# VISCERAL LEISHMANIASIS IN COMMUNITIES OF HAMER AND BENNA-TSEMAI DISTRICTS IN LOWER OMO VALLEY, SOUTH WEST ETHIOPIA: SEROPREVALENCE AND LEISHMANIN SKIN TEST SURVEY.


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**A Thesis Submitted to Jimma University College of Public Health and Medical Sciences Department of Medical Laboratory Sciences and Pathology in Partial Fulfillment of the Requirements for the Degree of Masters of Science in Medical Parasitology**

**JUNE 2014, JIMMA ETHIOPIA**

# Visceral Leishmaniasis in Communities of Hamer and Benna-Tsemai Districts, Lower Omo Valley Southwest Ethiopia: Sero-Epidemiological and Leishmanin Skin Test Survey.

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June 2014, 2014

## Abstract

***Background**: Visceral leishmaniasis is a systemic disease which is usually caused by Leishmania donovani or L. infantum, protozoan parasites that are transmitted by phlebotomine sandflies when blood-feeding on human and animal hosts. Eastern Africa is one of the world's main endemic areas, where it occurs in numerous foci in Eritrea, Ethiopia, Kenya, Somalia, Sudan and Uganda. In Ethiopia, Hamer and Benna-Tsemai districts in SNNPR-S are one of the areas which are presumed to be endemic for visceral leishmaniasis.*

***Objectives:** The objective of the study was to determine the prevalence of asymptomatic and symptomatic visceral leishmaniasis and also to determine the level of exposure (infection) to leishmania parasites in Hamer and Bena Tsemai districts in south western Ethiopia.*

***Methods:** Between 25th of July and August14th of 2013, we conducted a community based cross-sectional survey in selected villages of Hamer and Benna Tsemai districts in SNNPR-S. A total of 1760 individuals living in 440 households were included in the study. Socio-demographic and clinical data were collected from each of the participants; and venous blood was also collected for the detection of antibodies to visceral leishmaniasis using DAT. LST (Leishmanin Skin Test, also known as the Montenegro test) was performed to detect the exposure to the parasite.*

***Results**: A statistically significant variation in the rate of positive LST response was observed in different study sites and age groups. Positive LST response has shown an increasing trend with age. The DAT also showed a statistically significant variation among different study sites and age groups. High DAT positivity was observed in lower age groups. In general, the overall LST and DAT positivity were 8.6 and 1.8% respectively.*

***Conclusion and Recommendations**: Our study showed that asymptomatic VL infection in the study area is not negligible and could have a great contribution for anthropnotic transmission of the disease; thus, the regional health bureau specifically and MOH at large should take into consideration the implementation of a prevention and control strategy for VL. In addition to that we recommend an exhaustive survey, including a study of incidence and risk factors, which includes both sandfly investigation and epidemiology of the disease in one of the study kebele called cherkeka, represented by Ayro and Wisna villages in this study.*

## Acknowledgement

I take this opportunity to express my heartfelt gratitude to Professor Asrat Hailu, Country PI for Leishmaniasis East African Platform, who hasn't hesitated to spend his time and money helping me to achieve this work done. Your advice and comments will have a great positive influence in my future research and academic activities.

My heartfelt gratitude goes to my advisors Ato Tariku Belay and Ahemd Zenydin of Jimma University for their unlimited support, advice and comments during my work.

LRDL-AAU takes a great credit in this work; without their support of laboratory reagents, logistics and working environment it would have been impossible to achieve our goal.

I would like to express my appreciation to staffs of LRDL -AAU and AMH-LRTC: Hirut Wondimu, Welelta Shiferaw, Micheal Berhanu, Shewangizaw, Tedla Teferi and Dagmawie Tadesse for their unreserved effort during the field activity.

Finally I would like to thank Jimma university for giving me this chance and moreover for covering my personal expenses during this study.

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## Acronyms

| | |
|---|---|
| AAU | Addis Ababa University |
| AIDS | Acquired Immunodeficiency Syndrome |
| CL | Cutaneous Leishmaniasis |
| DAT | Direct Agglutination Test |
| DNA | Deoxy-ribonucleic acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| HIV | Human Immunodeficiency Virus |
| IFA | Immunofluorescence assay |
| LCL | Localized Cutaneous Leishmaniasis |
| LRDL | Leishimaniasis Research and Diagnostic Laboratory |
| LST | Leishmanin Skin Test |
| MCL | Muco-Cutaneous Leishmaniasis |
| PCR | Polymerase Chain Reaction |
| PKDL | Post Kala-azar Dermal Leishmaniasis |
| SbV | Pentavalent antimonials |
| SSG | Sodium Stibogluconate |
| VL | Visceral Leishmaniasis |
| WHO | World Health Organization |

# Chapter 1. Introduction

Visceral leishmaniasis(VL), also called Kala-azar, is a parasitic disease caused by members of the *Leishmania donovani complex* such as *L. donovani* and *L. infantum*(1). Transmitted by the bite of female phlebotomine sandfly, the parasite enters macrophages, where it multiplies and establishes the infection (2). A total of about 21 Leishmania spp. have been identified to be pathogenic to human. In most instances, they parasitize animals, and humans become infected incidentally when they enter an area of endemicity(3).

### 1.1.1 Geographical Distribution

VL is endemic in many parts of the tropics, subtropics and the Mediterranean basin, in settings ranging from rain forests in the Americas to deserts in western Asia, and from rural to peri-urban areas. It is endemic in more than 60 countries worldwide, including Southern Europe, North Africa, the Middle East, Central and South America and the Indian subcontinent. It is not endemic in South East Asia and Australia. The burden of disease (90% of cases) is borne by India, Bangladesh, Nepal, Sudan, and Brazil (4,5).

Eastern Africa is one of the world's main VL-endemic areas, where it occurs in numerous foci in Eritrea, Ethiopia, Kenya, Somalia, Sudan, South Sudan and Uganda. The number of VL cases in the region has increased dramatically in the past decade. A major contributing factor has been the ongoing armed conflicts causing wide-spread destruction of local housing and health infrastructures, increasing people's exposure to sandfly bites and making it hard to manage cases and deliver vector control activities(6).

As reviewed by Chappuis et al.(4), Ethiopia is one of the six countries (includes Bangladesh, Brazil, India, Nepal and Sudan) in which more than 90% of global VL cases occur. Within Ethiopia, VL is found in Tigray, Amhara, Oromia, Afar, Somali and SNNP-RS(7).

### 1.1.2 The Leishmania Parasite and Life Cycle

Leishmania parasite belongs to genus Leishmania, family Trypanosomatidae, and order Kinetoplastida, which consists of a set of organisms characterized by the presence of the kinetoplast. The genus Leishmania is divided into two subgenera on the basis of their development in sandflies. Growth of species of the subgenus Leishmania is restricted to parts of the alimentary tract of the natural vectors anterior to the pylorus at the junction of the midgut and

hindgut (suprapylarian development), whereas that of species of the subgenus Viannia occurs in both the midgut and the hindgut (peripylarian development)(1,2).

Leishmania parasite exists in two different morphological forms i.e. promastigotes (flagellate form) and amastigote (aflagellated form). Promastigotes develops inside the midgut of sandfly and become infective, non-dividing metacyclic promastigotes which are located near stomodeal valve (an invagination of the foregut into midgut). During blood feeding, metacylic promastigotes are regurgitated, and rapidly phagocytosed by one of several possible cell types that are found in the local environment at the site of biting. The various cell types may include neutrophils, tissue resident macrophages or dendritic cell (DC) or monocyte derived DCs (moDCs). After establishing an intracellular niche, metacyclic promastigotes are transformed to non motile amastigote form. These amastigotes replicate within the host cells, which rupture to release many amastigotes, allowing re-infection of phagocytes. The transmission is complete when infected phagocytes are taken up with blood meal of sand flies, where amastigotes then convert to promastigotes in the sandfly midgut(1,5)

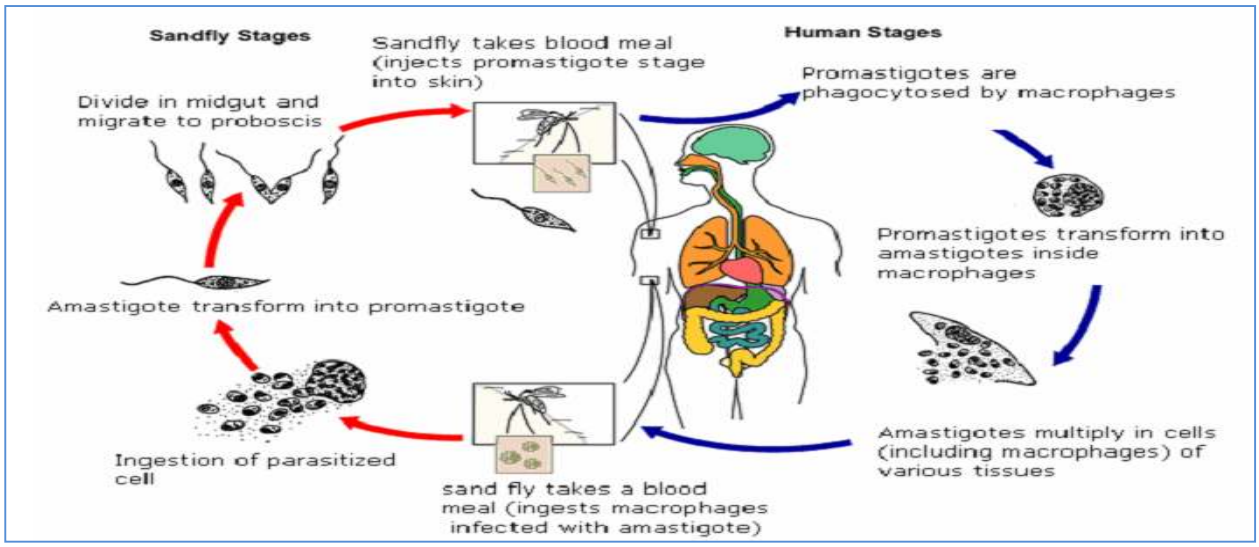


**Figure 1. Leishmania Parasite Life Cycle**

Available from www.cdc.org

### 1.1.3 The Sand Fly Vector

Phlebotomine sand flies are tiny blood-feeding insects of the family Psychodidae in the order Diptera with a body length of approximately 2−3 mm. They vary in color from silver-gray to almost black, and fold their wings into a characteristic V-shape when at rest. Phlebotominae sandflies occupy most of the zoogeographical regions of the world (Pale arctic, Oriental, Afro tropical, Nearctic, Neotrpical and Australasian). Each of the zoogeographical regions has its own characterstic sandfly fauna. Sandfly numbers are related to natural factors such as rainfall and temperature and may increase with global warming. Among all factors, temperature is considered to limit the worldwide distribution of sandflies. In extreme temperature, sandflies survive in diapauseThey are active nocturnally and rest in houses, cellars, caves and gaps among rocks in the daytime Sandflies live in dark, damp places, and are relatively weak fliers, with a range of only 50 -300 meters from the breeding site(3,9).

Unlike mosquitoes, they fly feebly and silently, and sneak upon a host using a peculiar hopping motion. Only female sandflies suck blood for egg production, and the bites are typically painful. To date, approximately 800 sand fly species have been recorded in five major genera and of these, proven vector species of Leishmania protozoa are classified into the genus Phlebotomus and Lutzomyia. The majority of the species play no part in the transmission of leishmaniasis in nature for several reasons; they may not feed on blood from humans and potential reservoir animals, and/or they may be incapable of supporting the development of Leishmania species. Less than 10% of sand flies have been incriminated as vector species of leishmaniasis, and only about 30 species have been demonstrated to have a vectoral capacity(1,3,10).

Based on circumstantial evidence, *P. orientalis* has been implicated as the vector of *L. donovani* in many endemic areas in Ethiopia including the lower Omo plains in the southwest and the Metema-Humra plains and also some other studies done have also implicated *P.martini* as the potential vector for *L.donovani* in southern Ethiopia(10).

### 1.1.4 Clinical Manifestation of Visceral Leishmaniasis

The infection L. donovani/L. infantum in man or dogs may be asymptomatic or may lead to fully blown kala-azar. A multitude of clinical features of the disease follow gradually, the most important being splenomegaly, recurring and irregular fever, anaemia, pancytopenia, weight loss, and weakness. Unlike malaria, there is no early dramatic fever to announce its arrival; the presentation is insidious, with symptoms appearing over a period of weeks or even months. Affected patients become progressively more anaemic, weak, cachectic, and susceptible to intercurrent infections. If untreated, it proves fatal within 2-3 yr; the cause of death is often intercurrent infections. VL affects not only the weakest in the community, such as children and those weakened by other diseases such as HIV and tuberculosis, but also healthy adults and economically productive social groups (1, 2, 4)

Post-kala-azar dermal leishmaniasis (PKDL) is a chronic rash seen in apparently cured kala-azar patients. It is seen in areas where *L. donovani* is endemic, i.e., in Asia (India, Nepal and Bangladesh) and in east Africa (Ethiopia, Kenya and Sudan). It is a late complication of visceral leishmaniasis which usually appears several months after treatment of a VL episode(12). In some parts of the world like India, the PKDL resembles lepromatous leprosy with verrucous, papilomatous, xanthomathous and gigantic nodular forms whereas in East Africa this disease is characterized by the appearance of macules, papules or nodules in the skin; the face is always affected but other parts of the body may also be involved. PKDL patients are suggested to be a human reservoir for anthroponotic transmission and it usually requires a long and expensive treatment(1,12,13).

Leishmania parasites have been recognized as opportunistic pathogens in immunosuppressed individuals, including those infected with human immunodeficiency virus type-1. Both the cellular and humoral responses to Leishmania are diminished in co-infected patients, leading to an increased risk of developing VL after Leishmania parasite infection, increased parasite load in blood and bone marrow, lower sensitivity of serological tests, and a higher rate of treatment failure. The clinical features can be atypical in severely immunosuppressed patients and the risk of treatment failure is high, whatever anti-leishmanial drug is used (1,4). Leishmaniasis and AIDS overlap in several sub-tropical and tropical regions around the world, including the Mediterranean area(1,14).

### 1.1.5 Immunity to Visceral Leishmaniasis

Immunopathogenesis of leishmaniasis relays to both parasite and host factors, which are interdependent. Inoculation of promastigotes is first point of Leishmania infection, which continues entering macrophages to escape host responses(15). Progressive intracellular infection by amastigotes depends on the maintenance of macrophages in an inert, inactivated state. Anti-leishmanial immunity is mediated by both innate and adaptive immune responses and requires effective activation of macrophages, dendritic cells (DCs), and antigen-specific CD4+ and CD8+ T cells (1,15).

The innate immune response to Leishmania is mediated by NK cells, cytokines and mononuclear and polymorphnuclear phagocytes. The production of IL-12 early in the course of infection by dendritic cells, leads to the early activation of NK cells and the production of IFN-Ɣ. The early NK-cell activation is also induced by chemokines (IP-10, MCP-1 and lymphotactin). Activated NK cells have been shown to be cytolytic for Leishmania-infected macrophages, but NK cell-derived IFN-Ɣ plays a more prominent role in host defence by activating macrophages to kill the intracellular parasite through the generation of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI). Activated polymorphnuclear leukocytes kill parasites primarily through oxidative mechanisms. Recently, it was shown that IFN-Ɣ and TFN-α participate in the early induction of NO and control of parasite replication early in infection (13,14).

Retrospective epidemiological studies indicate that most individuals with prior infection (subclinical or healed) are immune to a subsequent clinical infection. There is extensive evidence from experimental models that cellular immune mechanisms mediate acquired resistance to Leishmania infection, and human studies have generally confirmed this. Whereas anti-leishmanial antibodies, which are produced at a low level in CL and at a very high, level in VL, play no role in protection. A high antibody level is a marker of progressive disease in VL(13,14).

#### 1.1.5.1 Immune Evasion by Leishmania Parasite

Survival of Leishmania within the mammalian host depends on their ability to evade and manipulate the anti-leishmanial immune response, thus enabling them to reside long enough to establish infection. Since Leishmania are intracellular parasites, once inside the host cell, they evade the humoral immune response but become susceptible to attack by cell mediated

immunity. However, despite the presentation of leishmania antigens and the initiation of inflammatory responses, leishmania can reside within the host tissues for long periods by exploiting several mechanisms of evasion. There are numerous strategies utilized by leishmania to survive within mammalian hosts, some of which are common to other protozoan parasites such as *Toxoplasma gondii* and *Trypanosoma cruzi*. The various strategies employed by the invading pathogens demonstrate how leishmania parasites have evolved to successfully evade the immune responses designed to target and destroy them. Such evasion strategies include the modulation of macrophage function and the inhibition of antigen presentation and T cell stimulation (1,17).

### 1.1.6. Laboratory Diagnostic Methods for VL

Laboratory diagnosis of VL infection can be achieved through different methods; some of which are, microscopic detection of leishmanial amastigotes in stained smears of lymphnode, bone marrow or splenic aspirates, culture of the parasite, immunological tests for the detection of anti-leishmanial antibodies and leishmanial antigens and molecular techniques such as Polymerase Chain Reaction (PCR).

#### 1.1.6.1. Microscopy and Culture

Parasitological diagnosis remains the gold standard in leishmaniasis diagnosis because of its high specificity(18). Culture of parasite can improve the sensitivity of detection of parasite, but leishmania culture is rarely needed in routine clinical practice. However, cultures are required for (i) obtaining a sufficient number of organisms to use as antigen for immunologic diagnosis and speciation, (ii) obtaining parasites to be used in inoculating susceptible experimental animals, (iii) in vitro screening of drugs, and (iv) accurate diagnosis of the infection with the organism (as a supplement to other methods or to provide a diagnosis when routine methods have failed)(19). Microscopy is probably still the standard diagnostic approach at tertiary, secondary, or even primary health levels in areas of endemicity, because more sophisticated techniques are currently expensive and rarely available(18). However the sensitivity of microscopy and culture tends to be low and can be highly variable, depending on the number and dispersion of parasites in biopsy samples, and the sampling procedure; and in addition, microscopic examination requires invasive procedures and in vitro parasite isolation is difficult and time-consuming(20).

### 1.1.6.2. Serology and Other Immunological Techniques

Several serological approaches are commonly used in VL diagnosis. In particular, freeze-dried antigen-based direct agglutination tests (DAT) and commercially available immunochromatographic dipstick tests have increasingly become reference tests in operational settings since they have high sensitivity and specificity, are easy to use, and require minimal technological expertise or laboratory setup(18). Some of immunological methods currently in use are the Indirect Fluorescent Antibody Test (IFAT) which has a sensitivity range of 28.5 to 86.6 per cent; the DAT has a specificity of 72- 100% and a sensitivity range of 91-100%, and the Enzyme-linked immunosorbent Assay (ELISA) (21). In spite of all their benefits, immunological techniques on the contrary suffer from some limitations such as persistence of antibodies; certain individuals may have a high level of reactive antibodies in the absence of the organism (false positive); and conversely anti-parasite antibodies when induced may not be present until sometime after the initiation of infection (false negative). There is also possible cross-reactivity with other pathogens (malaria, trypanosomiasis, schistosomiasis and leprosy), and most serological tests cannot readily distinguish between current, subclinical or past infections. The performance of serological tests is particularly poor in patients co- infected with HIV(18,19,21,22).

Leishmanin skin test is an immunological technique that measures delayed type hypersensitivity reaction as skin indurations at the site of injection of killed stable fluid of Leishmanin after 48/72h. The LST is characteristically negative during active VL. The LST is useful to detect active MCL and CL. No cross-reaction occurs with Chagas' disease, but some cross-reactions are found with cases of glandular tuberculosis and lepromatous leprosy(18,19,21,22).

### 1.1.6.3. Molecular Techniques

Recently, several molecular biological techniques have been developed for the sensitive detection and identification of pathogens. The main approaches to nucleic acid-based detection are (I) hybridization using DNA probes (II) amplification techniques including the polymerase chain reaction (PCR) for the detection of DNA, nucleic acid sequence based amplification (NASBA) and reverse-transcriptase PCR (RT-PCR) for the detection of RNA. Although different molecular methods have successively been evaluated for leishmaniasis diagnosis (e.g.,

pulsed-field gel electrophoresis and multilocus enzyme electrophoresis), PCR-based assays currently constitute the main molecular diagnostic approach of researchers and health professionals(18). The specificity of the PCR can be adapted to specific needs by targeting conserved region of the gene. Various gene targets and nucleic acids can be used in PCR. The important gene targets are 18S-rRNA, small subunit ribosomal RNA (SSU rRNA), a repetitive genomic sequence of DNA, the miniexon (spliced ladder) gene repeat, the β-tubulin gene region, gp63 gene locus, internal transcribed spacer (ITS) regions; micro-satellite DNAs such as maxi and minicircles of kinetoplast DNA(19,20)

### 1.1.7 Visceral Leishmaniasis Treatment

Treatment of VL relies on specific anti-leishmanial drugs and the aggressive management of any concomitant bacterial or parasitic infections, anemia, hypovolemia (decreased blood volume) and malnutrition. Ideally, treatment for visceral leishmaniasis should cure the patient, reduce the risk for relapse and PKDL and reduce transmission of resistant parasites. The pentavalent antimonials sodium stibogluconate and meglumine antimoniate have been the first-line treatment for VL in many areas for more than 70 years(1,4). Amphotericin B deoxycholate and pentamidine have been used as second-line medicines. In the past 10 years, lipid formulations of amphotericin B, miltefosine and paromomycin have been approved for the treatment of visceral leishmaniasis; however, the efficacy and required dosage of several of these medicines have not been demonstrated in all endemic areas and may differ between these areas(1).

Sodium stibogluconate and meglumine antimoniate have been standard first-line medicines in most parts of the world (> 90%overall cure rate), but drug resistance is a major concern in the Bihar focus, India, and in Nepal, where unresponsiveness is as high as 60%, although this does not necessarily represent parasite resistance. Initial treatment of visceral leishmaniasis should be based on a daily injection of 20 mg/kg body weight .Injections are usually given for 28–30 days. Liposomal amphotericin B is the most extensively used formulation in visceral leishmaniasis. It is the reference treatment for visceral leishmaniasis in clinical practice in the Mediterranean region. There is limited experience in East Africa, where studies show lower (< 90%) cure rates at a total dose of 20 mg/kg, and where a total dose of 30 mg/kg given in 6–10 doses is now in limited use. In this region, studies are under way to determine the minimum effective total dose (1, 4). Visceral leishmaniasis negatively affects the response to antiretroviral treatment and is

difficult to cure in co- infected patients, especially those with CD4+ counts < 200 cells/ µl, who typically relapse. Antimonials are more toxic in HIV patients, necessitating careful monitoring for pancreatitis and cardiotoxicity. Amphotericin B deoxycholate or lipid formulations should be considered first and pentavalent antimonials only in areas of no significant resistance and when lipid formulations of amphotericin B are unavailable or unaffordable. Lipid formulations infused at a dose of 3–5 mg/kg daily or intermittently for 10 doses (days 1–5, 10, 17, 24, 31 and 38) up to a total dose of 40 mg/kg are recommended(1,22)

### 1.1.8 Control Measures

Concerning control measure for this deadly disease: There is no single method that can be used for all situations and one method may be successful in one place, but not in another. For efficient control, the ecology and epidemiology of the disease have to be understood and depending on the ecology (e.g. zoonotic or anthroponotic transmission), the control of either the reservoir or the vector is advisable. Presently, vector control is mainly used as a means to minimize the burden of the disease. The aim of a vector control program is to reduce or interrupt transmission of disease. An effective strategy for reducing human leishmaniasis is to control sandfly vectors, especially in domestic and peri-domestic transmission habitats. A number of control methods are available, including chemicals, environmental management and personal protection. Although some methods can have a strong independent effect on sandfly populations, it is highly recommended that sandfly control involve more than one method, in an integrated vector management approach(1,7)

## 1.2 Statement of the Problem

For many years, the public health impacts of the parasitic diseases have been grossly underestimated, mainly due to lack of awareness of its serious impact on health. Protozoan parasites of the genus Leishmania cause severe diseases that threaten human beings primarily in the tropical and subtropical areas. Leishmaniases have been considered tropical afflictions that together constitute one of the six entities on the World Health Organization/Tropical Disease Research (WHO/TDR) list of most important diseases. In eastern Africa only, VL causes at least 4,000 deaths annually, a loss of approximately 385,000 disability adjusted life years(24). The number of VL cases in the region has increased dramatically in the past decade. A major contributing factor has been the ongoing armed conflicts, which have caused widespread destruction of local housing and health infrastructures, increasing people's exposure to sandfly bites and making it hard to manage cases and deliver vector control activities. The conflicts have, in turn, induced massive movements of susceptible or infected populations into VL-endemic or non-endemic areas, respectively, triggering major epidemics. Due to the chronicity of underlying causes, it is likely that the case load will increase in the foreseeable future (25).Most VL infections occur in remote geographical areas where health facilities are not well established and where the infections often co-exist with malaria and other debilitating parasitic infections(1).

Ethiopia is one of the six countries (Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan) in which more than 90% of global Visceral Leishmaniasis (VL) cases occur and one of the ten countries with the highest estimated case counts, which together account for 70 to 75% of global estimated VL incidence. VL is growing health problem in Ethiopia, with endemic areas that are continually spreading. Geographically, VL is found in Tigray, Amhara, Oromia, Afar, Somali and SNNPR. Outbreaks of leishmaniasis have occurred in Ethiopia. Between 2005–2008G.C, a documented outbreak of VL occurred in Amhara Region (Libo Kemkem), with 2,500 cases and with a very high mortality rate. Omo valley, which is found in southern parts of the country, is in border with the areas which are known to be endemic for the disease such as Segen, Woito and Gelana river valley. When looking at the patient age and sex profile, and numbers of patients reporting to Arbaminch Hospital leishmaniasis research and treatment centre from our proposed study area, it appears that VL is typically endemic in the region with widespread infection and herd immunity. People living in the study area do not have access to diagnosis and treatment

service for this deadly disease; so the only option they left with are staying in their home suffering to death or travelling to Arbaminch hospital leishmaniasis treatment and research centre which is far away from their localities. Those patients who are unable visit the treatment centre stay in their home serving as a source of infection for other healthy individuals in the community there by maintaining the transmission cycle of the disease. In addition to the above problem currently the central government is implementing a large scale sugarcane plantation and factories which will possibly result in the influx of huge number of labor to the study area which may possibly upsurge the burden of the disease as it has happened in northern part of the country, Humera and Metema. Even though some efforts are underway to determine the burden of the disease in some parts of the country but in the lower Omo plains its magnitude remains undetermined, so this study will provide data on the magnitude of the disease and the vulnerable groups in the population so that it will help to design and implement a sound VL prevention and control strategy.

## Chapter 2 Literature Review

Leishmaniasis is endemic in more than 88 countries worldwide, in settings ranging from rain forests in the Americas to deserts in western Asia, and from rural to periurban areas including Southern Europe, North Africa, the Middle East, Central and South America and the Indian subcontinent. It is not endemic in South East Asia and Australia. The burden of disease (90% of cases) is borne by Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil, and Peru in the case of cutaneous leishmaniasis, and by India, Bangladesh, Nepal, Sudan, and Brazil in the case of visceral leishmaniasis(4,5,16,26).

Greater than 60% of the global burden of VL is attributed to south Asia(27). India, Nepal and Bangladesh harbor an estimated 67% of the global VL disease burden(4).Most of the estimated 200 to 400 thousand annual VL cases reported worldwide is located in South Asia (SA). In this Region the highest burden of the disease is concentrated in northern India (32,813 and 25,113 cases reported in 2005 and 2010, respectively), mainly Bihar State; eastern Bangladesh (6,892 cases in 2005 and 2,763 cases in 2010) and the south-eastern districts in Nepal (1,463 and 418 cases in 2005 and 2010, respectively)(28). A cross-sectional survey done in Bihar (India) using immunological technique has shown that Bihar-India is an area with a high VL incidence in southern Asia with an incidence rate ranging from 16.8 cases per 10,000 (90% CI: 9.3–30.6) to 35.6 cases per 10 000 persons per year (90% CI: 27.7–45.7)(29). VL is also a major public health problem in China. A retrospective study done by reviewing a VL cases notified between 2005 and 2010 based on the passive surveillance data has shown that a total of 2,450 VL cases were notified, with a mean of 408 cases per year. The number of VL cases and endemic countries had both increased in the period 2005-2010 in China(23).

Southern Europe is among the areas which are suffering from the affliction of this deadly disease. VL has been reported to be one of the emerging infectious diseases in Spain, Italy, Portugal, Hungary and Greece(29). In these areas the disease is zoonotic, dogs serving as the reservoir of infection and most of the patients had been found co- infected with HIV. Cases of co-infection have so far been reported in 35 countries world-wide, but most of the cases are from four countries; France, Italy, Portugal and Spain(30).

Southern America is among the areas which are affected by VL. A cross-sectional study done on a cohort of 946 individual in Brazil Amazon region with an objective of identifying individuals with symptomatic and/or asymptomatic infection due to Leishmania (L.) *infantum chagasi*; and aiming to determine the prevalence rate of infection at the beginning of the study using Leishmanin skin test (LST) and indirect fluorescent test has shown a prevalence rate of 12.6%(32).

Visceral leishimaniasis is a great public health problem in Africa especially in sub-Saharan region(23).Western Africa is among areas which are prone to this deadly disease. So far, in West Africa, the disease has been reported from Niger, Mali, Nigeria, Senegal, Cameroon, Burkina Faso, Mauritania, Gambia and Guinea(33).

Eastern Africa is one of the world's main VL_endemic areas, where it occurs in numerous foci in Eritrea, Ethiopia, Kenya, Somalia, Sudan and Uganda. The number of VL cases in the region has increased dramatically in the past decade. A major contributing factor has been the ongoing armed conflicts causing wide-spread destruction of local housing and health infrastructures, increasing people's exposure to sandfly bites and making it hard to manage cases and deliver vector control activities(6). During the 1980s visceral leshimaniasis has killed an estimated 100,000 people in Sudan(34). A recent study done in Eastern Gedaref state (Sudan), using LST and DAT, has shown that VL was a probable cause for 19% of all Deaths. This study has also showed that VL specific mortality rate was estimated to be 0.9/1000 per year(6).

As reviewed by Tonui et.al (35), VL was first reported in Kenya following an outbreak in the King's African Rifles troops encamped north of Lake Turkana in southwest Ethiopia in the 1940s. The disease in Kenya is caused by *L. donovani* and transmitted by *P. martini* though other vectors including *P. orientalis* have been reported. Since then Turkana, Baringo, Kitui, West Pokot, Machakos, Mwingi, Meru, Wajir, Mandera, Keiyo and Marakwet districts have been considered to be endemic for kala-azar. Baringo and the neighboring districts such as West Pokot were first identified as leishmaniases foci in 1955. Baringo district is the only foci where both VL and CL are known to occur in Kenya (11,35).

Even though there has not been an updated report of VL in Somalia, because of the political instability in the country, some reports made by non- governmental organizations shows that VL is endemic in some parts of the country. Areas of Somalia where VL has been reported include the coastal areas in the south of the country, the area along the Shebelle River in the south of Somalia, Lower Juba region and Baidoa in Bay region(36). The international humanitarian organization, MSF, which has been running a primary health-care project in the Huddur area since the year 2000, had witnessed a dramatic increase in the number of patients with VL admitted to its treatment center. The upsurge of VL was mostly reported in the Bakool region, where the average caseload that was previously stable at around 140 VL cases per year until September 2005, had increased seven-fold to a total of 1002 patients in year 2006 alone(37)

As reviewed by Chappuis et al. (4), Ethiopia is one of the six countries (Bangladesh, Brazil, India, Nepal and Sudan) in which more than 90% of global VL cases occur. Both CL and VL are growing health problems in Ethiopia, with endemic areas that are continually spreading. Geographically, VL is found in Tigray, Amhara, Oromia, Afar, Somali and SNNPR.(7). Historically the north-western part of Ethiopia is a known VL endemic focus. The area covers the semi-arid Humera and Metema plains in Tigray regional State bordering Sudan. In these areas a marked increase in VL incidence was observed during the 1970s when migrants from the non-endemic highlands began to arrive in the area to harvest crops in the area on the large scale agricultural farms introduced at the time.VL in the area predominantly affects young male migrant workers, who tend to sleep outdoors thereby increasing their exposure, and cases peak during the dry season from November to March (38). VL has been known to prevail in south west Ethiopia, a survey of the area which includes Hamer, Dassenech, Nyangatom has shown that level of positive skin tests for the groups tested ranged from over 64% for the three tribes collectively inhabiting the lower regions of the Omo Valley ( 500 m) to 6.4% for the Suri tribe, which lives at 1400 m (39). In 2005 MSF Greece identified an outbreak of VL in Libo kemkem woreda in Amhara regional state. The outbreak began in Bura kebele in 2003 with cases peaking in 2005 and occurring mainly in Libo Kemkem and Fogera woredas (38). A recent cross-sectional survey done in children of 4-15 years of age in Amhara regional state using LST and DAT has shown 9.9%

prevalence rate. The study also showed that there exists a positive association between VL positivity and increasing age and also being male(40).

Cases of VL have also been recorded in the Awash Valley, Afar regional states in the northeast. A cross-sectional LST survey carried out in middle course of the Awash valley involving 889 individuals has shown that an overall prevalence of 60% for males and 25% for females with increased positivity with age in both sexes(41). VL is also distributed throughout the lowlands of south Ethiopia with varying degree of endemicity. Important endemic foci include the Genale focus at Lake Abaya, the Segen Valley in Konso Woreda, and the Omo river plains. Visceral leishmaniasis survey done by Hailu et al. from July 1989 to June 1992 in eight localities of South and South west Ethiopia using DAT and Leishmanin skin test has shown a sero-prevalence rate of 1.8% to 27.8%(42).

Lower Omo valley which is found in southern parts of the country borders with the areas which are known to be endemic for the disease such as Segen, Woito and Gelana river valley. When looking at the patient age and sex profile, and numbers of patients reporting to AMH-LRTC, it appears that VL is typically endemic in the region with widespread infection and herd immunity. Efforts are underway to determine the burden of the disease in some parts of the country, but, in the lower Omo plains its magnitude remains undetermined, so this study will provide data on the magnitude of the exposure and infection by leishmania causing VL so that it will help to design and implement a sound VL control strategy.

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## 2.1 Significance of the Study

Hamer and Benna-Tsemai districts, like many rural areas of the country, are remote areas in southern Ethiopia with inadequate health facility. Independent studies carried out by other scholars have confirmed the existence of phelobotmine sandflies which are the potential vectors for the transmission of VL. Patient records from Arbaminch Hospital LRTC show that VL is becoming a public health problem in the region. People living in the study area do not have access to diagnosis and treatment service for this deadly disease; so the only option they are left with is staying in their home suffering to death or travelling to AMH-LRTC which is far away from their localities. Those patients who are unable visit the treatment centre stay in their home becoming a source of infection for the community; there by, maintaining the transmission cycle of the disease. So this study will provide data on the magnitude of exposure/infection to leishmania parasite causing visceral leishmaniasis. In addition, the result of this study will provide the central government a valuable information concerning the potential risk of VL in the area as it is planning to launch large scale sugar plantation farms and factories which will result in an influx of huge number of labor to the area. The study will also help the Zonal and Woreda health Bureaus specifically and the Ministry of Health and other concerned bodies at large, to plan and implement a sound VL prevention and control strategy.

# Chapter 3 Objectives

## 3.1 General Objective

To determine the level of exposure and prevalence of both asymptomatic and symptomatic infection of visceral leishmaniasis in selected communities of Hamer and Benna-Tsemai districts, lower Omo region in southern part of Ethiopia.

## 3.2 Specific Objective

- To determine the sero-prevalence of symptomatic and asymptomatic infections using serological techniques [DAT and RK39 chromatographic test]
- To determine the level of exposure to Leishmania by Leishmanin skin test(Montenegro)
- To identify vulnerable populations in the study area, and assess the risk of exposure/infection

# Chapter 4 Methods and Materials

## 4.1 Study Design

A community-based cross-sectional survey, using Direct Agglutination test, Rk39 rapid chromatographic test and Leishmanin skin test (Montenegro), was conducted from July 25, 2013 to August14, 2013 in selected populations of Hamer and Benna Tesmai districts in south Omo Zone of SNNP-RS, in southern Ethiopia.

## 4.2 Study Area and Population

The study was conducted in Hamer and Benna-Tsemai districts, Lower Omo region, South Omo Zone in Southern Nations, Nationalities & People's Regional State (SNNP-RS). Hamer woreda (district) is one of the 77 districts in SNNP-RS. It covers an area of 5,697.38sq.km and has 24 rural kebeles among which Chercheka, Besheda and Sinbile were surveyed. All the study kebeles are found below 1000m above sea level with the lowest being Cherkeka at 550m. The district is bordered by Kuraz woreda in the south, Konso Special wereda in northeast, and Oromia region in the east. Numerous ethnic groups have their homelands in this woreda. The Hamer people have their homeland in the western part, the Erbore people in the eastern part, the Tsemai in the northeastern, while the small homeland of the Birale people is an enclave within the territory of Hamer people. Based on figures published by the Central Statistical Agency in 2007, this Woreda has an estimated total population of 70,772 of whom 35,546 are men and 35,227 are women.

Benna-Tsemai is a district adjacent to Hamer woreda. It covers an area of 2,922.76 sq.km and has 23 rural kebeles among which Luka and Olu were surveyed. Olu is found 1285m above sea level whereas Luka is at 630m. According to CSA 2007 report the district has an estimated total population of 62,825 of which 31,788 are male and 31,037 are females. Most Tsamai are agro-pastoralists, herding cattle as well as growing crops. The district is bordered by Hamer Woreda in the south, by Konso Special wereda in the north east and by Bako Gazer woreda in the west. Key Afer is the central administrative town of the Woreda and has a total population of 2384. Most of the villages in this Woreda are located at the foot of the mountains in relatively elevated positions in order to minimize exposure to malaria, the main killer disease in the area. The main rainfall and the most productive agricultural season stretches from March to June(43).

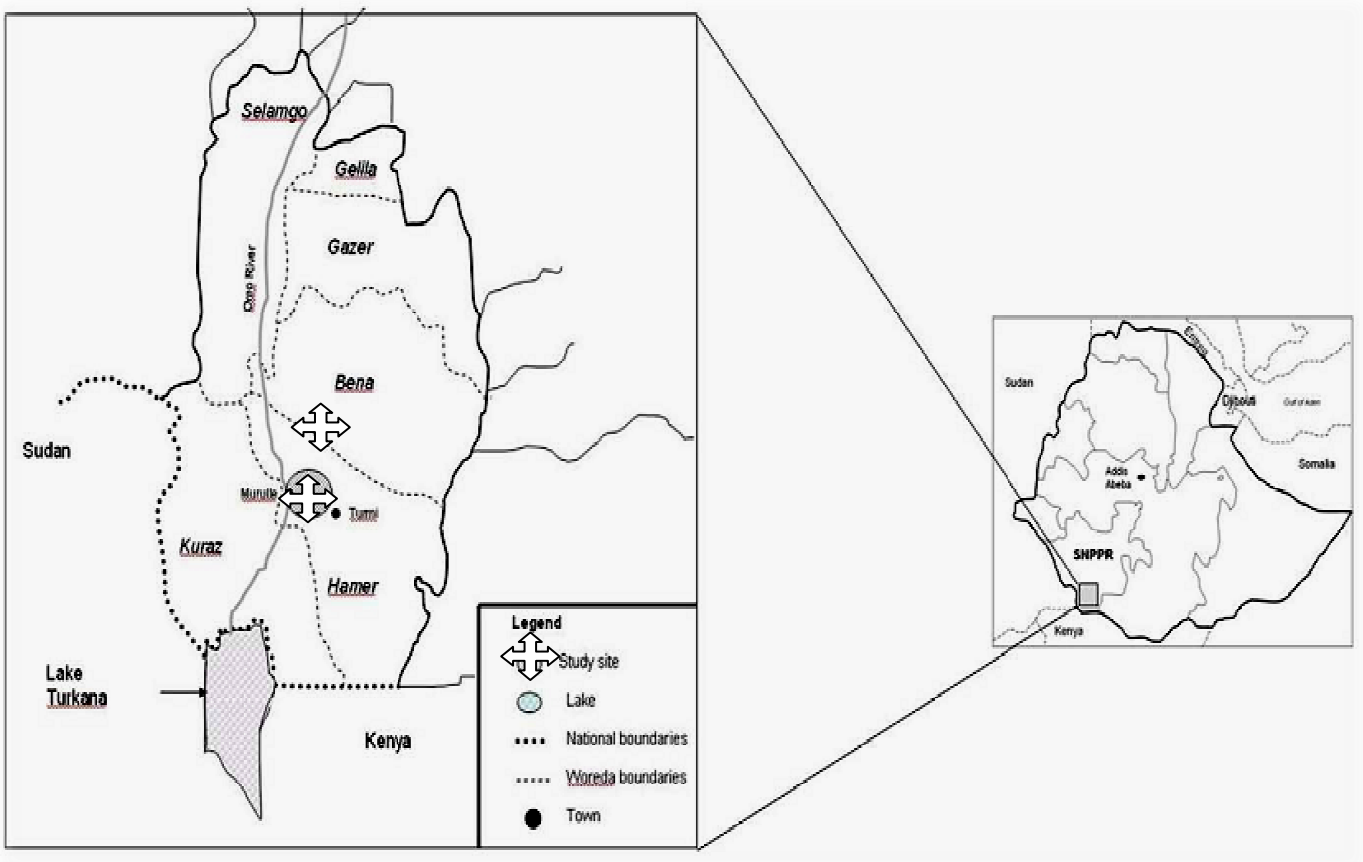



**Figure 2 Map of the study area**

Adopted from- PLoS ONE | www.plosone.org;

doi:10.1371/journal.pone.0012205.g001

## 4.3 Source Population and Study Population

The source populations of the study were residents of the villages in Hamer and Benna Tsemai districts where VL transmission was suspected to take place; based on patients' data from Arbaminch Hospital LRTC. Three kebeles from Hamer woreda (Besheda, Sinbile and Cherkeka) and two kebeles from Benna Tsemai woreda (Luka and Olu) were included in the study. The study population comprised individuals who were above two years of age living in households located in villages of the above-mentioned kebeles. A preliminary survey on spatial distribution of villages, households and the respective population sizes were carried out to identify villages (sub clusters) in geographical locations where VL has previously been diagnosed. Additional ecological parameters such as sandfly fauna were taken into account to select clusters that were

likely to be located in the transmission zone. Data on sandfly fauna was availed by the Leishmaniasis Research Group at Addis Ababa University.

## 4.4 Sample Size Determination

Sample size was calculated by using single population proportion formula and multiplying the result by a design effect of 1.5

$$n = DEFF \times \frac{(1.96)^2 \times (P)(1-P)}{d^2}$$

Where

DEFF=Design effect =1.5……. (8)

1.96=Z value for 95% confidence interval

P=Estimated prevalence; in our case which is =0.5

d=margin of error=3%

Taking 10% non response rate the final sample size was =1760

## 4.5 Preliminary Survey

Before the study sites were selected, a group of experts, which included principal investigator and senior scientist (Advisor), traveled to the localities. Discussions were held with the woreda Health Bureaus to facilitate the study. Although the Health Bureaus of both woredas knew of the presence of the disease, they have no documented case history as most of the suspected patients were referred to AMH- LRTC. After contacting the woreda administration and explaining the objective of the study, a site visit was made based on the patient registration data at AMH-LRTC. The population sizes of each kebele of the districts were documented. A meeting was also held with each kebele health extension workers to create awareness and discuss their involvement. After the reconnaissant assessment, representative study sites were selected as described below.

## 4.6 Sampling Technique/Sampling Procedure

Population sampling was carried out by a multi-staged cluster survey. Purposefully, sub-districts (kebeles) with registered VL cases at AMH-LRTC were selected for the study. Primary sampling units were randomly selected villages in each of purposefully selected sub-districts and these villages were used for sampling of households proportional to their size{pps} and finally individuals living within selected households and who are willing to participate in the study were included.

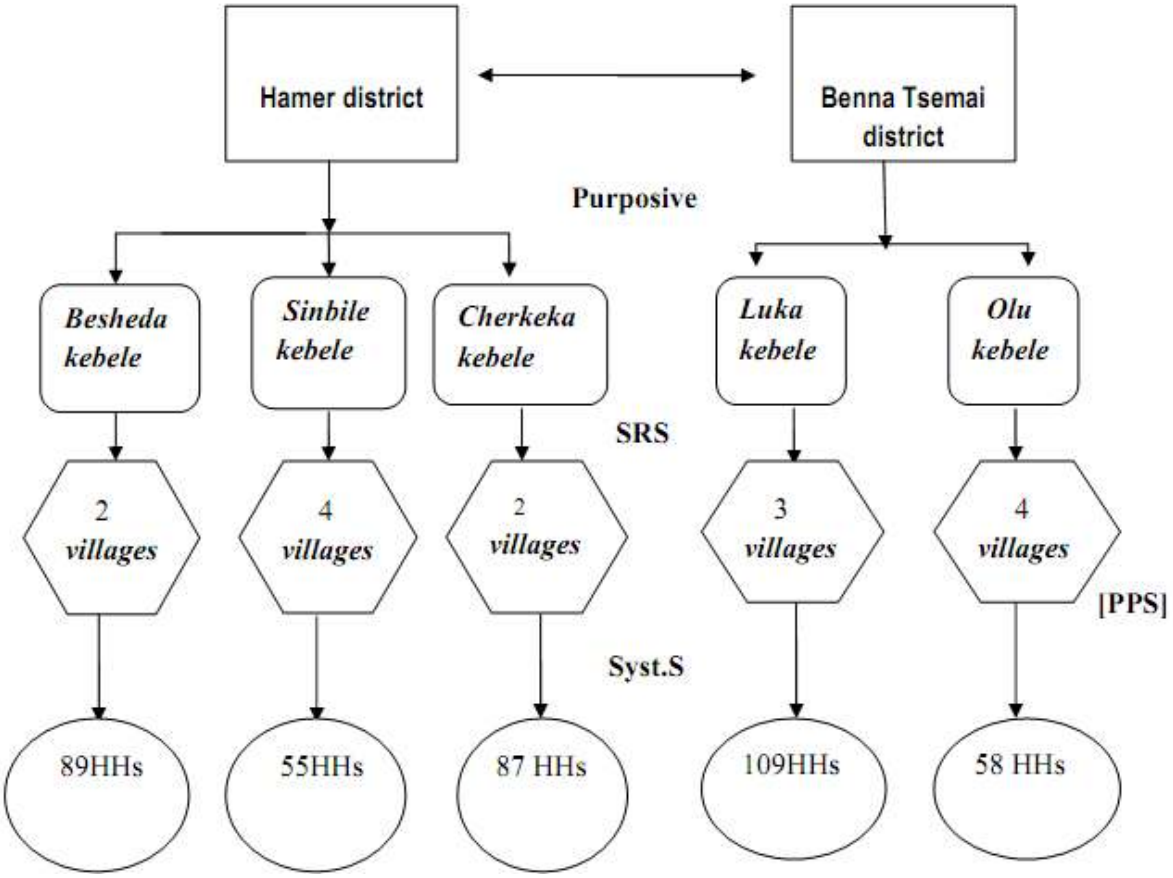



**Figure 3 Schematic Presentation of Sampling Technique**

## 4.7 Questionnaire and Clinical Examination

Socio-demographic characteristics were collected using pre-structured questionnaires. Informed consent to participate in the study was obtained from all adults and in the case of children from their guardians. An enrollment form containing past medical history, present complaints and other demographic data was completed for every individual.

A general clinical examination was conducted to each individual in the study population with particular reference to hepato-splenomegally, presence/absence of fever, enlargement of lymph nodes and presence of scars of previous cutaneous leishmaniasis and/ or PKDL. In sick looking subjects having complaints of abdominal pain/swelling and/or fever and weight loss, liver size was measured in the mid-clavicular line from the costal margin; the spleen size was assessed by measuring the distance between the coastal margins in the anterior axillary line to the tip of the spleen. Lymphadenopathy was classified as localized if found only at one site and generalized if found in two or more sites.

Inguinal lymphadenopathy was not included, as this is a common finding particularly in people who often walk barefoot. Malaria antibody detection was made using a rapid malaria test in all individuals who either had fever, looked ill or had splenomegally. Those with a positive result were treated either with chloroquine or coartem depending on the species detected.

For patients who fulfilled case definition of VL; i.e., individuals with fever for at least two weeks duration and testing positive in a rapid kala-azar test (DiaMed IT Leish-RK39) plus at least one of the following: splenomegaly, lymphadenopathies or history of weight loss were considered as suspected cases of VL and referred to Arba Minch Hospital LRTC for confirmation of the diagnosis (splenic aspiration or bone marrow punctures) and detailed investigations (physical examination, laboratory tests). Confirmed patients were treated at LRTC.

## 4.8 Sample Collection and Processing

The total blood volume collected was 5 ml from adults and 3 ml from children for all the tests. LST was performed and results documented while in the field, whereas all other tests were conducted at the facilities of LRDL-AAU.

## 4.9 Laboratory Procedures

### 4.9.1 Direct Agglutination Test

Blood sample was collected from a peripheral vein to obtain serum. Serially diluted serum samples were incubated with *Leishmania* antigen in V-shaped bottom microtitre plates. The plates were incubated at room temperature for 8-12 hours and then read visually. In samples with no anti-*Leishmania* antibodies, the antigen sediments to the bottom of the well and form a

small sharp blue dot and in those where anti-*Leishmania* antibodies were present agglutination was visible as a mat, a dot with frayed edges or an enlarged dot.(detail procedure is annexed).(40,44)

### 4.9.2 Leishmanin Skin Test (Montenegro test)

The Montenegro skin test (Leishmanin skin test) is a test for DTH specific to leishmania parasites. In this method, 0.5 ml of phenol-killed whole parasites ($5 \times 10^7$ promastigotes) was injected on the volar aspect of the forearm of the study subjects. After 48 to 72 h, the size of indurations was measured using the ball point method. Results were recorded as an average of two dimensional readings. Induration size of 5.0 mm and above was considered positive.(40,44)

## 4.10 Operational Definition

Subclinical case: A case with no VL history or sign and symptoms and test positive for DAT.

Active VL/Confirmed VL case: A case that is clinically and laboratory confirmed using parasitological examination.

VL suspect: A case that is clinically and laboratory confirmed (using DAT)

## 4.11 Data Analysis

Data was entered into excel sheets, checked for completeness, exported to SPSS and analyzed by the same. $\chi 2$ and the corresponding P-value were used to determine the statistical significance of the proportion/ratios obtained from the cross tabulated data .A P-value of $< 0.05$ was considered indicative of statistical significance.

## 4.12 Quality Control

A clear explanation of the purpose and procedures of the study was introduced to each participant to get the right information. A standard operating procedure (SOP) was strictly followed for sample collection, transport and storage. Prior to data collection a meeting was held with data collectors and a consensus was reached on data collection procedures .Questionnaires were filled by nurses who are trained by LEAP: Leishmaniasis East Africa Platform. All reagents that were used were checked a priori for their shelf life and storage condition before use. During sample analysis in the laboratory both positive and negative controls were run along with the test. For all types of tests, procedures followed were according to the manufacturers'

instructions. The LST antigen was validated at AMH-LRTC for potency in volunteer's formerly cured cutaneous and visceral leishmaniasis.

### 4.13 Ethical Consideration

Ethical clearance was obtained from Jimma University Research and Ethics Review Committee. Permission was also obtained from the respective woreda health department. A written informed consent was sought from each individual prior to involvement in the study. For children their parents were asked to involve them in the study. Information collected from each study participant was kept confidential and venous blood specimens collected were preserved anonymously. For patients who fulfilled case definition of VL detailed physical and laboratory examinations such as complete blood cell count, blood film and other tests were done in AMH-LRTC and were treated with SSG.

### 4.14 Limitation of the Study

Leishmanin skin test is a procedure which needs 48-72 hours for result reading; and thus some individuals failed to return back for LST reading within 72 hour time interval and some returned back after 72 hours. Tracking of these individuals was not feasible logistically as most of the individuals were shepherds and the area was remote and inaccessible.

# Chapter 5 Result

## 5.1Characteristic of the Study Population

A total of 1760 individuals living in 404 households, 975(55.3%) females and 785(44.7) males from different age groups, were included in the study. The minimum age of an individual included in the study was 2 years. Each study subject was subjected to physical examination, Leishmanin skin test and direct agglutination test for the presence of antibodies against visceral leishmaniasis LST was performed on 1600 subjects, of which 1268 had returned for the reading during the appropriate time; whereas 1760 individuals had been tested for the presence of antibodies to Leishmania using DAT. Most of the study subjects (58%) were from Hamer woreda and the remaining (42%) were from Benna Tsemai. Majority of the study subjects (44.1%) were less than 10 years of age followed by 10-20 years of age (24.1%). Those age 60 and above were 2.4% of the study subjects.

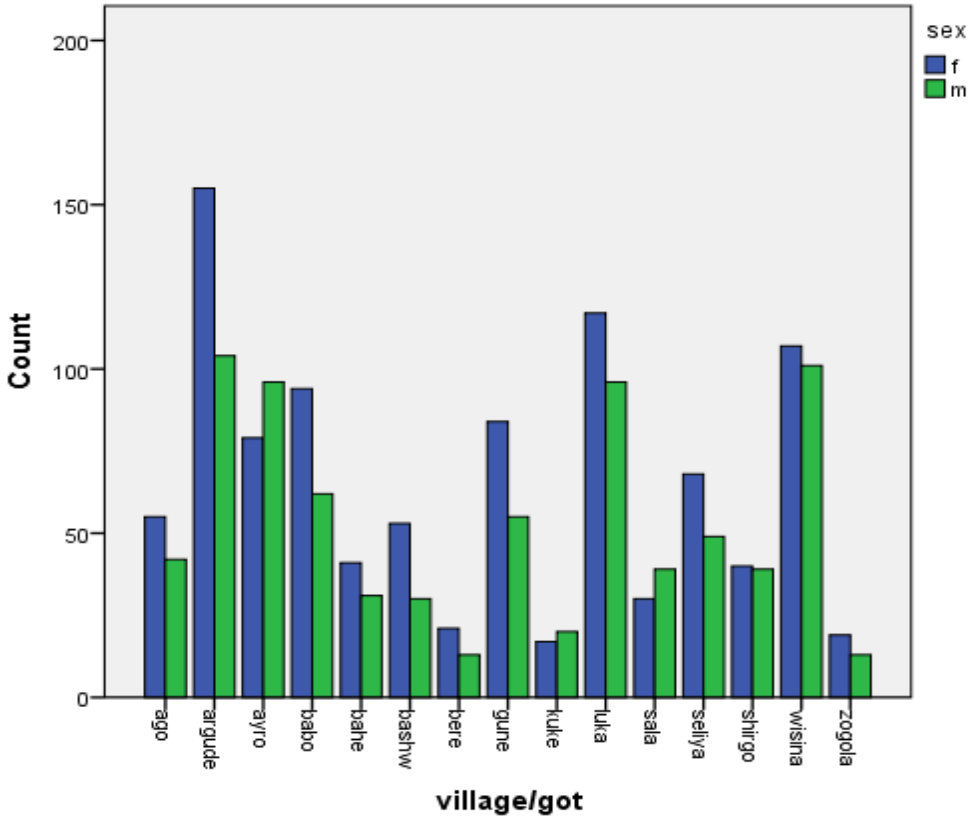
**Table1. Distribution of the study population by age and sex, July2013**

| Age group | | Sex | | | |
|---|---|---|---|---|---|
| | | F | | M | |
| | | Count | % | Count | N % |
| | < 10 | 355 | 36.4% | 349 | 44.1% |
| | 10 – 19 | 214 | 22% | 189 | 24.1% |
| | 20 – 29 | 119 | 12.2% | 100 | 12.7% |
| | 30 – 39 | 138 | 14.2% | 60 | 7.6% |
| | 40 – 49 | 87 | 8.9% | 48 | 6.1% |
| | 50 – 59 | 35 | 3.6% | 21 | 2.7% |
| | 60+ | 26 | 2.7% | 19 | 2.4% |
| | Total | 974 | 100% | 786 | 100% |

**Table2. Distribution of the study population by Age groups in Hamer and Benna, July2013**

| Age group | | Woreda | | | | Total |
|---|---|---|---|---|---|---|
| | | Benna Tesmay | | Hamer | | |
| | | Counts | % | Count | % | |
| | < 10 | 306 | 41.5% | 398 | 38.9% | 704 |
| | 10 - 19 | 186 | 25.2% | 217 | 21.2% | 403 |
| | 20 - 29 | 81 | 11% | 138 | 13.5% | 219 |
| | 30 - 39 | 77 | 10.4% | 121 | 11.8% | 198 |
| | 40 - 49 | 56 | 7.6% | 79 | 7.7% | 135 |
| | 50 - 59 | 20 | 2.7% | 36 | 3.5% | 56 |
| | 60+ | 12 | 1.6% | 33 | 3.2% | 45 |
| Total | | 738 | 100% | 1022 | 100% | 1760 |

**Figure4. Distribution of study participants by sub-clusters of villages and gender, July 2013**



## 5.2 Leishmanin Skin Test (LST)

Out of 1600 individuals on whom LST was performed 1268 have returned for the reading within 72 hours time interval. LST positivity has shown variation in different age groups [Table3/figure5], sex [Table 4/figure5] and also study sites [Table 5]. The overall LST positive rate was 8.6% (3.8% in males, 4.8% in females). The difference between males and females was statistically not significant. The highest rate of LST positivity was observed in age group of 20-29 and 50-59 (21.2% and 22.2) and also with in age group of 30-39 (21.1%) [Table 3]. The least positivity was observed within the age group of less than 10 (0.9%) followed by age group of 10-19 (5.9%). The difference in LST positivity by age group was found statistically significant($x^2$=114.8: p <0.001).

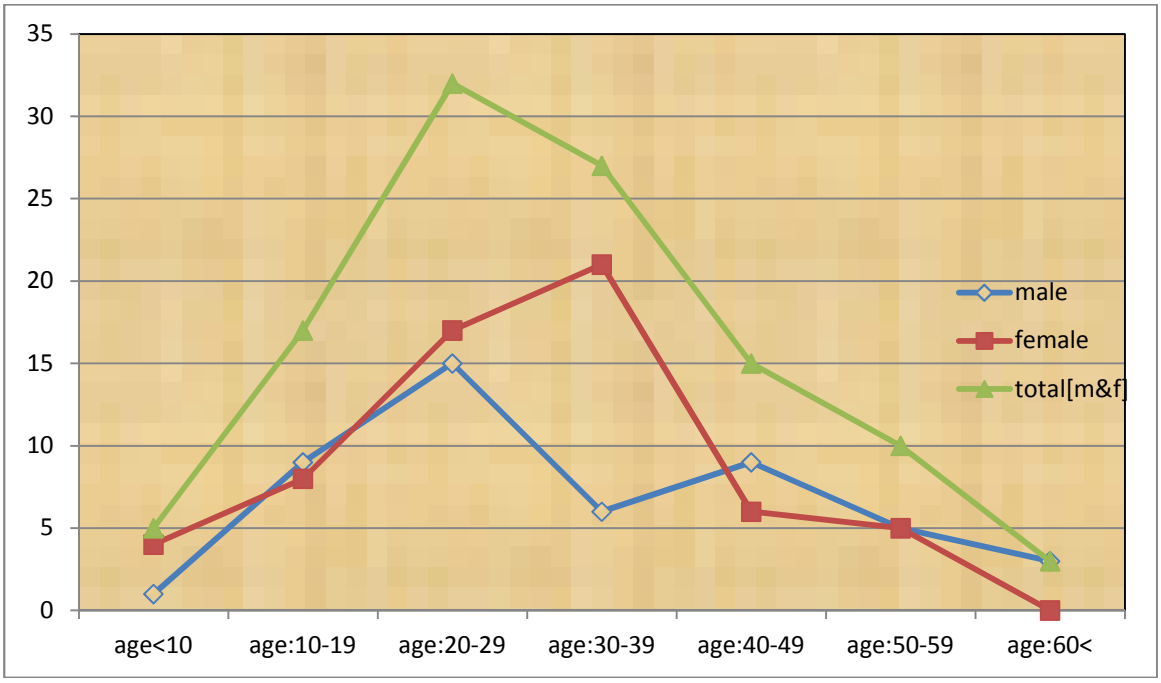
**Table 3 LST positivity in different age groups in the study population, July2013**

| Age group | | | LST | | Total |
|---|---|---|---|---|---|
| | | | positive | negative | |
| | < 10 | Count | 5 | 522 | 527 |
| | | % | 0.9% | 99.1% | 100.0% |
| | 10 - 19 | Count | 17 | 270 | 287 |
| | | % | 5.9% | 94.1% | 100.0% |
| | 20 - 29 | Count | 32 | 119 | 151 |
| | | % | 21.2% | 78.8% | 100.0% |
| | 30 - 39 | Count | 27 | 101 | 128 |
| | | % | 21.1% | 78.9% | 100.0% |
| | 40 - 49 | Count | 15 | 79 | 94 |
| | | % | 16.0% | 84.0% | 100.0% |
| | 50 - 59 | Count | 10 | 35 | 45 |
| | | % | 22.2% | 77.8% | 100.0% |
| | 60+ | Count | 3 | 33 | 36 |
| | | % | 8.3% | 91.7% | 100.0% |
| Total | | Count | 109 | 1159 | 1268 |
| | | % | 8.6% | 91.4% | 100.0% |

**Table 4. LST positivity by gender of the study population, July2013**

| Sex | Positive | Negative |
|---|---|---|
| | N[%] | N[%] |
| M | 48[8.5] | 520[91.5] |
| F | 61[8.7] | 639[91.3] |
| Total | 109[8.6] | 1159[91.4] |

**Figure 5. LST Positivity (count) by age group and sex in the study population, July2013**




Variations in the prevalence of LST positivity were also observed in different study sites [Table5]. The highest prevalence observed was in Wisna (23.1%) followed by Ayro (15.9%) and Luka (12%). No positive LST individuals were found in Bashe, Deliya and Kuke. The difference in leishmanin positivity by study sites (village) was statistically significant ($x^2$=81.8: p<0.001)

**Table 5.LST positivity by kebeles and respective villages in Hamer and Benna, July2013**

| Localities/kebeles | Villages | Tested | Positive[% | Statistics |
|---|---|---|---|---|
| Cherkeka | Ayro | 132 | 21[15.9] | $X^2$=81.8 |
| | Wisina | 156 | 36[23.1] | P<0.001 |
| Besheda | Argude | 196 | 15 [7.7] | |
| | Gune | 66 | 1 [1.5] | |
| Sinble | Ago | 63 | 3 [4.5] | |
| | Bashaw | 59 | 0 [0] | |
| | Bere | 25 | 1 [4] | |
| | Zogola | 18 | 1 [5.6] | |
| Luka | Babo | 123 | 5 [4.1] | |
| | Luka | 141 | 17 12] | |
| | Selya | 83 | 0 [0] | |
| Olu | Bahe | 56 | 4 [7.1] | |
| | Kuke | 32 | 0 [0] | |
| | Sala | 54 | 2 [3.7] | |
| | Shirgo | 64 | 3 [4.7] | |
| Total | | 1268 | 109[8.6] | |

## 5.3 Direct Agglutination Test (DAT)

### 5.3.1 DAT in different age groups and study sites

Individuals within the age group 10 -19 had the highest DAT positivity (2.5%) followed by age group 20-29(2.3%) [Table 6]. No DAT positive cases were found in the age groups 40-49 and greater than 60. Variations in the prevalence of DAT positivity were noted among different study sites [Table 7]. The highest positivity (5.1%) was observed in Ayro followed by Wisnia (4.8%) and Argude (3.1%). Among all DAT positive cases, two of them (one male and one female) had previous treatment history. The difference in DAT positivity by study sites (village) was statistically significant ($x2 = 38.5$; $P<0.05$). Our data also shows that 9(32.1%) of the DAT positive individuals have tested positive for LST [Table 8]

**Table 6 DAT positivity in different age groups in the study population, July2013**

| Age Group | Total Tested | Positive[%] |
|---|---|---|
| <10 | 704 | 13[1.8] |
| 10-19 | 403 | 10[2.5] |
| 20-29 | 219 | 5[2.3] |
| 30-39 | 198 | 3[1.5] |
| 40-49 | 135 | 0[0] |
| 50-59 | 56 | 1[1.8] |
| 60+ | 45 | 0[0] |
| Total | 1760 | 32[1.8] |

**Table 7. DAT positivity by kebeles and respective villages in Hamer and Benna, July2013**

| Localities/kebeles | Villages | Tested | Positive[%] |
|---|---|---|---|
| cherkeka | Ayro | 175 | 9 [5.1] |
| | Wisina | 208 | 10 [4.8] |
| Besheda | Argude | 255 | 8 [3.1] |
| | Gune | 138 | 0 [0] |
| Sinble | Ago | 97 | 0 [0] |
| | Bashaw | 83 | 0 [0] |
| | Bere | 34 | 0 [0] |
| | Zogola | 32 | 0 [0] |
| Luka | Babo | 151 | 2 [0] |
| | Luka | 213 | 0 [0] |
| | Selya | 117 | 2 [2] |
| Olu | Bahe | 72 | 0 [0] |
| | kuke | 37 | 0 [0] |
| | sala | 69 | 1 [1] |
| | shirgo | 79 | 0 [0] |
| Total | | 1760 | 32 [1.8] |

**Table 8 Prevalence of LST positivity in DAT positive and negative individuals in the study population, July2013**

| DAT | LST [N and % positive] | $X^2$, p-value |
|---|---|---|
| **DAT positive (n= 28)** | **9 (32.1%)** | **$X^2$= 20.2** |
| **DAT negative (n= 1240)** | **100 (8.1%)** | **p<0.0001** |

Active visceral leishmaniasis detection allowed us to detect two female cases from Hamer Woreda, Cherkeka Kebele, Wisina village both of them were less than five years of age. Upon arrival at AMH-LRTC they were diagnosed with Leucopenia [Total WBCs of $2.4\times10^3$ & $2.5\times10^3$], severe anemia [5.7g/dl &5.8g/dl] and also panacytopenia [$54\times 10^3$ & $55\times 10^3$]. The physical examination has also revealed that both subjects were emaciated, underweight [BMI of 13.8 & 15.2]. Both were treated with SSG 20 mg/kg for 30 days and discharged cured.

Our data also shows that 9 (32.1%) of DAT positive individuals tested positive on LST with the remaining 19 (67.9%) showing a negative result.

## Chapter 6 Discussion

In Ethiopia, Visceral leishmaniasis is an endemic disease of increasing public health concern. In areas where VL transmission is anthroponotic, asymptomatic persons might play a role as reservoirs, and even in areas where VL is zoonotic it is speculated that these persons could also contribute to transmission. Thus, it is important to determine the level of exposure and infection to Leishmania in VL endemic regions.

Leishmanin skin test is valuable tool in detecting exposure to Leishmania parasites in epidemiologic surveys, and its usefulness to detect asymptomatic infection has been shown by different authors in different disease-endemic areas(45). In this study, LST positivity has shown variation in different age groups, sex and also study sites. The highest positivity of LST was observed with in age group of 20-29 and 50-59 and also with in age group of 30-39. The least positivity rate was observed in the age group of less than 10 followed by age group of 10-19. The difference in LST positivity by age group was found statistically significant. The high proportion of positive LST in 20-29 and 50-59 years old is probably attributed to outdoor exposure to infective sand fly bites, associated with occupational activities specific to older children and adults. The low rate of skin test positivity in the under 10 year old indicates a decreased indoor exposure to infective sand fly bites, as most of the children spend the night indoor and some also use insecticide treated bed nets during sleeping hours. Some studies have shown that sleeping under insecticide treated bed nets potentially reduce VL incidence in Africa(46,47)

The high proportion of LST positivity observed in adulthood and in older age groups in this study and the relatively lower rate in those aged less than 10 is in line with data from other studies done in endemic localities of Humera and lower awash valley in Ethiopia and Baringo district of Kenya(28,37,43). LST positivity has also shown a marked difference in different study sites. The highest prevalence observed was in Wisnia followed by Ayro and Luka ; while no LST positive cases were found in Bashe, Seliya and Kuke. The difference in LST positivity by study sites (villages) was statistically significant. The difference observed in LST positivity in different study sites is probably attributed to micro-ecological differences such as altitude, soil type, presence and absence of termite mounds which serve as a resting place for sand fly vectors and in the type of vegetation that favor the breeding of insect vectors. All of the study sites with high LST positivity are found at altitudes less than 500m.The findings of this study are in

agreement with the study carried out in middle awash valley and also in the north west part of the country (37,40,41).

In this study, a slightly higher LST positivity was observed in females compared to the males even though this was not statistically significant. This finding is different from other studies carried out in Aba-Roba (Konso) by Hailu et al. (50), and in the middle awash valley by Ali et al (41) where LST positivity was found to be higher in the male population. Cultural differences might have played a role for this differences as females are highly involved in agricultural activities and also cattle herding which might make them vulnerable for infective sand fly bites, as one of the potential vector for the disease P. *orientalis* is presumed to stay close to cattle(49). Overall LST positivity observed in this study, 8.6%, is lower than those observed in lower awash valley, Afar region, and Aba-Roba plains (Konso). This could be due to micro-ecological differences; which are determining factors for infective insect vector breeding, life styles and wide use of insecticide treated bed net in our study area. Some studies have shown that insecticide treated bed nets which are used as malaria prevention strategy could help to prevent individuals from infective sand fly bites(11,47). Even though our study has shown lower LST positivity when compared with some studies carried out in many endemic areas of Ethiopia, the finding is comparable with the results of a study in Baringo district of Northern Kenya which had shown LST positivity of 10.5%(48).

The direct agglutination test (DAT) is a highly specific and sensitive test, which is simple to perform making it ideal for both field and laboratory use(21). WHO recommends DAT and LST as a valuable tool for visceral leishmaniasis in epidemiologic survey(23). In this study among those found DAT positive, two of them (one male and the other female) had a previous history of treatment. It was previously shown that DAT may detect antibodies in VL patients many years after treatment(23,51).

An increased number of DAT positivity which is observed in adult population, even though it is low compared with previous studies done elsewhere and Omo plains, is a typical characteristic of VL endemic area as children with immature immunity are more vulnerable to this deadly disease(23). Our finding of increased prevalence of DAT positive cases in those age groups (less

than 10 and 10-19) is in agreement with studies done in Aba Roba – Konso and also in eastern Sudan near Ethiopian border where almost greater than 60% of cases were aged less than 14 years(46,50,52).

Variations in the prevalence of DAT positivity were noted among different study sites. The highest positivity was observed in Ayro followed by Wisnia and Argude. Differences in altitude, soil type, vegetation and the presence and absence of termite mounds in each of the surveyed villages, and also the presence of large agricultural activity which is observed in Wisina and Ayro villages might have contributed to the increased DAT positive cases in the villages. Three of the villages with high DAT prevalence are found 400-600m above sea level, which is a favourable altitude for the infective sand fly vector breeding(53). People living in those high DAT prevalence villages have agricultural farms alongside Woito River and stay most of their time working on the farms which might increase encounters with infective sandfly bites. Even though DAT showed considerable variation among the study sites, the overall prevalence of 1.8% in this survey is lower than previous rates reported elsewhere and in Omo plains(42,54). This could probably due to the passive VL surveillance system which is implemented by AMH-LRTC and LDR-AAU in collaboration with DNDi, as most of the infected individuals which serve as a source for anthroponotic transmission of the disease have got the chance of effective treatment thereby increasing/expanding the herd immunity.

# CHAPTER 7 Conclusion and Recommendations

## 7.1 Conclusion

In this study, it is observed that Hamer and Benna Tsemai districts are remote areas in the southern part of the country with low health service coverage and poor living condition which makes them vulnerable to this deadly disease. Further, the present study also showed that asymptomatic infections of VL are quite common, and could possibly contribute to the anthroponotic transmission of the disease. This study also shows that transmission is taking place in and around their villages. The regional health bureau should take note of the potential risks of VL transmission in the region. The need to put in place sound visceral leishmaniasis prevention and control strategy is a matter of urgency. It is noteworthy that a continuing influx of migratory workers is increasing the population density in the areas around the huge sugar plantation schemes in Omo plains. Thus an increasing incidence of VL can be anticipated.

## 7.2 Recommendations

Visceral leishmaniasis seems to affect children in lower Omo plains; and thus a special attention should be given in both in treatment and prevention to young children.

One of the principal study sites, Cherkeka which is represented by Wisina and Ayro villages, appears to be a focus of VL transmission due to the fact that most of the asymptomatic as well as the symptomatic cases were found in the households of these communities. Thus it is recommended that an exhaustive survey which includes both entomological and epidemiological investigation should be done.

Even though there are some studies which generally incriminate *P. orientalis* as the potential candidate insect vector for the disease transmission in Omo plains, we recommend an entomological survey in the study sites and also an effort should be made to identify the reservoir hosts.

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## Annexes

### Annex I Questionnaire (Visceral Leishmaniasis Survey Format)

1. Identification

Date: -------- ---------- Code No: ----------

Name of patient: ----------Sex: ---------Age: ---------- woreda: ----

----------kebele: ----------village……..

Birth place: ----------Nationality/Ethnic group: ----------

Family size: ---------- Occupation: ----------

Marital status: Single----------Married----------Widow----------

Address: ---------- Duration of stay (at residence):----------

Previous place of residence (If new settler):----------

History of travel for the last 3 years:

Place: ----------Time/year/month: ----------

Place: ----------Time/year/month: ----------

Place: ----------Time/year/month: ----------

2. Present illness

| Sr.no | Clinical observations | Y/N | From Date | To Date |
|---|---|---|---|---|
| 01 | Fever | | | |
| 02 | Diarrhea | | | |
| 03 | Weight loss | | | |
| 04 | Abdominal swelling | | | |
| 05 | Pallor | | | |
| 06 | Splenomegaly | | | |
| 07 | Hepatomegaly | | | |
| 08 | Lymphadenopathy | | | |
| 09 | Wasting | | | |
| 10 | PKDL (if any) | | | |
| 11 | Other symptoms(cough, loss of appetite, joint pains ,headache) | | | |

**Y=yes**

**N=no**

**Annex I I Result Recording Format**

1. Serological Examination

DAT Positive---------- Titer----------

Negative---------- Titer----------

2. Leishmanin skin test

LST after 48/72 hours (*L. infantum*) ----------mm

3. Hematological examination

WBC X $10^3$----------

Hgb/Hct----------

PLT x$10^3$----------

RBC x$10^6$----------

5. Parasitological examination

BF (Hemoparasite) ----------

Other (specify) ----------

## Annex III Consent Form (English)

A study of visceral leishmaniasis in Hmer and Benna -Tsemai districts in lower omo valley (southern Ethiopia):

We came from Jimma University College of public health and medical sciences .The aim of the study is to know Burden of infection in the community so that it will help to implement a sound control Program. Visceral leishmaniasis is transmitted by insect vector and it affects all the population. The examination includes skin (LST) and laboratory tests. The standard diagnosis will be made by taking 2-5ml of blood and 0.1ml of the antigen will be injected intradermally in the volar surface of forearm by syringe and sterile needle. The diagnosis procedures we are going to perform aren't experimental but they are the routine procedures for this disease. At the end of the study a report will be prepared and it will be confidential. The report will not mention your identification like name and your address.

If you well understand the above in formations, we invite you to participate in the study.

I the under signed, will like to confirm that, as I give consent to participate in the study, it is with clear understanding and recognition of:

1. The objective of the study
2. The examination and treatment included in the study
3. My right to resign from the study during any stage of the study

I confirmed my agreement with my signature after the detailed objective of the study has been explained to me in the language I understand well.

Signature---------- Signature----------

(Participant's/patients/Guardian) (Investigator)

Date---------- Date----------

**Annex IV Consent Form (Amharic version)**

**<u>የፈቃደኝነት መጠየቂያ ቅፅ</u>**

**እኛ የመጣነው ከጅማ ዩኒቨርሲቲ ሲሆን የመጣንበትም ዓላማ በካላዘር በሽታ ላይ ጥናት ለማካሄድ ነው፡፡ በሽታው የሚተላለፈው በትንኝ ሲሆን ማንኛውንም ሰው እድሜና ፆታ ሳይለይ ያጠቃል፡፡ የጥናቱ ዓላማ በሽታው ምንያክል በዚህ ህብረተሰብ ውስጥ እንደተሰራጨ ማወቅ ሲሆን በተጨማሪም በበሽታው ለተጠቁ ሰዎች አጠቃላይ ምርመራ እና ህክምና ያለክፍያ በነፃ ይደረጋል፡፡ ምርመራውን ለማካሄድ ከ2-5ml ደም ከእርስዎ የምንወስድ ሲሆን በእጅዎ ቆዳ ላይም በበሽታው ከዚህ በፊት ተጠቅተው መሆኑ ለማወቅ የሚረዳ ምርመራም ይካሄዳል፡፡ በእርሶ ላይ የሚደረገው ምርመራ ከዚህ በፊት በሽታውን በተመለከተ ሲደረግ የነበረ ምርመራ ሲሆን አዲስ የምርመራ አይነት አለመሆኑን እናረጋግጥሎታለን፡፡ የምርመራውን ውጤት የማወቅ መብት ያሎት ሲሆን የእርሶን ማንነት የሚገልፅ ስምም ሆነ አድራሻ የማንጠቀም መሆኑን እንገልፅሎታለን፡፡**

**የተሳታፊው ፍቃድ መግለጫ**

**ግልጽ ስለሆነልኝ ለመሳተፍ ተስማምቻለሁ**

**ስም**

_____

**ፊርማ**

_____

**ቀን**

_____

**ምርምሩን የሚያካሂደው ባለሞያ**

**ስም**

_____

**ፊርማ**

_____

**ቀን**

_____

**Cell -0911768691**

## Annex V Subject Information Sheet (English)

First of all we want to thank you for giving your time to undergo this conversation. Having saying this we will give you enough information about the study that we are going to do and please listen with full attention. Finally if there is any question/ ambiguity you can ask and get information.

### Aim of the Study

The study is on the disease called visceral leishmaniasis (kalazar) that can infect humans, with no restriction on age sex and ethnicity/race. The aim of this study is to determine the burden of the infection in this locality.

### Subject's role

If they are voluntary to participate in this study, study participants required to give blood that is used only to check whether they are infected with the parasite or not. And also they will give other socio demographic information that is related with the study.

### Subject's right

Study participants have the right to know their result, and if they are not interested in the study they can out from the study at any time.

### Subject's benefit

Participating in this study doesn't give any other unique benefit for study subjects. But those

Subjects who have positive result will be treated without any cost.

### Harm

Study participants do not get any harm by participating in this study. There may feel some Discomfort during blood collection but this does not produce series pain.

### Confidentiality

All the data obtained from the participants including blood results will be kept strictly Confidential by using only code numbers.

## Annex VI Subject Information sheet (Amharic)

**ለተሣታፊዎች የሚሰጥ መረጃ**

በቅድሚያ ጊዜዎን መስዋዕት አድርገው ይህን ንግግር እንድናደርግ ስለፈቀዱልን በጣም እናመሠግናለን፡፡ ከዚህ በመቀጠል ስለጥናቱ መረጃ ስለምንሰጥዎ ልብ ብለው ያድምጡ፡፡

**<u>የጥናቱ ዓላማ</u>**

ጥናቱ የሚካሄደው በተለምዶ ከላዘር በሚባለው በሽታ ላይ ሲሆን ይህም በሽታ በዚህ አካባቢ ላይ ምን ያህል እንደተሠራጨ ለማወቅ ይረዳል፡፡

**<u>የተሣታፊዎች ድርሻ</u>**

ተሣታፊዎች በጥናቱ ለመሣተፍ ፈቃደኛ ከሆኑ ለጥናቱ ዓላማ የሚሆን ደም መሥጠት እና ከጥናቱ ጋር የተያያዙ አንዳንድ ሞያዊ ጥያቄዎች ሲቀርብላቸው መረጃ መስጠት ይጠበቅባቸዋል፡፡

**<u>የተሣታፊዎች መብት</u>**

ተሣታፊዎች ፍቃደኛ ካልሆኑ በጥናቱ ላይ ለመሣተፍ አይገደዱም እንዲሁም በጥናቱ ላይ የተሣተፈ ማንኛውም ሠው የምርመራውን ውጤት የማወቅ መብት አለው፡፡

**<u>ተሣታፊዎች የሚያገኙት ጥቅም</u>**

ተሣታፊዎች እዚህ ጥናት ውስጥ በመሣተፍባቸው የሚያገኙት የተለየ ጥቅም የለም፡፡ ነገር ግን በሽታው መጠቃታቸው በምርመራ ከታወቀ ያለምን ወጭ ህም ያገኛሉ፡፡

**<u>ተሣታፊዎች የሚደርስባቸው ጉዳት</u>**

ተሣታፊዎች እዚህ ጥናት ውስጥ በመካተታቸው የሚደርሥባቸው ጉዳት የለም፡፡ ነገር ግን ደም በሚሰጡበት ጊዜ አነስተኛ ህመም ሊሠማቸው ይችላል ይህ ማለት ግን ለጊዜው የሚሠማቸው እንጂ ምንም አይነት ጉዳት የሚያስከትልባቸው አይደለም፡፡

**<u>የውጤትና መረጃ አያያዝ</u>**

ከታሣታፊዎች የሚገኝ ማኛውም መረጃ የደም ውጤትን ጨምሮ በሚስጥር የሚያዝ ይሆናል፡፡ ይህም ተሣታፊዎች የሚለዩት በስማቸው ሣይሆን በመለያ ቁጥር በመሆኑ ጥናቱን ከሚያካሄደው ሰው ውጪ ሌላ ሠው የማወቅ ዕድል አይኖረውም፡፡

## Annex VI Laboratory Procedures

### A. Direct Agglutination Test (DAT)

Blood can be collected from the finger to blot on to filter paper (Whatman No.3) or from the vein to obtain serum or plasma. Serial dilutions of the patient's blood sample eluted from filter paper, or directly from serum, are incubated with Leishmania antigen in V-shaped microtitre plates. The plates are incubated at room temperature for 8-12 hours and then read visually. If no anti-Leishmania antibodies are present, the antigen will sediment to the bottom of the well and form a small sharp blue dot. If anti-Leishmania antibodies are present in the blood, they will react with the antigen and the agglutination will be visible as a blue mat, a dot with frayed edges, or an enlarged dot.

1, Materials

Freeze dried Leishmania antigen (FD), Phosphate Buffered Saline (PBS) pH 7.2 or normal saline, Gelatin powder, 2-mercaptoethanol (2-ME), Micro-titre plates V-shaped with self adhesive sealers or covers

1µl pipette, 50µl pipette, 50µl multi-channel pipette, 100µl multi-channel pipette, Disposable tips for pipettes, Negative and positive serum samples

2, Serum diluent

Normal saline solution or PBS containing 0.2% gelatin

Sodium nitrite could be added to the buffer as a preservative (Optional)

Prepare fresh serum diluents every time the test is done and add 2-ME to make a 0.78%

NB. Take care when handling 2-mercaptoethanol as it is a toxic substance

## Test Procedure

1. Dispense 50µl of SD into all the wells except column 2 where you dispense 100µl of sample diluents.

2. Add 1µl of either test or control serum samples into column 2

*Serum dilutions*

1. Using a multi-channel pipette mix column 2 thoroughly and transfer 50µl from column 2 to column 3, mix thoroughly and transfer 50µl from column 3 to column 4, repeat this until column12 (giving a dilution of 1:102,400) and discard the last 50µl Column 2 is used as the FD antigen control since it contains only the antigen and the sample diluents. Any auto-agglutination of the antigen will be seen in this row.

2. Add 50µl of antigen suspension in each and every well of the plate using a multi-channel dispenser pipette

3. Cover the plates and rotate the plate gently by hand for one minute by swirling.

4. Leave the plates on an equal level surface and let incubate overnight at room temperature

*Reading the plates*

1. Put the plates on a white background and determine the titre by comparing the agglutination button to the control in column 1

2. The titer is the highest dilution of the serum sample that gives a positive reaction and it is usually a well before that which is identical to the antigen control.

NB. Once reconstituted, the antigen should be stored at $4^0$ C for not more than 1 week

Serial two fold dilutions (starting 1:100) from the serum samples are made in a V-shaped micro titer plate. The antigen is added and the plates are read the next day. Agglutination is visible as a blue/purple mat in the wells of the micro titer plate. Serum is diluted from row A to row G;Row H is used as negative control.

*Interpretation of DAT Results*

DAT positive patients (patients with titers of 1:12,800 and above) are sent for admission and further parasitological investigations carried out

DAT negative patients (patients with titers below 1:12,800) are evaluated for other diseases and told to return for repeat DAT after 2 - 3 weeks if they still feel sick. By then their titer might have risen if they have VL.

DAT borderline patients (patients with titers of 1:6400 and 1:12,800) are either evaluated for other diseases or told to return for repeat DAT after 1 - 2 weeks, or they are admitted for parasitological investigations.