INDIRECT HEMAGGLUTINATION ASSAY FOR DIAGNOSTIC AND EPIDEMIOLOGICAL STUDIES OF VISCERAL LEISHMANIASIS IN ETHIOPIA

A Thesis Submitted to Graduate Studies program Addis Ababa University in Partial Fulfillment of the Requirements for Masters Degree in Parasitology

By

Tadesse Kebede

June 2005

ADDIS ABABA UNIVERSITY
GRADUATE STUDIES PROGRAMME

INDIRECT HEMAGGLUTINATION ASSAY FOR DIAGNOSTIC AND EPIDEMIOLOGICAL STUDIES OF VISCERAL LEISHMANIASIS IN ETHIOPIA

By

Tadesse Kebede

ADVISOR: Hsrat Hailu (prof.)

June 2005

ADDIS ABABA UNIVERSITY
GRADUATE STUDIES PROGRAMME

INDIRECT HEMAGGLUTINATION ASSAY FOR DIAGNOSTIC AND EPIDEMIOLOGICAL STUDIES OF VISCERAL LEISHMANIASIS IN ETHIOPIA

By

Tadesse Kebede

Approved by the Board of Examiners Signature

_____ _____

Adviser:

1. _____ _____

Examiners:

1. External_____ _____

   Internal_____ _____

June 2005

AKNOWLEDGEMENTS

It is my great pleasure here to acknowledge Prof. Asrat Hailu, my adviser, for his close supervision, invaluable advice, very encouraging assistance and concern. Had it not been for his financial assistance, this work would have not been realized.

I would like to extend my gratitude to collaborators of the study Dr. Yibeltal Asefa and Dr. Yosef chane of Kahsay Abara hospital, Dr. Ermias Diro of Gonder Hospital, and Dr. Kusito Kursha Abaroba Health Center and Dr. Nega Berhe of Aklilu Lemma Institute of Pathobiology for their contribution during the sample collection.

I also like to thank Prof. Eyasu Makonnan for his cooperation for me to use the core laboratory. My thanks also go to W/o Woinshet Mekonen for her immense contribution during the collection of data. She had to stay away home on many week ends for getting things done in the lab. I wish to express my gratitude to W/t Bethelhem Tefera, Ato Tesfaye Getachew, W/o Bysassu Gebre Medhin, W/t Addis Yifru, Ato Nega Negussie, and Ato Mulugeta Gichle for their unreserved technical assistance. My thanks also goes to all the Aklilu Lemma IPB animal attendantes, Ato Girma Tadesse and Ato Teshome Aynalem, in particular, for keeping the experimental animals safe and for their cooperation during the collection of blood.

My appreciation goes to all my instructors, especially Prof. Beyene Petros and Dr. Mekuria Lakew, for helping me understand the basics of Immunology and parasitology during my study.

I would like to thank the Aklilu Lemma Institute of Pathobiology and the Core Laboratory of the Medical Faculty for the facilities I have been offered. I also gratefully acknowledge School of Graduate Studies for partly funding the research and The Department of Biology of AAU for letting me have the chance to conduct the study and facilitating my work.

I would like to extend my gratitude to family and friends for their moral support and assistance through out my study period.

Finally my special appreciation is also extended to my wife Elizabeth Teklu for her encouragement and support and my kids for sharing their time with me. Their patience has been tried while dad was not at home most of the times.

LIST OF ABBREVATIONS

AIDS	Acquired Immuno Deficiency Syndrome
BCG	Bacillus Calmette Guerin
CD	Cluster differentiation
CL	Cutaneous leishmaniasis
DAT	Direct Agglutination Test
DCL	Diffuse cutaneous leishmaniasis
DDT	Dichloro Diphenyl Trichloroethane
DNA	Deoxyribo nucleac acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme Linked Immunosorbent Assay
FAST	Fast Agglutination Screening Test
FCS	Fetal calf serum
FN	False negative
FP	False positive
HIV	Human Immunodeficiency Virus
IFAT	Indirect Immunofluorescent Antibody Test
IFN-γ	Interferon-γ
IgG	ImmunoglobulinG
IHA	Indirect hemagglutination Assay
IL	Interleukin
LCL	Localized cutaneous leishmaniasis
LST	Leishmanin skin test
MCL	Mucocutaneous leishmaniasis
NNN	Novy MacNeal Nicole
PBS	Physiological buffered saline
PCR	Polymerase Chain Reaction
PKDL	Post Kala-azar dermal leishmaniasis
PPV	Positive predictive value
NPV	Negative predictive value

rpm	Rotation per minute
RR	Relative Risk
SRBC	Sheep red blood cell
TB	Tuberculosis
Th	T helper cells
TN	True negative
TNF-α	Tumor necrosis factor-α
TP	True positive
TSS	Tropical splenomegaly syndrome
VL	Visceral Leishmaniasis
WB	Western blot
WHO	World Health Organization

TABLE OF CONTENTS PAGES

LIST OF TABLES PAGES

LIST OF FIGURES PAGES

Abstract

Visceral leishmaniasis (VL) is potentially a fatal disease that is caused by intracellular protozoan parasites of the *L. donovani spp*. To date, there has been no reliable diagnostic test for active infection of VL. Therefore, there is an urgent need for a simple, rapid, and reliable diagnostic test with high sensitivity and specificity for clinical practice and VL control. To determine the diagnostic and sero-epidemiological screening performance of Indirect Hemagglutination, serum samples were obtained from endemic areas of Ethiopia and evaluated using antigens prepared from an isolate of *L. donovani* (MHOM/SD/68/1S) promastigote stage. Serum samples of 77 suspected VL and 51 controls for diagnostic; and for epidemiological study 1217 samples involving 57 VL suspected, and 1085 samples including 132 samples from previously treated VL patients were tested by IHA at various cut-off titers, DAT, and FAST. Spleen and/or lymph node aspiration with subsequent smear and/or culture test confirmed the presence of parasite in 37 and 19 individuals in diagnostic and epidemiological study subjects, respectively. IHA test performed for comparison was able to detect anti-leishmanial antibodies in 94.6% and 100% of the confirmed cases of VL (at 1:64 cut-off titer) in diagnostic and epidemiological study subjects, respectively. Among 1085 sera, IHA (at 1:64 cut-off titer) test was positive in 10.6% of previously untreated asymptomatic individuals. Whereas out of 132 previously treated VL patients, 72% were found to be IHA positive at 1:64 dilutions. IHA at cut-off titers 1:64 and 1:128 showed a sensitivity of 94.5% and 86.5%, a specificity of 60% and 77.5% respectively in the diagnostic study and a sensitivity of 100% and 100%, specificity of 33.3% and 50% in the epidemiological study. The result showed IHA to be sensitive, rapid, and simple test for the

diagnosis and epidemiological screening of visceral leishmaniasis. Further evaluation of IHA test should be conducted with careful use of the gold standard.

Keywords: Indirect Hemagglutination test; Visceral Leishmaniasis; Ethiopia; seroepidemiology

MCL is mainly caused by the species of *L. b. braziliensis, L. b. panamensis, L. b. guyanensis* and *L. aethiopia* (Desjeux, 1996). In immunocompromised patients mucosal lesions caused by *L. d. donovani, L. major and L. d. infantum* have also been reported in the Old World (Desjeux, 1996).

Visceral leishmaniasis is the most severe form of leishmaniasis, resulting from multiplication of *Leishmania* amastigotes within the entire reticuloendothelial system causing visceralizing disease. The disease is mainly caused by *L. d. donovani*, *L. d. infantum*, and *L. d. chagasi* (Mauricio *et al*., 2000).

The World Health Organization considers leishmaniasis as one of the most important parasitic diseases (WHO, 1990). Currently, leishmaniasis affects some 12 million people, mostly children and young adults, in 88 countries in all continents except Australia (WHO, 1998), out of which 72 are developing countries, 13 of them being least developed (Desjeux, 1996). More than 350 million people are exposed to the infection (WHO, 1998) and the annual incidence is about 2 million cases (Enk *et al*., 2003).

World wide, vector borne transmission is the most common mode of transmission (Iqbal *et al*., 2002). Other modes of transmission i.e., parentral, congenital, sexual, transfusion-associated, and laboratory-acquired transmission are more relevant in Human Immunodeficiency Virus (HIV) positive patients (Rosenthal, 1988; Alvar, 1994; Magill *et al*., 1995).

Despite its wide geographic distribution, human leishmaniasis is often focal within endemic areas. Outbreaks of the disease depend on the ecological relationship between human activity, vector and reservoir hosts (Ashford, 2000). There is evidence in many countries that urbanization, agricultural development, deforestation, irrigation and more presently HIV (Desjeux *et al*., 2001), contributed to increased transmission and spread of the disease. In developing countries like Ethiopia, the spread of HIV is believed to worsen the health impacts of endemic tropical diseases like leishmaniasis, increasing the overall

morbidity and mortality. The *Leishmania*/HIV co-infection is therefore considered as an opportunistic and emerging disease (Alvar, 1994; WHO, 1996).

This emergence might be correlated with invasion by humans of zoonotic foci and migration of infected humans or domestic animals beyond their normal range (Ashford, 2000). The resurgence of leishmaniasis, and its emergence in newer geographic areas, besides changing the clinical profile of infected patient, has put forward newer challenges in the areas of diagnosis, treatment, and disease control.

In addition the currently available diagnostic tools however have their own limitations, which can seriously compromise the success of the case detection strategy especially for active infection of visceral leishmaniasis. The routine diagnosis of leishmaniasis relies on microscopic detection of *Leishmania* amastigote in Giemsa stained aspirates from lymph node, bone marrow, spleen or liver, in slit skin smears or in peripheral blood. The method is relatively simple and cheap, but has limited sensitivity (Weiss, 1995). Similarly serological tests based on the detection of anti-leishmanial antibodies are costly and are not yet reliable under field conditions.

More importantly, the ability to detect asymptomatic infection or disease at an early sub clinical stage is needed to improve intervention, to potentially direct chemotherapy to the appropriate individuals, and to better determine the population at risk and the determinants of disease. Therefore, there is an urgent need for a simple, cheap, rapid and accurate test with high sensitivity and specificity (Sundar, 2003; de Beer *et al*., 1991).

1.2 Epidemiology of VL

Visceral Leishmaniasis is endemic in 62 countries with a total of 200 million people at risk. Globally, there are an estimated 500,000 new cases annually, 90% are found in Bangladesh, Brazil, India, Nepal and Sudan (Desjeux, 1996). However, official figures are likely to underestimate the real prevalence of the disease (Thakur, 2000). Similarly, there are no accurate mortality data, apart from the annual WHO report, which recorded

57,000 deaths due to VL in 1999 (WHO, 2000) and estimated around 59,000 in 2001 (Rijal *et al*., 2004). VL is responsible for significant morbidity and high rates of mortality if left untreated mainly due to profound cachexia, secondary infections and/or haemorrhage (Berman, 1988). In treated VL, reported case fatality rate varies between 5-15% (Zijlstra, 1995).

The factors determining susceptibility or resistance to visceral leishmaniasis remain unclear. However, parasite strain, dose of infection, site of inoculation, sex of the host, pregnancy, acquired immunosuppression, and most importantly genetically determined predispositions of the host (Kafetzis, 2003) are reported to increase the risk of *Leishmania*-infected people developing clinical illness. Both the disease incidence and its severity are linked to poverty, for example malnutrition was found to be a risk factor for VL (Cerf *et al*., 1987), and high case fatality rates have been reported in the war-torn areas of Southern Sudan (Seaman *et al*., 1996) where socioeconomic conditions were worse.

Since 1993, *Leishmania*-endemic regions have expanded; accompanied by a sharp increase in the number of recorded cases (Arias *et al*., 1996). In Eastern India, deadly epidemics occurred where greater than 200,000 people are believed to have contracted the disease since 1994 (WHO, 1998). This might be due to an increased vector population following cessation of DDT spraying, together with an available human reservoir of infection in the form of post kala-azar dermal leishmaniasis, active visceral leishmaniasis cases including asymptomatic carriers.

This increase has led to a marked shift in the age pattern of VL infection, from infants to adults (Santos-Gomes *et al*., 2000), primarily in immunocompromised adults (Alvar *et al*., 1997). The decline in childhood visceral leishmaniasis in Southern Europe in the second half of the 20th century is related to social changes. This gave rise to a less frequent exposure at a young age as well as a lowered susceptibility to disease through nutritional intervention and immune improvements (Moral *et al*., 2002).

1.3 Life Cycle and Transmission of *Leishmania*

The life cycle of *Leishmania* species consists of two different morphological stages, the amastigote and the promastigote. When a female sand fly bites an infected human or an animal reservoir, it picks *Leishmania* amastigotes inside macrophages, with the host's blood. In the sand fly's midgut, the amastigotes differentiate into procyclic promastigotes that rapidly divide. After a few days, the parasites cease dividing and migrate to the proboscis of the insect. When infected sand fly rips up the epidermis and gains access to dermal capillaries of mammalian host, the promastigotes are injected into the bite wound.

Once in the host, the macrophages or mononuclear phagocytes take up the promastigotes where they rapidly transform again into amastigotes and initiate the infectious process. The parasite proliferates by binary fission within the parasitophorous vacoules and lysis the host cells to release the amastigotes, which further infect neighboring competent cells. This ecological cycle continues to another when another sand fly comes and feed on this host.

Zoonotic and anthroponotic forms of transmission characteristics are designated in VL. In the former the parasite is maintained in an animal reservoir and man is an occasional host. In the later, no animal reservoir exists, and the parasite is exclusively maintained in a man-vector-man cycle. Two types of are distinguished:

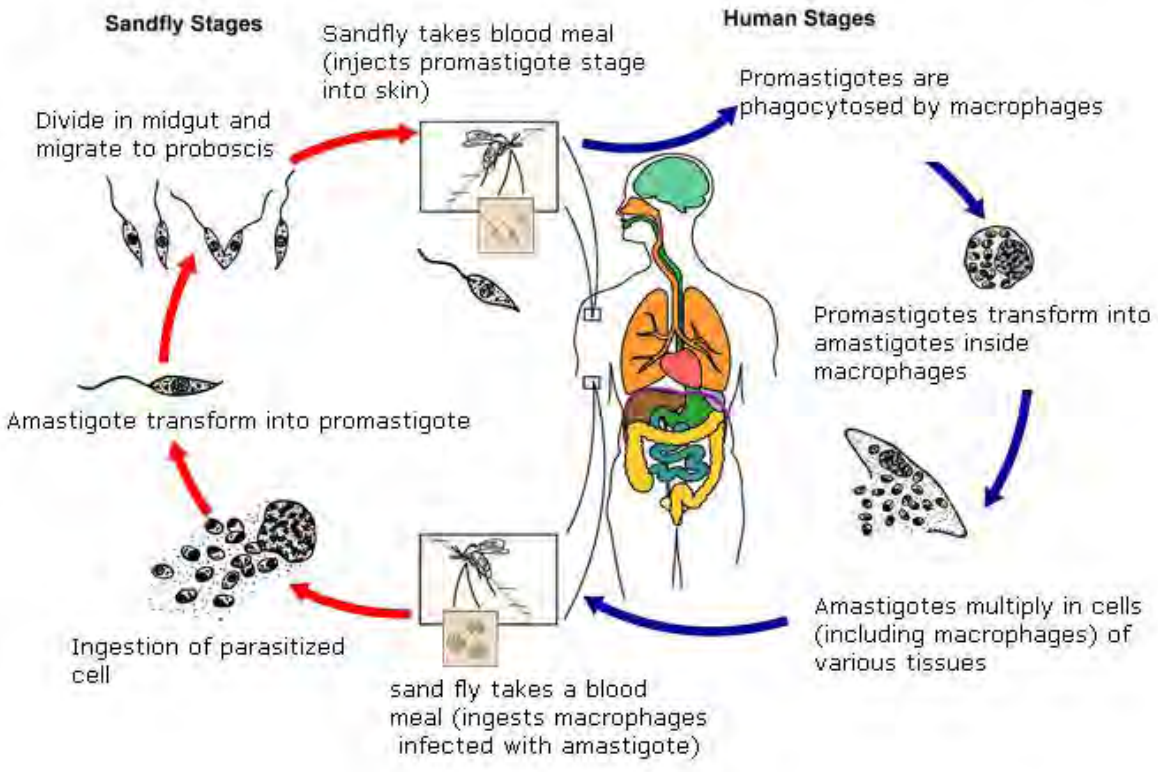


Fig. 1 Life cycle of *Leishmania* species. Phlebotomine sand flies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes. In the sand fly's midgut, the parasites differentiate into promastigotes, which multiply and migrate to the proboscis. The sand flies inject the infective stage, promastigotes, during blood meals. Promastigotes that reach the puncture wound are phagocytized by macrophages and transform into amastigotes. Amastigotes multiply in infected cells and affect different tissues, depending in part on the *Leishmania* species. This originates the clinical manifestations of leishmaniasis.

Source: http://www.dpd.cdc.gov/dpdx/html/leishmaniasis.htm

1.3 Visceral Leishmaniasis

Visceral leishmaniasis (kala-azar) covers a broad spectrum of severity and disease manifestations. The term kala-azar generally conjures up the classic image of profoundly cachectic, febrile patients who are heavily infected with parasites and have life-threatening disease in the absence of treatment. It is one of the tropical diseases that have a chronic course over a period of several months with severe symptoms.

A more acute course of the disease is described in the immunologically naïve population (Hashim *et al*., 1994). Classic kala-azar presents as a prolonged but irregular fever, weight loss, emaciation, malaise, and discoloration of the skin (Barbosa-de-Deus *et al*., 2002). It may also be found as a transient and milder visceral disease and a subclinical infection with constitutional symptoms but without progression to the full-blown disease (Magill *et al*., 1993).

The abnormal laboratory findings associated with advanced disease include pancytopenia, anemia, leucopenia (neutropenia, marked eosinopenia, relative lymphocytosis and monocytosis), thrombocytopenia which may cause uncontrolled epistaxis or bleeding from other sites of the body, as well as hypergammaglobulinemia (chiefly involving IgG, from polyclonal B cell activation) and hypersplenism, autoimmune hemolysis, and bleeding (Handman, 2001). Peripheral lymphadenopathy and hepatosplenomegaly are findings on physical examination that result from reticuloendothelial cell hyperplasia. Generally spleen is soft, non tender and more enlarged than the liver, typically 5-15cm below the left costal margin (Berman, 1997). Splenomegaly may be absent in 5% of the cases (Hashim *et al*., 1994).

Several related species of *Leishmania*, i.e. *L. d. donovani* in East Africa and on the Indian subcontinent, *L. d. infantum* in the Meditrranean region, the Middle East, and Asia and *L. d. chagasi*, in South America appear to home to visceral organs and lead to marked alterations in the function of the spleen, liver, and bone marrow (Berman, 1997; Handman, 2001). Each disease tends to be associated with a specific species of *Leishmania*. In the Mediterranean region, VL is due to *L. d. infantum* and occurs as a sporadic disease mainly in children younger than 5. A similar disease occurs in South America and is caused by the highly related parasite, *L. d. chagasi* (Berman, 1997).

Exceptional cases have been described in individuals infected with dermotrophic strains *L. tropica* and *L. aethiopica* (Oren *et al*., 1991; Ozbel *et al*., 1995), causing viscerotropic leishmaniasis, to distinguish it from visceral leishmaniasis which is frequently considered

to be the same as classic kala-azar, presumably depending on host factor. In such cases, the affected persons had light parasite burdens and differ from classical VL in the variable pathologies observed and also exhibit nonspecific manifestations of visceral infection (fatigue, fever, and gastrointestinal symptoms) and low anti-leishmanial antibody titers (Magill *et al*., 1993; Ozbel *et al*., 1995).

Some patients may develop infiltrative or nodular lesion of the skin, post-kala-azar dermal leishmaniasis (PKDL) typically is most prominent on the face, which develops during therapy or within a few months to years thereafter (Handman, 2001). In eastern Sudan around 56% of the VL patients were reported to develop PKDL after treatment (Zijlstra *et al*., 1995).

Close relationship between VL and PKDL was observed, and strains of *L. d. donovani* causing the original visceral infection were shown to be responsible for PKDL. As a result it is believed that, persons with PKDL can serve as reservoir hosts of infection (Addy and Nandy, 1992; Herwaldt, 1999).

1.3.1 Pathophysiology

The pathogenetic mechanisms of the disease are not fully understood, but in the organs spleen, liver and bone marrow, marked cellular alterations are clearly exhibited as a result of reticuloendothelial cell hyperplasia of histiocytes, which leads to hypertrophy especially in spleen and liver. Bone marrow and lymph nodes are filled with infected macrophages, and develop concomitant leukopenia and anemia. Histologically, a granuloma composed of a prominent infiltration of lymphocytes, epithelioid cells, and parasites are the hallmark of the syndrome (Handman, 2001).

The spleen gradually enlarges to enormous proportions, eventually extending into the pelvis. The capsule is thickened and more deeply the sinuses are dilated. It is common to see erythrophagocytosis by histiocyte, and the anemia so typical of kala-azar may in part be the result of the sequestration of red cells. Kupffer cells of liver filled with amastigote

are swollen and become hyperplastic, with centrilobular necrosis and fatty infiltrations. In late stage or chronic disease, increased hepatic fibrosis may give nodular cirrhosis. Lymphadenopathy, especially of the mesentric gland reveals large numbers of parasite filled macrophages (Handman, 2001).

The bone marrow is often filled with parasitized histiocytes, which replace the normal marrow elements resulting in anemia. Thus many patients with visceral leishmaniasis become cachectic. Death in visceral leishmaniasis is often secondary to bacterial or viral infections in debilitated patients with advanced disease (Berman, 1988).

1.3.2 Immunology

Local inflammation is initiated immediately after infection of parasite, which involves local accumulation of cells to clear damaged tissue and to initiate wound healing. At early stage of leishmaniasis infection, wound is infiltrated by neutrophilic and eosinophilc granulocytyes, followed by a wave of inflammatory macrophages, which within a few days predominate in the area of the lesion. By the time infection appears, extra cellular promastigotes are mostly dead or opsonized by serum complement. Opsonization triggers endocytosis by resident cells, which promastigotes use as safe targets (Greil *et al*., 1998). Langerhans cells could initially function as a safe habitat, transporting the parasites from the infected skin to the draining lymph node (Blank *et al*., 1996).

The interaction between *Leishmania* parasite with macrophage and dendritic cells undoubtedly forms the core of the antileishmanial immune response (Bogdan *et al*., 1996). *Leishmania* soon after infection utilize modulation of antigen presentation and cytokine production by macrophages, thereby delaying the initiation of an effective immune response (Bogdan *et al*., 1996). There is apparent cellular anergy to *Leishmania* antigens (Sacks *et al*., 1987), which may result in inappropriate antigen presentation and communication between the antigen-presenting cells and T-cells and from the induction of cytokines with macrophage inactivating properties (Engwerda *et al*., 2000), this is also dependent on the type of T-cell response.

Extensive studies with experimental models have shown that the outcome of infection is critically dependent on the activation of one of the two subsets of $CD4^+$ T cells, Th1 and Th2 (Reiner *et al*., 1995), which mediate different immunological effector functions. Cure or resistance in leishmaniasis is associated with Th1 response characterized by the appropriate production of IL-12, TNF-α and IFN-γ leading to parasite destruction by macrophage. On the other hand disease progression has been related to a Th2 type of response with the immunoregulatory cytokine production IL-10, which plays an important role in downmodulation of immune responses i.e., early IL-4 production, IL-12 insensitivity, and a feature of IFN-γ induced macrophage activation (Brodskyn *et al*., 2001; Bottler *et al*., 2001). However, in human leishmaniasis, a clear functional dichotomy in $CD4^+$ T cells has not definitely been documented (Solbach and Laskay, 2000).

1.4 HIV/VL Co-infection

In the last two decades, visceral leishmaniasis has been recognized as an opportunistic disease in the immunocompromised patients infected with human immunodeficiency virus (Choi and Lerner, 2001). Recently, there has been an increase in overlapping of visceral leishmaniasis and HIV infection due to the spread of the HIV pandemic (Solbach and Laskay, 2000). As a result, the number of people with *Leishmania*-HIV co-infection increased (Ashford, 2000; WHO, 2000).

With the emergence of HIV epidemic in the early 1980s, the disease spectrum caused by the *Leishmania* parasites has changed significantly. Species that traditionally caused only one type of disease has been shown to cause other diseases (Herwaldt, 1999). Strains of *Leishmania* that seem to be avirulent in immunocompetent individuals can cause disease in immunocompromised hosts. These strains react differently to drugs, as evidenced by relapse, and may transmit themselves in novel ways (Alvar *et al*., 1997). Furthermore, leishmaniasis as a childhood disease has been drastically altered due to HIV-leishmaniasis co-infection. In Spain before 1985, it has been estimated that approximately two thirds of

leishmaniasis cases were among patients less than 15 years of age. However, since then, almost 80 percent of the cases have been in adult immunodepressed patients, 60 percent of them being HIV positive (Herwaldt, 1999).

Most of the burden of HIV, leishmaniasis, and probably of co-infection are reported in Africa, India, and South America where the true impact is not entirely known (WHO, 1996). In Europe, it is estimated that, 70% of adult cases of VL are associated with HIV infection, and up to 9% of people with AIDS suffer from newly acquired or reactivated VL (Alvar *et al*., 1997; WHO, 1998).

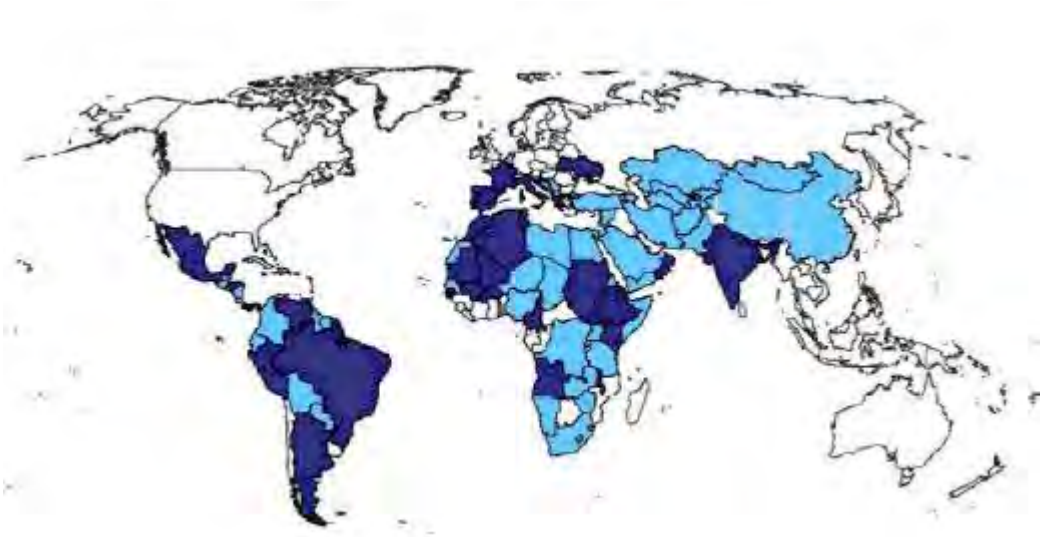


Fig. 2. Worldwide distribution of leishmaniasis (■) and countries reporting *Leishmania*/HIVco-infection (■)

Source: Desjeux and Alvar (2003)

A person with HIV infection whose immune system is suppressed, and who is bitten by a sand fly infected with *Leishmania*, will develop severe leishmaniasis. Since both HIV and *Leishmania* organisms can invade and replicate within macrophages, it is possible that interactions between these pathogens can exacerbate the process of HIV infection (Bernier *et al*., 1995). VL, once developed in the HIV-infected person, accelerates HIV replication and impairs the patient's condition by further immunosuppression. As a consequence, most of the patients (95%) quickly become AIDS patients with opportunistic disease such

as tuberculosis, candidiasis, pneumonia due to *Pneumocystis carinii*, or toxoplasmosis (WHO, 2000).

It is supposed that *Leishmania* infection increases human immunodeficiency virus (HIV) replication in seropositive individuals. This might be partly due to a Th2 type of immune activation, as demonstrated by higher plasma levels of IL-4, -6 and -10 in HIV-*Leishmania* coinfected patients than in HIV alone infected individuals (Preiser *et al*., 1996). The inability of antibody response to control the parasite and the absence of specific T-cell immunity to *Leishmania* species shown by a significant drop in the $CD4^+$ cell count during active VL, and maintained thereafter in co-infected individuals (Medrano *et al*., 1998) would explain the high frequency of relapses in HIV-*Leishmania* co-infected patients (Moreno *et al*., 2000).

HIV/*Leishmania* co-infected patients also remain problematic in the diagnosis of leishmaniasis. Amastigotes are found in the peripheral blood in 50% of cases and they may be found in unusual locations such as the lungs, larynx, gastrointestinal tract, the rectum and cerebrospinal fluid (Alvar *et al*., 1997) as a result parasitological tests give a negative result. Similarly the observed low level of anti-leishmanial antibodies in these patients decreases the sensitivity of serological tests.

1.5 Leishmaniasis in Ethiopia

Leishmmaniasis is one of the most important vector–borne diseases in Ethiopia. Four species of *Leishmania*: *L. d. donovani* (causing VL), *L. aethiopia, L. major* and *L. tropica* (causing CL) are responsible for human leishmaniasis (Hailu and Frommel, 1993).

Different species of sand fly vector are known to exist in Ethiopia. Species known to transmit the different forms of leishmaniasis include: *Phleobotamus martini*, *P.celiae, P. orientalis, P. pedifer, P. longipes* and *P. duboscqi* (Gebre-Michael and Lane, 1996; Hailu *et al*., 1995; Ashford *et al*., 1973; Gebre-Michael *et al*., 1993). Species found in VL endemic foci include: *P. duboscqi, P. rodhaini, P. mireillae, P. saevus, P. alexanderi and*

P. orientalis assumed to be potential vectors of VL (Gebre-Michael & Lane, 1996; Balkew *et al*., 1999; Gebre- Michael & Balkew, 2002).

1.5.1 Visceral Leishmaniasis in Ethiopia

Of the various forms of leishmaniasis, VL is regarded as the major public health problem in Ethiopia, responsible for significant morbidity and high rates of mortality. It is caused by *L. d. donovani* and endemic with a patchy distribution in the Southern and North western arid and semi-arid areas of the country (Maru, 1979; Ayele and Ali, 1984; WHO, 1996), with characteristic variation in magnitudes of endemicity (Hailu *et al*., 1996). Important endemic foci include the Gelana focus east of Lake Abaya, the Segen valley, Omo River plains and Metema & Humera plains in Northwestern Ethiopia (Hailu *et al*., 2004). In Segen Valley (Aba-Roba focus in Konso Wereda) VL is maintained in extra- and peri-domestic settings.

The data from active and passive case findings and epidemiological surveys provided evidence on the occurrence of the disease in several isolated foci where it mainly affect children and young adults (Hailu and Frommel, 1993). Although the countrywide incidence and prevalence rates are not known, for Aba-Roba focus in Southwest Ethiopia, the prevalence was estimated to be 6.9 per 1,000 (Ali and Ashford, 1994).

Outbreaks of VL in Ethiopia were reported since 1970, among annual migrant workers, which appears to correspond to an extensive programme of agricultural development in northwestern Ethiopia (Mengesha and Abuhoy, 1978; Maru, 1979; Berhe *et al*., 2001). The most recent outbreak started again in the northwestern lowland, in 1995. 56 cases were detected in a population of highland settlers and is currently producing 600-800 new cases annually (Hailu and Berhe, 2002). Although more males than females are infected with VL globally (WHO, 1990), the very high male to female ratio 11:1 (Lyons *et al*., 2003) reported in Tigray reflects the high numbers of males that make up the migrant workers who are at risk of contracting VL. Outbreaks have also been reported from the

neighbouring country Sudan, causing 100,000 deaths in the last 10–15 years (Herwaldt, 1999).

Several factors, both environmental and ecological, may have contributed to the increase in VL incidence. These include soil type, rainfall, expansion of towns and villages, establishment of new settlements, and increased agriculture and population movements. These might have led to an increase in sand fly vector and naive host contact. Even if environmental risk factors are associated with transmission cycle, sex might also have a correlation with male infection as experimental evidences have indicated (Travi *et al*., 2002).

The current dramatic increase in the burden posed by VL in Ethiopia is alarming, and mainly correlated with the continued spread of HIV. In 2002, 6.4% of adults were estimated to be HIV-positive (UNAIDS *et al*., 2002). Sporadic epidemic of visceral leishmaniasis including the spread of HIV infection to areas where VL is endemic has become a major challenge to the control of VL, complicating diagnosis and treatment.

HIV and *Leishmania* co-infection in Africa was first reported in Ethiopia (Berhe *et al*., 1995). The number of cases are increasing (WHO, 2000; Wolday *et al*., 2001), many are dying undiagnosed. There is also very little information on co-infection regarding estimation of true numbers, clinical presentation or outcome in Africa, where the causative organism is *L. d. donovani* (WHO, 1998; Wolday *et al*., 2001).

In Europe, where *L. d. infantum* is the causative organism (WHO, 1998; Desjeux, 1999), it was found that individuals co-infected can present with vague and atypical symptoms of VL; absence of hepatosplenomegaly, non-specific gastrointestinal problems, with parasites often found outside the reticulo-endothelial system and negative serology (Desjeux, 1999; Pintado *et al*., 2001).

1.6 Control Measure of Visceral Leishmaniasis

The control measures rely both on chemotherapy to alleviate disease and vector control to reduce transmission. The highest priority is given to case-detection and treatment of VL and PKDL cases, and to vector control through individual protection by impregnated bed nets and peridomestic spraying mainly in the anthroponotic transmission of VL.

The most recent difficulties in eliminating VL are co-infection with HIV and resistance to pentavalent antimony (Murray, 2001) which has been used worldwide for the past 60 years. These problems join the long standing obstacles in VL include: poverty, malnutrition, migration of non-immune refugees, inadequate vector control, and the lack of vaccine. There are also common problems in diagnosis, unavailable or unaffordable drugs, and until recently lack of oral therapy (Murray, 2001). On the other hand, most infections remain entirely asymptomatic or subclinical as a result there may be millions of new infections each year. These impede efforts made in disease management and control.

1.6.1 Vector control

Vector control may be useful under certain conditions but are not applicable in every epidemiological setting and require infrastructure and vigilance beyond the capability of many VL endemic countries. In the developing countries, the use of personal protection with repellants, permethrin-impregnated bed nets, and suitable clothing significantly reduce the domestic transmission of leishmanial infection (Bern *et al*., 2000; Campbell-Lendrum *et al*., 2001). The killing of all stray and domestic infected dogs is also required for a successful intervention on the reservoir-vector-host chain (Grech *et al*., 2000) mainly in zoonotic transmission of VL. Noxious plants are also harmful to sand flies, and may provide local protection against sand fly bites (Schlein *et al*., 2001).

1.6.2 Treatment

The first line standard anti-leishmanial treatment, pentavalent antimony compounds meglumine antimonate (Glucantime) and sodium stibogluconate (Pentostam) (antimonials), are over a half-century old and have extensively been used world wide (Maltezou, *et al*., 2000). These agents not only require repeated injections for a month, which require lengthy hospitalizations, but also remain expensive, associated with side effects and not always available (Herwaldt, 1999). Relapses are mainly observed among patients treated with shorter courses and drug resistance is also becoming common in certain areas like in India (Sundar *et al*., 2000) that make their use ineffective. Recently the orally active drug, multifosine development against visceral leishmaniasis is a break through, but has not been widely used. However, its possible side effects are not determined and are not affordable for most.

For HIV/*Leishmania* co-infected patients, the preferred anti-leishmanial agent as well as the optimal duration of treatment has not yet been established. In addition there are no uniform guidelines regarding the use of maintenance regimens to prevent relapse (Herwaldt, 1999; Pintado *et al*., 2001).

1.6.3 Diagnosis of visceral leishmaniasis

The diagnosis of VL is complex because of its clinical features which are also shared by a host of other commonly occurring diseases like malaria, typhoid, and tuberculosis. Sequestration of the parasite in the spleen, bone marrow, or lymph node further complicates the situation (Sundar and Rai, 2002) or during AIDS-related illnesses (WHO, 1998; Desjeux, 1998) many of these diseases can be present along with VL in cases of co-infection.

1.6.3.1 Parasitological Methods

Clinically, VL may be confirmed by direct examination and/or culture of aspirate material. These techniques are the methods of reference to confirm active infections by leishmaniasis (Kohanteb *et al*., 1987). Nevertheless, in epidemiological research they are inappropriate (Riera *et al*., 2004) due to low sensitivity and the invasive, painful, and even hazardous nature of the techniques (Kar, 1995).

Microscopic demonstration of parasites from Giemsa-stained smears made from spleen, liver, bone marrow, and lymph node, aspirate material is the most common VL diagnostic test. Splenic aspiration is generally accepted as the most sensitive method, however, it requires certain precautions, and it is ethically acceptable only in case of clinical suspicion of the disease (Zijlstra *et al*., 1992), and has several contraindications like severe anemia, restless children, pregnancy, and bleeding tendency. The splenic puncture may lead to bleeding that may be fatal, from a soft and enlarged spleen. To avoid the risk of excessive blood loss, splenic puncture should be avoided in patients with platelets count of less than 40,000 platelets/µl and a prothrombin time of more than 5 second over the control.

The microscopic method is relatively simple and cheep, but there is no possibility to distinguish among *Leishmania* amastigotes belonging to the different species (Weiss, 1995; Osman *et al*., 1997). The sensitivity of smears from splenic, bone marrow, and lymph node aspirate was estimated to have greater than 90%, 70%, and 50% or lower respectively (Zijlstra *et al*., 1992; Sundar and Rai, 2002), but when bone marrow aspirate repeated and used during the first onset of the disease, is one of the most sensitive method (93.5%) (WHO, 2000).

Leishmania parasites can be cultured in a range of media where they grow as promastigotes. Parasite can also be demonstrated from *in vivo* culture, after inoculation of laboratory animals (such as hamsters, mice or guinea pigs) with infected specimen

(Marsden, 1986). Animal inoculation is not usually employed as a diagnostic test, since several months may be required to obtain a positive result (Sundar and Rai, 2002).

The sensitivity of detection of parasite from aspirate material can be improved by culture especially for treated patients or during relapses. Culture also required for accurate diagnosis of infection with the organism (as a supplement to other methods), obtaining a sufficient number of organisms for various laboratory uses or to provide a diagnosis when routine methods have failed. However, *Leishmania* culture is rarely used in routine clinical practice, since it requires expensive equipment, expertise, and longer time (Singh and Sivakumar, 2003).

One of the major drawbacks of culturing, especially under field conditions, is its sensitivity to bacterial or fungal contamination. However, in some instances it is very difficult to demonstrate the presence of the parasite, such as in patients with mucocutaneous leishmaniasis and visceral leishmaniasis co-infected with HIV (Alvar *et al*., 1997). In these patients immunodiagnosis becomes an important alternative for demonstrating the presence of the parasite (Kar, 1995).

Due to the limitations inherent in the techniques used for detection of parasites, new approaches such as DNA hybridization have been attempted since the early 1980s. Although these methods have considerable sensitivity like detecting as few as 50 to 100 parasites, their potential use in routine diagnosis is hampered by the complex procedure of hybridization (Sundar and Rai, 2002).

Molecular approach is capable of detecting nucleic acids unique to the parasite (Singh and Sivakumar, 2003), and appear to offer the promise of accurate non-invasive tools for the diagnosis of leishmaniasis (Kafetzis, 2003). Over the past years, the value of polymerase chain reaction (PCR) for the diagnosis of visceral leishmaniasis has been assessed using different clinical specimens (peripheral blood, bone marrow, and spleen) (Salotra *et al*., 2001; Spanakos *et al*., 2002). The detection of persistent parasite DNA in infected tissues

by means of PCR may also be used as a marker for the risk of relapse after initial cure. However, this technique can only be used in referral laboratories.

1.6.3.2 Immunodiagnostics

For several decades, nonspecific methods, which depend upon raised globulin levels, have been used in the diagnosis of VL. Some of the tests used for detecting these nonspecific immunoglobulins are Napier's formol gel or aldehyde test and the Chopra antimony test. These tests are highly unreliable because of lack of specificity, as well as varying sensitivities (Sundar and Rai, 2002). To day, most epidemiological methods on VL are performed through immunological methods which include leishmanin skin test (LST), antigen detection, and antibody detection.

The leishmanin skin test (LST) measures a delayed type hypersensitivity (DTH) reaction for detecting cell-mediated immunity in *Leishmania* infections (Riera *et al*., 2004) to an antigen prepared from an in vitro parasite culture, various species of *Leishmania* may be used. The Montenegro skin test for delayed type hypersensitivity is usually negative during active VL but becomes positive after treatment (Osman, 1997) or recovery (Liew and O'Donell, 1993).

The LST has been shown to be a useful tool for surveys, epidemiological investigations and also used to quantify transmission of leishmaniasis. A positive LST was believed to remain so for most of the rest of a person's life, probably indicating an immune status, which prevents further development of leishmaniasis (Pampiglione *et al*., 1975).

When antibody production is impaired, an antigen detection test is useful and has shown good prognostic value and is currently under evaluation (Rijal *et el*., 2004). A recently developed nitrocellulose dipstick test that detects antibody to the recombinant amastigote antigen K39 is highly sensitive and specific for the diagnosis of acute VL in clinical settings, but it is not yet widely used in part because of concerns about cost (Bern *et al*.,

2000). Detection of leishmanial antigen in urine through a latex agglutination test (katex) seems to be promising for both diagnosis and prognosis (Rijal *et el*., 2004).

Specific serological techniques are based on the determination of antibodies produced against the circulating *Leishmania*-specific antigens. Antibody detection tests are simple and non-invasive for detecting active leishmaniasis when large amounts of specific antibodies are present. In subclinical infections, it is difficult to ascertain the proportion of individuals that are infected, because antibody levels are low and serological tests show low sensitivity (Badaro *et al*., 1983). Indirect immunological methods may be useful, but should always be judged carefully against clinical data. Most of the serological methods for diagnosis use antigen preparations that lack sensitivity (Singh and Sivakumar, 2003). Previous attempts to use antigens in serological diagnostic tests for VL have been hampered by low sensitivity, specificity, and test reproducibility (Badaro *et al*., 1983).

Several immunodiagnostic methods that are more sensitive and specific have been developed and evaluated for the diagnosis of VL. These include the direct agglutination test (DAT) (Meredith *et al*., 1995; Rab and Evans, 1997; Boelaert *et al*., 1999; Mbati *et al*., 1999), enzyme-linked immunosorbent assay (ELISA) (Fargeas *et al*., 1996; Zijlstra *et al*., 1998; Raj *et al*., 1999; Kaul *et al*., 2000), immunoblot analysis (Mary *et al*., 1992; Salotra *et al*., 1999), and immunofluorescent-antibody tests (Walton *et al*., 1972).

Among the serological assays recently developed, the DAT, in which serum antibodies agglutinate stained parasites, seemed most appropriate for use in the field (Sinha and Sehgal, 1994; Shiddo *et al*., 1995). However, the relatively long incubation period (18 hrs), the fact that serial dilutions of blood or serum must be made (test less suitable for screening large numbers of samples), variation between batches, the high cost of commercially available antigen (Davies *et al*., 2003) and the limited stability of the aqueous antigen and lower specificity are the major limiting factors (Harith *et al*., 1988).

In order to avoid some of the problems, fast agglutination screening test (FAST) was developed (Schoone *et al*., 2001). The test is based on the DAT, but combines a high

parasite antigen concentration with a smaller test volume. Furthermore, it requires only one serum dilution and the results can be read within three to four hours. Evaluation under laboratory condition in the Netherlands and Ethiopia showed a high sensitivity and specificity comparable to the current DAT (Schoone *et al*., 2001; Hailu *et al*., 2002).

The Enzyme Linked Immunosorbent Assay (ELISA) is a valuable tool in the serodiagnosis of leishmaniasis. The test is useful for laboratory analysis as well as for field applications. However, the sensitivity and specificity of ELISA is greatly influenced by the antigen used (Singh and Sivakumar, 2003). Among the serological tests, Western blot (WB) is highly sensitive technique when low antibody titers are present, and provides broader information about the parasite's antigenic profile (Kar, 1995). Moreover, western blot analysis showed a large diversity in the antibody response to visceral leishmaniasis and permitted detection of antibodies to *Leishmania* in serum (Kar, 1995).

In HIV patients, the diagnosis of VL is hampered by its coexistence with other opportunistic infections as well as the poor sensitivity of serological tests. New serological tests [IFAT, ELISA and direct agglutination test (DAT)] for determining leishmaniasis infection do not function as well in immunocompromised patients who do not make antibodies to infections. In Europe, more than 50% of these patients show no detectable levels of anti-leishmanial antibodies (Pintado *et al*., 2001). In these situations, two or more tests must be used, unfortunately making the diagnostic procedure more expensive and less reliable. As a result, in countries where VL is endemic, there is a basic need for cheap, easy, and field applicable diagnostic test.

On the other hand uncertainty about DAT's specificity created more reliance on parasitological test for borderline titers. Furthermore, failure to treat DAT positive but parasitologically negative patients by some clinicians, created urgent need for VL diagnostics with better specificity and high sensitivity.

Therefore, this work has focused on the evaluation of the performance of an alternative serological test, the indirect hemagglutination Assay (IHA), in the detection of antibodies

to visceral leishmaniasis parasite antigens. The sensitivity, specificity, positive predictive value, and negative predictive value of the assay in the diagnosis and epidemiological studies of VL in Ethiopia were determined.

IHA involves the agglutination of red blood cells by antibodies specifically directed to soluble parasite antigen that have been previously adsorbed or otherwise attached to the red blood cell surface (Bray and Lainson, 1967). Erythrocytes of man, rabbit, fowl, and sheep are a class of cells, which can be readily obtained in bulk amounts with little effort (Talwar, 1983). They are particles of almost uniform size and shape. The most important feature of the particles is that they are red in color and hence agglutination is very easy to observe. The end point titer can be estimated as the last dilution before a clear sharp-edge red spot identical to the one observed in the antigen control well. The non-agglutinated cells settle to the bottom as a clear red button, while agglutinated cells settle as pink diffuse mat. Since the visualization is so simple, the test can be carried out on microscale using microtitre plate. This makes the test quick and suitable for application in the field where many samples have to be tested at a time.

2. OBJECTIVES

2.1 General Objective

To evaluate the performance of Indirect Hemagglutination test and to contribute to improved case detection both in diagnostic and sero-epidemiological screening of visceral leishmaniasis in Ethiopia.

2.2 Specific Objectives

1) To evaluate the diagnostic value of IHA test in VL patients
2) To assess the use of IHA for sero-epidemiological screening of VL in endemic population.
3) To compare the diagnostic value of IHA test with parasitological method, DAT and FAST
4) To evaluate diagnostic performance of IHA test in HIV/VL co infection.

3 Materials and Methods

All test sera were obtained from serum bank of Aklilu Lemma Institute of Patobiology, of which 19 were clinically (and parasitologically confirmed) diagnosed of having VL, 132 formerly VL treated patients, 38 sub clinical cases based on their reaction with DAT and 1028 samples from apparently healthy individuals in the endemic area, 7 known tuberculosis cases, 13 CL confirmed cases and 10 healthy control from non-endemic area.

Additional sera from 77 subjects with splenomegaly and fever of at least 2 weeks duration and considered as VL suspects were also included from Mykadra (humara). Clinical suspects of VL with positive DAT were presumed as cases of VL (if previously untreated) and as probable cases of relapse or unresponsiveness (if treated before). Smear and culture of spleen and lymph node aspirate were obtained from all presumed cases of VL at Kahsay Abera Hospital, Humara by experienced physical. Diagnosis was confirmed by demonstration of amastigotes in Giemsa stained smear of aspirates or promastigotes in NNN cultures maintained for 3 weeks. For each smear at least 5000 microscopic fields were examined under oil immersion (magnification 1000X). Cases of parasitological findings were treated with a standard regimen of sodium stibogluconate 20mg/kg body weight per day for 30 days. Cases with negative parasitological results were referred to a VL clinic of the district for further follow-up. 21 subjects diagnosed of having malaria were also included in the study. Parachek, rapid test for *P. falciparum* malaria (Orchid Biomedical systems, Verna, Goa 403722, India) and thick and thin film were used to confirm malaria.

The study was ethically cleared by the Aklilu Lemma Institute of Patobiology Ethical Clearance Committee and National Ethical Clearance Committee (NECC) of the Ethiopian Science and Technology Commission. Informed consent (form annexed) was obtained from each study participant.

3.1 Indirect hemagglutination (IHA) test

3.1.1 Antigen preparation

From an isolate of *L. donovani* (MHOM/SD/68/1S), promastigote stage were grown in RPMI-1640 (SIGMA) complete medium supplemented with 15 % fetal calf serum (SIGMA), 2 mM L-glutamine, streptomycin 100µg/ml, and penicillin 100u/ml. Parasite recovered in stationary growth phase were harvested, washed by centrifugation five times with physiological saline solution (PH 7.4), at 4^0C. The parasites were disrupted by resuspention in sterile distilled water, followed by 5 freeze-thaw cycle at -196^0C and 37^0C and a final ultrasonication at amplitude of 5λ. The suspension was centrifuged at 10,000 rpm for 30 minute at 4^0C. The supernatant was analyzed for protein concentration using Lowry *et al*. (1951) method and stored in aliquots at -196^0C until use.

3.1.2 Sheep Red Blood Cell preparation

Sheep blood was aseptically collected by venipuncture into equal volumes of Alsever's solution (pH 6.1) (Talwar, 1983). The fresh red blood cells were washed five times with phosphate buffer saline (PBS) (pH 7.2) and packed by centrifugation. 25ml of packed cells were resuspended to 200ml PBS (pH7.2) and placed in 500ml conical flask. 50ml commercial formalin (40% formaldehyde) was introduced into a length of dialysis tubing two-third full, which is then tied off so that air is excluded. The filled dialysis tube was submerged into the red cell suspension and gently agitated at 100 rpm on a shaker at room temperature. After three hours the swollen dialysis sac was punctured and the formalin allowed to escape and removed. The dark brown suspension produced was filtered through muslin to remove seam and debris after overnight gentle mixing. The cells were washed with PBS (pH 7.2) five times to remove the formalin and finally 50% suspension (packed cell volume to total volume) in PBS (pH 7.2) was prepared and stored at 4^0C until use.w5

3.1.3 Sensitization and coating of sheep red blood cells

SRBCs were sensitized by mixing 0.5ml packed cells in 10ml PBS (pH7.2) with 100µl 25% gluteraldehyde and incubated at 37^0C water bath for 20 minutes, then washed three times by centrifugation at 3000 rpm for 5 minutes at 4^0c (GPR, BECKMAN, centrifuge), and finally resuspended with 10ml PBS (pH7.2). 500µl packed sensitized sheep red blood cells were coated with equal volume of 500µl antigen 15mg/ml (stock) in 4ml coating buffer (PBS pH 6.4), and the mixture were incubated at 37^0C water bath for 30 minutes. The coated cells were washed and centrifuged at 3000 rpm for 5 minutes three times with PBS (pH 7.2) containing 1% heat inactivated fetal calf serum (FCS) (serum saline) and finally suspended in the same serum saline to 1% cell suspension ready to use for the test (Morsy et al., 1990).

3.1.4 IHA test procedure

50µl test sera were adsorbed by mixing with 50µl 10% sensitized SRBCs and incubated for 30 minutes at room temperature, and centrifuged at 3000 rpm for 5 minutes to avoid non-specific (group agglutinins) reaction. To a V-shaped microwell plate (Greiner, Frickenhausen, Germany), 50µl PBS (pH 7.2) was distributed to each well and 50µl of adsorbed test sera diluted 1/16 in PBS (pH 7.2) were added to the 2nd row and double fold serial dilutions of sera were made by transferring 50µl mixture to the series of wells (1:32, 1:64, 1:128,…, 1:4096). 50µl of 1% sensitized and coated SRBCs was added to each well of the microwell plate and kept on the bench undisturbed from 3 hours to overnight at room temperature. The hemagglutination results were read by settling patterns. Agglutination titer greater than 50% of controls was taken as positive.

3.2 Direct Agglutination Test

3.2.1 Antigen preparation

DAT was used for VL screening of study subjects for anti-*Leishmania* antibodies, using the *L. donovani* (MHOM/SD/68/1S) test antigen prepared at Aklilu Lemma Institute of Pathobiology. The antigen was prepared following the procedure of Harith *et al*. (1988). The log phase promastigotes were harvested and washed five times with cold Locke solution (PH 7.4), at 4^0C. The pellet was treated with 0.4% trypsin at 37^0C for 45 minutes. The trypsin was removed by centrifugation, and the pellet washed five times with cold Locke solution. The treated promastigotes were then fixed overnight with 2% formaldehyde solution at 4^0C. Following fixation, the promastigotes were washed twice with cold (4^0C) saline (0.15 M sodium chloride, 0.056M sodium citrate, pH 7.4). The promastigotes were then stained with 0.1% Coomassie brilliant blue for 90 minutes, washed three times with cold saline, and resuspended at a concentration of 4.5 x 10^7 promastigotes/mL with citrate saline containing 0.4% (wt/vol) formaldehyde solution. The antigen was stored at 4^0C until use. The quantity of the antigen was standardized with the commercially available antigen using a positive standard serum sample and other VL serum samples.

3.2.2 DAT test procedure

100µl and 50µl serum diluent (0.9% (wt/vol) sodium chloride with 0.78% (vol/vol) ß-mercaptoethanol were added to V-shaped microwell plate (Greiner, Frickenhausen, Germany). 1µl serum sample was added to wells containing 100µl serum diluent and mixed thoroughly by pipetting up and down 8 times, and 50µl of the mixture was transferred for serial dilution. 50µl antigen was added to each well of the microwell plate. The microwell plate was covered and kept on a level surface at ambient temperature, the result was read and interpreted after 20-24 hours (Harith *et al*., 1988). A titer of 1: 1600 was used as the threshold for seropositivity.

3.3 FAST test procedure

FAST test for leishmanial serology was performed according to the manufacturer's instruction (Kininklijk Institute voor de Tropen, Biomedical Research, The Netherlands, Amsterdam). Briefly, vials containing 2 X 10^8 promastigotes of the antigen were reconstituted in 1.0ml normal saline. Using V-shaped microtiter plate (Greiner, Frickenhausen, Germany), 20µl of this antigen was mixed with 20µl of serum diluted 1:100 in normal saline containing 0.78% mercaptoethanol. Plates were kept at room temperature for 3hrs. Test results were read as negative (compact blue dot) or positive (diffuse blue mat).

3.4 HIV testing

An initial HIV test was made using ELISA test kit (Vironostica, HIV Uni-Form II Ag/Ab, BioMerieux bv, Boseind 15, 5281 RM Boxtel, The Netherlands) in duplicate following the manufacturer's instruction, and finally Uni-gold (Trinity Biotech PLC, Ireland) was used for confirmation.

3.5 Data analysis

Data were entered into d-base file (D-base IV plus, Borland International 1993). For analysis of data, SPSS-11.5 and EPI INFO 6, ver, 6.4 (CDC, USA) were used. Agreement was studied on both a categorical (IHA positive-negative at 1:64, 1:128, and 1:256 dilution, and DAT positive-negative at 1:1600 dilutions) and a discrete numerical scale. Discrete numerical IHA results were expressed as end titers, on a scale from 4 to 13, a transformation from the dilution to the corresponding number of twofold serial dilutions. A negative IHA result was coded 4, the first dilution (1:16), dilution (1:32) was coded as 5 and so on to code 13 for the dilution 1:8192.

Sensitivity was calculated as (TP)100/(TP + FN), specificity was calculated as (TN)100/(TN + FP), positive predictive value (PPV) was calculated as (TP)100/(TP + FP), and negative predictive value (NPV) was calculated as (TN)100/(TN + FN). Where TP is

the number of patients with true-positive results, FN is the number of patients with false-negative results. TN is the number of patients with true-negative results, and FP is the number of patients with false-positive results. In this work DAT was used as a reference for IHA validation with our antigen.

For comparison of groups, kappa and chi-square (uncorrected) were used. For determination of validity of IHA, results were compared with parasitological data, DAT and FAST. The measures of performance were: sensitivity, specificity, positive predictive value, negative predictive value, and percent agreement.

4 RESULTS

Indirect hemagglutination test was evaluated for the diagnosis and epidemiological study of VL in two groups of subjects that are shown in Table 1. The diagnostic study was from Northern Ethiopia, Maykadra (Humara) and the epidemiological study was from Southern Ethiopia, Abaroba (Konso). A total of 214 subjects (116 from Abaroba and 98 from Maykadra) were found to have had an enlarged spleen and/or liver. Of these, 134 were considered as VL suspects based on the presence of fever for at least 2 weeks in duration and hepato-splenomegaly.

Table 1. Summary of study population

	Diagnostic study	Epidemiolog-ical study	Total
No. of subjects examined	98	1217	1315
No. of sera screened	128[§]	1217	1345
No. of subjects with hepato-splenomegaly	98	116	214
No. of subjects suspected as VL cases[†]	77	57	134
No. of sera tested			
IHA (1:64)	128	1217	1345
(1:128)	128	1217	1345
(1:256)	128	1217	1345
DAT	128	1217	1345
FAST	127	1217	1344
HIV	71	-	71
No. of patients subjected to aspiration	77*	25[¶]	102

[†] Defined with splenomegaly and fever of at least 2 weeks duration. *Spleen aspirated cases, ¶ lymph node aspirated cases. [§] Include CL, malaria, TB, and non-endemic healthy control sera.

Over all 1345 sera were used for different serological tests that include: IHA, DAT and FAST. 71 sera were tested for HIV antibody. Aspirations of spleen and/or lymph node were made for 102 suspected VL patients to demonstrate parasites either in smear and/or culture of aspirated materials.

4.1 Diagnostic Study

To evaluate the anti-*Leishmania* antibody detection capacity of IHA in the diagnosis of visceral leishmaniasis, sera from patients with i) clinically suspected VL (n=77), ii) documented CL (n=13), iii) parasitologically confirmed malaria (n=21), iv) documented TB (n=7), and v) non-endemic healthy controls (n=10) were used (Table 2).

The result of the IHA test was compared with parasitological methods, DAT, and FAST, the commonly used diagnostic techniques for visceral leishmaniasis. Cut-off titer for positivity and negativity of IHA test was assigned based on checkerboard titration on known positive and negative samples as described on the material and methods section.

In an attempt to determine level of cross reactivity in IHA test, sera from individuals with malaria, TB and CL were employed. IHA showed cross reactions with sera from patients with malaria at 1:64 (n=4) and at 1:128 (n=1) dilutions, and TB at 1:64 dilutions (n=1). Even if the sera of these individuals were found to be negative by DAT and FAST no parasitological methods were employed to confirm VL. In addition the sera of the 10 healthy controls from non-endemic area and the 13 CL cases were non-reactive for IHA test, DAT and FAST (Table 2).

Table 2. Serologic reactivities of patients with documented VL, suspected VL, and other infections by IHA, DAT, and FAST for detection of anti-*Leishmania* antibodies

Study group	No. (%) seropositive				
	IHA (1:64)	IHA (1:128)	IHA (1:256)	DAT	FAST
VL - positive (n=37)	35 (94.6)	32 (86.5)	20 (54.1)	35 (94.6)	32 (86.5)
- negative (n=40)	16 (40.0)	9 (22.5)	3 (7.5)	14 (35.0)	13 (32.5)
CL (n=13)	0	0	0	0	0
Malaria (n=21)	4 (19.0)	1 (4.8)	0	0	0
TB (n=7)	1 (14.3)	0	0	0	0
Healthy control (n=10)	0	0	0	0	0

All clinically suspected individuals were subjected to spleen and lymph node aspiration, and parasitologically 48.1% (37/77) of VL suspected patients were confirmed to have VL by demonstration of parasites in smear and/or culture. IHA test detected 94.6% (35/37), 86.5% (32/37), and 54.1% (20/37) of the confirmed VL patients at 1:64, 1:128, and 1:256 dilutions respectively. However, 2, 5, and 17 sera were non-reactive to IHA test at the above cut-off titers respectively. DAT detected 94.6% (35/37) of documented VL patients and failed to detect 2 VL confirmed patients. However, FAST detected 88.9% (32/36) of confirmed VL patients and failed to detect 4 VL confirmed patients (Table 3). One confirmed and one suspected VL patients were not tested for FAST technique.

Table 3. IHA at various cut-off titers, DAT, and FAST comparison with parasitological methods in clinically suspected VL patients

		Parasitology			Measure of intrinsic validity	
		+	-	**Total**		
IHA(1:64)	+	35	16	**51**	Sensitivity = 94.6%	[80.5, 99.1]
					Specificity = 60.0%	[43.4, 74.7]
	-	2	24	**26**	PPV = 68.6%	[54.0, 80.5]
					NPV = 92.3%	[73.4, 98.7]
Total		**37**	**40**	**77**	κ = 0.538	
IHA (1:128)	+	32	9	**41**	Sensitivity = 86.5%	[70.4, 94.9]
					Specificity = 77.5%	[61.1, 88.6]
	-	5	31	**36**	PPV = 78.0%	[62.0, 88.9]
					NPV = 86.1%	[69.7, 94.8]
Total		**37**	**40**	**77**	κ = 0.637	
IHA(1: 256)	+	20	3	**23**	Sensitivity = 54.1%	[37.1, 70.2]
					Specificity = 92.5%	[78.5, 98.0]
	-	17	37	**54**	PPV = 87.0%	[65.3, 96.6]
					NPV = 68.5%	[54.3, 80.1]
Total		**37**	**40**	**77**	κ = 0.472	
DAT	+	35	14	**49**	Sensitivity = 94.6%	[80.5, 99.1]
					Specificity = 65.0%	[48.3, 78.9]
	-	2	26	**28**	PPV = 71.4%	[56.5, 83.0]
					NPV = 92.9%	[75.0, 98.8]
Total		**37**	**40**	**77**	κ = 0.582	
FAST	+	32	13	**45**	Sensitivity = 88.9%	[73.0, 96.4]
					Specificity = 66.7%	[49.7, 80.4]
	-	4	26	**30**	PPV = 71.1%	[55.5, 83.2]
					NPV = 86.7%	[68.4, 95.6]
Total		**36**	**39**	**75**	κ = 0.550	

N.B: Numbers in brackets are 95% CI

When IHA test is compared with parasitological methods, percent agreement of 76.6%, 81.8%, and 74.0% was observed at 1:64, 1:128, and 1:256 cut-off titers respectively, which show fair to moderate agreement with coefficient of kappa value 0.472 - 0.637. Table 4, shows sensitivity, specificity, positive predictive value, and negative predictive value of serological tests compared to parasitological methods.

Table 4. Sensitivity and specificity of serological tests

	DAT	FAST	IHA (1:64)	IHA (1:128)	IHA (1:256)
Sensitivity	94.6% [80.5, 99.1]	88.9% [73.0, 96.4]	94.6% [80.5, 99.1]	86.5% [70.4, 94.9]	54.1% [37.1, 70.2]
Specificity	65.0% [48.3, 78.9]	66.7% [49.7, 80.4]	60.0% [43.4, 74.7]	77.5% [61.1, 88.6]	92.5% [78.5, 98.0]
PPV	71.4% [56.5, 83.0]	71.1% [55.5, 83.2]	68.6% [54.0, 80.5]	78.0% [62.0, 88.9]	87.0% [65.3, 96.6]
NPV	92.9% [75.0, 98.8]	86.7% [68.4, 95.6]	92.3% [73.4, 98.7]	86.1% [69.7, 94.8]	68.5% [54.3, 80.1]

Sensitivity, specificity, positive predictive value and negative predictive value calculation was described in materials and methods section.
N.B: Numbers in brackets are 95% CI.

In clinically suspected VL patients, the overall seroprevalence of anti-leishmanial antibodies detected by IHA test at various cut-off titers were compared with DAT. IHA test detected 91.8% (45/49), 80.6% (40/49), and 44.9% (22/49) of DAT positive sera. However, IHA test failed to detect 4, 9, and 27 DAT positive samples at dilutions 1:64, 1:128, and 1:256 respectively, and 6 and 1 DAT negative samples were labeled IHA test positive at 1:64 and >1:128 dilutions respectively. As a result moderate agreement was observed between the two tests at the cut-off titer 1:128 ($\kappa = 0.736$) and 1:64 ($\kappa = 0.715$), however, poor agreement was showed at 1:256 ($\kappa = 0.345$) (Table 5).

Table 5. IHA test at various cut-off titers compared with DAT in the diagnosis of visceral leishmaniasis

		DAT			Measure of agreement
		+	-	**Total**	
IHA (1:64)	+	45	6	**51**	% agreement = 87.0
	-	4	22	**26**	κ = 0.715
Total		**49**	**28**	**77**	
IHA (1:128)	+	40	1	**41**	% agreement = 87.0
	-	9	27	**36**	κ = 0.736
Total		**49**	**28**	**77**	
IHA (1: 256)	+	22	1	**23**	% agreement = 63.6
	-	27	27	**54**	κ = 0.345
Total		**49**	**28**	**77**	

HIV antibodies were screened for 71 suspected VL cases and 28 were seropositive, out of which 18 (64.3%) were confirmed VL patients, and 43 were HIV negative of which 17 (39.5%) were confirmed VL patients. The performance of IHA test in the detection of anti-leishmanial antibodies in HIV negative confirmed and suspected VL cases were compared with parasitological methods. As it was shown in table 6, IHA test was positive in 17 HIV negative VL patients at 1:64 and 1:128 cut-off titers. However, IHA detected only 11 of the 17 HIV negative VL patients at 1:256 cut-off titer. The two tests showed excellent agreement at 1:128 cut-off titer (κ = 0.769) and moderate agreement at 1:64 and 1:256 cut-off titers with kappa coefficient of 0.559 and 0.641 respectively.

Table 6. IHA test at various cut-off compared with parasitological methods in the diagnosis of HIV negative VL suspects

		Parasitologically			
		+	-	**Total**	Measure of intrinsic validity at 95% CI
IHA (1:64)	+	17	10	**27**	Sensitivity = 100.0% [77.1, 100.0] Specificity = 61.5% [40.7, 79.1]
	-	0	16	**16**	PPV = 63.0% [42.5, 79.9] NPV = 100.0% [75.9, 100.0]
Total		**17**	**26**	**43**	κ = 0.559
IHA (1:128)	+	17	5	**22**	Sensitivity = 100.0% [71.1, 100.0] Specificity = 80.8% [60.0, 92.7]
	-	0	21	**21**	PPV = 77.3% [54.2, 91.3] NPV = 100.0% [80.8, 100.0]
Total		**17**	**26**	**43**	κ = 0.769
IHA (1: 256)	+	11	1	**12**	Sensitivity = 64.7% [38.6, 84.7] Specificity = 96.2% [78.4, 99.8]
	-	6	25	**31**	PPV = 91.7% [59.8, 99.6] NPV = 80.6% [61.9, 91.9]
Total		**17**	**26**	**43**	κ = 0.641

N.B: Numbers in brackets are 95% CI.

In table 7, the performance of IHA test in HIV/VL co-infected patients compared with parasitological method. Out of 28 HIV positive VL suspects 18 were confirmed to have VL, and IHA test was positive in 88.9% (16/18), 72.2% (13/18), and 38.9% (7/18) of HIV/VL co-infected patients at 1:64, 1:128, and 1:256 cut-off titers respectively. Among these cut-off titers, fair agreement was shown between the two tests at 1:64 cut-off titer with kappa coefficient of 0.417.

Table 7. Diagnostic performance of IHA test at various cut-off titers compared with parasitological methods in HIV positive VL suspects

		Parasitologically				
		+	-	**Total**	Measure of intrinsic validity at 95% CI	
IHA (1:64)	+	16	5	**21**	Sensitivity = 88.9%	[63.9, 98.1]
					Specificity = 50.0%	[20.1, 79.9]
	-	2	5	**7**	PPV = 76.2%	[52.4, 90.9]
					NPV = 71.4%	[30.3, 94.9]
Total		**18**	**10**	**28**	κ = 0.417	
IHA (1:128)	+	13	3	**16**	Sensitivity = 72.2%	[46.4, 89.3]
					Specificity = 70.0%	[35.4, 91.9]
	-	5	7	**12**	PPV = 81.3%	[53.7, 95.0]
					NPV = 58.3%	[28.6, 83.5]
Total		**18**	**10**	**28**	κ = 0.404	
IHA (1: 256)	+	7	1	**8**	Sensitivity = 38.9%	[18.3, 63.9]
					Specificity = 90.0%	[54.1, 99.5]
	-	11	9	**20**	PPV = 87.5%	[46.7, 99.3]
					NPV = 45.0%	[23.8, 68.8]
Total		**18**	**10**	**28**	κ = 0.236	

N.B: Numbers in brackets are 95% CI.

Comparing the diagnostic value of IHA test with DAT in HIV negative VL confirmed and suspected cases, IHA test was positive in 61.4%, 50.0%, and 27.3% of the 44 patients. IHA test was positive in 95.8%, 91.7%, and 50% of the 24 DAT positive sera. Consequently IHA failed to detect antibodies to *Leishmania* antigen in 1, 2, and 12 HIV negative with suspected and confirmed VL patients at different cut-off titers. The two tests showed strong agreement at 1:128 cut of titer with kappa coefficient of 0.909 and at 1:64 cut-off titer with kappa coefficient of 0.768; and fair agreement at 1:256 cut-off titer with kappa coefficient of 0.476 (Table 8).

Table 8. IHA test performance at various cut-off titers compared with DAT for the detection of anti-*Leishmania* antibodies in HIV negative VL suspects

		DAT			
		+	-	**Total**	Measure of agreement
IHA (1:64)	+	23	4	**27**	% agreement = 88.6
	-	1	16	**17**	κ = 0.768
Total		**24**	**20**	**44**	
IHA (1:128)	+	22	0	**22**	% agreement = 95.5
	-	2	20	**22**	κ = 0.909
Total		**22**	**20**	**44**	
IHA (1: 256)	+	12	0	**12**	% agreement = 72.7
	-	12	20	**32**	κ = 0.476
Total		**24**	**20**	**44**	

when diagnostic value of IHA test compared with DAT in HIV positive VL confirmed and suspects, IHA test detected 86.4%, 68.2%, and 31.8% of the 22 DAT positive sera (Table 9). In this group the two tests showed a fair agreement only at 1:64 cut-off titer with kappa coefficient of 0.43. This might indicate in HIV positive VL suspects and confirmed cases, the two tests show poor agreement compared to the HIV negative cases.

Table 9. Performance of IHA test at various cut-off titers compared with DAT in HIV positive VL suspects

		DAT			
		+	-	**Total**	Measure of agreement
IHA (1:64)	+	19	2	**21**	% agreement = 81.5
	-	3	3	**6**	κ = 0.430
Total		**22**	**5**	**27**	
IHA (1:128)	+	15	1	**16**	% agreement = 70.4
	-	7	4	**11**	κ = 0.329
Total		**22**	**5**	**27**	
IHA (1: 256)	+	7	1	**8**	% agreement = 40.7
	-	15	4	**19**	κ = 0.057
Total		**22**	**5**	**27**	

4.2 Epidemiological Study

In an epidemiological study conducted in Feb, 2000 a total of 116 subjects were found to have had an enlarged spleen and/or liver. Of these, 57 were considered as VL suspects based on the presence of fever for at least 2 weeks in duration (Table 1). Out of these 57 suspected cases, lymph node aspiration was done for 25 subjects based on the clinical symptoms of the patients and 19 were confirmed to have VL by direct demonstration of *Leishmania* amastigotes in tissue samples.

All 19 samples with documented VL were shown to have significant levels of antibodies to *Leishmania* antigen by IHA test at various cut-off titer, DAT and FAST, except one serum sample that is classified as IHA test false-negative at cut-off titer 1:256 compared to the parasitological result (Table 10). Six parasitologically negative, but clinically

suspected VL cases subjected to serological tests gave 3 positive and 3 negative results except for IHA at 1:64 dilutions that gave 4 positive and 2 negative results.

The measure of association for IHA titer 1:64, 1:28, 1:256 compared to parasitological data showed sensitivity of 100.0%, 100.0%, and 94.7%, specificity of 33.3%, 50.0%, and 50.0%, positive predictive value of 82.6%, 86.4, and 85.7%, and negative predictive value of 100.0%, 100.0%, and 75.0% respectively. When compared with parasitological data, DAT or FAST presented a sensitivity of 100.0%, a specificity of 50.0%, a PPV of 86.4%, and NPV of 100.0%. As a result, fair to moderate agreement was observed among the serological tests compared to parasitological method with kappa value 0.432-603 (Table 10).

Table 10. IHA test result at various cut-off titers, DAT, and FAST in comparison with parasitological methods in aspirated subjects

		Parasitological				
		+	-	**Total**	Measure of intrinsic validity at 95% CI	
IHA (1:64)	+	19	4	**23**	Sensitivity = 100.0%	[79.1, 100.0]
					Specificity = 33.3%	[6.0, 75.9]
	-	0	2	**2**	PPV = 82.6%	[60.5, 94.3]
					NPV = 100.0%	[19.8, 100.0]
Total		**19**	**6**	**25**	κ = 0.432	
IHA (1:128)	+	19	3	**22**	Sensitivity = 100.0%	[79.1, 100.0]
					Specificity = 50.0%	[13.9, 86.1]
	-	0	3	**3**	PPV = 86.4%	[64.0, 96.4]
					NPV = 100.0%	[31.0, 100.0]
Total		**19**	**6**	**25**	κ = 0.603	
IHA (1: 256)	+	18	3	**21**	Sensitivity = 94.7%	[71.9, 99.7]
					Specificity = 50.0%	[13.9, 86.1]
	-	1	3	**4**	PPV = 85.7%	[62.6, 96.2]
					NPV = 75.0%	[21.9, 98.7]
Total		**19**	**6**	**25**	κ = 0.505	
DAT/FAST	+	19	3	**22**	Sensitivity = 100.0%	[79.1, 100.0]
					Specificity = 50.0%	[13.9, 86.1]
	-	0	3	**3**	PPV = 86.4%	[64.0, 96.4]
					NPV = 100.0%	[31.0, 100.0]
Total		**19**	**9**	**25**	κ = 0.603	

N.B: Numbers in brackets are 95% CI.

In a community survey conducted at Abaroba area where VL is endemic, 1217 sera were tested using IHA test, DAT and FAST, out of which 145, 135, and 88 sera showed significant levels of antibodies to *Leishmania* by IHA at a cut-off titer 1:64, 1:128 and 1:256 respectively. However, DAT showed 153 sera positive. In this case, large numbers of discrepant results were observed between DAT and IHA that include: 65, 21, and 12 samples that were IHA test positive but DAT negative at IHA cut-off titer 1:64, 1:128, and 1:256 respectively. On the other hand 8, 18, and 65 DAT positive sera were non-reactive to IHA test at cut-off titer 1:64, 1:128, and 1:256 respectively. As a result, DAT show a prevalence of 12.5% while IHA show a prevalence of 17.3%, 12.8%, and 8.2% respectively. This indicated that, in the community DAT and IHA (at 1:128 cut-off titer) showed similar anti-leishmanial antibodies prevalence. With respect to FAST, 64 negative sera tested IHA test positive at cut-off titer1:64 (data not shown). IHA test compared to DAT showed an excellent agreement of 96.8% and 94.0% at 1:128 ($\kappa = 0.885$) and 1:64 ($\kappa = 0.765$) cut-off titers respectively; and moderate agreement of 93.7% at 1:256 cut-off titer ($\kappa = 0.662$) (Table 11).

Table 11. Performance of IHA test at various cut-off in comparison with DAT for detection of anti-leishmanial antibodies in the study population

		DAT			Measure of agreement and prevalence
		+	-	**Total**	
IHA (1:64)	+	145	65	**210**	Prevalence (DAT) = 12.5%
	-	8	999	**1007**	Prevalence (IHA) = 17.3%
Total		**153**	**1064**	**1217**	% agreement = 94.0 κ = 0.765
IHA (1:128)	+	135	21	**156**	Prevalence (DAT) = 12.5%
	-	18	1043	**1061**	Prevalence (IHA) = 12.8%
Total		**153**	**1064**	**1217**	% agreement = 96.8 κ = 0.885
IHA (1: 256)	+	88	12	**100**	Prevalence (DAT) = 12.5%
	-	65	1052	**1117**	Prevalence (IHA) = 8.2%
Total		**153**	**1064**	**1217**	% agreement = 93.7 κ = 0.662

The performance of IHA test and DAT for the detection of antibodies to *Leishmania* antigen in confirmed VL patients, VL suspected, and in endemic control groups excluding previously treated subjects are indicated in Table 12. IHA test was positive in 100% (19/19), 100% (19/19%), and 94.7% (18/19) of confirmed VL; 31.6% (12/38), 26.3 (10/38), and 23.7% (9/38) of clinical suspected cases; and 8.8% (91/1037), 4.3% (45/1037), and 2.1% (22/1037) of endemic control at 1:64, 1:128, 1:256 cut-off titer respectively.

Table 12. Sero-prevalence of anti-leishmanial antibodies based on different IHA cut-off titer and DAT in subjects with and without symptoms of VL and controls

	No. tested	DAT/FAST pos. No. (%)	No. (%) positive in different IHA cut-off titer		
			(1:64)	(1:128)	(1:256
VL patients	19	19 (100)	19 (100)	19 (100)	18 (94.7)
Clinical suspects	38	8 (21.1)	12 (31.6)	10 (26.3)	9 (23.7)
Endemic controls	1037	36 (3.5)	91 (8.8)	45 (4.3)	22 (2.1)

Table excludes all individuals with past history of treatment for VL.

Seroprevalence of anti-leishmanial antibody profile assayed by IHA and DAT in treated VL cases was summarized in Table 13. Out of 132 previously treated visceral leishmaniasis patients 73.5% were DAT positive, 72.0%, 67.4%, and 25.8% sera were IHA test positive at 1:64, 1:128 and 1:256 cut-off titer respectively. However, 4 DAT negative samples were IHA positive at 1:64 cut-off titer, and at 1:128 and 1:256 cut-off titers DAT negative IHA test positive sera were 3 (data not shown).

Table 13. Sero-prevalence of anti-leishmanial antibodies based on various IHA cut-off titers by previous history of treatment for VL

Past history of VL	No. tested	DAT/FAST pos. No. (%)	No. (%) positive in different IHA cut-off titer		
			(1:64)	(1:128)	(1:256)
No treatment	1085	56 (5.2)	115 (10.6)	67 (6.2)	44 (4.1)
Previously treated	132	97 (73.5)	95 (72)	89 (65.2)	34 (25.8)
Total	1217	153 (12.6)	210 (17.3)	156 (12.8)	48 (3.9)

The prevalence of ant-leishmanial antibodies depend clearly on age and sex. Serologic profile of IHA showed distinct variations among age groups. The sero-conversion had occurred at the mid-age of 4.5 to 14.5 and peak sero-prevalence sustained from mid-age of 14.5 to 34.5 (Figure 3). Twice as many males as females were sero-positive in IHA test (Figure 4), this difference was statistically significant (OR = 2.4; 95% confidence interval [1.75, 3.38]; uncorrected $\chi^2 = 31.7$; $P < 0.05$).

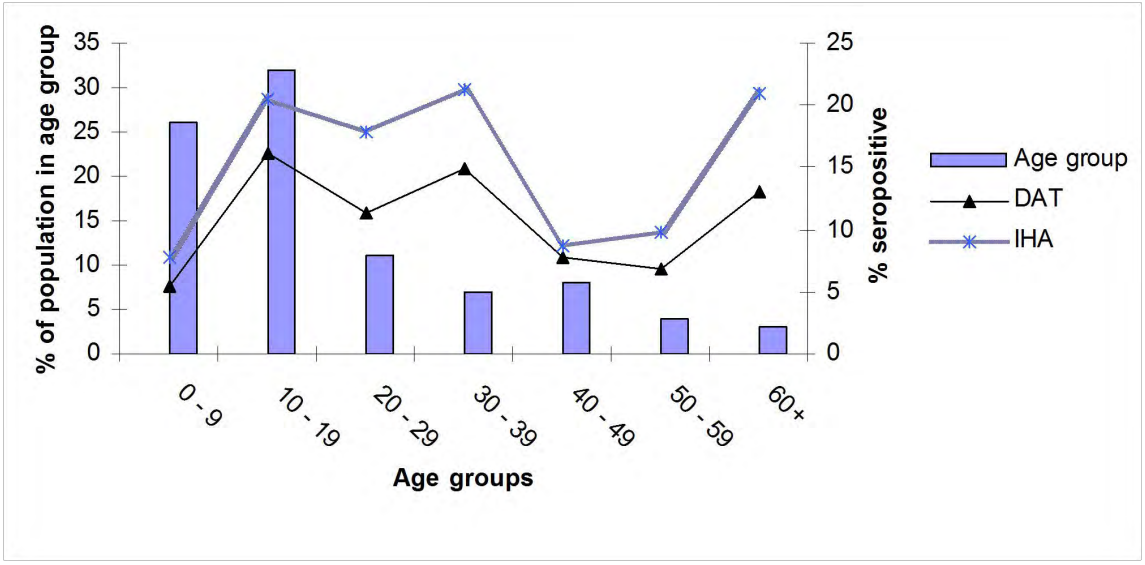
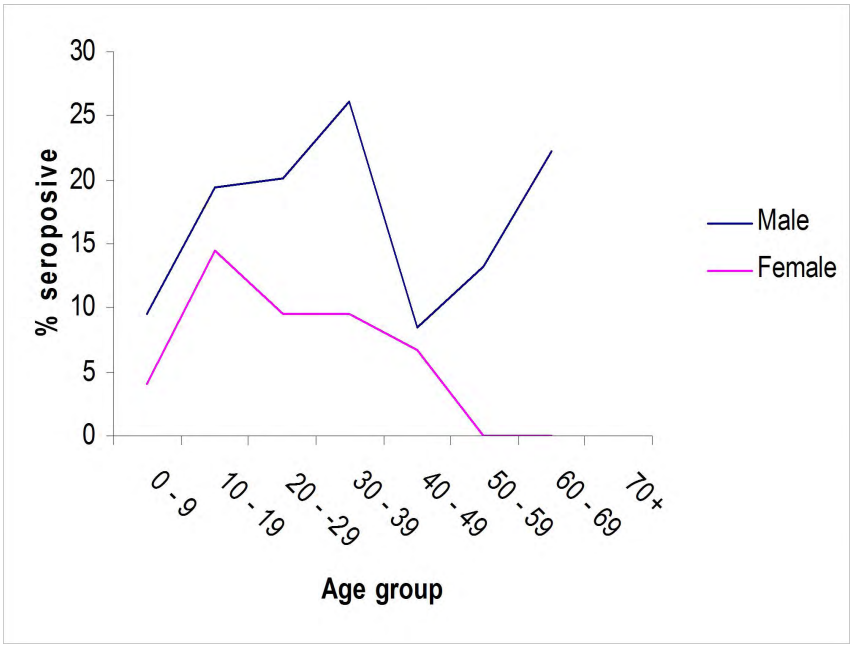


Fig. 3. Seroprevalence of anti-leishmanial antibodies measured by IHA (at 1:128 cut-off titer) and DAT shown by age group. Bars are percent of population tested in each age group; lines show percentage seropositive by IHA and DAT in the different age groups.



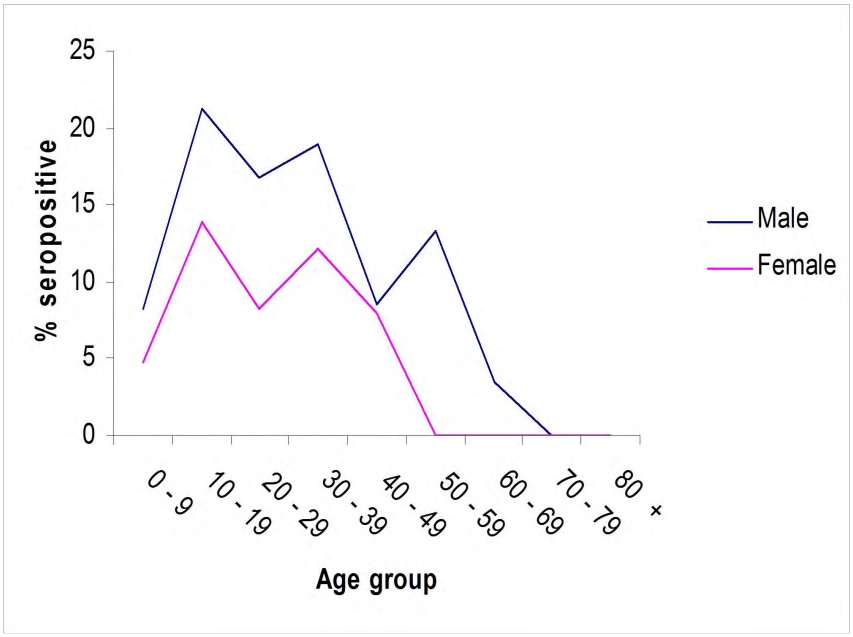


Fig. 4. Overall sero-prevalence of anti-leishmanial antibodies measured by IHA (at 1:128 cut-off titer) (top) and DAT (bottom). Lines show percentage seropositive by sex in the different age groups.

5 DISCUSSION

In the diagnosis of VL, serology is an indirect supportive method, while parasite demonstration is confirmatory (Bryceson, 1996). The parasitological tests, involving invasive procedures of organ aspiration, are very specific (100%), but their sensitivity varies from 50 - 60% in aspirates of enlarged lymph nodes, over 60-80% in bone marrow aspirates to 95% in splenic aspirate (Zijlstra *et al*. 1992; Bryceson, 1996; Thakur, 1997). However, parasitological diagnosis presents practical difficulties, given the scarcity of amastigotes, shortage of trained staff and modest facilities available at district hospitals.

During VL, although not protective high titers of anti-leishmanial antibodies are present (Braz *et al*., 2002). As a result, development of sensitive serological test with sufficiently high specificity can replace parasitology for clinical practice and for VL control. It is less invasive for the patient, relatively easy, and many samples can be processed simultaneously. In this respect, this work evaluates the performance of one of the serological tests, IHA test in the diagnosis and epidemiological studies of visceral leishmaniasis.

In the diagnostic study, the combined parasitological examinations (culture and/or smear), confirmed the presence of *Leishmania* parasite in 48.1% of clinically suspected VL patients. IHA test (at 1:64 cut-off titer), DAT, and FAST tests showed positive reaction in 66.2%, 63.6%, and 60.0%, of the suspected patients respectively, which show relatively high sensitivity in IHA compared to DAT and FAST. Of 37 parasitologically confirmed VL patients, IHA test (at 1:64 cut-off titer) and DAT sensitivity appeared to be similar while, IHA test (at 1:128 cut-off titer) and FAST test showed sensitivity of 86.5% and 88.9% respectively.

The sensitivity of IHA test at 1:64 dilutions is comparable to that of DAT, 94.6% than FAST techniques in clinically suspected VL patients. Of the three serological tests, IHA (at 1:64 cut-off titer) was as sensitive as DAT and relatively 60% specific as DAT for the

detection of anti-*Leishmania* antibodies (Table 5). However, FAST showed lower sensitivity of 89% and similar specificity as that of DAT 65%.

Despite the fact that, IHA at 1:256 and 1:128 cut-off titers showed a better specificity of 93% and 78%, the sensitivity was low, i.e., 54% and 87% respectively. As the cut-off titer of IHA increases the test failed to detect specific antibodies. In 2 patients with documented VL, the IHA test failed to detect anti-leishmanial antibodies. This is possibly due to removal of the specific anti-leishmanial antibodies by sensitized SRBCs that used for absorption of non-specific antibodies. The crude parasite lysates or undefined antigens used could also be responsible for low specificity of IHA. Furthermore, the weak antibody responses elicited, ethnic background, the severity of infection and time since infection may contribute to the failure to detect anti-leishmanial antibodies in two patients as previous studies indicated (Shiddo *et al*., 1995).

Lack of gold standard, exact knowledge of the true disease status of the tested individuals can seriously under- or overestimate (Valenstein, 1990) the result of the new test. In this study, a combination of parasitological examination of lymph node and splenic aspirate was used as the golden standard with a high sensitivity. As a result, the IHA specificity might be underestimated. However, with our particular set of serum samples and antigen prepared from *L. donovani* promastigotes, optimal sensitivity and specificity for IHA was established at a cut-off titer 1:64 or 1:128 (see table 5). However, the choice of cut-off titer with regard to sensitivity and specificity depends on diagnostic or epidemiological purpose.

Several authors reported that DAT showed high specificity in the range of 90 - 100% on healthy controls from endemic areas (Abdel Hameed *et al*. 1989; Schaefer *et al*. 1995; Shiddo *et al*. 1995; Boelaert *et al*. 1999), although healthy controls in these studies were, however, not representative for the clinical setting. In this study, the specificity was found to be 65%. The highest specificity of IHA was 93% at 1:256 dilutions, whereas the sensitivity was very low (54%). At the cut-off titers of IHA 1:64 and 1:128, the specificities were 60% and 78% respectively.

The weakness of serological tests including IHA specificity in clinically suspected persons is might be partially due to inherent weakness of the imperfect gold standard. Parasitology, or response to treatment could not make a gold standard for *L. donovani* infection in humans, given the substantial proportion of asymptomatic infection in the community (Schaefer *et al*., 1995; Seaman *et al*., 1992; Zijlstra *et al*., 1994) and to a lesser degree, the persistence of the serological response in treated VL patients (Hailu 1990). In clinical suspects with negative parasitology, Zijlstra *et al*. (1991) estimated the specificity of DAT to be 72%. That is why some clinicians do not consider a positive DAT result as sufficient evidence for starting treatment. Instead the clinicians prefer to combine DAT and parasitology. Similarly, the 78% specificity of IHA at the cut-off titer 1:128 requires further study in spectrum of a clinic situation in which a test for VL disease will be applied. In earlier study, IHA showed sensitivity of 100% and specificity of 86% for sera from VL patients (Iqbal *et al*., 2002). This was in contrast to the present finding, where a sensitivity of 94.6% or 87% and specificity of 60% and 77.5% respectively at 1:64 and 1:128 cut-off titer in the diagnostic study.

IHA showed positive reactions for patients with malaria and TB. Such non-specific cross-reactions have been reported earlier (Iqbal *et al*., 2002; Bardaro *et al*., 1996; Sinha and Sehgal, 1994; Zijlstra *et al*., 1992). In VL endemic area, malaria is co-endemic, tropical splenomegaly syndrome (TSS) is also prevalent. As a result, VL and TSS patients will normally report with complaints of enlarged spleen and with or without fever. In this study, there is no evidence to suggest that confirmed malaria and IHA positive cases are due to false positive reaction, however, they might have sub-clinical infection.

In this setting, the availability of a diagnostic tool for VL such as IHA, or DAT is crucial. The main inconvenience in IHA was the use of crude antigen that might share common epitopes with other parasite antigens. Previously it was suggested that, antibodies against malaria, TB cross react in the IHA for the anti-leishmanial antibodies at dilutions up to 1:128. The diagnostic titer should be raised to 256 in order to increase specificity of the

test in areas where such parasites occurs (Morsy *et al*., 1990). However, the sensitivity of the test decreases.

In clinically suspected persons with a negative IHA, the disease can be ruled out with a certain degree of confidence but there will be a need for scheduled follow-up. However, with the presently available evidence, treatment can not be recommended if a clinically suspected person has a positive IHA based on the IHA result alone. In an endemic situation there is a need for parasitological analysis. In an epidemic situation, however, treatment based on the IHA result might be justified.

In this study 89%, 72%, and 39% serum samples of HIV/VL coinfected patients were positive in the IHA at 1:64, 1:128, and 1:256 cut-off titers respectively. It was previously reported that 89% of HIV/VL coinfection were DAT positive (Oskam *et al*., 1999). This is in agreement with present study of IHA 89% at 1:64 cut-off titer. It was also reported that in HIV/VL coinfection the results with serology are variable, ranging from 6 – 82% of the serum samples being positive (Reviewed in: Gari-Toussaint *et al*., 1994; Alvar *et al*., 1997). Others also suggested that HIV patients, who had acquired their *Leishmania* infection before being infected with HIV, may produce high antibody titers, whereas patients who acquired the HIV infection before the *Leishmania*, may be impeded in their antibody response. In contrast to the situation in Italy (Gradoni *et al*., 1993), a high percentage of Ethiopian HIV/VL coinfected patients are considered to contract *Leishmania* first.

In this study it was shown that the IHA and DAT tests showed 96% agreement (strong) in HIV negative VL cases and 70% agreement (poor) in HIV positives VL cases. Unlike what happens in immunocompetent patients indirect hemagglutination showed lower sensitivity (78%) than DAT (82%) in HIV positive VL cases which is in agreement to the study of Lopez-Velez *et al*. (1995).

The indirect hemagglutination test is a fast (3 hours of incubation) and simple technique, showing high sensitivity and can be applied in diagnostic and

epidemiological studies. However, among the main disadvantage of the IHA is that it shares with DAT the persistence of antibody titers after apparent cure (Hailu, 1990), but avoids long incubation of 18 hours.

In the epidemiological study, the prevalence of anti-leishmanial antibody detected by IHA (at1:128 cut-off titer) and DAT were almost identical, however, it is higher than that of DAT at a cut-off titer 1:64. In 25 suspected VL patients, 2 were excluded by IHA (at 1:64 cut-off titer) and 3 by IHA (1:128 cut-off titer), 3 by DAT and FAST. However, IHA (at 1:256 cut-off titer) excluded 4 patients including 1 confirmed VL patient. This might be due to the presence of samples from patients with recent infections, in those the antibody levels might be low (Shiddo *et al*., 1995) or due to removal of specific anti-leishmanial antibodies during absorption of non-specific reactions from the sera.

In Table 12, it was shown that out of 1035 asymptomatic individuals, 91, 45, and 22 were IHA seropositive at a cut-off titer 1:64, 1:128, and 1:256 respectively, and 36 were DAT/FAST seropositive. This could be a reflection of positive serological test in the absence of clinical illness (e.g., past infection or preclinical VL) or cross-reactivity with other infectious organisms (Badaro *et al*., 1996).

It is well documented that individuals with VL may remain seropositive by conventional serological tests for months or even years following successful treatment of the active infection (Hailu, 1990; Zijlstra *et al*., 1991; Oskam *et al*., 1999; Hailu *et al*., 2002). In this respect, DAT and FAST were 74% seropositive in VL patients many years after treatment, and IHA was seropositive in 72%, 65%, and 26% of previously treated VL patients at a cut-off titer 1:64, 1:128, and 1:256. Therefore, IHA as a sole diagnostic test for patients with clinically suspected VL is not sufficient. Its main inconvenience is that as a crude antigen, it may share common epitopes with other infectious disease (Badaro *et al*., 1986).

Other serological tests like ELISA have also been used for diagnosis and epidemiological screening of visceral leishmaniasis, although the specificity depends upon a particular antigen used. The most commonly used antigen is crude soluble

antigen derived by lysing the *Leishmania* promastigotes. Its sensitivity ranges from 80-100%, but like that of IHA test presented herein, cross-reactions with sera from patients with malaria and Tuberculosis have been recorded (Sundar and Rai, 2002; Sinha and Sehgal, 1994).

The prevalence of anti-leishmanial antibodies in endemic areas is clearly age dependent, showing an increase with age. This age profile of serology probably shows the pattern of life time exposure and herd immunity rather than pattern of recent infection. Statistically significant difference was also observed by IHA test between males and females as previously shown (Hailu *et al*., 2002). Adult males reacted more commonly than adult females. This is might be due to the higher reaction rate among adult males whose ordinary activity at least in a rural area, are different from those of children and women who spend more time indoors and in the village (Moral *et al*., 2002).

Even if, the availability of IHA can help to exclude significant number of subjects with signs and symptoms of VL from being subjected to organ aspiration, IHA test was shown to have important limitations. Of these false positive results in healthy controls from endemic areas, cross-reactivity, repeated sheep bleeding, preparation of cells, the need for electricity and cold chain severely limit its application in the field.

6. CONCLUSIONS AND RECOMMENDATION

6.1 CONCLUSIONS

The control measures of leishmaniasis relys on sensitive, specific, reproducible and cheap diagnostic test. The currently available diagnostic tools however have their own limitations, which can seriously compromise the success of the case detection strategy. Parasitology has a weak sensitivity, in lymph node and bone marrow aspirates. Splenic aspirate perform better, but are hardly feasible in district hospitals. Promising serological tests, as the DAT, have been developed but diagnostic algorithm for their use by the health services are not yet available. Development of serological test with high sensitivity and specificity is needed for clinical practice and for VL control. Furthermore serological tests have additional advantage of being less invasive for patients.

Compared to the available serological tests IHA test was found to be relatively simple, cheap, and fast (of 3 hrs compared to18hrs incubation of DAT). However, it shares certain similarities with other serological tests, like cross-reactivity, failure to distinguish current, subclinical and past infections.

The IHA test was shown to have similar sensitivity, specificity, PPV, and NPV with DAT at 1:64 cut-off titer, whereas the IHA test at a titer of 1:128 was found to be comparable to FAST. Although the IHA test at 1:256 titer was more specific, the sensitivity was very low. Therefore, the IHA test at 1:64 titer could be used for diagnostic test. In the evaluation of the use of IHA test in epidemiological study, the IHA test showed similar sensitivity and specificity with DAT and FAST at a cut-off titer 1:128.

IHA and DAT showed strong agreement in HIV negative VL patients at a cut-off titer 1:128 than at 1:64 and 1:256. On the other hand in HIV positive VL subjects, IHA at various cut-off titer and DAT exhibited moderate to poor agreement.

Thus the IHA test could be an alternative for DAT and parasitology in the clinical diagnosis and epidemiological studies of VL at least in epidemic VL situation. Considering the number and diversity of samples tested in this study, the results obtained with the IHA test was encouraging. However, further research is needed on the application of IHA for detection of VL disease in persons with clinical symptoms.

Whenever the IHA test is used in a decision, the cut-off titer for diagnosis should be carefully weighted against its predictive values according to the prevailing prevalence rates. The cut-off for decision making depends furthermore on the case-mix, and might vary depending on whether the test is used for active case detection in the community, or for passive case detection.

The IHA technique is rapid, simple, inexpensive, and can be applied for a large-scale studies and routine use.

Cross-reactivity, positive result in healthy individuals from endemic area repeated fresh red blood cell preparation and the need for electricity are the important drawback of IHA test.

6.2 RECOMENDATIONS

Development of more sensitive and specific diagnostic test is fundamental in the control and management of visceral leishmaniasis. Based on the above findings, the following studies are recommended:

- Study in improvement of antigen preparation for IHA test.
- Looking for techniques that could minimize cross-reactivity.
- Further evaluation of IHA in terms of reproducibility and repeatability in the diagnostic and epidemiological screening of VL.
- Further research on test validation of IHA with careful attention on the choice of gold standard.
- Prospective analysis of the IHA test in HIV patients living in endemic areas of VL.

7 REFERENCES

Abdel-hameed AA, Harith AE, Abdel-Rahim IM. (1989) Potential of a direct agglutination test (DAT) for the detection of visceral leishmaniasis in a known endemic area in Sudan. Trop Med Parasitol 40:470-471.

Addy M, Nandy A. (1992) Ten years of kala-azar in West Bengal, part I. did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas? Bull World Health Organ 70:341-346.

Ali A, Ashford RW. (1994) Visceral leishmaniasis in Ethiopia. IV. Prevalence incidence and relation of infection to disease in an endemic area. Ann Trop Med Parasitol 88:289-293.

Alvar J, Canavate C, Gutierrez-Solar B, Jimenez M, Laguna F, Lopez-Velez R, Molina R, Moreno J. (1997) *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. Clin Microbiol Rev 10:298-319.

Alvar J. (1994) Leishmaniasis and AIDS co-infection: The Spanish example. Parasitol Today 10:160-163.

Arias JR, Monteiro PS, Zicker F. (1996) The re-emerging of visceral leishmaniasis in Brazil. Emerg Infect Dis 2:145-146.

Ashford RW, Bray MA, Hutchinson M, Bray RS. (1973) Epidemiology of cutaneous leishmaniasis in Ethiopia. Trans R Soc Trop Med Hyg 67:568-601.

Ashford RW. (2000) The leishmaniases as emerging and reemerging zoonoses. Int J Parasitol 30:1269-1281.

Ayele T, Ali A. (1984) The distribution of visceral leishmaniasis in Ethiopia. Am J Trop Med Hyg 33:548-552.

Badaro R, Benson D, Eulalio MC, Freire M, Cunha S, Netto EM, Pedral-Sampaio D, Madureira D, Burns JM, Houghton RL, David JR, Reed SG. (1996) rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. J Infect Dis 173:758-761.

Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, Teixeira R, Johnson WD Jr. (1986) New perspectives on a subclinical form of visceral leishmaniasis. J Infect Dis 154:1003-1011.

Badaro R, Reed SG, Carvalho EM. (1983) Immunofluorescent antibody test in American visceral leishmaniasis: sensitivity and specificity of different morphological forms of two *Leishmania* species. Am J Trop Med Hyg 32:480-484.

Balkew M, Hailu A, Berhe N, Gemetchu T. (1999) Leishmaniasis in the lower Omo plains, south-western Ethiopia: the sand fly fauna. Ethiop Med J 37:31-40.

Barbosa-de-Deus R, dos Mares-Guia ML, Nunes AZ, Costa KM, Junqueira RG, Mayrink W, Genaro O, Tavares CAP. (2002) *Leishmania major*-like antigen for specific and sensitive serodiagnosis of human and canine visceral leishmaniasis. Clin Diag Lab Immunol 9:1361-1366.

Bardaro R, Benson D, Evlalio C, FreireM, Cunha S, Netto EM, Pedral-Sampaio D, Madureira C, Burns M, Houghton RL, David JR, Reed SG. (1996) rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. J Infect Dis 173:758-761.

Berhe N, Hailu A, Abraham Y, Tadesse Y, Breivik K, Abebe Y. (2001) Inter-current and nosocomial infections among visceral leishmaniasis patients in Ethiopia: an observational study. Acta Trop 80:87-95.

Berhe N, Hailu A, Wolday D, Negesse Y, Cenini P, Frommel D. (1995) Ethiopian visceral leishmaniasis patients co-infected with human immunodeflciency virus. Trans R Soc Trop Med Hyg 89:205-207.

Berman JD, Gallalee JF, Gallalee JV. (1988) Pharmacokinetics of pentavalent antimony (pentostam) in hamsters. Am J trop Med Hyg 39:41-47.

Berman JD. (1997) Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin Infect Dis 24:684-703.

Bern C, Jha SN, Joshi AB, Thakur GD, Bista MB. (2000) Use of the recombinant k39 dipstick test and the direct agglutination test in a setting endemic for visceral leishmaniasis in Nepal. Am J Trop Med Hyg 63:153-157.

Bernier R, Turco SJ, Olivier M, Tremblay M. (1995) Activation of human immunodeficiency virus type-1 in monocytoid cells by the protozoan parasite *Leishmania donovani*. J Virol 69:7282-7285.

Blank C, Bogdan C, Bauer C, Erb K, Moll H. (1996) Murine epidermal Langerhans cells do not express inducible nitric oxide synthase. Eur J Immunol 26:792-796.

Boelaert M, El Safi S, Jacquet D, De Muynck A, Stuyft PVD, Le Ray D. (1999) Operational validation of the direct agglutination test for diagnosis of visceral leishmaniasis. Am J Trop Med Hyg 60:129-134.

Bogdan C, Gessner A, Solbach W, Rollinghoff M. (1996) Invasion, control and persistence of Leishmania parasites. Curr Opin Immunol 8:517-525.

Bottler RLA, Dutra WO, Martins FA, Gontije B, Carvalho E, Barral-Netto M, Barral A, Almeida RP, Mayrink W, Locksley R, Gollob KJ. (2001) Flow cytometric determination of cellular sources and frequencies of key cytokine-producing lymphocytes directed against recombinant LACK and soluble leishmanial antigen in human cutaneous leishmaniasis. Infect Immun 69:3232-3239.

Bray RS, Lainson R. **(**1967) Studies on the immunology and serology of leishmaniasis. V. The use of particles as vehicles in passive agglutination tests. Trans R Soc Trop Med Hyg 61:490-505.

Braz RF, Nascimento ET, Martins DR, Wilson ME, Pearson RD, Reed SG, Jeronimo SM. (2002) The sensitivity and specificity of Leishmania chagasi recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. Am J Trop Med Hyg 67:344-348.

Brodskyn C, DeKrey GK, Titus RG. (2001) Influence of costimulatory molecules on immune response to *Leishmania major* by human cells *in vitro*. Infect Immun 69:665-672.

Bryceson AD. (1996) Leishmaniasis. In: Manson's Tropical Diseases, Cokk GC, (ed), 20th edn, Saunders, London, Philadelphia, Toronto, pp. 1213-1246.

Campbell-Lendrum D**,** Dujardin JP, Martinez E, Feliciangeli MD, Perez JE, Silans LN, Desjeux P. (2001) Domestic and peridomestic transmission of American cutaneous leishmaniasis: changing epidemiological patterns present new control opportunities. Mem Inst Oswaldo Cruz 96:159–162.

Cerf BJ, Jones TC, Badaro R, Sampaio D, Teixeira R, Johnson WD. (1987) Malnutrition as a risk factor for severe visceral leishmaniasis. J Infect Dis 156:1030-1033.

Choi CM, Lerner EA. (2001) Leishmaniasis as an emerging infection. J Investig Dermatol Symp Proc 6:175-182.

Davies CR, Kaye P, Croft SL, Sundar S. (2003) Leishmaniasis: new approach to diseases control. BMJ 326: 377-382.

de Beer P, EI Harith A, Deng LL, Semiao Santos SJ, Chantal B, van Grootheest M. (1991) A killing disease epidemic among displaced Sudanese population identified as visceral leishmaniasis. Am J Trap Med Hyg 44:283-289.

Desjeux P, Alvar J. (2003) Leishmania/HIV co-infections: epidemiology in Europe. Ann Trop Med Parasitol. 97: 3–15.

Desjeux p, UNAIDS. (1998) Leishmania and HIV in Gridlock. Document WHO/CTD/LEISH/98.9 Add. I, UNAIDS/98.23. Geneva: World Health Organisation.

Desjeux P. (1996) Leishmaniasis. Public health aspects and control. Clinics in Dermatol 14:417-423.

Desjeux P. (1999) Global control and *Leishmania*/HIV co-infection. Clin Dermatol 17:317–325.

Desjeux P. (2001) The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg 95:239-243.

Engwerda CR, Kaye PM. (2000) Organ-specific immune response associated with infectious disease. Immunol Today 21:73-78.

Enk CD,Gardlo K, Hochberg M, Ingber A, Ruzicka T. (2003) Cutaneous leishmaniasis. Hautarzt 54:506-512.

Fargeas C, Hommel M, Maingon R, Dourado C, Monsigny M, Mayer R. (1996) Synthetic peptide-based enzyme-linked immunosorbent assay for serodiagnosis of visceral leishmaniasis. J Clin Microbiol 34:241-248.

Gari-Toussaint M, Lelievre A, Marty P, Le Fichoux Y. (1994) Contribution of serological tests to the diagnosis of visceral leishmaniasis in patients infected with the human immunodefIciency virus. Trans R Soc Trop Med Hyg 88:301-302.

Gebre-Michael T, Balkew M. (2002) On the occurrence of *Phelebotomus (Paraphelebotomus) mireillae* (Diptera: Psychodidae) in Ethiopia. Ann Trop Med Parasitol 96:421-425.

Gebre-Michael T, Lane RP. (1996) The Roles of *Phelebotomus martini* and *P. celiae* (Diptera: Phlebotominae) as vectors of visceral leishmaniasis in the Aba-Roba focus, Southern Ethiopia. Med Vet Entomol 10:53-62.

Gebre-Michael T, Partlong F, Lane RP. (1993) *Phelebotomus (Phelebotomus) duboscqi* (Diptera: Phlebotominae), naturally infected with *Leishmania major* in southern Ethiopia. Trans R Soc Trop Med Hyg 87:10-11.

Gradoni L, Scalone A, Gramiccia M. (1993) HIV-Leishmania co-infections in Italy: serological data as an indication of the sequence of acquisition of the two infections. Trans R Soc Trop Med Hyg 87:94-96.

Grech V, Mizzi J, Mangion M, Vella C. (2000) Visceral leishmaniasis in Malta: an 18 year paediatric, population based study. Arch Dis Child 82:381-385.

Greil J, Bodendorfer B, Rollinghoff M, Solbach W. (1998) Application of recombinant granulocyte-macrophage colony-stimulating factor has a detrimental effect in experimental murine leishmaniasis. Eur J Immunol 18:1527-1533.

Hailu A, Balkew M, Berhe N, Meredith SE, Gemetchu T. (1995) Is *Phlebotomus* (*Larroussius*) *orientalis* a vector of visceral leishmaniasis in South-West Ethiopia? Acta Tropica 60:15-20.

Hailu A, Berhe N, Sisay Z, Abraham I, Medhin G. (1996) Seroepidemiological and leishmanin skin test surveys of visceral leishmaniasis in South and Southwest Ethiopia. Ethiop Med J 34:11-23.

Hailu A, Berhe N. (2002) The performance of direct agglutination tests (DAT) in the diagnosis of visceral leishmaniasis among Ethiopian patients with HIV co-infection. Ann Trop Med Parasitol 96:25-30.

Hailu A, Frommel D. (1993) Leishmaniasis in Ethiopia. In: *The Ecology of Health and disease in Ethiopia*. Kloos, H. and Zein, Z. A. (eds.). Boulder, West View Press. pp.375-388.

Hailu A, Gebre-Michael T, Berhe N, Balkew M.(2004) Leishmaniasis in Ethiopia. In: The Ecology of Health and Disease in Ethiopia, Kloos H and Zein A(eds), 3rd edn, (In Press)

Hailu A, Kroon CCM, Schoone GJ, Berhe N, Schallig HDFH, Kager PA. (2002) Sera-epidemiological assessment and diagnosis of visceral leishmaniasis in an endemic locality using Fast Agglutination Screening Test (FAST). Acta tropica 83:93-101.

Hailu A. (1990) Pre- and post-treatment antibody levels in visceral leishmaniasis. Trans R Soc Trap Med Hyg 84:673-675.

Handman E. (2001) Leishmaniasis: current status of vaccine development. Clin Microbiol Rev 14:229-243.

Harith A, Kolk AH, Leeuwenburg J, Muigai R, Huigen E, Jelsma T, Kager PA. (1988) Improvement of a direct agglutination test for field studies of visceral leishmaniasis. J Clin Microbiol 26:1321-1325.

Hashim FA, Ali MS, Satti M, el HA, Ghalib HW, el SS, el HI. (1994) An outbreak of acute kala-azar in a nomadic tribe in western Sudan: features of the disease in a previously non-immune population. Trans R Soc Trap Med Hyg 88:431-432.

Herwaldt BL. (1999) Leishmaniasis. Lancet 354:1191-1199.

Iqbal J, Hira PR, Saroj G, Philip R, Al-Ali F, Madda PJ, Sher A. (2002) Imported visceral leishmaniasis: Diagnostic dilemmas and comparative analysis of three assays. J Clin Microbiol 40:475-479.

Kafetzis DA. (2003) An overview of paediatric leishmaniasis. J Postgrad Med 49:31-38.

Kar K. (1995) Serodiagnosis of leishmaniasis. Crit Rev Microbiol 21:123-152.

Kaul P, Malla N, Kaur S, Mahajan RC, Ganguly NK. (2000) Evaluation of a 200-kDa amastigote-specific antigen of *L. donovani* by enzyme-linked immunosorbent assay (ELISA) for the diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg 94:173-175.

Kohanteb J, Ardehali SM, Rezai HR. (1987) Detection of *Leishmania donovani* soluble antigen and antibody in the urine of visceral leishmaniasis patients. Trans R Soc Trop Med Hyg 81:578-580.

Liew FY, O'Donnell CA, (1993) Immunology of Leishmaniasis Adv Parasitol 2:161-259.

Lopez-Velez R, \laguna F, Alvar J, Perez-Molina JA, Molina R, Matrinez P, VillarrubiaJ. (1995) Parasitic culture of Buffy coat for diagnosis of visceral leishmaniasis in human immunodeficiency virus-infected patients. J Clin Microbiol 33:937-939.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951) Protein measurement with the Folin phenol reagent. J Bioi Chem 37:171-182.

Lyons S, Veeken H, Long J. (2003) Visceral Leishmaniasis and HIV in Tigray, Ethiopia. Trop Med Intern health 8:733-739.

Magill AJ, (1995) Epidemiology of leishmaniasis. Dermatol Clin 13:505-523.

Magill AJ, Grogl M, Gasser JrR, Sun W, Oster CN. (1993) Visceral infection caused by *Leishmania tropica* in veterans of operation desert storm. N Engl J Med 328:1383-1387.

Maltezou HC, Siafas C, Mavrikou M, Spyridis P, Stavrinadis C, Karpathios T, Kafetzis DA. (2000) Visceral leishmaniasis during childhood in southern Greece. Clin Infect Dis 31:1139-1143.

Marsden PD. (1986) Mucosal leishmaniasis (espundia Escomel 1911). Trans R Soc Trop Med Hyg 80:859-876.

Maru M. (1979) Clinical and laboratory features and treatment of visceral leishmaniasis in hospitalized patients in northwestern Ethiopia. Am J Trop Med Hyg 28:15–18.

Mary C, Lamouroux D, Dunan S, Quilici M. (1992) Western blot analysis of antibodies to *Leishmania infantum* antigens: potential of the 14-kD and 16-kD antigens for diagnosis and epidemiologic purposes. Am J Trop Med Hyg 47:764-771.

Mauricio IL, Stothard JR, Miles MA. (2000) The strange case of *Leishmania chagasi*. Parasitol Today 16:188-189.

Mbati PA, Githure JI, Kagai JM, Kirigi G, Kibati F, Wasunna K, Koech DK. (1999) Evaluation of a standardized direct agglutination test (DAT) for the diagnosis of visceral leishmaniasis in Kenya. Ann Trop Med Parasitol 93:703-710.

Medrano FJ, Rey C, Leal M, Canavate C, Rubio A, Sanchez-Quijano A, Alvart J, Lissen E. (1998) Dynamics of serum cytokines in patients with visceral leishmaniasis and HIV-l co-infection. Clin Exp Immunol 114:403-407.

Mengesha B, Abuhoy M. (1978) Kala-azar among labor migrants in Metema-Humera region of Ethiopia. Trop Geogl Med 30:199–206.

Meredith SEO, Kroon NCM, Sondorp E, Seaman J, Goris MGA, van Ingen CW, Oosting H, Schoone GJ, Terpstra WJ, Oskam L. (1995) *Leish*-KIT, a stable direct

agglutination test based on freeze-dried antigen for serodiagnosis of visceral leishmaniasis. J Clin Microbiol 33:1742-1745.

Momeni AZ, Jalayer T, Emamjomeh M, Khamesipour A, Zicker F, Ghassemi RL, Dowlati Y, Sharifi I, Aminjavaheri M, Shafiei A, Alimohammadian MH, Hashemi-Fesharki R, Nasseri K, Godal T, Smith PG, Modabber F. (1999) A randomised, double-blind, controlled trial of a killed L. major vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. Vaccine. 17:466-472.

Moral L, Rubio EM, Moya M. (2002) A leishmanin skin test survey in the human population of I'Alacanti Region (Spain): implications for the epidemiology of *Leishmania infantum* infection in southern Europe. Trans R Soc Trop Med Hyg 96:129-132.

Moreno J, Canavate C, Chamizo C, Laguna F, Alvar J. (2000) HIV-*Leishmania infantum* co-infection: humoral and cellular immune responses to the parasite after chemotherapy. Trans R Soc Trop Med Hyg 94:328-332.

Morsy TA, Ahmed MM, Younis TA, Arafa MM. (1990) On the specificity of the indirect haemagglutination in diagnosis of visceral leishmaniasis. J Egypt Soc Parasitol 20:231-234.

Murray HW. (2001) Clinical and experimental advances in treatment of visceral leishmaniasis. Antimicrob Agent Chemother 45:2185-2197.

Oren R, Schnur LF, Yehuda DB, Mayner V, Okon E, Rachmilewitz EA. (1991) Visceral leishmaniasis: a difficult diagnosis and unusual causative agent. J Infect Dis 164:746–749.

Oskam L, Nieuwenhuijs JL, Hailu A. (1999) Evaluation of the direct agglutination test (DAT) using freeze-dried antigen for the detection of anti-*Leishmania* antibodies in serum samples from various patient groups in Ethiopia. Trans R Soc Trop Med Hyg 93:275-277.

Osman OF, Oskam L, Zijlstra EE, Kroon NCM, Schoone GJ, Khalil E-TAG,, El-Hassan AM, Kager PA. (1997) Evaluation of PCR for diagnosis of visceral leishmaniasis. J Clin Microbiol 35:2454-2457.

Ozbel Y, Turgay N, Ozensoy S, Ozbilgin A, Alkan MZ, Ozcel MA, Jaffe CL, Schnur L, Oskam L, Abranches P. (1995) Epidemiology, diagnosis and control of leishmaniasis in the Mediterranean region. Ann Trop Med Parasitol 89:89-93.

Pampiglione S, Manson-Bahr PEC, La Placa M, Bogatti MA, Micheloni F. (1975) Studies in Mediterranean leishmaniasis. 3. The leishmanin skin test in kala-azar. Trans R Soc Trop Med Hyg 69:60-68.

Pearson RD, de Queiroz S. (1996) Clinical spectrum of Leishmaniasis. Clin Infect Dis 22:1-13.

Pintado V, Martin-Rabadan P, Rivera ML, Moreno S, Bouza E. (2001) Visceral leishmaniasis in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. A comparative study. Medicine 80:54-73.

Preiser W, Cacopardo B, Nigro L, Braner J, Nunnari A, Doerr HW, Weber F. (1996) Immunological findings in HIV-Leishmania coinfection. Intervirol 39:285-288.

Rab MA, Evans DA. (1997) Detection of anti-*Leishmania* antibodies in blood collected on filter paper by the direct agglutination test. Trans R Soc Trop Med Hyg 91:713-715.

Raj VS, Ghosh A, Dole VS, Madhubala R, Myler PJ, Stuart KD. (1999) Serodiagnosis of leishmaniasis with recombinant ORFF antigen. Am J Trop Med Hyg 61:482-487.

Reiner SL and Locksly R. (1995) The regulation of immunity to *Leishmania major*. Annu Rev Immunol 13: 151-177.

Riera C, Fisa R, Udina M, Gallego M, Portus M. (2004) Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg 98:102-110.

Rijal S, Boelaert M, Regmi S, Karki BM, Jacquet D, Singh R, Chance ML, Chappuis F, Hommel M, Desjeux P, Van der Stuyft P, Le Ray D, Koirala S. (2004) Evaluation of a urinary antigen-based latex agglutination test in the diagnosis of kala-azar in Eastern Nepal. Trop Med Int Health 9:724-729.

Rosenthal PS, Chaisson RE, Hadley WK, Leech JH. (1988) Rectal leishmaniasis in a patient with acquired immunodeficiency syndrome Am J Med 84:307-310.

Sacks DL, Lal SL, Shrivastava SN, Blackwell J, Neva FA. (1987) Analysis of T cell responsiveness in Indian kala-azar. J Immunol 138:908-913.

Salotra P, Raina A, Negi NS. (1999) Immunoblot analysis of the antibody response to antigens of *Leishmania donovani* in Indian kala-azar. Br J Biomed Sci 56:263-267.

Salotra P, Sreenivas G, Pogue GP, Lee N, Nakhasi HL, Ramesh V, Negi NS. (2001) Development of a species-specific PCR assay for detection of *Leishmania donovani* in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. J Clin Microbiol 39:849-854.

Santos-Gomes G, Gomes-Pereira S, Campino L, Araujo MD, Abranches P. (2000) Performance of immunoblotting in diagnosis of visceral leishmaniasis in human immunodeficiency virus-*Leishmania spp*.-co-infected patients. J Clin Microbiol 38: 175-178.

Schaefer KU, Kurtzhals JA, Gachihi GS, Muller AS, Kager PA. (1995) A prospective sero-epidemiological study of visceral leishmaniasis in Baringo District, Rift Valley Province, Kenya. Trans R Soc Trop Med Hyg 89:471-475.

Schlein Y, Jacobson RL, Muller GC. (2001) Sand fly feeding on noxious plants: a potential method for the control of leishmaniasis. Am J Trop Med Hyg 65:300-303.

Schoone GJ, Hailu A, Kroon CCM, Nieuwenhuys JL, Schalling HDFH, Oskam L. (2001) A fast agglutination screening test (FAST) for the detection of anti-*Leishmania* antibodies. Trans R Soc Trop Med Hyg 95:400-401.

Seaman J, Ashford RW, Schorscher J, Dereure J. (1992) visceral leishmaniasis in southern Sudan: status of health nvillages in epidemic conditions Ann Trop Med Parasitol 5:481-486.

Seaman J, Mercer AJ, Sondorp E. (1996) The epidemic of visceral leishmaniasis in western Upper Nile, southern Sudan: course and impact from 1984 to 1994. Int J Epidemiol 25:862-871.

Shiddo SA, Mohamed AA, Huldt G, Loftenius A, Nilsson L, Jonsson J, Ouchterlony O, Thorstensson R. (1995) Visceral leishmaniasis in Somalia. Circulating antibodies as measured by DAT, immunofluorescence and ELISA. Trop Geogr Med 47:68-73.

Singh S, Sivakumar R. (2003) Recent advances in the diagnosis of leishmaniasis. J Postgrad Med 49:55-60.

Sinha R, Sehgal S. (1994) Comparative evaluation of serological test in Indian kala-azar. J Trop Med Hyg 97:333-340.

Solbach W, Laskay T. (2000) The host response to *Leishmania* infection. Adv Immunol 74:275-317.

Spanakos G, Patsoula E, Kremastinou T, Saroglou G, Vakalis N. (2002) Development of a PCR-based method for diagnosis of *Leishmania* in blood samples. Mol Cell Probes 16:415-420.

Sundar S, More DK, Singh MK, Singh VP, Sharam S, Makharia A, Kumar PC, Murray HW. (2000) Failure of pentostam antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis 31:1104-1107.

Sundar S, Rai M. (2002) Laboratory Diagnosis of Visceral Leishmaniasis. Clin Diagn Lab Immunol 9:951-958.

Sundar S, Reed SG, Singh VP, Kumar PCK, Murray HW. (1998) Rapid accurate field diagnosis of Indian visceral leishmaniasis. Lancet 351:563-565.

Sundar S. (2003) Diagnosis of kala-azar -An important stride. JAPI 51:753-755

Talwar GP. (1983) A hand book of practical Immunology. Vikas Publishing house, pp:139-257.

Thakur CP. (1997) A comparison of intercostal and abdominal routes of splenic aspiration and bone marrow aspiration in the diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg 91:668-670.

Thakur CP. (2000) Socioeconomics of visceral leishmaniasis in Bihar (India). Tran R Soc Trop Med Hyg 94:156-157.

Travi BL, Osorio Y, Melby PC, Chandrasekar B, Arteaga L, Saravia NG. (2002) Gender is a major determinant of the clinical evolution and immune response in hamsters infected with *Leishmania spp*. Infect Immun 70:2288-2296.

UNAIDS, UNICEF and World Health Organization. (2002) Epidemiological Fact Sheet on HIV/AIDS and Sexually Transmitted Diseases: Ethiopia. WHO, Geneva.

Valenstein PN. (1990) Evaluating diagnostic tests with imperfect standard. Am J Clin Path 93:252-256.

Valenzuela JG, Belaid Y, Garfield MK, Mendez S, Kamhawi S, Rowton ED, Sacks DL, Ribeiro JMC. (2001) Toward a defined anti-*Leishmania* vaccine targeting vector antigens: characterization of a protective salivary protein. J Exp Med 194:331-342.

Walton BC, Brooks VM, Arojone I. (1972) Serodiagnosisi of American leishmaniasis by indirect fluorescent antibody test. Am J Trop Med Hyg 21:296-299.

Weiss JB. (1995) DNA probes and PCR for diagnosis of parasitic infections. Clin Microbiol Rev 8:113-130.

WHO (1990) Control of the leishmaniasis: report of a WHO expert committee. WHO Tech Rep Ser 793:1–158.

WHO (1996) The Leishmaniasis and Leishmaniasis/HIV co-infections. Fact Sheet #116.

WHO (1998) *Leishmania* and HIV in gridlock. WHO/UNAIDS report. Geneva: World Health Organization:15-25.

WHO (2000) *Leishmania*/HIV co-infection. South-western Europe 1990-1998. Geneva: World Health Organization, mimeographed document WHO/LEISH/2000.42.

Wolday D, Berhe N, Akuffo H, Desjeux H, Britton S. (2001) Emerging Leishmania/HIV co infection in Africa. Med Microbiol Immunol 190:65-67.

Zijlstra EE, Ali MS, el Hassan AM, el Toum IA, Satti M, Ghalib HW, Kager PA. (1991) Direct agglutination test for diagnosis and sero-epidemiological survey of kala-azar in the Sudan. Trans R Soc Trop Med Hyg 85:474-476.

Zijlstra EE, Ali MS, el Hassan AM, el Toum IA, Satti M, Ghalib HW, Kager PA. (1992) Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. Trans R Soc Trop Med Hyg 86:505-507.

Zijlstra EE, Daifalla NS, Kager PA, Khalil EAG, El-Hassan AM, Reed SG, Ghalib HW. (1998) rK39 enzyme-linked immunosorbent assay for diagnosis of *Leishmania donovani* infection. Clin Diagn Lab Immunol 5:717-720.

Zijlstra EE, El Hassan AM, Ghalib HW, Ismael A. (1994) Epidemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical disease and post-kala-azar dermal leishmaniasis. Am J Trop Med Hyg 51:826-836.

Zijlstra EE, El-Hassan AM, Ismael A. (1995) Endemic kala-azar in eastern Sudan: post-kala-azar dermal leishmaniasis. Am J Trop Med Hyg 52:299-305.