

**ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES**

**Visceral leishmaniasis in Bira Abo, a kebele in Addis Zemen:
Sero-epidemiological and Leishmanin Skin Test Survey**

**By
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*A thesis submitted to the School of Graduate Studies of Addis Ababa
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Advisor: Prof. Asrat Hailu,DMIP,AAU

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List of Abbreviations

MCL	Mucocutaneous Leishmaniasis
VL	Visceral leishmaniasis
CL	Cutaneous leishmaniasis
PKDL	Post Kala-azar Dermal Leishmaniasis'
GPI	Glycosylphos-phatidylinositol
PPG	Proteophosphoglycans
GIPLs	Glycosylinositol phospolipids
DAT	Direct Agglutination Test
LST	Leishmanin Skin Test
CSA	Crude soluble antigen
rK39	Immunochromato-graphic strip test
ELISA	Enzyme Linked Immunosorbent Assay
SDS-PAGE	Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis
FBS	Fetal bovine serum

Abstract

Visceral leishmaniasis is a serious public health problem of global importance, with a total of 200 million people at risk, an estimated 500 000 symptomatic new cases each year worldwide. The aim of this study was to determine the prevalence and exposure of Visceral leishmaniasis in Bira Abo kebele, one of the localities in Addis Zemen (Northern Ethiopia) in June 2006. Sero-epidemiological and Leishmanin skin test survey of VL was carried out. A total of 1280 subjects comprising 709 males and 571 females and 30 previously treated VL patients were included in the study. After clinical screening, 1280 sera were tested by Direct Agglutination Test (DAT) to determine prevalence of leishmanial antibody. The rate varied from 2.9% to 14.1% among the different localities. The overall DAT positivity was 8.4%. Leishmanin Skin Test (LST) was used to determine exposure, with rate varied from 6.2% to 28.6% and with 12.3% over all prevalence. The difference in leishmanin positivity by study sites and sero prevalence by sex were all statistically significant ($\chi^2=67.59$; $P < 0.01$ and $\chi^2 = 14.76$; $P < 0.05$ respectively). Out of sero positive individuals 7 were young children (<5 years), who had no history of travel out of Bira Abo, suggesting that transmission occurred in the study site. Hence, there is a need to implement a sound control program.

positive for *M. bovis*. The results indicated that *M. tuberculosis* is the causative species for tuberculous lymphadenitis in Dera.

1 INTRODUCTION

1.1 Overview of Global epidemiology

The leishmaniasis are a group of diseases with a broad range of clinical manifestations caused by several species of parasites belonging to the genus *Leishmania*. There are three clinical forms of Leishmaniasis: visceral Leishmaniasis (VL), cutaneous leishmaniasis, mucocutaneous leishmaniasis (MCL), disseminated cutaneous leishmaniasis (DCL) and Post Kala-Azar Dermal leishmaniasis (PKDL) (WHO, 1998).

Leishmaniasis affects more than 12 million people worldwide and is found in 88 countries (22 in the New World and 66 in the Old World). More than 90 percent of the cutaneous cases occur in Afghanistan, Saudi Arabia, Algeria, Brazil, Iran, Iraq, Syria and Sudan. Mucocutaneous form is mostly found in Latin America. Approximately 350 million people live in the area of active parasite transmission (WHO, 2000).

Visceral leishmaniasis also known as Kala-azar is a human systemic disease caused by parasitic protozoan species of the genus *Leishmania*. The etiological agents belong to the *Leishmania donovani* complex, *L.d donovani*, *L.d infantum* and *L.d archibaldi* in the old world and *L.d chagasi* in the new world. The old world species are transmitted by the sandfly vector *Phlebotomus sp.* Humans, wild animals and domestic dogs are known to act as reservoir hosts, the parasite enters macrophages, where it multiplies and establishes the infection (WHO, 1990; Handman *et al.*, 2000).

Visceral leishmaniasis is prevalent throughout the tropical and sub-tropical regions of Africa, Asia, the Mediterranean, Southern Europe (old world) and South and Central America (new world). It is endemic in 62 countries, with a total of 200 million people at risk, an estimated 500 000 symptomatic new cases each year worldwide, and 41 000 recorded deaths in the year 2000 (WHO, 2000). Over 90% of cases of visceral leishmaniasis occur in five countries: India (especially the Ganges and Brahmaputra plains), Bangladesh, Nepal, Sudan, and northeastern Brazil (WHO, 2005).

With the emergence of the HIV/AIDS epidemic in the early 1980s, the disease spectrum caused by the Leishmania parasites has changed significantly (Herwaldt *et al.*, 1999; Gillis *et al.*, 1995; WHO, 2000, Sulahian *et al.*, 1997).

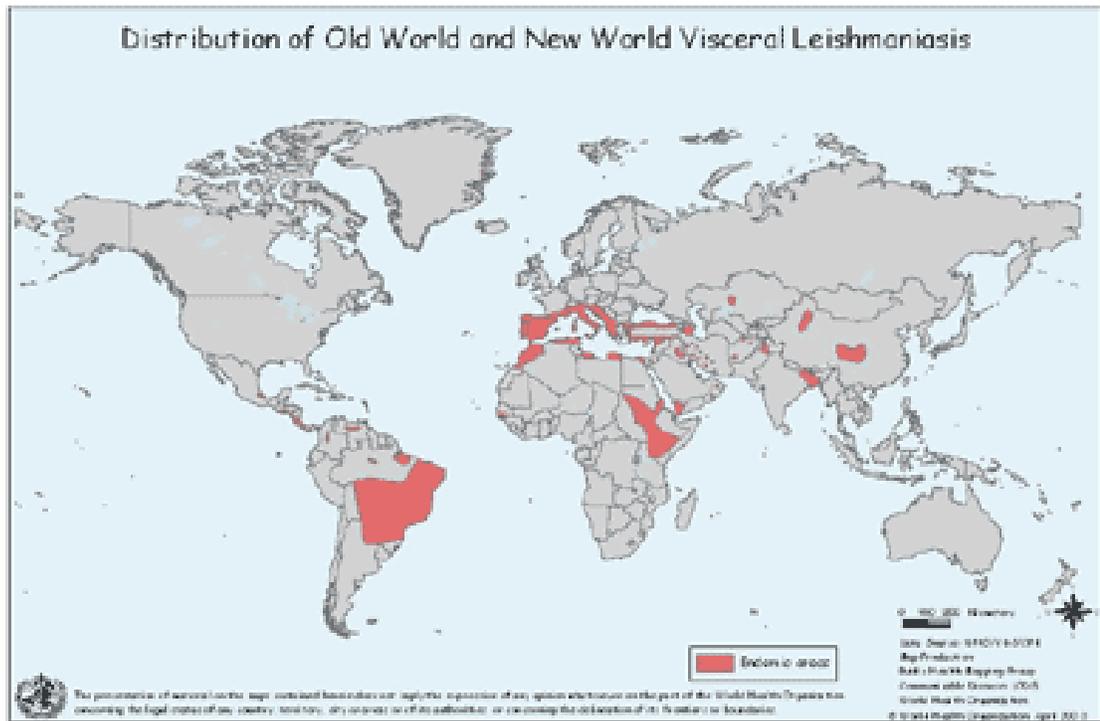


Figure 1. Distribution of old world and new world leishmaniasis

The World Health Organization (WHO) estimates that 25%-70% of adult VL cases in southern Europe are related to HIV, while between 2% and 9% of AIDS patients are at risk of experiencing newly acquired or reactivated VL (Alvar *et al.*, 1994). WHO estimates that AIDS increases the risk of VL by 100-1000 times in endemic regions (Sulahian *et al.*, 1997). Symptomatic visceral leishmaniasis is commonly fatal if untreated (WHO, 1998).

Visceral leishmaniasis reportedly occurs in 62 countries, more than half of the recent cases have been in Sudan and India. The epidemic in southern Sudan reportedly began in 1984 in the Western Upper Nile province but was first recognized by medical personnel in 1988. It has occurred in an area that was not previously considered a focus for visceral leishmaniasis; the area's infrastructure and civilians have been severely affected by the civil war that erupted again in 1983. Medecins Sans Frontieres-Holland estimates that the excess mortality

attributable to visceral leishmaniasis in Western Upper Nile is about 100,000 deaths among about 300,000 persons at risk for this syndrome (Perea *et al.*, 1991).

On the other hand, visceral leishmaniasis (VL) was observed in children in Bakool region, Somalia, an area where VL has not been reported before. Marlet *et al.*(2003) describe the extent of the problem in this war- and famine-stricken area. A retrospective analysis was done of all cases admitted to a VL treatment centre between July 2000 and August 2001. Patients with longstanding fever, splenomegaly and a positive direct agglutination test were treated as suspected VL cases. In 1 year, 230 serologically-positive cases were diagnosed as VL. Parasitological confirmation was attempted and obtained in 2 cases. In addition, in a serological survey of 161 healthy displaced persons, 15% were positive by the leishmanin skin test and 3 (2%) were positive by the DAT. Food insecurity might have contributed to the emergence and detection of VL in this area(Marlet *et al.*,2003).

Even though first case of VL documented in 1942,the outbreak that occurred in Addis Zemen and its surrounding localities such as Bira Abo was the first of its kind in Ethiopia.

1.2 Epidemiology in Ethiopia

Visceral Leishmaniasis, which is fatal in untreated patients, has been a cause of major epidemic which killed thousands of people. Kala-azar, caused by *Leishmania donovani*, is known in Ethiopia from the work of Cole *et al.* (1942) who recorded cases near Lake Rudolph on the Kenya border, and Tekle *et al.* (1970) who diagnosed the disease in migrant workers on the agricultural schemes at Metema and Humera, in the lowlands on the Sudan border. This area also adjoins the vast VL endemic area of the Sudan, where the VL epidemic claimed the lives of many thousands (Seaman *et al.*, 1996).Based on clinical, serological and leishmanin skin test survey conducted in June 1995, overall proportion of leishmanin skin test positives in the different settlements in Humera varied from 12.5% to 28.5%, with an average estimate of 20%, which was characterized with male preponderance and a gradual increase in age (Berhe *et al.* Unpublished).The proportion of leishmanin skin test positives among farm laborers who immigrated from the Sudan was 63.2%(Berhe *et al.* Unpublished).In Humera lowlands, leishmanin skin test rates of up to 43% have been reported among 530 farm laborers (Fuller *et al.*, 1976).

VL is distributed throughout the lowlands of Ethiopia with varying degree of endemicity. Important endemic foci include the Genale focus at Lake Abaya, the Segen Valley in Konso Woreda, the Omo river plains and the Metema and Humera plains in Northwest Ethiopia (Hailu *et al.*, 1993)

According to a survey made by Fuller *et al.* (1979), the distribution of positive leishmanin skin test was found to be greatest. Over 60% of the positive cases were from the southern parts of the Omo River. However 20% were from the Southern parts of the former Illubabur and Kefa administrative regions. Farther to the north-west, and among the people inhabiting the area between the Omo River and Chew Bahir, the skin test positivity was 20%-30%. They also diagnosed five parasitologically proven cases of kala-azar; two of them came from Konso, near the Segen River.

Ali *et al.* (2002) screened for overt cases of Leishmaniasis in the middle course of the Awash Valley. A cross-sectional leishmanin skin test survey was undertaken in 926 individuals. The overall prevalence of leishmanin positivity among 889 individuals was about 40% of the male and about 25% of the females. Positivity appeared to be increased with age in both sexes.

In a study conducted in Aba Roba, a VL endemic focus in Konso Wereda, the authors reported on the leishmanin skin test pattern. Some 50% of the people above 5 years of age were positive and the annual incidence of skin test transformation in the same age group was almost 15% (Ali and Ashford, 1993).

A repeated serological study of VL was carried out in a cohort of people in Aba Roba by Ali and Ashford (1994). They tested the people 3 times, at 6-month interval. 609 people were examined in round 1, and 667 in each of the 6-monthly follow-ups. Sero positivity increased

with age and males had higher rates than females. 68 individuals (11.2%) were sero-positive in round 1 and 42(6.3%) and 45 (6.7%) in two follow-ups.

On their extensive survey of visceral Leishmaniasis from July 1989 to June 1992, Hailu *et al.* (1996) carried sero-epidemiological and leishmanin skin test in eight localities of South and South west Ethiopia. A total number of 4870 subjects comprising semi-pastoral nomads, peasants and farm laborers were included in the study. Area of high and low LST positivity were identified with rates varying from 1.0%-80.5%. Using ELISA sero-prevalence were determined, and the rate varied from 1.8% to 27.8%.

In a study in Belessa, east of Gondar in the Northern Highlands, the authors found six cases of VL. But it was confirmed that only 2 who didn't have any history of travel had acquired their infection in Belessa. The report showed for the first time in Ethiopia, the possibility that Viscera Leishmaniasis might intrude into the highlands (Ashford *et al.*, 1973).

The lowlands of Metema and Setit Humera are also endemic for visceral Leishmaniasis. The first VL patient with post kala-azar dermal Leishmaniasis (PKDL) and HIV co-infection was reported from the work of Hailu *et al.* (2003). The first seven cases of HIV and Leishmania co-infection to be described in Africa occurred in Ethiopia (Berhe *et al.*, 1995). The incidence of visceral leishmaniasis (VL) in Ethiopia has dramatically increased over the last 10 years, coinciding with the advent of HIV epidemic (Hailu *et al.*, 2002).

In Ethiopia the incriminated vectors of leishmaniasis, from which parasites have been detected and/or isolated and identified so far, include *P. (Synphlebotomus) martini*, *P. (Sy.) celiae* and *P. (Larrousius) orientalis* for *L. donovani* from foci in the south and southwest (Hailu *et al.*, 1995; Gebre-Michael and Lane, 1996).

1.3 The genus Leishmania

The genus *Leishmania* belongs to the *Trypanosomatidae* family of the order *Kinetoplastidae*, which consists of a set of organisms characterized by the presence of the kinetoplast. The genus *Leishmania* consists of digenetic parasites (they cycle between a mammalian host and an insect vector) and is divided into two subgenera, *Leishmania* and *Viannia*, on the basis of

the localization of the parasite during its development within the alimentary tract of the infected sandfly (Lainson *et al.*, 1987). Parasites of the subgenus *Leishmania* are restricted to America, Asia, Europe and Africa, whereas parasites of subgenus *Viannia* are distributed in the American tropics and sub-tropics (WHO, 1990).

1.4 Lifecycle

The transmission of the parasite *Leishmania* is caused by the bite of an infected female sandfly. They are digenetic parasites: they need to pass through two hosts (i) invertebrate (sandfly) and (ii) vertebrate (mammals, including humans). *Leishmania* parasites can take two morphological appearances, promastigote (long flagellated parasite) and amastigote (round, intracellular parasite, without free flagellum) (WHO, 1990).

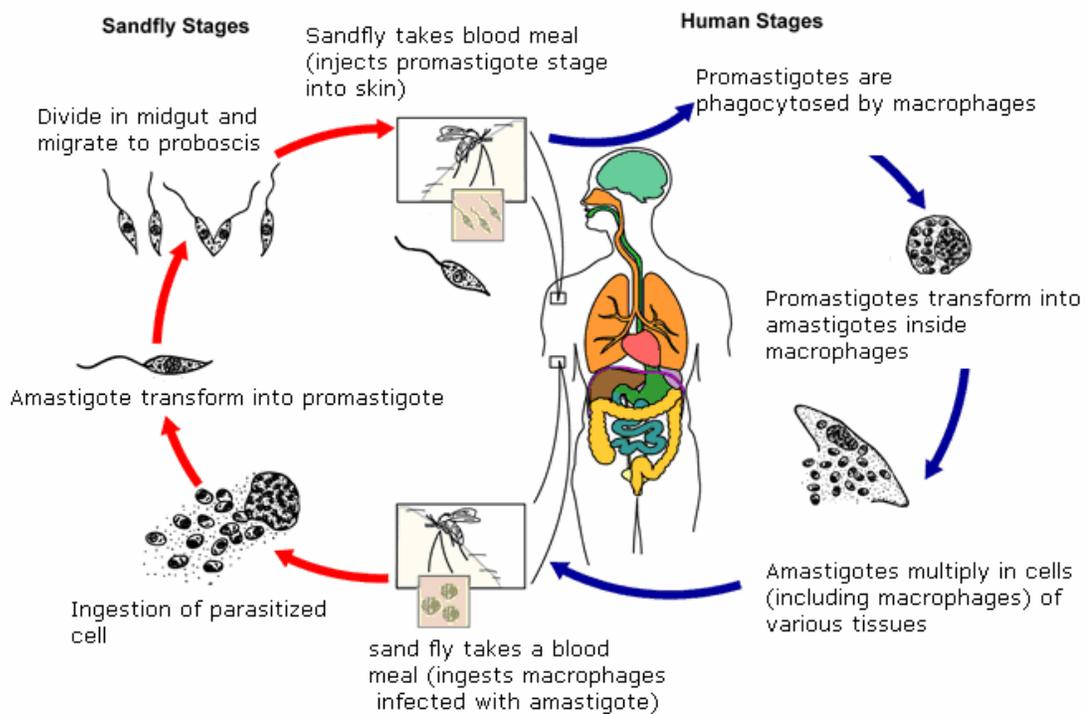


Figure1. Life cycle of VL
1.4.1 Invertebrate (sandfly)

When a female sandfly takes a blood meal from a *Leishmania*-infected mammal, amastigotes are ingested by the insect, they will have to survive, multiply and transform. They are ingested as amastigotes and driven into the abdominal midgut of the fly where they are captured together with the blood meal in the peritrophic membrane which is secreted by gut cells. Within the sandfly, amastigotes undergo several divisions and progressively change to long slender nectomonads. This stage of promastigote development is one of replication and is referred to as the procyclic stage. Before the blood meal is completely digested, the anterior part of the peritrophic membrane disintegrates and exiting parasites migrate toward the anterior part of the midgut and the cardiac valve. In the peritrophic membrane the *gp63* protease clears the haemoglobin around the parasites, and subsequently the chitinolytic enzymes of the promastigotes lyse the chitin framework of the membrane. This function of *gp63* is important because haemoglobin is known to inhibit the secretion of chitinolytic enzymes (Sacks *et al.*, 1989). Non-infective promastigotes, which are continuously dividing, attach to the microvilli of the thoracic midgut through lipophosphoglycan (LPG) and transform into infective promastigotes with shorter body and longer flagellum. Increasing numbers of slender, non-replicating, rapidly moving promastigotes can be observed in the lumen of the anterior midgut and foregut. It is this highly infective promastigote form which will be introduced into the mammalian host (Kelleher *et al.*, 1995).

1.4.2 Vertebrate (mammals, including humans)

Parasites will be injected into the mammalian host. In the vertebrate host, parasites are quickly taken up by tissue phagocytes, monocytes and neutrophils, attracted to the biting site due to the damage caused by the sandfly. Within the macrophage, the parasite loses its flagellum and transforms into a non-motile amastigote form. The amastigote survives and replicates in the very acidic environment of the phagolysosome. Infected cells can be lysed by the multiplying amastigotes, which will be freed to infect nearby cells. The parasites spread to the organs of the mononuclear phagocytic system, giving rise to VL. When a sandfly takes a blood meal from an infected host, it acquires either free amastigotes, or amastigote-infected macrophages and the life cycle can start again (Wilson *et al.*, 1987).

1.5 VL: clinical signs and symptoms

In endemic areas of VL, the disease is chronic and onset is gradual. Although people of all ages are susceptible, children below the age of 15 are more commonly affected. In sporadic and epidemic cases of VL, the disease is usually acute and symptoms appear suddenly with people of all ages being at risk except those who have conferred immunity due to a past infection. The symptoms of VL may also vary between individuals and according to geographical foci. However, some of the common symptoms include high undulating fever often with two or even three peaks in 24 hours and drenching sweats, which can easily be misdiagnosed as malaria, chills, rigors, weight loss, fatigue, poor appetite, cough, burning feet, insomnia, abdominal pain, joint pain, anorexia, epistaxis and diarrhea (WHO, 1998).

Clinical signs include splenomegaly, hepatomegaly and lymphadenopathy. The incubation period is highly variable. The disease can appear anything between ten days to over one year (Evans *et al.*, 1993). Even longer incubation periods have been documented (WHO, 1996).

1.6 Leishmania /HIV co-infection

Leishmania/HIV co-infection is emerging as an extremely serious, new disease and it is increasingly frequent. Although people are often bitten by sandflies infected with *Leishmania* protozoa, most do not develop the disease. However, among persons who are immunosuppressed (e.g. as a result of advanced HIV infections, immunosuppressive treatment for organ transplants, hematological malignancy, auto-immune diseases), cases quickly evolve to a full clinical presentation of severe leishmaniasis. AIDS and VL are locked in a vicious circle of mutual reinforcement. On the one hand, VL quickly accelerates the onset of AIDS (with opportunistic diseases such as tuberculosis or pneumonia) and shortens the life expectancy of HIV-infected people. On the other hand, HIV spurs the spread of VL. AIDS increases the risk of VL by 100-1000 times in endemic areas (Sulahan *et al.*, 1997). These two diseases produces cumulative deficiency of the immune response since *Leishmania* parasites and HIV destroy the same cells, exponentially increasing disease severity and consequences. VL is considered a major contributor to a fatal outcome in co-infected patients. Leishmaniasis can be transmitted directly person to person through the sharing of needles, as is often the case among intravenous drug users. This group is the main population at risk for co-infection. (WHO, 2004)

1.7 PKDL

After recovery, the patients may develop a sequel to the infection (chronic CL form) called 'post kala-azar dermal leishmaniasis' (PKDL). PKDL develops as a result of an influx of immunocompetent cells into the skin, which harbors parasite. In India, the PKDL resembles lepromatous leprosy with verrucous, papilomatous, xanthomatous and gigantic nodular forms. 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 (El-Hassan *et al.*, 1992).

1.8 Pathogenesis (parasite invasion mechanism)

The protozoan parasite *Leishmania* as an obligate intracellular parasite resides within an acidified phagolysosome of the vertebrate host macrophages. Disease manifestation of the *Leishmania* infection requires mechanisms, which allow the parasite to get into the macrophage, replicate and resist at least innate and acquired defense. *Leishmania* promastigotes bind to some surface molecules like CR1 (major) and CR3 and C3b of macrophage before they are internalized. Additional macrophage ligand for promastigote implicated includes mannose fructose receptor and parasite surface glycoprotein (Guy *et al.*, 1993). The internalization pathway of amastigotes is poorly defined. There are strong possibilities that *Leishmania* might bind to Toll-Like Receptors (TLR) on macrophages and dendritic cells even if the mechanism is not known (Hawasthi *et al.*, 2004).

After vertebrate infection, infective metacyclic *Leishmania* must resist the action of complement, resist host defenses such as oxidants and hydrolytic enzymes, inhibit macrophage activation, and differentiate to the amastigote stage, which is adapted for long-term survival and replication within the acidified phagolysosome (Spath *et al.*, 2003).

The *Leishmania* promastigotes are covered with dense surface glycocalyx that is composed of several molecules attached by glycosylphosphatidylinositol (GPI) anchor like surface protease gp63, proteophosphoglycans (PPGs), glycosylinositol phospholipids (GIPLs) and phosphoglycan called LPG (Beverley *et al.*, 1998). All these molecules appear to play certain roles in *Leishmania* infection of macrophages. They are referred as invasive/ evasive determinants as they help to successfully establish intracellular parasitism following sequential events: A) Evasion of humoral lytic factors B) Attachment of parasites to macrophages followed by their intracellular entry into these phagocytes c) Intracellular survival of the endocytosed parasites D) Promastigote-to-amastigote differentiation, and E) Replication of the amastigote. The development of leishmaniasis is thus dependent upon the spreading of amastigote to infect additional cells (Chang *et al.*, 1990).

Even though much remains to be elucidated the ectometalloprotease gp63 is known to help promastigotes enter host cell and for survival both before host cell infection (evading humoral lytic factors) and for intraphagolysosomal survival (degrading immunoglobulins, complement factors, and lysosomal proteins) (McGwire and Chang, 1994). The LPG was also implicated to be important for establishment of host cell infection and survival in insect vector and inside host cells. Resistance to complement lysis, transient inhibition of phagolysosomal fusion, and resistance to oxidant could implicate these roles of LPG (Beverley *et al.*, 1998). Other biochemical processes important for parasite survival in the highly acidic and hydrolytic environment of the phagolysosome include the activation of a heat shock response (Wiesgigi and Clos, 2001) and the stage-specific expression of specific nutrient transporters. Furthermore a study on *L. mexicana* provided strong evidence that mannan accumulation is important for parasite differentiation and survival in macrophages (Ralton *et al.*, 2003).

1.9 Immune response to VL

Immunity against *Leishmania* is cell-mediated. The parasites escape the humoral immune response of hosts by residing in the phagolysosomes of macrophages. Therefore, antibodies

have no effect on the infection and may even be detrimental to the host. Macrophages employ a number of defense strategies against the infecting parasites. Phagocytosis of a foreign body by the macrophage results in an oxidative burst. NAD(P)H oxidase in the plasma membrane is activated, transferring protons to molecular oxygen and forming highly reactive superoxide, hydrogen peroxide, and hydroxyl radicals at the site of phagocytosis, which interact with pathogen phospholipid membranes . Another macrophage defense mechanism is the acidification of the vesicle formed by the fusion of the phagosome and endosome by a proton ATPase. The acidic environment promotes protein denaturation, which leaves the protein, as well as DNA, RNA, and carbohydrates, susceptible to degradation by acid hydrolases (Reiner, 1994).

T helper cells also play a role in the immune response. The expansion of TH 1 clones protects during infection, while TH 2 cell expansion exacerbates the disease. IL-12 production by dendritic cells and macrophages causes naive T cells to differentiate into TH 1 cells and induces the production of IFN- γ by T cells and natural killer (NK) cells. IFN- γ conjunction with TNF- α , produced by the infected macrophages, activates the inducible nitric oxide synthetase (iNOS) gene, resulting in the production of nitric oxide (NO), which is toxic to the parasite . While TH 1 cell expansion is occurring, TH 2 cell expansion must be kept in check. IL-4 regulates TH 2 cell differentiation, which confers susceptibility to *Leishmania* by down regulating IL- 12, IFN- γ production, and IL-12 receptor expression and inhibiting macrophage NO production (Kane and Mosser, 2000).

1.10 Diagnosis of Visceral Leishmaniasis

1.10.1 Microscopic examination

The commonly used method for diagnosing VL has been the demonstration of parasites in splenic, bone marrow, lymph nodes, liver aspirate and the buffy coat of peripheral blood. The sensitivity of the bone marrow smear is about 60 to 85%. Splenic aspirate is one of the most valuable and preferable method with a sensitivity exceeding 95%. The risk of splenic puncture is bleeding from a soft and enlarged spleen. To avoid the risk of excessive blood

loss, splenic puncture should be avoided in patients with a platelet count of less than 40,000 platelets/ μ l and a prothrombin time of more than 5 seconds (Hockmeyer *et al.*, 1981).

Culture of parasite can improve the sensitivity of detection of parasite, but leishmania culture is rarely needed in routine clinical practice. Leishmania strains can be maintained as promastigotes in artificial culture medium. The culture media used may be monophasic (Schneider's insect medium, M199, or Grace's medium) or diphasic (Novy-McNeal Nicolle medium and To-bies medium) (Sundar *et al.*, 2001).

1.10.2 Animal Inoculation

The parasite can also be demonstrated after inoculation of laboratory animals (such as hamsters, mice or guinea pigs) with infected specimen. Animal inoculation is not usually employed as a diagnostic test, since several months may be required to obtain a positive result. Golden hamster is the animal of choice for maintaining *L. donovani* complex. It can be infected via many routes, including across mucous membranes, but intraperitoneal and intrasplenic routes are preferred. After inoculation, the animal is examined weekly for signs of infection, such as, hepatosplenomegaly. Amastigotes can be harvested by biopsy from the spleen and the liver of an animal (Marsden, 1986).

1.10.3 DNA detection method (polymerase chain reaction)

The development of PCR has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis. In recent years, PCR-based diagnostic methods with a wide range of sensitivities and specificities have been described. In a study reported from India, in which a species-specific primer for *L. donovani* (LDI primer) was used, the sensitivity of PCR with whole blood from VL patients was 96% and *Leishmania* DNA was detected in skin specimens from 45 of 48 patients with PKDL (sensitivity, 93.8%) (Piarroux *et al.*, 1994).

1.10.4 Immunodiagnosis

1.10.4.1 Antigen detection

A new latex agglutination test (KATEX) for detecting leishmanial antigen in urine of patients with VL has showed sensitivities between 68 and 100% and a specificity of 100% in preliminary trials. The antigen is detected quite early during the infection and the results of animal experiments suggest that the amount of detectable antigen tends to decline rapidly following chemotherapy. The test performed better than any of the serological tests when compared to microscopy. Large field trials are under way to evaluate its utility for the diagnosis and prognosis of VL (Attar *et al.*, 2001).

1.10.4.1 Antibody detection

1.10.4.1.1 Napier's formol gel

The formol gel test has the advantage of being cheap and simple to perform. Serum is mixed with one drop of 30% formaldehyde. A positive reaction is shown if the mixture solidifies and forms a white opaque precipitate within 20 minutes. A positive test can not be detected until 3 months after infection and becomes negative 6 months after cure. The test is non-specific since it is based on detecting raised levels of IgG and IgM which also result from other infections such as African trypanosomiasis, malaria and schistosomiasis (Bray, 1976).

1.10.4.1.2 DAT

In 1988, a modified DAT was reported to be useful in kala-azar and is being used in several countries of endemicity. In this test the antigen preparation consists of whole organisms and a serological response (mainly IgG) recognizes surface-born antigens of the parasite. Use of a 0.8% of 0.1 M 2-mercaptoethanol in the sample diluent further improves its performance. DAT in various studies has shown to be 91 to 100% sensitive and 72 to 100% specific. It is easy to perform, cost effective and adapted for field condition. Thus one of the most widely used an immunological test that has been applied in diagnostic and epidemiological studies (Harith *et al.*, 1988; Boelaert *et al.*, 1999).

In contrast to the observations made in Europe, Hailu *et al* confirmed that DAT in Ethiopia remain reasonably sensitive in the diagnosis of VL during HIV co-infection (Hailu and Berhe, 2002).

Direct Agglutination Test (DAT) has proven to be a very important sero-diagnostic tool, combining high level of intrinsic validity and ease of performance. The assessment of the diagnostic potential of DAT showed that the test has 91 to 100% sensitive to pick up VL cases. Thus on the ground of its simplicity, accuracy and safety the technique is recommended as a vital screening tool as an alternative field diagnosis method for Visceral Leishmaniasis in Ethiopia (Hailu et al., 2002).

1.10.4.1.3 ELISA

ELISA is highly sensitive but its specificity depends upon the antigen used. Several antigens have been tried. The commonly used antigen is a crude soluble antigen (CSA). The sensitivity of ELISA using these concentrations of CSA is reported to range from 80 to 100%, but cross-reactions with sera from patients with trypanosomiasis, tuberculosis, and toxoplasmosis have been recorded. On the other hand, when various selective antigenic masses (116 kDa, 72 kDa, and 66 kDa) were used, a specificity of 100% could be achieved, but only at the cost of sensitivity, which went down to as low as 37.5% (Vinayak *et al.*, 1994).

1.10.4.1.4 Immunochromato-graphic strip test (rK39)

A promising ready-to-use immunochromato-graphic strip test based on rK39 antigen has been developed as a rapid test for use in difficult field conditions. In the first extensive field trial in 323 patients, they found the strip test to be 100% sensitive and 98% specific. Anti-rK39 IgG may be present in serum for an extended period after successful treatment for VL; thus, patients with suspected relapse of VL with a past history of infection would not be candidates for diagnosis by strip testing. It cross-react with malaria, typhoid fever, or tuberculosis. Notwithstanding these limitations, the rK39 immunochromatographic strip test has proved to be versatile in predicting acute infection, and it is the only available format for diagnosis of VL with acceptable sensitivity and specificity levels which is also inexpensive, simple and easy to perform (Sundar *et al.*, 1998).

1.10.4.1.5 Western blotting

For western blotting, proteins are originally separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitro-cellulose or nylon membrane. If there are antibodies present in the patient sample, they react with the components of the membrane and the antibody-antigen complex can be detected as in ELISA.

Using Western blotting, one can find even minor antigenic differences among various organisms and thus detect cross-reactive antigens. However, the process is time consuming, technically cumbersome, and expensive (Singh, 1996).

1.10.5 Leishmanin (Montenegro) test

Leishmanin is a killed suspension of whole ($0.5-1 \times 10^8$ /ml) or disrupted ($250 \mu\text{g}$ protein/ml) promastigotes in pyrogen-free phenol saline. Leishmanin Skin Test is usually used as an indicator of the prevalence of cutaneous and mucocutaneous Leishmaniasis in human and animal populations and also in protectively successful cure of visceral leishmaniasis. During active kala-azar disease there will be no or negligible cell mediated immune response. 0.1 ml solution is inoculated into the volar surface of the forearm. The area is measured 48-72 h later. The test is negative in acute cases of VL due to the absence of DTH and is positive only in cases where kala-azar has been cured. Overall it is a good test for epidemiological surveys of a population to identify groups at risk of infection (Palma and Gutierrez, 1991; Weigle *et al.*, 1987; Singh, 1999).

1.11 Treatment of VL

Treatment of VL mainly relies on the pentavalent antimonials sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime), the first -line drugs except when resistance exists, and the usual dose is 20 mg/kg/day for 30 days. They are expensive and need to be given by injection. The second-line drugs in case of resistance -amphotericin B and pentamidine, used in cases unresponsive to antimonials, need careful management to avoid serious side effects. They are used intravenously over several hours on alternate days from 0.1 mg/kg/day up to 1 mg/kg/day with a maximum total dose of 3g. For VL, aminosidine, alone or in association with pentavalent antimonials, has shown good efficacy but it is still under evaluation. Amphotericin B, included in liposomes, has proven to be very efficient but its use is still limited and expensive (Desjeux, 1996 ;HO, 1998).

1.12 Control of Leishmaniase s

Control measures of leishmaniasis are complicated by variety of different *Leishmania* species, diverse clinical manifestation and the unique epidemiologic pattern by each parasite species. Current control measures are environmental-oriented (vector control, reservoir control and epidemiologic surveillance) and also based on chemotherapy (use of leishmanicidal drugs). These are meant for prevention and containment of the disease. Problems associated with the methods are (i) the fact that vector control utilizing large scale insecticide spraying in developing countries is costly and not feasible; (ii) *Leishmania* spp. readily acquires resistance to antimonial drugs (which despite their toxic properties remain the treatment of choice); (iii) response to treatment varies considerably depending on the parasite species involved (*L. donovani* vs. *L. aethiopica*) and the clinical form or stage of the disease (Girmaldi and Tesh,1993). In spite of these there is a consensus that in longer term, vaccines ought to become a major tool in the control of the disease. Furthermore, determination of appropriate reservoir hosts, if any, and mode of transmission, development of appropriate diagnostic methods and designing a policy with respect to the prevention and control of the drug resistance together with formulation of effective drugs are very important to control leishmaniasis (Handman, 2001).

1.13 Risk Factors for Leishmaniasis

Increasing risk factors are making leishmaniasis a growing public health concern for many countries around the world. Risk factors associated with visceral leishmaniasis include urbanization, deforestation, new settlements, migration, cross-border movement, domestication of the transmission cycle, and agricultural development. Other than the factors mentioned above, factors like AIDS and other immunosuppressive conditions, malnutrition, socioeconomic and environmental factors are assumed to be most important risk factors to visceral leishmaniasis (Desjeux ,2001).

1.14 Statement of the problem

Leishmaniasis forced itself upon medical attention as an increasingly significant problem over the last decade. Because of its importance, Leishmaniasis is considered as one of the 6

diseases selected by WHO. It ranks only second to malaria among human protozoan diseases. Symptomatic visceral leishmaniasis is commonly fatal if untreated (WHO, 1990).

For many years, the public health impact of visceral leishmaniasis has been grossly underestimated, mainly due to lack of awareness of the serious impact of health over the last many years. Endemic regions have been spreading further and there has been a sharp increase in the number of cases of the disease, hence linking visceral leishmaniasis to poverty, economic development and various environmental changes such as deforestation, urbanization, migration of people into endemic areas, HIV etc.

VL often exists in areas that are remote, health facilities are absent or undeveloped, tools for screening and identification of patients are inadequate, and above all with no or few trained man power. Due to lack of up-to-date information even most critical cases are neither treated nor reported, so they act as a reservoir of infection (mainly in areas where transmission is man to man), passing on the parasite to family and neighbors through the bite of sand fly.

In Ethiopia the first case of VL was documented in 1942 in the southern part of the country (Cole and Gosgrove, 1942). Since then the disease has spread to become endemic in the Segen, Woito and Gelana river valley.

Libo Kemkem district, where the first VL outbreak occurred, is found in NW Ethiopia. The District has never been endemic to VL until the recent epidemic reported in April 2005. The outbreak claimed a large number of lives before its cause was identified. The outbreak started at least as early as 2004, but was initially misdiagnosed as malaria. Bira Abo is a Kebele in Libo Kemkem which was attacked by VL.

The outbreak seems to suggest the existence of the spectrum of the disease and thousands of residents are currently at risk of acquiring the disease. Since the true burden was not known in the study localities, describing of the burden of disease and infection in place and time was necessary.

Even though VL had remained endemic in Ethiopia since at least 1942, its magnitude in Bira Abo, one of the surrounding localities in Addis Zemen was not well known.

In this survey, we studied leishmanin skin test ,serological screening of the population and determined the prevalence of VL, so that it will have paramount importance in designing a sound VL control strategy, which will help to prevent epidemics from continuing.

2 OBJECTIVES

2.1 General Objective

- To determine the prevalence and exposure of Visceral leishmaniasis in Bira Abo kebele, one of the localities in Addis Zemen.

2.2 Specific Objective

- To determine the sero- prevalence of Visceral leishmaniasis.
- To determine the level of exposure to Leishmania.
- To determine the prevalence of Visceral leishmaniasis
- To determine the presence of Intestinal Parasites in DAT positive cases.
- To determine the presence of anemia and leucopenia in DAT positive cases.

3 MATERIALS AND METHODS

3.1 Study design

Cross-sectional surveys using leishmanin skin test (LST) and Direct Agglutination Test (DAT) were performed involving all residents of Bira Abo Kebele. This survey was carried in June, 2006.

3.2 study area and Population

Libo Kemkem district which is found South Gonder Zone (NW Ethiopia), has a total population of 196,813 and 32 *kebeles*. The capital of the district is Addis Zemen Town which is located along the major route of Sudan-Metema-Gondar-Bahir Dar-Addis Ababa. The health infrastructure in this district includes 1 HC, 10 HP and a few private drug vendors. The specific study site, Bira Abo, is a kebele with six villages (“Gots”) including Abo Bahir, Walka Mender, Gedm, Ambye, Aba Foye and Sinko Bahir.

3.2 Study Subjects

It is community-based study which included 1280 residents of Bira Abo. 1280 participants above the age of 2 years were enrolled in the study and benefited from both the diagnosis and the treatment.

3.3 Study Subjects

1280 sample size was required in the study. This ample size was calculated using the WHO recommended statistical formula for health studies. (Lwanga et al.,1991).

$$n = Z^2 P (1-P) / d^2 , \text{where}$$

n= number of study subjects enrolled in the study

Z=test statistic which allows us to calculate our result with 95% confidence (1.96)

d = the level of precision (5%) expressed as a fraction of 1 so that the sample can estimate the proportion to within 5% of the points (0.0274)

P=proportion (prevalence) to be used on estimates which was expressed in decimal (We used maximum prevalence=50%)

3.4 Questionnaire

Information about socio-demographic profile (sex, age, address, occupation etc.) and present complaints (fever, wasting, weight loss etc) was collected using questionnaires. The questionnaires were filled with physician and an experienced nurse trained in VL by MSF Holland.

3.5 Sample collection and processing

From all study subjects venous blood was collected using 5ml sterile disposable syringe and needle. The blood sample was divided into two, one part with anticoagulant which was intended for hematologic examination and the other for serological test (DAT). The blood for DAT was allowed to stand for 30 minutes and sera were separated. Stools were also collected from DAT positives. All tests were performed on the same day of their collection.

3.6 Laboratory (Diagnostic) Techniques

3.6.1 Leishmanin Skin Test (LST)

Leishmania infantum derived LST antigen was obtained from Istituto Suoeriore di Sanita (Rome, Italy). 0.1ml of the antigen was injected intradermally in the volar surface of the forearm and 0.1ml of control reagent was injected at 10cm from the test area using 1.0ml sterile disposable syringe and needle (0.50mm × 16mm). Induration sizes were determined 72 hours after administration, using the ball point. Results were recorded as an average of two dimensional readings. Indurations size of 5.0mm and above were considered positive, as done elsewhere (Manson-Bahr, 1987).

3.6.2 Direct Agglutination Test (DAT)

The DAT was performed as described by Harith and others (Harith et al., 1988). Briefly, 50 µL of two-fold serially diluted serum samples in physiologic saline (0.15 M NaCl) containing 1% heat-inactivated fetal bovine serum (FBS) was applied to each well of a V-shaped microtiter plate. The initial serum dilution was 1:200. The DAT antigen (50 µL) was then

added and after two minutes of gentle shaking on a level surface, the plate was covered with a lid and was left overnight at room temperature. The control wells contained physiologic saline plus 1% heat-inactivated FBS and DAT antigen. The results were judged against a white background. The end point titer was determined in reference to a clear sharp-edged blue spot which was observed in the control wells. Sample with a titer $\geq 1:3,200$ was considered positive.

3.6.3 Hematological examinations and stool examinations (Hemoglobin determination and white blood cell)

Blood film and direct stool examination, Hemoglobin determination, WBC count, blood film and direct stool examination were performed for all DAT positive cases.

3.7 Data processing and Statistical analysis

All data were entered, cleaned and analyzed using EPI Info (CDC/Atlanta, WHO/Geneva, 1991) ver 6.04 and SPSS 11.01(SPSS Inc., 1996). χ^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.

3.8 Ethical Consideration

This community-based study was carried out after ethical clearance is obtained from ethical committee of Addis Ababa University and Amhara regional governmental Health bureau. Written consents were obtained from all household members and guardians of children. Clinically suspected VL with DAT positives were sent to Gondar Hospital for further diagnosis and treatment.

4 RESULT

4.1 Characteristics of the study population

The socio-demographic Characteristics of the population are given in the table 1 and figure 3. A total of 1280 individuals of which 709(55.4%) males and 571 (44.6%) females, greater than 2 years of age were identified from 6 representative sites for physical examination, leishmanin skin test and serology (DAT).The male to female ratio was 1.2:1.Most of the study subjects (30.3%) came from Gedm followed by 22.8% from Walka Mender and the least (3.8%) came from Ambye (Table 1). In addition, the majority (12.4 %) of the study subjects were in 5-9 years old, followed by 12 % in 15-19 years but the least (6.5 %) was in 10-14 years old(Figure 3).

Table 1. The distribution of sex in different study sites (villages) in Bira Abo in June 2006.

Sex	Study villages						Total
	Abo Bahir	Walka Mender	Gedm	Ambye	Aba Foye	Sinko Bahir	
Male n (%)	134(55.6)	153 (52.4)	208 (53.6)	16(44.4)	166 (60.4)	32 (66.7)	709 (55.4)
Female n (%)	107(44.4)	139 (47.6)	180 (46.4)	20(55.6)	109 (39.6)	16 (33.3)	571 (44.6)
Total	241	292	388	36	275	48	1280

n=Total number of study subjects.

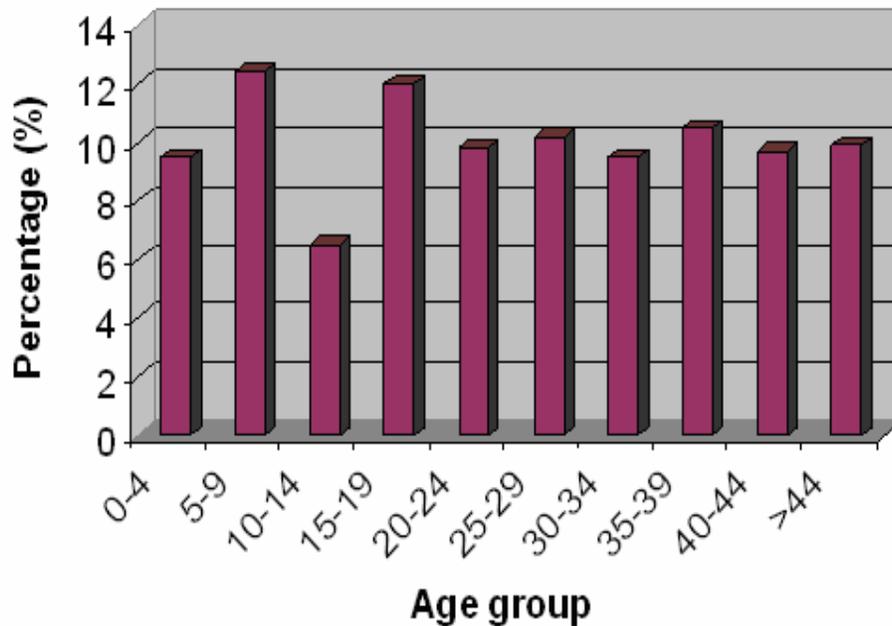


Figure 3. Percentage distribution of study subjects in different age group in Bira Abo in June 2006.

4.2 Leishmanin skin test (LST)

Leishmanin skin test was performed in 1023 study subjects, of which 925 returned for reading of the test. Induration size of >5mm was recorded as positive. Leishmanin skin test varied from 28.6% in the age group 25-29 to 6.2% in the age group 5-9. Leishmanin skin test positivity increased steadily to 25-29 years old (28.6%) and immediately falls to 11.5% in the age group 30-34% but again it increases. Variation in the prevalence of leishmanin positivity were noted among the different study sites. Sinko Bahir had the highest prevalence (25 %), followed by Abo Bahir (19.8%), while the least prevalence was in Aba Foye (8.5%). The difference in leishmanin positivity by study sites (village) was statistically significant ($\chi^2 = 67.59$; $P < 0.0001$) (Table 2)

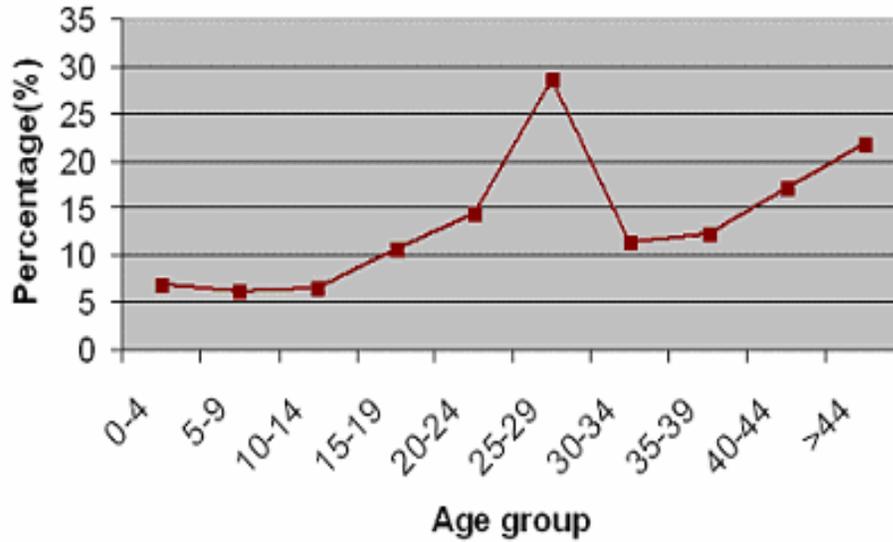


Figure 4. Percentage distribution of Leishmanin skin test positivity in different age groups in Bira Abo in June 2006.

Table 2. Percentage distribution of Leishmanin skin test positivity in different study sites in in Bira Abo in June 2006.

Village	No tested	Positive	%
Abo Bahir	172	34	19.8
Walka Mender	223	36	16.1
Gedm	301	29	9.6
Ambye	22	4	18.1
Aba Foye	176	15	8.5
Sinko Bahir	32	8	25.0

4.3 Direct Agglutination Test (DAT)

4.3.1 DAT in different age, study sites and occupation

The highest percentage (16.0%) of DAT positive was in the age group 25-29, followed by the age group 20-24. But the least percentage (5.8%) was noted in less than 4 years old (Figure 5). Variation in the prevalence of DAT positivity were noted among the different study sites. Abo Bahir had the highest prevalence (14.1%), followed by Ambye (11.1 %), while the least prevalence was in Aba Foye (2.9 %). The difference in DAT by study sites (village) was statistically significant ($\chi^2 = 67.59$; $P < 0.05$). The overall DAT positive was 8.4% (Table 3).

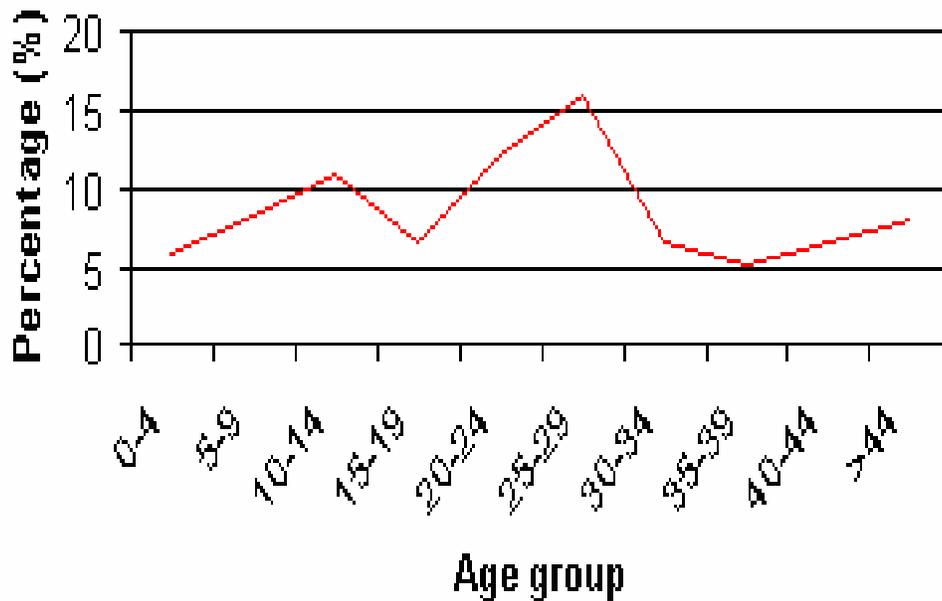


Figure 5..Percentage distribution of Direct Agglutination Test (DAT) in different age groups in Bira Abo in June 2006.

Table 3. Percentage distribution of direct agglutination test (DAT) in different study sites (villages) in Bira Abo in June 2006.

Village	Number tested	Positive	%
Abo Bahir	241	34	14.1
Walka Mender	292	30	10.3
Gedm	388	29	7.5
Ambye	36	4	11.1
Aba Foye	275	8	2.9
Sinko Bahir	48	3	6.3
Total	1280	108	8.4

Table 4. Percentage distribution of DATpositives in different occupations and in different study sites in Bira Abo in 2006.

Age group	Farmer n (%)	Student n (%)	Pre-school n (%)	Total n (%)
Abo Bahir	20(34.5)	3(12.5)	11(42.3)	34(31.5)
Walka Mender	16(27.6)	8(33.3)	6(23.1)	30(27.8)
Gedm	15(25.9)	9(37.5)	5(19.2)	29(26.9)
Ambye	1(1.7)	2(8.3)	1(3.8)	4(3.7)
Aba Foye	5(8.6)	1(4.2)	2(7.7)	8(7.4)

Sinko Bahir	1(1.7)	1(4.2)	1(3.8)	3(2.8)
<i>Total</i>	58(100)	24(100)	26(100)	108(100)

In term of occupation, farmers constituted above half 58 (53.7%) of the DAT positives. The percentages of DAT positive students and pre-school children were 22.2% (24) and 24.1(26) % respectively. It is apparent from Table 4.3.1-c that 34.5 % of the farmers were from Abo bahir, followed by 27.6% from Walka Mender but the least 1.7% both from Ambye and Sinko Bahir. The highest prevalence of DAT positive cases in students (37.5%) was from Gedm , but in pre-school children the highest percentage (42.3%) was from Abo Bahir.

4.3.2 DAT in treated and untreated study subjects

Among 108 DAT positive cases, 74 were males and 34 were females. The 24 DAT positive cases in males had previous history of past treatment , however 50 DAT positive cases in males were untreated. Among the DAT positive females 6 cases were only treated, but 28 cases were untreated. Among the treated males, the highest number (9) were from Abo Bahir but 1 was from Ambye. Seventeen treated males were from Gedm but no untreated male cases was from Sinko Bahir. In females 6 cases who had previous history of treatment were from Abo Bahir but no untreated cases came from Ambye and Sinko Bahir. The association of sex with sero-prevalence was statistically significant ($\chi^2 = 14.76$; $P < 0.05$) (Table 5).

Table 5. Percentage distribution of Direct Agglutination Test (DAT) in both sexes in treated and untreated study subjects in different villages in Bira Abo in 2006.

Address	Male				Female			
	Treated	%	Untreated	%	Treated	%	Untreated	%
Abo Bahir	9	37.5	15	30	1	16.7	9	32.1
Walka Mender	7	29.2	14	28	2	33.3	7	25
Gedm	2	8.3	17	34	2	33.3	8	28.6
Ambye	1	4.2	2	4	1	16.7	0	0
Aba Foye	2	8.3	2	4	0	0	4	14.3
<i>Sinko Bahir</i>	3	12.5	0	0	0	0	0	0
<i>Total</i>	24	100	50	100	6	100	28	100

n= Total number of study subjects for the different group.

Table 6. Percentage distribution (frequency) of DAT titer in treated VL cases in different month interval (less than 3 months, 3-6 months and above 6 months)

DAT titer	<3 month	3-6 months	> 6 months
	n (%)	n (%)	n (%)
Borderline positive	1(16.7)	3(15.8)	-
Low titer positive	2(33.3)	8(42.1)	2(40)
High titer positive	3(50)	8(42.1)	3(60)
Total	6	19	5

Borderline positive=1/800 and 1/1,600.

Low-titer positive=1/3,200 and 1/6,400.

High titer positive= \geq 12,800.

Including previously treated VL patients a total of 108 sera tested DAT positive .All the 108 sera were fully tittered Using DAT .The DAT titer of 108 positive sera were equal to or above DAT titer scale 4.Among the previously VL treated cases 18(60%) were LST positive. The highest percentage (63.3%) of previously treated VL cases was in between 3-6 months. In those treated before 3 months 50% had DAT high titer. But in those treated 3-6 months, both low titer positive and high titer positive cases had equal percentage (42.1%)

Table 7. Percentage distribution of symptomatic and asymptomatic study subjects in treated and untreated cases in Bira Abo in 2006.

DAT titer	Symptomatic	%	Asymptomatic	%
	n		n	
Previously treated	11	55	19	21.6
Untreated	9	45	69	78.4
<i>Total</i>	20	1.5	88	85.5

Symptomatic cases = all subjects with splenomegaly and fever of at least 2 weeks duration.

Asymptomatic cases = those with a single clinical sign (symptom) or without clinical sign and symptom.

Among the 20 symptomatic cases 9 had high titer and 9 had low titer. In case of asymptomatic cases 50 were border line positive and 35 cases had low titer positive.

4.4 LST and DAT

Comparing the prevalence of LST in 108 DAT positive cases, Leishmanin skin test positivity was highest in Ambye (100%) and Sinko in Bahir (100%). But taking in to consideration the large number of study subjects Walka Mender (79.2%) had the highest prevalence, followed by Abo Bahir (50%). The overall prevalence of LST in VL cases was 61.8 %.

Table 8. Percentage distribution of Leishmanin Skin Test (LST) in DAT positive study subjects in different study sites in Bira Abo in 2006.

Village	No tested	Positive	% Positive
Abo Bahir	26	13	50
Walka Mender	24	19	79.2
Gedm	26	12	46.2
Ambye	3	3	100
Aba Foye	7	5	71.4
Sinko Bahir	3	3	100
Total	89	55	61.8

Table 9. Percentage distribution of LST in treated and untreated study subjects among the DAT positive study subjects in Bira Abo in 2006.

Age group	Treated			Untreated		
	No tested	Positive	%	No tested	Positive	%
0-4	1	1	100	5	3	60
5-9	3	3	100	5	1	20
10-14	5	2	40	10	4	40

15-19	0	0	0	6	5	83.3
20-24	2	1	50	6	3	50
25-29	3	2	66.7	2	2	100
30-34	3	3	100	10	5	50
35-39	4	3	75	6	4	66.7
40-44	3	2	66.7	6	5	83.3
>44	0	0	0	9	6	66.7
Total	24	17	70.8	65	38	58.5

n= Total number of study subjects for the different group.

The highest percentage (16.7%) of LST positive was in 35-39 years old. In the treated study subjects LST positive was also observed in less than 5 (0-4) years old. In untreated study subjects the highest percentages (15.4%) of LST positive were in both 10-14 and 30-34 years old. In addition 3 cases in less than 5 years old among the untreated study subjects were LST positive. We also observed that there were cluster of both DAT and LST positive cases in the family. In one family there were 5 DAT positive cases. On the other hand in each of 8 families, there were 3 DAT positive cases. In other 8 families there were 2 DAT positive cases each. Concerning LST, 3 LST positive cases were found in each of the 4 families and 2 LST positive cases in each of 13 families.

4.5 Hemoglobin determination and WBC count

In the bellow table, 31.5% of DAT positives had WBC count above 4000.35.2% of DAT positive also had a WBC count between 2001-4000/mm² .Sever leucopenia of 2000/mm² or less was observed in 23.1% of DAT positive. There was large percentage (92 %) of anemic patients among the severe leucopenic cases. In general from 108 DAT positive cases 50% of the cases were also anemic.

Table 10. Percentage distribution (frequency) of Hemoglobin determination and WBC count in 108 DAT positive study subjects in Bira Abo in 2006.

Age group	WBC<2000		WBC		WBC>4000	
	Cell/m ³		2000-4000 Cell/m ³		Cell/m ³	
	Hb<11g/dl n (%)	Hb>11g/dl n (%)	Hb<11g/dl n (%)	Hb>11g/dl n (%)	Hb<11g/dl n (%)	Hb>11g/dl n (%)
Abo Bahir	11(32.4)	2(66.7)	3(20)	8(34.8)	2(40)	8(27.6)
Walka Mender	11(32.4)	-	2(13.3)	7(30.4)	1(20)	9(31)
Gedm	8(23.5)	-	7(46.7)	4(17.4)	-	10(34.5)
Ambye	1(2.9)	-	-	1(4.3)	1(20)	1(3.4)
Aba Foye	1(2.9)	1(33.3)	2(13.3)	3(13)	1(20)	1(3.4)
Sinko Bahir	2(5.9)	-	1(6.7)	-	-	-
Total	34	3	15	23	5	29

WBC=white blood cell in Cell/m³

Hb= hemoglobin expressed in g/dl.

Hb<11=anemic case.

4.6 Stool examination for intestinal parasites

Among the DAT positive cases, high percentage (36.7%) parasite positive cases were from Walka Mender, followed by Sinko Bahir (33.3%).The least percentage (13 %) was from Gedem. We found only 13 cases of *A.lumbricoids ova*, 8 cases of *E.histolytica* or *E.dispar trophozoits*, 4 cases of *Hookworm species ova* and 3 cases of *S.mansoni ova*. All stool examination positive cases were treated.

Table 11. Prevalence of intestinal parasites among DAT positive cases in Bira Abo in 2006.

Study sites	Number tested	Positive	% Positive
Abo Bahir	34	9	26.5
Walka Mender	30	11	36.7
Gedem	29	4	13.8

Ambye	4	1	25
Aba Foye	8	2	25
Sinko Bahir	3	1	33.3
Total	108	28	25.9

4.7 Frequency (Percentage) of clinical features, malaria parasites and VL cases

Based on physical examination and history of the study subjects, among the previously treated cases, 15 (50%) were febrile and 11(36.7%) had enlarged spleen.34.6% of untreated cases were febrile and 26.9% had splenomegaly. In those having previous history of treatment, 4 febrile cases were in 10-14 years old and 3 cases with enlarged spleen were in 25-29 years old. Six febrile untreated cases were in 0-4 years old and 4 with splenomegaly were in both 5-9 and 20-24 years old.

In those previously treated subjects we found 3 PKDL cases. We performed blood film examination for all DAT positive cases but only 3 were positive for *P.vivax trophozoite*.

After clinical screening, eleven DAT positive subjects with clinical signs and symptoms were selected and sent with one of their family to Gondar Hospital. From 3 DAT positive individuals parasite was demonstrated from spleen aspirate.

5 DISCUSSION

VL is known to prevail in undetermined magnitude in the various localities of Ethiopia. In the area where the epidemiology of VL has been soundly established, the disease is considered to be alarming, contributing to about a third of the crude mortality rate in the absence of provision for early diagnosis and treatment (Ali and Ashford, 1994).

A total of 1280 individuals of which 709 males and 571 females, greater than 2 years of age were identified from the 6 representative sites for physical examination and serology. But LST was performed on only 1023 individuals. The male to female ratio was 1.2:1.

Among the 1023 study subjects who received LST antigen, 505 were males and 385 were females. Of the 126 LST positives, 72.2% were males and 27.8% were females. Male was strongly associated with LST ($\chi^2 = 53.81$; $P < 0.05$). The variation in skin test reaction in sex implies difference in exposure of males to female due to occupation and that the access of women to health care has been limited due to cultural barriers. Some experimental murine models suggest that women are less likely to develop the clinical symptoms of VL than exposed men (Evans *et al.*, 1992). However this phenomenon remains unexplained. Serological and clinical data also suggest, however, that males were slightly more exposed than females, possibly due whilst working in the fields surrounding the villages. The low prevalence of Visceral Leishmaniasis in young children (less than 5 years) is probably due to a lower level of exposure to the parasite, which is consistent with the low reactivity of their sera to Leishmania antigen. Children are usually entirely covered by a blanket at night, which might prevent them from being bitten by infective sandflies. The male preponderance and LST positivity was also in agreement with the finding of Ali *et al.* (2002). In their study, the overall prevalence of leishmanin positivity was about 40% of the male and about 25% of the females tested positive for the antigen.

Leishmanin skin test varied 6.2% in the age group 5-9 to 28.6% in 25-29 years old. Leishmanin skin test positivity increased steadily to 25-29 years old (28.6%) and after

immediately falls to 11.5% in the age group 30-34%, again it increases steadily. The high proportion of positive LST in 25-29 years old, favored outdoor exposure to transmission, associated with activities specific to older children and adults. In addition, the existence of positive leishmanin reaction in children aged less than 5 was an indication of established focal transmission within the study sites (villages). In other study, in an extensive survey of visceral Leishmaniasis from July 1989 to June 1992 in eight localities of south and south west Ethiopia, Hailu *et al.* (1996) have reported LST rates varying from 1.0%-80.5%.

In our survey, variation in the prevalence of leishmanin positivity was noted among the different study sites. Abo Bahir had the highest prevalence (14.1%), followed by Ambye (11.1 %), while the least prevalence was in Aba Foye (2.9 %). The difference in leishmanin positivity by study sites (village) was statistically significant ($\chi^2 = 67.59$; $P < 0.05$). The wide variation in leishmanin response in the respective study sites might (could) be explained by the presence of black clay soil in which cracks during the dry seasons are thought to provide favorable breeding and resting microhabitat for the vector.

The marked ecological variation influencing presence of conducive conditions for potential vectors to perpetuate the transmission of Viscera Leishmaniasis.

The leishmanin skin test has been shown to be a valuable tool to measure exposure to leishmanial parasites. Although the role of non-pathogenic leishmania or cross-reaction of the antigen with related organisms was not known (Leeuwenburg *et al.*, 1981), the overall LST positivity in Bira Abo was 12.3%. It was very low, compared with other findings of Fuller *et al.* (1979). They reported skin test rate as high as 61%. It was also true with the finding of Hailu *et al.* (1996), who reported high LST rate. But on their former study in Humera lowlands, Fuller *et al.* (1976) have reported leishmanin skin test rates of up to 43% among 530 farm laborers.

On the contrary, the overall prevalence (12.3%) in this study was higher than the lowest reported community prevalence in VL endemic areas like 10% in Sardinia (Gramiccia *et al.*, 1990). Moreover, our finding was comparable with the work of Berhe *et al.* (Unpublished), Based on clinical, serological and leishmanin skin test survey conducted in

June 1995, they found overall proportion of leishmanin skin test positives in the different settlements in Humera varied from 12.5% to 28.5%, with an average estimate of 20%, which was characterized with male preponderance and a gradual increase in age.

The highest percentage (16.0%) of DAT positive was in the age group 25-29, followed by the age group 20-24(12%). But the least percentage (5.8%) was noted in less than 5(0-4) years old. In addition, all less than 5 years had no history of travel outside of Bira Abo. This also suggests that transmission occurred in the study sites, or this may indicate an anthroponotic or domestizoon transmission in the localities (Bira Abo).

We found 108 DAT positive cases which included 30 treated and 78 untreated cases. In our finding all the previously treated subjects were DAT positive, and only 6.2% of untreated subjects were DAT positive. It is obvious that DAT detects antibodies in VL patients many years after treatment (Hailu, 1990). DAT percentage varied from 2.9% to 14.1% among the different localities. The overall DAT positivity was 8.4%. The overall prevalence of serology (8.4%) in this study was comparable with the finding of Ali and Ashford (1994) which was carried out in Aba Roba. The authorS found that 68 individuals (11.2%) were sero-positive in round 1 and 42(6.3%) and 45 (6.7%) in two follow-ups. But it was lower than the work of Hailu *et al.* (1996). From a total number of 4870 subjects, sero prevalence rate varied from 1.8% to 27.8%.

In term of occupation, farmers constituted above half (53.7%) of the DAT positive subjects. The percentage of students and pre-school children were 22.2% and 24 % respectively. It is apparent from the table 4 that 34.5 % of the farmers were from Abo Bahir, followed by 27.6% from Walka Mender but the least (1.7%) were both from Ambye and Sinko Bahir. The highest prevalence of DAT positive cases among students (33.3%) were from both Walka mender and Gedm, but in pre-school children, the majority (42.3 %) were from Abo Bahir. Occupation wasn't statistically associated with DAT ($X^2=22.37$; $P=0.31$)

Comparing the prevalence of LST in 108 DAT positive cases, Leishmanin skin test positivity was highest in both Ambye (100%) and Sinko Bahir (100%). But taking into consideration the

large number of study subjects tested, Walka Mender (79.2%) had the highest prevalence, followed by Abo Bahir (50%).The prevalence of LST in VL cases was 61.8 %. The occurrence of LST positive in VL cases was in line with the previous information made by Badaro *et al.*(1986b).If positive leishmanin skin test indicates immunity against leishmania, a concomitant positive serology in leishmanin positivity might simply show re-exposure to infection in immunocompetent individuals (Manson-Bahr., 1961a).In another study ,Murray *et al.*(2001) indicated that previously infected persons (whether they were asymptomatic or have been treated) show skin-test reactivity and antigen-specific T-cell responses and appear to be resistant to reinfection.

From the total 108 DAT positive, 74 were males and 34 were females. Thirty (27.8%) DAT positive subjects had past history of kala-azar, of which 24 were males and 6 were females. On the contrary 78 subjects were untreated, which included 50 males and 28 females.

In those previously treated subjects, we found 3 PKDL cases. PKDL is a common complication following kala-azar (Visceral Leishmaniasis).PKDL, develops as a result of an influx of immunocompetent cells into the skin, which harbors parasite. In majority fosi PKDL heals spontaneously and in others symptoms are sever and persist for years so that they serve as source for VL transmission (El-Hassan *et al.*,1992).

Concerning hematological examinations, leucopenia and anemia were the most frequent laboratory findings. More than 31.5% of DAT positive cases had WBC count above 4000.35.2% of patients also had a WBC count 2001-4000/mm² .Severe leucopenia of 2000/mm² or less was observed in 23.1% of DAT positive subjects. There were large percentage (92 %) of anemic patients among the sever leucopenic cases. In general, from 108 DAT positive cases, 50% were suffering from anemia. Leucopenia of 2000 cells/mm² or less was observed in 22.2% of DAT positive cases.

It is to be noted that, only a fraction of subjects with Leishmania reactive antibody are affected by severe visceral leishmaniasis. So most of the infections remain entirely

asymptomatic or subclinical (Guerin et al., 2002). All study subjects with splenomegaly and fever of at least 2 weeks duration were considered as VL suspect. The sera of these subjects were tested with DAT. In our study 88 cases (81.5%) were asymptomatic. But the rest 20(18.5%) were symptomatic with splenomegaly and fever of at least 2 weeks duration. In Brazil the majority of patients who develop VL are malnourished children with mean age of 3 years (Badaró *et al.*, 1986b). Another study indicated that, in persons with asymptomatic intracellular infection, recrudescence can occur even decades later, especially if CD4+ T-cell-mediated immunity is disturbed. Reactivated (opportunistic) kala-azar is now well recognized in immunosuppressed patients and in those with advanced human immunodeficiency virus (HIV) disease (Murray *et al.*, 2002).

Since “sterile” immunity (complete eradication) probably seldom develops in persons with visceral infection, residual parasites, tucked away within macrophages but capable of reaching the circulation, probably persist for the lifetime of the host. Given the sheer size of the population of persons with asymptomatic visceral leishmaniasis and the fact that there is such a population in Bira Abo, these persons may well represent an important and unrecognized, substrate for the sandfly, making control of transmission all the more difficult (Costa *et al.*, 2002). Walsh *et al.* (1993) summarized the reason why the parasitic-driven process remain asymptomatic in certain subjects and cause a lethal disease in others. They suggested that environmental factors that affect the fly ecology and the presence of other animals permissive to the leishmania lifecycle, human activities that increase exposure to sandfly, poor economic condition, malnutrition and impaired reactivity of the immune system have been shown to increase the risk of Visceral Leishmaniasis

Eleven DAT positive subjects with clinical signs and symptoms were selected and sent with one of their family to Gondar Hospital. And 3 DAT positive individuals were treated after parasite was demonstrated from spleen aspirate.

We also observed that there were cluster of both DAT and LST positive cases in the family. In one family there were 5 DAT positive cases. On the other hand in each of 8 families, there were 3 DAT positive cases. In other 8 families there were 2 DAT positive cases each. Concerning LST, 3 LST positive cases were found in each of the 4 families and 2 LST

positive cases in each of 13 families. Though it is hard to tell the exact reason, this is possibly due to the evidence that host genetic background may be important in determining susceptibility or resistance to infection. A single gene (the Lsh gene, recently renamed Nramp-1 or natural resistance associated macrophage protein gene) was found to control this resistance in mice (Bradly *et al.*, 1979).

One of the differential diagnosis in Visceral Leishmaniasis endemic area is malaria. Although, almost all cases in Bira Abo had a history of malaria, only 3 were positive for *P.vivax* trophozoite. But we couldn't see an objective finding to suggest that VL cases run increased risk of malaria infection. However John *et al.* (1986) postulated a protective effect of splenomegaly due to chronic malaria against leishmanial infection.

In areas where VL is endemic most infected individuals remain asymptomatic and also there is a possibility of missing early cases so that there were many more cases and our finding represent the tip of the iceberg.

6 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In this study, after screening 1280 study subjects in Bira Abo the overall DAT positive rate was 8.4% and the overall LST positive rate was 12.3%.The number of people infected but asymptomatic is much higher than the number infected and presenting with clinical illness .Eleven DAT positive subjects with clinical signs and symptoms were selected and sent with one of their family to Gondar Hospital. But only 3 DAT positive individuals were treated after parasite was demonstrated from spleen aspirate.

We found 7 DAT positive young children (< 5 years) who had no history of travel out of Bira Abo. This suggests that transmission occur in the study site (Bira Abo).

With all the evidences observed in this study, we can definitely say that visceral leishmaniasis is a major health problem in Bira Abo. The existence of untreated cases of VL (DAT positive cases) and PKDL are considered to be reservoir and disseminate the causative parasite. Therefore ,the immediate establishment of sound control program integrated with other vector borne diseases is very crucial.

6.2 Recommendations

Based on the findings of this study, the following recommendations are forwarded:

1. In our study we confirmed the existence of visceral leishmaniasis in Bira Abo. But the vector(s) as well as the reservoir(s) host responsible for the transmission of VL are still unknown. So it is very important to conduct both vector(s) as well as reservoir(s) host studies.
2. DAT positive and PKDL cases serve as reservoir for VL transmission. Though treatment of PKDL cases need a very long time, cases must be detected and treated.
3. Taking the burden of VL into consideration, immediate interventions are needed. It is also advisable if it is integrated with other control programs aimed against vector-borne diseases.

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APPENDIX

Appendix I: Questionnaire (Visceral Leishmaniasis Survey Format) (Addis Zemen and its surrounding areas)

1. Identification

Date: -----

Code No: -----

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

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

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

Marital status: Single-----Married-----Widow/Widower-----

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)			
	Other symptoms(cough, loss of appetite, joint pains ,headache)			

3. Past Medical History

A) Past history of kala-azar? Yes-----No-----

If yes, date of diagnosis/treatment-----

B) Past history of kala-azar in the family? Yes-----No-----

If yes relation to the patient-----

C) Past history of malaria? Yes-----No-----

If yes, since when-----

D) Other past medical history-----

Appendix I I: Result recording format

1. Serological Examination

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

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

2. Leishmanin skin test

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

3. Hematological examinations

WBC x 10³-----

Hgb/Hct-----

PLT x10³-----

RBC x10⁶-----

4. Parasitological examination

BF (Hemoparasite) -----

Direct stool examination-----

Concentration-----

Appendix III Consent Form (English)

A study of visceral leishmaniasis in Addis Zemen and surrounding (Northern Ethiopia): Sero-epidemiological and leishmanin skin test survey.

We came from Addis Ababa University medical Faculty .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. We came to study about visceral leishmaniasis. 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 procedure we are going to perform and the drugs you are going to receive aren't experimental but they are the routine procedures for this disease.

If the diagnosis is confirmed, you will receive the necessary drug(s) to cure you from the 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 informations, 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-----

