mixed likewise. After 2 hours of incubation followed by extensive wash with $EL_X$ 800 Bioelisa Washer (biokit, Highland Park, USA) to remove unbound proteins, 100µL/well of biotinylated detection antibodies was added and the absorbance of the streptavidin-HRP complex measured with $EL_X$ 800 Bioelisa Reader (biokit, Highland Park , USA) at 450/630 nm. Serum cytokine levels were calculated by interpolating the standard curve for absorbance readings of test samples calculated from standards of known concentrations provided with the kits and the results were expressed as picograms (pg) of cytokine/ml, based on the standard curves of the respective cytokine. The range of minimum sensitivity of each cytokines was 2pg/ml for IL-10, 4Pg/ml for IFN-γ, 156.3pg/ml for TGFB1 and 2pg/ml for IL-2.

### 4.9. Patient care

Patients with positive parasitology or having other signs of VL, in instances in which parasitological examination is not feasible, was treated in accordance with current country/hospital policy and WHO guidelines. The diagnostic and therapeutic procedures used for patients enrolled in the study were standard of care for the respective hospital. The HIV status of VL patients was an important parameter in deciding what treatments to offer. Thus, all confirmed VL patients was tested for HIV by PICT procedures if not previously examined under VCT.

### 4.10. Ethical considerations

All individuals included in this report provided written informed consent to participate in the study before clinical samples were collected. Consent for inclusion of young children, was obtained from parents or guardians and assent from children under the ages of 12-17 years were obtained from the minor (themselves). Examination of patients and sample collection was performed as per the national guidelines and procedures of Ethiopia. The study was reviewed and approved by the ethical review committee at the Medical Faculty of Jimma University.

### 4.11. Data Quality assurance

Questionnaire and informed consent forms were translated from English to Amharic and back to English to reaffirm consistency. Two individuals who were able to spoke and write English and Amharic with a professional background was translated the Questionnaire and informed consent forms from English to Amharic individually. Two of them were discussed in the translated material and consensuses were reached. Another neutral person was translating it back to English where amendments and corrections were made.

Every day, data collection outputs were cross-checked for completeness and consistency. Standard operational procedure tools were strictly used for sample collection, transportation and storage. Special emphasis was given during coding (labeling) of the data sheet as well as the collected blood samples. All reagents were checked for proper storage conditions and shelf life. To avoid measurement bias, internal quality control was run along with the test sample. All tests were performed according to the manufacturer's instructions. Any deviation from the recommended procedure was recorded.

As the interpretation of RDT and microscopy results were subjective; it was recommended that at least two individuals read the test results independently. In this type of agreement study,

blinding was necessary to ensure the independence of test results in the evaluation. Laboratory staffs were blinded to the above test results and vice versa.

### 4.12. Statistical analysis

The data were processed using the IBM SPSS Statistics 20 Full statistical program (version 20.0) and Microsoft Excel (Office 2013) data analysis and chart tools. Initially, difference in values of laboratory parameters and cytokine levels were compared between groups using the nonparametric statistics and, when applicable, the median, interquartile [IQ] range and 95% confidence intervals were calculated for descriptive analysis of the data. The level of significance was set at $P \leq 0.05$. GroupWise comparison of average values of cytokines was performed using nonparametric statistics (Mann-Whitney U test or the Wilcoxon Matched-Pairs Signed-Rank Test ([Kruskal-Wallis test for two groups]) was used to examine whether continuous variables from two or multiple groups, respectively, originated from the same distribution. Spearman's ($r_s$) rank correlations were computed to assess statistical dependence between cytokine levels and the corresponding patient demographic and/or laboratory and clinical characteristics and between each cytokine pair.

# CHPTER FIVE: RESULTS

## 5.1. Socio- demographic characteristics

A total of 48 VL seropositive individuals who had been attending Arba Minch hospital LRTC Center from October 2015 to July 2016 were included in the study. All patients had the diagnosis of VL confirmed by rK39 serology and/or splenic or bone marrow aspiration of parasites. The median age of the participant's was 18.5 (interquartile range (IQ) 10.00-25.00) and within the age ranges of 4.00-58.00 years. Higher proportions of the subjects were male (male (n=38) and female (n=10)). Pairwise comparison of clinical and laboratory findings of patients with active disease presented hematologic disorders, especially a decrease in platelet, WBC, RBC and law Hemoglobin (Platelets 98,000/$mm^3$ ;interquartile range (IQ) 65,250-153,000/$mm^3$, WBC 1,900/$mm^3$; interquartile range (IQ) 1,400-3,000/$mm^3$, RBC 3.12x $10^6$/µL interquartile range (IQ) 2.86-3.51x $10^6$/µL and Hemoglobin 7.25g/dl; interquartile range (IQ)6.35-8.63 g/dl) before treatment (day 0) as compared to after treatment, patients recovery, (Platelets 260,000/$mm^3$ ;interquartile range (IQ) 174,250-307,500/$mm^3$, WBC 3,550/$mm^3$; interquartile range (IQ) 2,700-4,530/$mm^3$, RBC 3.87x $10^6$/µL interquartile range (IQ) 3.46-4.39x $10^6$/µL and Hemoglobin 9.35g/dl; interquartile range (IQ) 8.65-10.45g/dl) or showed signs of recovery of these hematologic parameters. The spleen palpation shows a significant decrease with median size of 11.00cm (interquartile range (IQ) 7.25-15.75cm) compared to after treatment 5.00cm (interquartile range (IQ) 1.5-8.00cm) [Table-2].

Table 2: Description of the study population by Socio-demographic, clinical and Laboratory parameters (Median and Interquartile (IQ) range)

| | Median | Interquartile range(IQ) |
|---|---|---|
| Age(n=48) | 18.50 | 10.00-25.00 (Min. 5.00-Max. 58.00) |
| Sex -Male(n=38) -Female(n=10) | | |
| Axillary Temperature(n=48) | 36.60 | 35.93-37.25 |
| Weight(n=48) | 44.00 | 25.5-50.00 |
| Spleen size day0(n=48) : Spleen size EOT (n=46) | 11.00: 5.00 | 7.25-15.75 : 1.5-8.00 |
| Hb at day0(n=48) : Hb at EOT(n=46) | 7.25 : 9.35 | 6.35-8.63 : 8.65-10.45 |
| RBC at day0(n=48) : RBC at EOT(n=46) | 3.12 : 3.87 | 2.86-3.51 : 3.46-4.39 |
| WBC at day0(n=48) : WBC at EOT(n=46) | 1.90 : 3.55 | 1.4-3.00 : 2.7-4.53 |
| Platelet at day0(n=48) : Platelet at EOT (n=46) | 98.00 : 260.00 | 65.25-153.00 : 174.25-307.50 |

Statistically significant Differences in the absolute counts of WBC, Platelet and measurement of Hemoglobin (Hb) were observed by comparing their values before treatment at day 0 and during

treatment at day 7, 14, 18, 18(EOT) and 30(EOT) days ($P \leq 0.001$). Significantly decreased values of WBC, Hb and Platelet was measured before treatment with a median count of 1,850/mm$^3$, 7.2 mg/dl and 98,000/mm3 compared to during treatment at day 14, which is 3,250/mm3, 8.95mg/dl and 255,000/mm3, and at end of treatment(D30) 4,500/mm$^3$, 11.3mg/dl and 287,000/mm3 respectively. The step by step increments of the above laboratory findings shows the patients progress throughout the treatment periods; in addition, the observed data indicates the patients' hematological indicators becoming near to normal range around 14 days after treatment taken (Fig-3); the pooled weight of the patients didn't showed significant change before and after treatment, since our patients had different duration of illness, measurement of weight change in individual data would be indicator of VL patients progress.

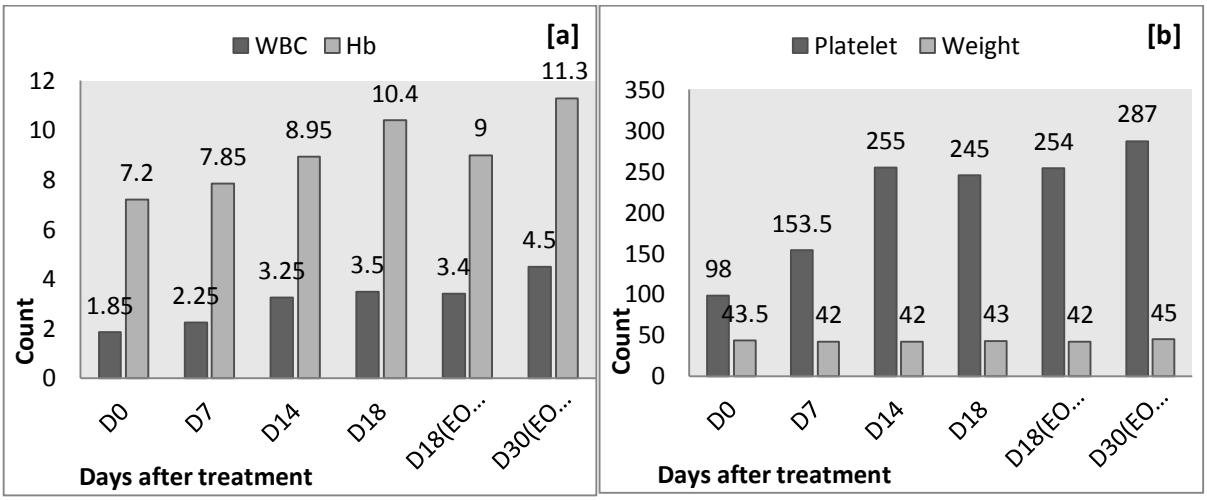



**Figure 2: Laboratory parameters before, during and after treatment:**
The x-axis shows time ranges in days after treatment of visceral leishmaniasis patients, i.e., D0 = immediately before treatment, EOT= End of Treatment after 18 and/or 30 days according to the drug of choice (PM + SSG and/or SSG alone). Each bar represents the pooled data (median) of VL patients and the data represent [a] WBC count in x10$^3$/mm$^3$ and Hemoglobin (Hb) in mg/dl and [b] Platelet count and weight in kg.

## 5.2. Tests of data normality

Test of normality was done by Kolmogorov-Smirnov analysis (Sig. P Value<0.000) and descriptive statistics for Histogram, P-P plot, Q-Q plot (Fig. 2.), and Stem-and-leaf plot shows the data for IL-10, TGFB1, IFN-Γ and IL-2 do not assume following the normal distribution (deviate from the normal curve). The tests of normality overlay a normal curve on actual data, to assess the fit. A significant test means the fit is poor. For the standard alloy, the test is not significant; they fit the normal curve well. However, for the premium alloy, the test is

significant; they fit the normal curve poorly. Therefore the differences between the above groups were done by using nonparametric tests.

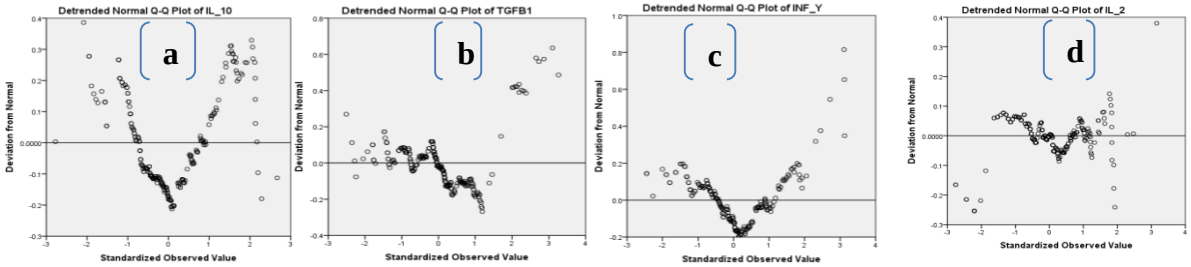
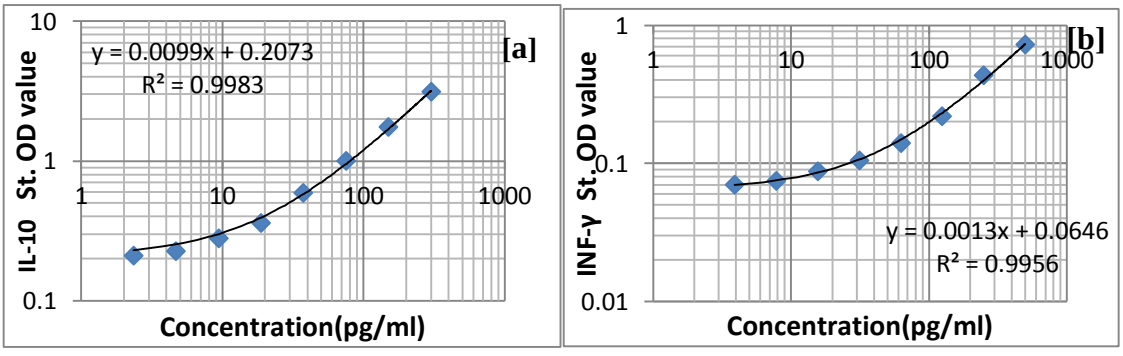



**Figure 3: Tests of data normality:**

The tests of normality on cytokine level actual data, to assess the fit. A Q-Q plot is displayed for IL-10(a), TGFB (b), IFN-Γ(c) and IL-2 (d). The horizontal line in the plot represents expected values when the data are normally distributed. The standardized observed bearing values deviate markedly from that line, especially as Cytokine Level increases.

## 5.3. Correlation of immune response with serum cytokine concentration

Initially, the sensitivity of the assay was determined by serially diluted known amount of Standards (2-300pg/ml for IL-10, 4-500Pg/ml for IFN-γ, 156.3-10,000pg/ml for TGFB1 and 2-250pg/ml for IL-2). The mean standard curve was linear over 8 log range of each cytokine concentrations with correlation coefficient ($r^2$) of 0.998, 0.995, 0.999 and 0.999 for IL-10, IFN-γ, TGFB1 and IL-2 respectively. Serum cytokine levels were calculated by interpolating the standard curve for absorbance readings (Y= O.D. value) of test samples calculated from standards of known concentrations(X=Concentration) provided with the kits and the results were expressed as picograms (pg) of cytokine/ml, based on the standard curves of the respective cytokine (Fig - 4).



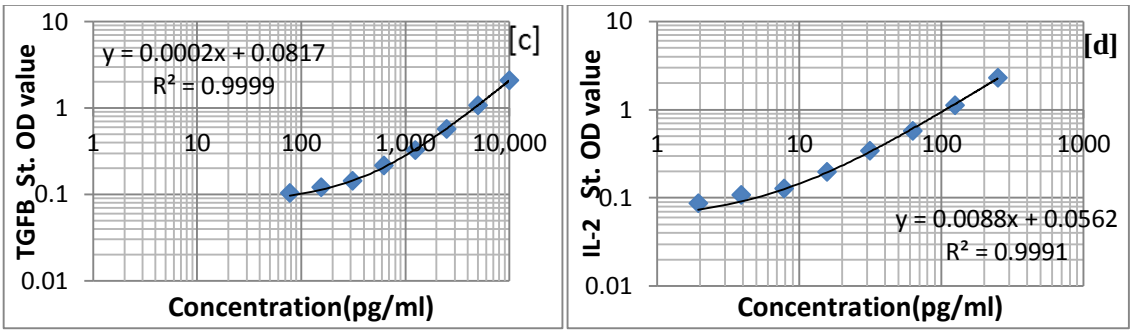



**Figure 4: Standard curves of each cytokine concentrations:**
Standard curves of each Cytokines obtained by measuring the O.D. values against the standard curve range concentration [a] Standard curve of IL-10 [b] Standard curve of INF-γ [c] Standard curve of TGFB and [d] Standard curve of IL-2 expressed as standards concentrations (pg/ml) per plate (96 reactions).

High concentration of IL-10 and INF-γ cytokines were observed in sera of VL patients (n=49) before treatment when compared with during treatment, after treatment and healthy controls (n=20) for IL-10 and after treatment and health controls for INF-γ (Fig-5 & 6). Additionally, our data shows serum concentration levels of IL-10 cytokines decreased sharply to below detection limit after start of treatment within seven days; but IFN-γ were high before treatment and at medium concentration level during and after treatment.

Differences in the level of cytokines before treatment at day 0 and 7, 14, 18, 30 days during treatment and 120 days after treatment were statistically significant ($P \leq 0.000$) for IL-10 Increased levels of IL-10 was measured before treatment, with a median concentration of 40.76 pg/mL (interquartile [IQ] range 15.52−64.85 pg/mL) at day 0; and with a sharply decrease after starting treatment to below detection limit throughout weekly follow-up schedules and 120 day after treatment (Fig-5 &6) ; with the exception of one patient who had IL-10 concentration of 98.30pg/ml at 120 days after treatment initially treated with combination treatment of PM + SSG. Then this patient was having splenic aspirate grades of +2 and treated with SSG alone due to absence of AmBisome. After 30 days of treatment the splenic aspirate result was not changed and his IL-10 level was 50.00pg/ml. Finally the patient was treated with AmBisome and discharged with initial cure according to microscopic negative result.

The level of TGFB1 had variable significance at day 7, 18, and 31($P < 0.05$) with a median concentration of 861.18 pg/mL (interquartile [IQ] range 611.18−1152.36 pg/mL) at day 0; and 1117.06 pg/mL (interquartile [IQ] range 696.47−1343.53pg/mL), 1131.76 pg/mL (interquartile [IQ] range 834.71−1522.94pg/mL) and 1114.12 pg/mL (interquartile [IQ] range 984.71−1502.35 pg/mL) at day 7,18 and 31 respectively ;but not significant at day 14 and 120.

The Level of IFN-y at day 0 for VL patients (n=49) was significantly ( P < 0.05) increased, with a median concentration of 47.44pg/mL (interquartile [IQ] range 21.92−134.63 pg/mL) compared to the consecutive follow up periods 33.27pg/mL (interquartile [IQ] range 19.73−68.49 pg/mL) at day 7, 36.68pg/mL(interquartile [IQ] range 17.44−63.00pg/mL) at day 14, 34.87pg/mL (interquartile [IQ] range 21.28−73.96 pg/mL) at day 18, 39.54pg/Ml (interquartile [IQ] range 28.77−78.26pg/mL) at day 30, 37.71pg/mL(interquartile [IQ] range13.85−74.88pg/mL) 120 days after treatment and Health control(n=20) with median of 8.00pg/mL (IQ range 2.62 −10.69pg/mL) (fig-5 & 6).

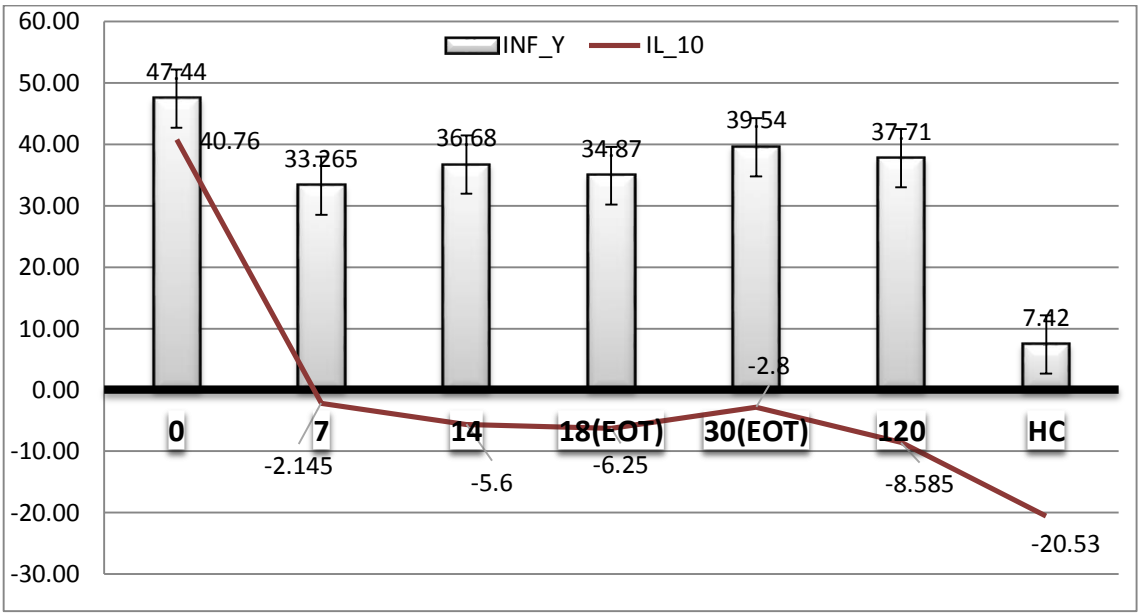



**Figure 5: Correlation of immune response with serum cytokines concentration:**

Each bar represents the pooled data (median) of VL patients and the data represent IFN-γ serum concentration and the Line represents the pooled data (median) of VL patients IL-10 serum concentration. Both cytokines IL-10 & IFN-γ before treatment at day 0 (n=49) median serum concentrations are compared with respect to during treatment at day 7 (n=49), 14(n=46), 18((EOT) n=47) and 30((EOT) n=11), after treatment at day 120 (n=26) and Health Controls HC (20). Values are given as concentration in pg/ml. The statistical significances are given among active VL (day 0) vs on treatment (day 7 & 14) *, active VL (day 0) vs EOT (day &30) **, active VL (day 0) vs after treatment (day120) *** and active VL (day 0) vs Health Controls****. (*, **, ***, p<0.05; and ****, p<0.001) for IFN-γ and (*, **, ***, ****, p<0.001) for IL-10.

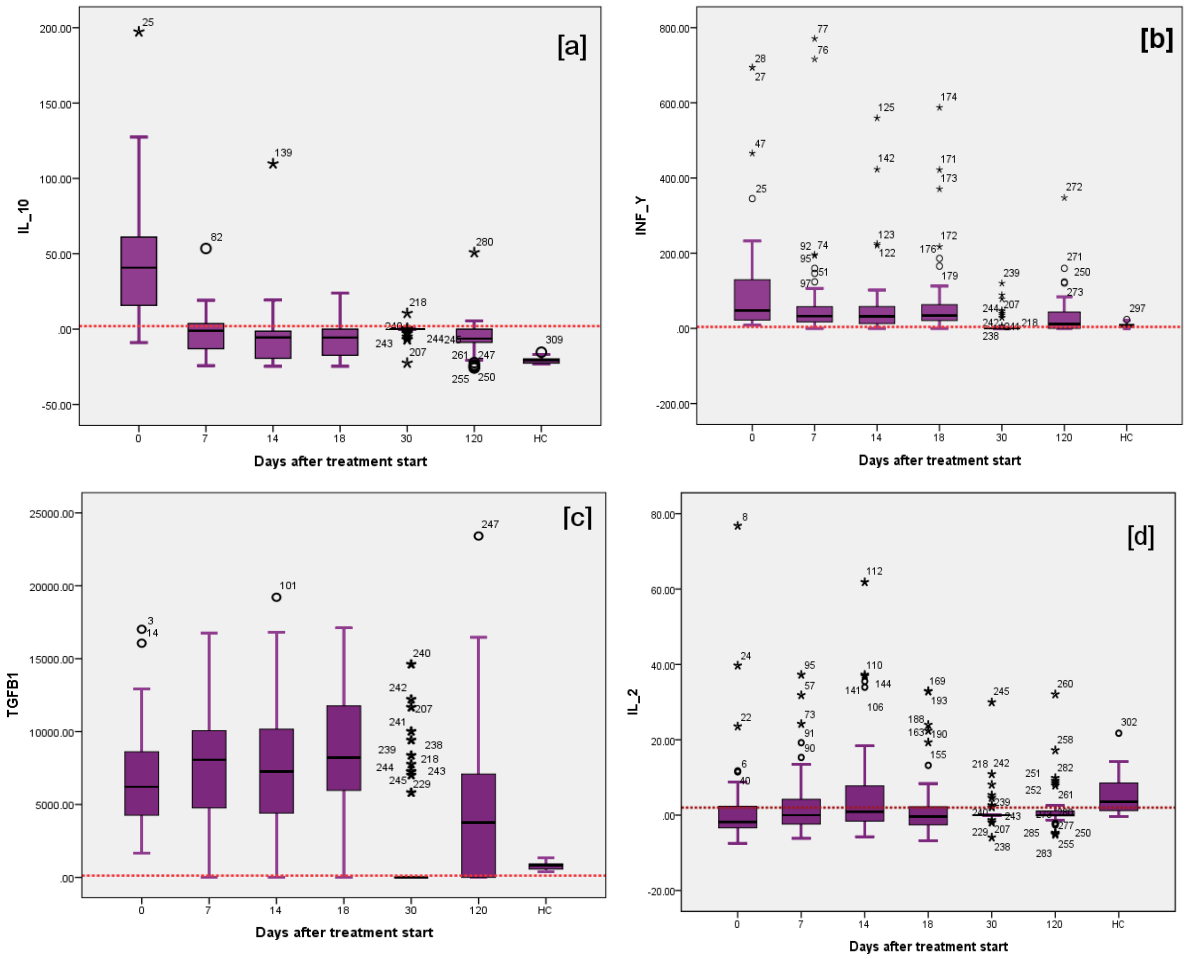



**Figure 6: Serum levels of different cytokines:**
Serum levels of IL-10 [a], IFN-γ [b], TGFB1 [c] and IL-2 [d] were observed by ELISA from active VL patients before treatment (day 0,n=49) at different stages during treatment (day 7,n=46, day 14;n=47,day 18;n=45, day and 30;n=11) and after treatment (day 120;n=26) are shown. Health Control (HC; n=20) serum samples collected from non-endemic area also included. Boxplots show the median (thick line across the box), interquartile range (vertical ends of the box), extremes (*), outliers (0), and whiskers (lines extending from the box to the highest and lowest values excluding the outliers and extremes). Extremes are cases with more than 3.0 box lengths from the upper or lower edge of the box. Outliers are cases with 1.5−3.0 box lengths from the edges of the box. The Horizontal reference line shows the minimum sensitivities of each cytokines (2pg/ml for IL-10 and IL-2, 4pg/ml for IFN-γ and 156.3pg/ml for TGFB).

## 5.4. Comparison of Microscopic parasite grade and serum cytokine levels

Levels of IL-10, IFN-γ, TGFβ and IL-2 were measured in serum samples of VL Patients (n = 48) at base line before treatment and end of treatment (EOT). Patients expressing higher levels of serum IL-10 seemed to harbor higher levels of parasites before the patients were treated. Serum concentrations of both IL-10 and IFN- γ were significantly higher in patients with active VL (Fig-7). The overall circulating level were found to correlate strongly and significantly with splenic aspiration parasite grade (r = 0.56; p = 0.000 for IL-10). The overall correlation, however, was

not so strong for the other three cytokines tested (r = 0.07 for IFN-γ, 0.21 for TGFβ and 0.09 for IL-2). The median serum concentration of IL-10 with regards to splenic aspiration parasite grade were 77.4pg/ml, 19pg/ml, 24.1pg/ml, 38.6pg/ml, 58.1pg/ml, 37.2pg/ml and below detectable level (-5.1pg/ml) at +1, +2, +3, +4, +5, +6 and end of treatment (EOT) respectively (Fig-7). Our result shows the level of IL-10 were high during active VL regardless of microscopic parasite grading but below detectable level at end of treatment; suggesting that undetectable level of IL-10 correlates with absence of parasite and clinical cure.

The median serum concentration of IFN-γ with regards to splenic aspiration parasite grade were 121.4pg/ml, 59.4pg/ml, 162.6pg/ml, 35.3pg/ml, 36.3pg/ml, 45.4pg/ml and 43.6pg/ml at +1, +2, +3, +4, +5, +6 and end of treatment (EOT) respectively (Fig-7). There was a tendency of higher median serum IFN- γ being measured at lower parasitaemia, e.g., at 1+ and 3+.

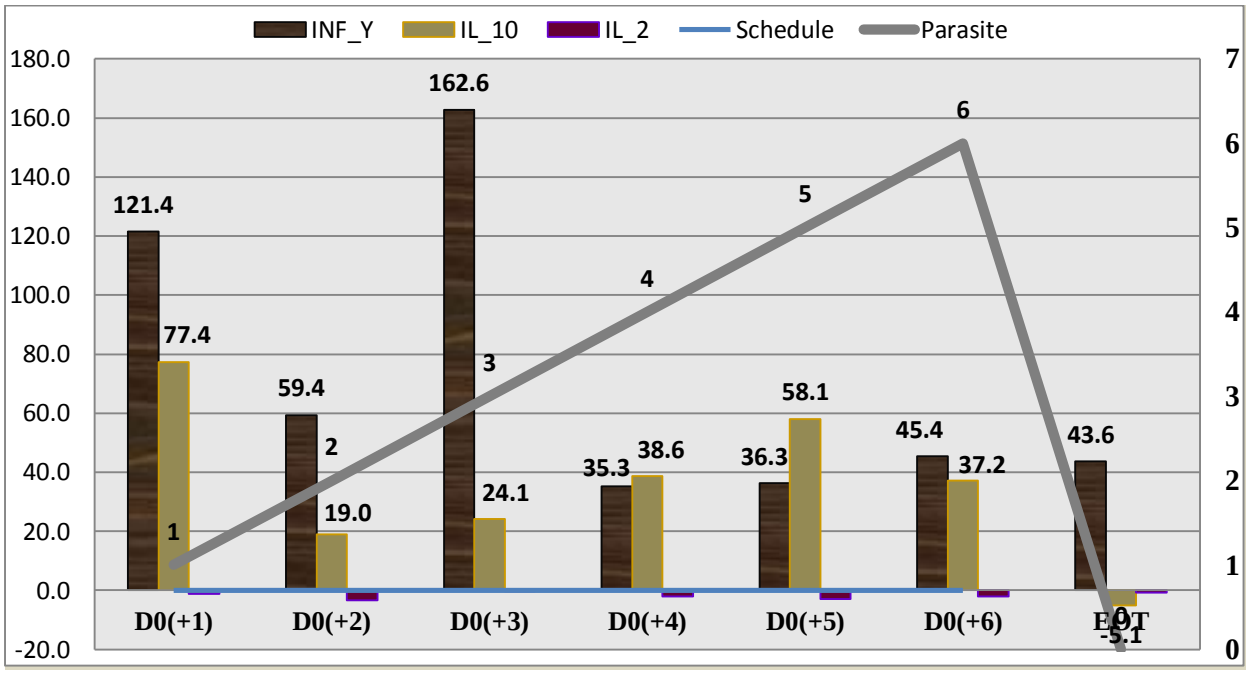



**Figure 7: Comparison of Microscopic parasite grade and serum cytokine levels:**
The x-axis shows time ranges in days after treatment of visceral leishmaniasis patients, i.e., D0 = immediately before treatment, EOT= End of Treatment after 18 and/or 30 days according to the drug of choice (PM + SSG and/or SSG alone). Statistically significant differences (SSDs) are based on P values $\leq$ 0.05. **IL-10** : SSDs observed between visceral leishmaniasis patients before treatment at day 0 and cured visceral leishmaniasis patients at 18 or 30 days after treatment and parasite load by splenic aspiration; the IL-10 level is below detectable level after treatment. **IFN-γ**: SSDs observed between visceral leishmaniasis patients at day 0 before treatment and cured visceral leishmaniasis patients at 18 or 30 days after treatment and parasite load by splenic aspiration. **IL-2**: the over all test does not show SSDs between visceral leishmaniasis patients before treatment at day 0 and cured visceral leishmaniasis patients at 18 or 30 days after treatment and parasite load by splenic aspiration; the concentration level of IL-2 also below minimum detection limit before treatment and after treatment;(Nonparametric tests:Independent samples median test and Independent samples Kruskal-Wallis test).

## 5.5. Comparison of antibody O.D. values with serum cytokine levels

Pairwise comparison of the level of Antibody (O.D. value) with IL-10, IFN-γ and IL-2 levels were performed. Both antibody level and Cytokine concentrations (IL-10 and IFN-γ) were significantly high before treatment compared to the level of IL-2, which is below detection limit. IL-10 concentration was sharply decreased to below detection limit at day seven (7) and throughout the consecutive follow-up schedules; but IFN-γ concentration and antibody levels (IgG + IgM) were not significantly decreased throughout the follow-up periods during treatment; but both decreased significantly at 120 days after treatment (Fig-8). The antibody levels of VL patients were at higher level compared to health controls throughout treatment and follow-up periods.

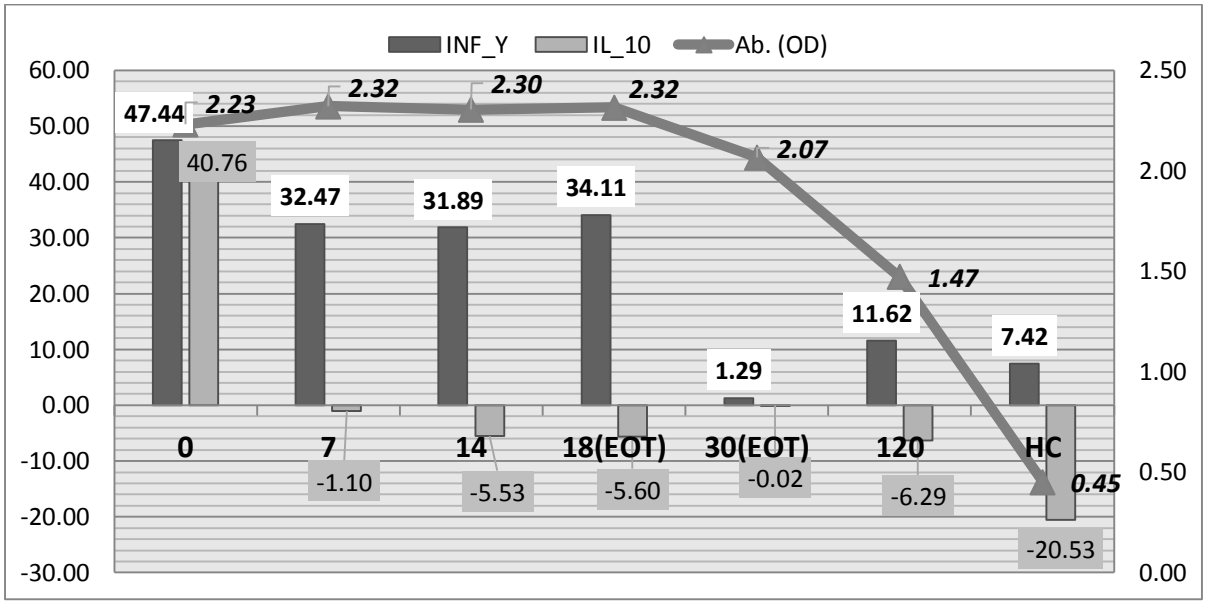



**Figure 8: Comparison of antibody O.D. values with serum cytokine levels:**
Antibody levels (O.D. value) in VL patients and health control (n=20) against IFN-γ and IL-10 levels. Each bar represents the pooled data (median) of IFN-γ and IL-10 and the line chart shows the level of antibody (O.D. values) with respect to the consecutive VL patient schedules. Significance values indicate the statistical paired difference of antibody level (O.D. value) with IFN-γ and IL-10 among active VL (day 0), on treatment (day 7 & 14) *, EOT (day 18 & 30) **, after treatment (day120) *** and normal group ****. (*, ** (day 18), ***, ****, $p \leq 0.001$; and ** (day30), $p<0.005$) for IFN-γ and (*, $p<0.001$) for IL-10 and below detection limit after the start of treatment (95% Confidence Interval of the Difference). Overall Statistical Significance Differences (SSDs) of antibody level (O.D. value) vs IFN-γ and antibody level (OD value) vs IL-10 shows the correlation is significant at the $p<0.05$ and $p<0.01$ level (2-tailed), respectively.

## 5.6. Comparison of Microscopic parasite grade and antibody O.D. value

Production of high level of immunoglobulin's can be used as a surrogate marker of cellular immune responses developed by the patients. With this perspective, we evaluated anti-leishmania total IgG+IgM levels during active VL before treatment, at different times during treatment and

120 days post treatment. High level of anti-leishmania antibodies was present in all VL patients regardless of spleen aspirate microscopic parasite grade before treatment and at different time during treatment. However; a significant decrease of antibody level observed at 120 days post treatment with median O.D. values of 1.47 (Fig-9).

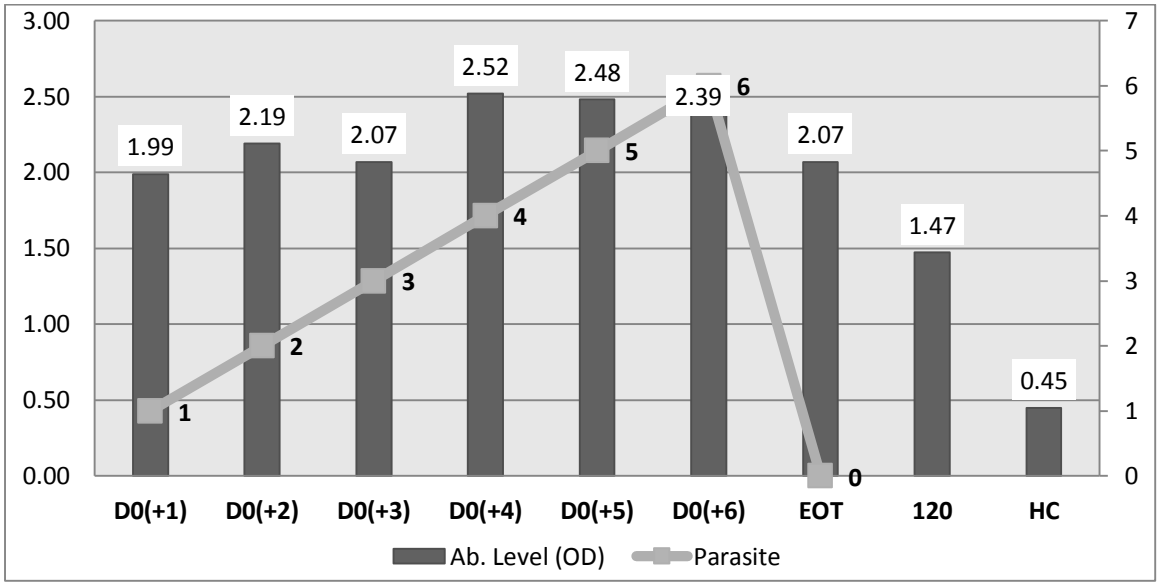



**Figure 9: Comparison of Microscopic parasite grade and antibody O.D. value:**
Antibody levels (O.D. values) in active VL patients and amastigote grades of splenic aspiration. Each bar represents the pooled data (median value) of patient's antibody level. The line chart shows the splenic aspirate microscopic parasite amastigote grades from +1to +6 at day 0 and zero (0) parasite at EOT. Scoring of parasite load is on a logarithmic scale from +1 to +6, were 0 is no parasites per 1000 microscopic fields (1000x ), +1 is 1–10 parasites per 1000 fields, and +6 is >100 parasites per field. Statistical Significance Differences (SSDs) of antibody level (OD value) before treatment and after treatment vs Parasite grade after treatment indicate the difference between the antibody level and parasite grades at $p<0.001$level.

# CHAPTER SIX: DISCUSSION

The present study investigated the correlation among different cytokine concentrations, and antibody levels in serum samples, clinical and laboratory parameters associated with VL patients and assessed their interplay in order to explore the time-dependent alterations of their immune components in defining the disease outcome. Interestingly, this results revealed a strong correlation of increasing IL-10 and IFN-γ with different clinical and laboratory parameters implying disease manifestations to be linked with the increased immunological markers as well as symptoms like duration of illnesses, splenomegaly and pancytopenia. Herewith, we studied 48 patients with presentation of active VL by evaluating the patterns of their splenic aspirate parasitic grade, cytokine concentrations and antibody levels before treatment, during treatment at different times and post treatment in order to identify biomarkers of disease state and to assess responses to treatment. cytokines can be used as markers in epidemiological studies conducted in endemic areas to distinguish between different clinical forms of VL(57).

For the clinical aspects, the high prevalence of palpable spleen and severe cytopenia among true VL cases with rk39 positive test are the most significant aspects that may increase clinical suspicion of VL. The recombinant antigen, rK39 is specific for antibodies against VL caused by *L. donovani* complex members. It is highly sensitive and predictive for onset of acute disease(50). Although the sensitivity of the test is high, it is entirely influenced by the antigen used in the test(34). In contrast, rK39 does not show detectable antibodies in cutaneous or mucocutaneous leishmaniasis(51). In this data all patients had palpable spleen range from 3 to 24cm with median size of 12cm and 100% of the participants showed rk39 rapid test positive result.

In this study, the levels of the serum cytokines IL-10, IFN-γ, and TGFB were higher before treatment of active VL patients than control individuals (fig-6). This is in agreement with a study in south-western Ethiopia, serum cytokines (IFN-γ, IL-12, and IL-15) in symptomatic VL patients were significantly higher than in patients with asymptomatic leishmania infection and healthy controls in the same endemic area with significant decrease of IFN-γ and all mediators was observed after treatment of VL patients (20,36). Plasma concentrations of IFN-γ and IL-10 were elevated in VL(36). Levels of serum cytokines, IL-10, IL-12, and IFN-γ , were higher in patients than in family members and control individuals(58).

IL-10 has been well known for its role in VL pathogenesis and a significant correlation was already reported between parasite load and plasma IL-10 levels in VL patients(60). The

abundance of *Treg* cells, along with IL-10 and TGFβ, in VL patients observed, may be the main reason for large numbers of parasites inflicting disease aggravation(59). TGFβ has been shown to impart down-regulatory effects on macrophages and its blockade could restrict parasite progression in these cells(61). Previous studies including this paper had documented elevated levels of IL-10 in VL patients(20,36,57,60,62) and its association with pathology. Thus, IL-10 is well established as a critical component in susceptibility to Leishmania infection that contributes to the pathogenesis of VL. This data demonstrates that IL-10 production, rather than any other cytokine, is a clear correlate of severity of VL in human.

Levels of IL-10, a Th2-type cytokine that inhibits Th1 cell and macrophage activation, were significantly higher in the serum of individuals with active VL patients before treatment, compared to patients who were on treatment, post treatment and health controls. These results are in agreement with those of plasma levels of IL-10 show a marked decrease in most patients already 30 days after end of treatment(62). Production of IL-10 has been suggested to be important for the survival and persistence of the parasite inside macrophages(37,57). In addition, IL-10 protects against the side effects of an exaggerated inflammatory response, playing an important role in the regulation of the inflammatory response; all Th cells share the important task of controlling over exuberant immune responses by means of IL-10 production(30,31). In this study the observed sharp fall in IL-10 levels following start of treatment within seven days is coincident to the control of parasite multiplication lending support to an important role of this cytokine in human VL.

IL-10 concentration was sharply decreased to below detection limit at day seven (7) and throughout the consecutive follow-up schedules; but IFN-γ concentration and antibody levels (IgG + IgM) were not significantly decreased throughout the follow-up periods during treatment; but both decreased significantly at 120 days after treatment (Fig-5). The high levels of IFN-γ observed during active VL are sharply decreased in most patients by 30 days post treatment and are almost vanished 120 days after treatment(62). High antibody titers and immune complex formation in VL may contribute to the elevated IL-10 levels observed and participate in the progressive decline in the immune status of VL patients(38).

Clinical outcome of the disease is a consequence of the complex interaction between the pathogen and the host and survival of pathogen largely depends on the type of cytokine (Th1 or Th2) being produced by host immune cells on encounter(60). We therefore analyzed the major Th1/Th2

cytokines in VL patients to understand the correlation of immune response with levels of antibody. This study revealed significantly elevated levels, strongly correlating with an increasing antibody levels before treatment with decreasing order of both IFN-γ and antibody level 120 days after treatment in VL patients. Since total plasma cytokine profiles might not be a conclusive representation of infection(60) this study revealed significantly elevated levels of IL-10, IFN- γ and TGFβ in VL patients. One patient had come back with VL relapse 120 days after treatment. The patient presented with high serum concentrations of IL-10, IFN- γ, TGFβ as well as high titres of antileishmanial antibody to rk-39. Albeit in one patient, this data gives anecdotal evidence that these cytokines (IL-10, IFN- γ, TGFβ) might be useful biomarkers of prognosis and could be used to assess responses to treatment.

For an effective control of VL from endemic areas; reliable and rapid diagnostic and prognostic tools are desperately needed. Suggesting that; a combination of non-invasive active case detection methods like rk39; with better clinical investigation targeting IL-10 may be a method to improve VL therapy and disease outcome.

**Strength and limitation of the study**

This study sheds light on the magnitude of serum cytokine levels, microscopic parasite load and levels of antibody in VL patients at different stages of treatment; the test kits utilized had high specificity and sensitivity. However, limited flow of patients and long follow-up periods of the participants to the study site leads to prolonged extension of the paper. Measuring other important cytokines (e.g., IL-4, IL-5, IL-6, IL-12 and TNF-a) would provide better results and conclusion.

# CHAPTER SEVEN: CONCLUSION AND RECOMMENDATIONS

## 7.1. Conclusion

Regardless of microscopic parasite load and levels of antibody; remarkably the elevated levels of IL-10 in VL serum significantly decreased to below detectable limit after seven days of treatment. Taken together, these results suggest that cytokines can be used as markers of disease in epidemiological studies conducted in endemic areas to distinguish between the different clinical stages of VL. Further, measuring circulating cytokines concentrations could be used in combination with other clinical criteria as biomarkers of the infectious stages of VL.

## 7.2. Recommendations

- ✓ Further investigation of other cytokines (e.g., IL-4, IL-5, IL-6, IL-12 and TNF-a) and immunological markers should be conducted by including HIV-VL co-infected patients.
- ✓ Cytokine concentration, levels of antibody and microscopic parasite grades needs to be compared in the study area with molecular (rPCR) parasite load measurement to find the best non-invasive procedure for VL out-come evaluation.

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# Jimma University, Institute of Health, Faculty of Health Sciences, School of Medical Laboratory Sciences

## ANNEXES

### ANNEX 1: Subject information sheet

Dear participants: good morning/good afternoon?

My name is Dagimawie Tadesse and I am pursuing a second degree program at Jimma University. I am conducting a research on the parasite *Leishmania donovani* which causes a severe disease that is fatal without appropriate treatment. The main objective of this study is to detect and quantify *L. donovani* parasitaemia by using real time PCR before, during and after treatment in patients infected with visceral Leishmaniasis. You are one among the many such patients that we are inviting to participate in the planned study. Therefore, you are kindly requested to participate in this study. Your participation in this study is completely on voluntary basis and you have the right to refuse from participation. If you agree to participate in this study, in addition to the routine blood sample for laboratory test, about 6 ml blood specimen will be collected from you. During collection of blood you may feel minimal discomfort. Your response and the laboratory results would be kept confidential.

I would like to inform you again your participation is very essential not only for the successful accomplishment of the study but also for producing relevant information which will be helpful to design appropriate strategies for the prevention of the disease.

Do you agree to participate in this study? Yes, continue _____No, thank you! _____

Thank you for your cooperation!!!

## Subject information sheet (Amharic version)

## ጅማ ዩኒቨርሲቲ፡ የጤና ሲንስቲቲዩት፡ የጤና ሳይንስ ፋካልቲ □ስቦፊቶፊ ት/ክፍል

**ስተሳታፊዎች ስለጥናቱ መረጃ መስጫ ቅፅ**

**ውድ የመጠይቁ/ የጥናቱ ተሳታፋ□ች እንደምን አደራችሁ/ዋላችሁ፡፡**

እኔ ስሜ □ልማዊ ታ□ሠ እባላለሁኝ ፡ የሁስተኛ ዲግሬ ትምህርት በጅማ ዩኒቨርሲቲ እ□ተከታተልኩኝ ስሆን በአሁን ሰዓት የመመረቂያ ጥናታዊ ፅሁፍ ሌሽማኒያሲስ በሚባል ጥቸኛ ህ□ስ ሳ□ እ□ስፈሁ እቸኛለሁ፡፡ □ህም ዓቸኛ ህዋስ ስስፈሳጊ ህክምና ከልተደረገለት ከፍተኛ በሽታ በማምጣት ደገድሳል፡፡ የጥናቱም ዋና ዓላማ በርስዎ ደም ዉስጥ ሊሽማኒያ ደኖቫኒ የሚባለዉን ጥገኛ ህዋስ መድሀኒት ከመዉሰድዎ በፌት፤ በማዉሰድ ላይ ስያሉ ስና ከወሰዱ በኃላ መጠኑን ለማየት □ና ስርጭቱን ለመቁጠር ነዉ፡፡ እናም እርስዎ የዚህ ጥናት ተሳታፊ ስንዲሆኑ እና ስስፈላጊውን መረጃ እንዲሰጡኝ ስል በታላቅ ትህትና እጠይቃለሁ፡፡ በዚህ ጥናት ዉስጥ የመሳተፍ ዉይም ያለመሳተፍ ሙሉ መብትዎ የተጠበቀ ነዉ፡፡ በዚህ ጥናት ለመሳተፍ ፍቃደኛ ከሆኑ ስርሶ የሚጠየቁት የደም ናሙና ስንዲሰግሱ ሲሆን የሚሰግሱት የደም ናሙና መጠን ለመ□በኛ ምረመሬ ከሚሰጡት በ3 ሚሲ ይጨምራል (5ሚሲ) ደም በመስጠት በዚህ ጥናት ዉስጥ የተሸለ የምርመሬ ዘዴና መሳርያ ለማግኘት የሚደረገዉን ጥረት ያግዛሉ፡፡ ደም በሚወሰድበት ጊዜም ትንሽ የህመም ስሜት ሊሰማዎት ይችላል ይህ ማለት ግን ስጊዜው የሚሰማዎት እንጂ በእርስዎ ላይ ችግር የሚፈጥር አይ□ስም ::

ከዚህ በተጨማሪ እርስዎ የሚሰጡት መረጃ እንዲሁም የደም ናሙና ውጤት በታ·ማኝነት እንደም□ብቅ በት□ሚ ሳረ□ቢዓ ል□ □ል□ስሁ፡፡

በመፌ ረሽም እርስዎ የሚሰጡት መረጃ ስንዲሁም የደም ናሙና ውጤቱ ከጥናቱ መሳካት ባለፌ በስፋት በሽታውን ከመከላከል ስንፃርም በጎ ጎኑ የሳቀ መሆኑን ሳረጋግጥልዎ እወፌለሁ፡፡

በዚህ ጥናት ዉስጥ ለመሳተፍ ፍቃደኛ ነዎት? ስዎን □ቀዓ ሉ_____

ስይይስሁም ስመሰግናስሁ_____

ስትብብርዎ እ□□ በጣም ስመሰግናስሁ!!

## ANNEX 2: Assent form for inclusion in the study

### A. For participation in the study

The undersigned, confirm that, I give my assent to participate in the study with a clear understanding of the objectives and conditions of the study and with the recognition of my right to withdraw from the study if I change my mind. I, _____ do hereby give my assent to Mr./Ms./Mrs./Dr _____ to include me in the proposed research. I have been given the necessary information and understand that it will be necessary that there are no additional risks involved in participating in this research. I have also been assured that I can withdraw my assent at any time without penalty or loss of the benefit of treatment. The study has been explained to me in the language I understand.

I agree to participate

Patient/guardian

Print Name: ..... Date: .....

Sign Name: .....Time: .....

Child

Print Name: ..... Date: .....

Sign Name: .....Time: .....

Doctor/Person Conducting the Informed Consent

Print Name: ..... Date: .....

Sign Name: ..... Time: .....

Witness

Print Name: ..... Date: .....

Sign Name: ..... Time: .....

Contact person:

Name: _____

Address: _____

**በጥናት ዉስጥ ለመሳተፍ የስምምነት ቅፅ**

እኔ ስምና ፊርማዬ ከዚህ በታች የተቀመጠዉ በዚህ ጥናት ዉስጥ ለመሳተፍ ሙሉ ፍቃደኝነቴን ስገልፅ የጥናቱ ዓላማና ሂደት በሚገባ ተረድቼ ሲሆን በተጨማሪም ሀሳቤን/ስምምነቴን ትቼ ከጥናቱ ዉስጥ ለመዉጣት ሙሉ መብት አንዳለኝ ተረድቻለሁ፡፡ አኔ----------
--በጥናቱ ዉስጥ ለመሳተፍ ሙሉ ስምምነቴን ለፕ/ዶ/ር ---------- አገልግለሁ፡፡ በዚህም መሰረት ከጥናቱ ጋር በተያያዘ ዙሪያ □ሚዘ□ ምንም አይነት ተዛማች ጉዳት ወይም ተፅዕኖ እንደሌለው በሚገባ ተረድቻለሁ፡፡ በተጫማሪም በጥናቱ የመሳተፍ ስምምነቴን በፈለኩ ጊዜ የማቋረጥና በማቋረጤም ቅጣት ወይም በመደበኛ በማገኘዉ የሕክምና አገልግሎት ላይ ተፅእኖ አንደማይደርስብኝና በምንም መልኩ የሚገባኝን የሕክምና አገዛ ሁሉ አንደማይነፈገኝ በመረዳት ነዉ፡፡ በመጨረሻም የጥናቱ ጠቅላላ ሂደት አፍ በፈተሁበት ወይንም በደንብ በምረዳዉ ቋንቋ ግልፅ ተደርጎ ተነግሮኛል፡፡ አኔም በሚገባ ተረድቻለሁ፡፡

**ታካሚ/አስ□ማሚ**

ስም ፡ .....ቀን ፡ .....

ኝርማ :..... ሰዓት ፡ .....

**ል□**

ስም ፡ .....ቀን ፡ .....

ኝርማ :..... ሰዓት ፡ .....

**የስምምነቱን ቅፅ ያስፈረመዉ ዶ/ር ወይም ባለሙያ**

ስም ፡ .....ቀን ፡ .....

ኝርማ ፡ .....ሰዓት: .....

**ምስ□ር (□ማኝ)**

ስም ፡ .....ቀን ፡ .....

ኝርማ:.....ሰዓት ፡ .....

**ተ□ሪ**

ስም:..... ቀን ፡ .....

አድራሻ :.....ሰዓት ፡ .....

## ANNEX 3: Consent to store blood samples left over from the study

I give permission for the blood sample that I have provided (or my child's blood sample) for this research to be stored for additional analyses in the future, if needed. No human genetic testing will be performed on these samples. These samples will be stored at the Leishmaniasis Research & Diagnostic Laboratory of Addis Ababa University in Black Lion Specialized Hospital.

All samples will be identified only by the identification number given to each sample. If unused within a period of 10 years, they will be discarded. I understand it is my right to refuse permission for these samples to be kept and used in the future, and if I do refuse to give permission there will not be any penalty to me or loss of benefits.

Patient/ (Parent/Lawful Guardian for < 18 years)

Print Name: ..... Date: .....

Sign Name: .....Time.....

Child

Print Name: ..... Date: .....

Sign Name: .....Time: .....

Doctor/Person Conducting the Informed Consent

Print Name: ..... Date: .....

Sign Name: ..... Time: .....

Witness

Print Name: ..... Date: .....

Sign Name: ..... Time: .....

Contact person:

Name: _____

Address: _____

**ከጥናቱ የተረፈዉን የደም ናሙና ለማስቀመጥ የስምምነት መግለጫ**

በዚህ ጥናት ዉስጥ በመሳተፍ ከሰጠሁት የደም ናሙና ዉስጥ ጥናቱ ሲያልቅ የሚተርፈዉን ተቀምጦ ወደፊት ለሚደረግ ተጨማሪ ጥናት አንዲዉል ፈቅጃለሁ፡፡ በሰጠሁት የደም ናሙና ላይ የዘር መለመል ጥናት አይደረግም፡፡ ናሙናዎቹ የሚቀመጡት በአድስ አበባ ዩኒቨርሲቲ ጥቁር አንበሳ አጠቃላይ ሆስፒተል ሌሽማንያሲስ ጥናትና ምርመራ ማዕከል ዉስጥ ነዉ፡፡ ናሙናዎቹ በሙሉ የራሳቸዉ የሆነ የመለያ ቁጥር ብቻ ይኖራቸዋል፡፡ በአስር አመት ዉስጥም ስራ ላ□ ካል□ለ □ወ□ቱል፡፡

ከጥናቱ የተረፈዉ ናሙና አንዳይቀመጥና ወደፊት ለሚደረገዉ ጥናት አንዳይዉል የመወሰን መብት አለኝ፡፡ በተጫማሪም በጥናቱ የመሳተፍ ስምምነቱን በፈለኩ ጊዜ የማቋረጥና በማቋረ□,ም ቅጣት ወይም በመደበኛ በማገኝዉ የሕክምና አገልግሎት ላይ ተፅዕኖ አንደማይደርስብኝና በምንም መልኩ የሚገባኝን የሕክምና አገዛ ሁሉ አንደማይነፈገኝ በመረዳት ነዉ፡፡

**□ካሚ(ቤተሰብ ወይም አስታማሚ ከ18 ዓመት በታች ለሆኑ)**

ስም ፡ .....ቀን ፡ .....

ኝርማ: .....ሰዓት: .....

**ል□**

ስም ፡ .....ቀን ፡ .....

ኝርማ :..... ሰዓት ፡ .....

**የስምምነቱን ቅፅ ያስፈረመዉ**

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**ምስ□ር (□ማኝ)**

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ኝርማ:.....ሰዓት ፡ .....

**ተ□ሪ**

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አድራሻ:.....ሰዓት: .....

## ANNEX 4: Source documents and case report forms

### A. Enrollment criteria:

**a**. Age > 5 years?

**b**. VL positive?

If a and b = YES, and the patient will be asked for informed consent

**c**. Both informed consent forms signed?

If c is YES, patient will be included in the study

### B. Patient demographics and eligibility criteria

PATIENT INITIALS: ----------

Date of enrollment: -------- /---------- /---------- (dd/MMM/yyyy)

Date of signed informed consent: ----------/---------- /---------- (dd/MMM/yyyy)

### C. Patient profile:

1. Sex: male [ ] female [ ]
2. Date of birth ---------/---------/---------- (dd/MMM/yyyy) or Age: _____ years
3. Occupation: _____
4. Ethnic group (or ethnicity): _____
5. Place of residence: _____

Have you been in an area thought to have VL transmission? □ Yes □ No

If Yes, where?

Locality _____. How long were you there? _____

Locality _____. How long were you there? _____

Locality _____. How long were you there? _____

### D. Clinical signs:

6. Temperature: ------ºC on admission; Date: ____/_____/_____ (dd/MMM/yyyy)

Weight: ------- (kg); Height: _____. ____ (cm) BMI: ____.___ (kg/m2)

In children: MUAC: _____. (cm); Spleen size: _____. ____ (cm)

**E. Clinical laboratory results:**

7. Routine blood tests (on admission and at end of treatment)

Date: ___/____/___(dd/MMM/yyyy) on admission

Hb level _____ (g/dl)

RBC count _____

WBC count _____

Platelet counts _____

Lymphocyte percent _____

**Result at end of treatment and/or as required**

Date: ___/____/___(dd/MMM/yyyy)

Hb level _____ (g/dl)

RBC count _____

WBC count _____

Platelet counts _____

Lymphocyte percent _____

**Other diagnostic tests and results:**

Specify: _____ □ Positive □ Negative □ Indeterminate

Specify: _____ □ Positive □ Negative □ Indeterminate

Specify: _____ □ Positive □ Negative □ Indeterminate

**8. Splenic aspiration:** Date: ____/_____/_____ (dd/MMM/yyyy)

Not done []

Negative []

Positive []

**9. HIV test results (VCT/ PICT):**

Date blood drawn for HIV test: ____/_____/_____ (dd/MMM/yyyy)

Not done []

Negative []

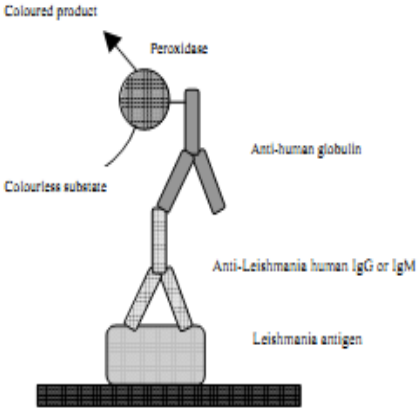
Positive []

## ANNEX 5: Laboratory test protocol used for the detection of IgG +IgM Antibodies to Leishmania Donovani in Human serum

T1000: Indirect immunoenzyme assay to test IgG+IgM antibodies against Leishmania infantum in human serum. 96 tests.

**PRINCIPLE OF THE TEST:**

The ELISA method is based upon the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. Unbound immunoglobulins are washed off. An enzyme-labelled anti-human globulin binds the antigen-antibody complex in a second step. After a new washing step, bound conjugate is developed with the aid of a substrate solution (TMB) to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution.




**KIT FEATURES:**

All reagents, except for the washing solution, are supplied ready to use. Serum dilution solution and conjugate are coloured to help in the performance of the technique.
Sample predilution is not necessary.
Break-apart individual wells are supplied, so that the same number of wells is consumed than the number of tests performed.

**KIT CONTENTS:**

1 VIRCELL LEISHMANIA PLATE: 1 96-wells plate coated with antigen of Leishmania infantum.

2 VIRCELL SERUM DILUENT: 25 ml of serum dilution solution: a blue coloured phosphate buffer containing protein stabilizers and Proclin. Ready to use.

3 VIRCELL IgG+IgM POSITIVE CONTROL: 500 µl of positive control serum containing Proclin.

4 VIRCELL IgG+IgM CUT OFF CONTROL: 500 µl of cut off control serum containing Proclin.

5 VIRCELL IgG+IgM NEGATIVE CONTROL: 500 µl of negative control serum containing Proclin.

6 VIRCELL IgG+IgM CONJUGATE: 15 ml of anti-human peroxidase conjugate dilution in a red-coloured Proclin-containing buffer. Ready to use.

7 VIRCELL TMB SUBSTRATE SOLUTION: 15 ml of substrate solution containing tetramethylbenzidine (TMB). Ready to use.

8 VIRCELL STOP REAGENT: 15 ml of stopping solution: 0.5 M sulphuric acid.

9 VIRCELL WASH BUFFER: 50 ml of 20x washing solution: a phosphate buffer containing TweenR-20 and Proclin.

Store at 2-8ºC and check expiration date.

## Materials required but not supplied:

- Precision micropipettes 5 and 100 µl.
- Eight channel micropipette 100 µl.
- ELISA plate washer.
- Thermostatized incubator/water bath.
- ELISA plate spectrophotometer with a 450 nm measuring filter and a 620nm reference filter. Alternatively, an ELISA automated processor.
- Distilled water.

## SPECIMEN COLLECTION AND HANDLING:

Blood should be collected aseptically using venipuncture techniques by qualified personnel. Use of sterile or aseptic techniques will preserve the integrity of the specimen. Serum samples are to be refrigerated (2-8ºC) upon collection or frozen (-20ºC) if the test cannot be performed within 7 days. Samples should not be repeatedly frozen and thawed. Do not use hyperlipemic, hemolysed or contaminated sera. Samples containing particles should be clarified by centrifugation. **Do not use plasma.**

**PRELIMINARY PREPARATION OF THE REAGENTS:**

Only the washing solution must be prepared in advance. Fill 50 ml of 20x washing solution up to 1 litter with distilled water. Should salt crystals form in the washing concentrate during storage, warm the solution to 37ºC before diluting. Once diluted, store at 2-8ºC.

**TEST PROCEDURE:**

1. Set incubator/water bath to $37^{o}c \pm 1^{o}c$
2. Bring all reagents to room temperature before use (approximately 1 hour), without removing the plate from the bag.
3. Shake all components
4. Remove the plate [1] from the package. Determine the numbers of wells to be employed counting in four wells for the controls: two for the cut off serum and one each for the negative and positive sera. Wells not required for the test should be returned to the pouch, which then be sealed.
5. Add 100uL of serum dilution solution [2] to all wells. Add 5uL of each sample, 5uL of positive control [3], 5uL of cut off serum[4] (cut off serum in duplicate) and 5uL of negative control[5] in to the corresponding wells. If the assay is performed manually, shake the plate in a plate shaker (2 min.) in order to achieve a homogenous mixture of the reagents. If for some reason correct shaking cannot be guaranteed, a pre dilution of the sample in a separate tube or plate should be made. Mix homogenously with the pipette and dispense 105uL of each diluted sample to the wells [1].
6. Cover with a sealing sheet and incubate at $37^{o}c \pm 1^{0}c$ for 45 min.
7. Remove the seal, aspirate liquid from all wells and wash five times with 0.3 ml of washing solution [9] per well. Drain off any remaining liquid.
8. Immediately add 100uL of conjugate solution [6] into each well.
9. Cover with a sealing sheet and incubate at $37^{o}c \pm 1^{0}c$ for 30 min.
10. Remove the seal, aspirate Liqid from all wells and wash five times with 0.3 ml of washing solution [9] per well. Drain off any remaining liquid.
11. Immediately add 100uL of substrate solution [7] in to each wells.
12. Incubate at room temperature for 29 min protected from light.
13. Add immediately 50uL of stopping solution [8] into all wells.
14. Read with a spectrophotometer at 450/620 nm within 1 hour of stopping.

## INTERNAL QUALITY CONTROL:

Each batch is subjected to internal quality control (Q.C.) testing before batch release complying with specifications stricter than validation protocol for users. Final Q.C. results for each particular lot are available. The control material is traceable to reference sera panels internally validated.

## VALIDATION PROTOCOL FOR USERS:

Positive, negative and cut off controls must be run with each test run. It allows the validation of the ssay and kit. Optical densities (O.D.) must fall in the following ranges. Otherwise, the test is invalid and must be repeated.

| CONTROL | O.D. |
|---|---|
| POSITIVE CONTROL | >0.9 |
| NEGATIVE CONTROL | <0.55 |
| CUT OFF CONTROL | <0.7 x(D.O. POSITIVE CONTROL) |
| | >1.5 x(D.O. NEGATIVE CONTROL) |

## INTERPRETATION OF RESULTS:

Calculate the mean O.D. for cut off serum.

Antibody index= (sample O.D. / cut off serum mean O.D.) x 10

| INDEX | INTERPRETATION |
|---|---|
| < 9 | Negative |
| 9-11 | Equivocal |
| >11 | Positive |

Samples with equivocal results must be retested and/or a new sample obtained for confirmation.
Samples with indexes below 9 are considered as not having antibodies against Leishmania.
Samples with indexes above 11 are considered as having antibodies against Leishmania.

## LIMITATIONS:

1. This kit is intended to be used with human serum.
2. The user of this kit is advised to carefully read and understand the package insert. Strict adherence to the protocol is necessary to obtain reliable test results. In particular, correct sample and reagent pipetting, along with careful washing and timing of the incubation steps is essential for accurate results.
3. The results of samples should be used in conjunction with clinical evaluation and other diagnostic procedures. A definitive diagnosis should be made by isolation techniques.
4. This test will not indicate the site of infection. It is not intended to replace isolation.

5. Lack of significant rise in antibody level does not exclude the possibility of infection.

## PERFORMANCE:

## SENSITIVITY AND SPECIFICITY:

141 serum samples from donors were assayed with LEISHMANIA ELISA IgG+IgM against a immunofluorescence kit. The results were as follows:

| | SAMPLE NR | SENSITIVITY | SPECIFICITY |
|---|---|---|---|
| IgG+IgM | 141 | 97% | 99% |

Indeterminate values were omitted from the final calculations

## INTRA-ASSAY PRECISION:

**3** sera were individually pipetted 10 times each serum in a single assay performed the same operator in essentially unchanged conditions. The results were as follows:

| SERUM | N | %C.V. |
|---|---|---|
| PC | 10 | 1.95 |
| NC | 10 | 6.06 |
| CO | 10 | 2.11 |

C.V. Coefficient of variation

## INTER-ASSAY PRECISION:

3 sera were individually pipetted on 5 consecutive days by 2 different operators. The results were as follows:

| SERUM | N | % C.V. |
|---|---|---|
| PC | 10 | 2.16 |
| NC | 10 | 6.65 |
| CO | 10 | 2.61 |

C.V. Coefficient of variation

## CROSS REACTIVITY AND INTERFERENCES:

3 samples known to be positive for *Toxoplasma gondii* were assayed for IgG+IgM testing. The results of the test demonstrated the specific reaction of the kit with no cross reaction or interferences with the referred specimens. In visceral leishmaniosis immune response of high intensity and can be detected by ELISA. In endemic areas cross-reactions with *Trypanosoma cruzi* may be expected, therefore serological results must be confirmed by alternative techniques.

# ANNEX 6: Laboratory test protocol used for Cytokine Enzyme Linked Immunossorbent Assay (ELISA)

**Protocol: ELISA Protocol Using ELISA Ready-Set-Go!**

eBioscience Ready-SET-Go! ELISA reagent sets (with or without high-affinity binding microwell plates) contain the necessary reagents, buffers and diluents for performing quantitative enzyme linked immunosorbent assays (ELISA). These ELISA reagent sets are specifically engineered for accurate and precise measurement of protein levels from samples including serum, plasma, and cell culture supernatants.

The following protocol is a general guideline for the ELISA Ready-Set-Go!

**Materials Provided**

• Please refer to the Certificate of Analysis (C of A) for components

**Other Materials Needed**

• Buffers*

- Wash Buffer: 1x PBS, 0.05% Tween-20 (or eBioscience ELISA Wash Buffer Powder Cat. No. 00-0400)

- Stop Solution: 1M $H_3PO_4$ (recommended) or 2N $H_2SO_4$

• Pipettes

• Refrigerator & frost-free -20°C freezer

• 96-well plate (Corning Costar 9018 or NUNC Maxisorp®**)**

**NOTE: The use of ELISA plates which are not high affinity protein binding plates will result in suboptimal performance, e.g., low signal or inconsistent data. Do not use tissue culture plates or low protein absorption plates. Use only the Corning Costar 9018 or NUNC Maxisorp (Cat. No. 44-2404) 96-well plates).**

• 96-well ELISA plate reader (microplate spectrophotometer)

• ELISA plate washer (highly recommended)

**NOTE: To ensure optimal results from this Uncoated ELISA Set, please only use the components included in the set. Exchanging of components is not recommended as a change in signal may occur.**

**Time Requirements**

- 1overnight incubation
- 4½-hour incubations
- 1 hour washing and analyzing samples

**Experimental Procedure**

1. Coat Corning Costar 9018 (or Nunc MaxisorpTM) ELISA plate with 100 µL/well of capture antibody in 1X Coating Buffer (dilute as noted on C of A, which is included with the reagent set). Seal the plate and incubate overnight at 4°C.

2. Aspirate wells and wash 3 times with >250 µL/well Wash Buffer*. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes. Blot plate on absorbent paper to remove any residual buffer.

3. Dilute 1 part 5X ELISA/ELISPOT Diluent with 4 parts DI water*. Block wells with 200 µL/well of 1X ELISA/ELISPOT Diluent. Incubate at room temperature for 1 hour.

4. Optional: Aspirate and wash at least once with Wash Buffer.

5. Using 1X ELISA/ELISPOT Diluent *, dilute standards as noted on the C of A to prepare the top concentration of the standard. Add 100 µL/well of top standard concentration to the appropriate wells. Perform 2-fold serial dilutions of the top standards to make the standard curve for a total of 8 points. Add 100 µL/well of your samples to the appropriate wells. Seal the plate and incubate at room temperature for 2 hours (or overnight at 4°C for maximal sensitivity).

6. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes**.

7. Add 100 µL/well of detection antibody diluted in 1X ELISA/ELISPOT Diluent * (dilute as noted on C of A). Seal the plate and incubate at room temperature for 1 hour.

8. Aspirate/wash as in step 2. Repeat for a total of 3-5 washes**.

9. Add 100 µL/well of Avidin-HRP* diluted in 1X ELISA/ELISPOT Diluent (dilute as noted on C of A). Seal the plate and incubate at room temperature for 30 minutes.

10. Aspirate and wash as in step 2. In this wash step, soak wells in Wash Buffer* for 1 to 2 minutes prior to aspiration. Repeat for a total of 5-7 washes**.

11. Add 100 µL/well of 1X TMB Solution to each well. Incubate plate at room temperature for 15 minutes.

12. Add 50 µL of Stop Solution to each well.

13. Read plate at 450 nm. If wavelength subtraction is available, subtract the values of 570 nm from those of 450 nm and analyze data.

**NOTES:**

*** Be certain that no sodium azide is present in the solutions used in this assay, as this inhibit HRP enzyme activity.**

****The number of washes in the protocol was adapted to an automatic plate washer. This can be decreased when using other methods but should be tested empirically. Allowing time for soaking (~ 1 minute) during each wash step increases the effectiveness of the washes.**

## Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university and that all sources of materials used for the thesis have been correctly acknowledged.

Name _____

Signature _____

## Assurance of principal investigator

The undersigned agrees to accept responsibility for the scientific ethical and technical conduct of the research project and for provision of required progress reports as per terms and conditions of the college of Public Health and Medical Science in effect at the time of grant is forwarded as the result of this application.

Name of the student: _____

Date._____ Signature _____

**APPROVAL OF ADVISORS**

This thesis has been submitted with my approval as University advisor.

Name of the first advisor: _____

Date._____ Signature _____

Date of submission: _____

Name of the second advisor: _____

Date._____ Signature _____

Date of submission: _____

Name of the third advisor: _____

Date._____ Signature _____

Date of submission: _____

Name of the fourth advisor: _____

Date._____ Signature _____

Date of submission: _____