ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

FACULTY OF LIFE SCIENCES

LABORATORY BASED COMPARATIVE EVALUATION OF THE PERFORMANCE OF rK28 AND rk39 RAPID TESTS AND DAT IN THE DIAGNOSIS OF VISCERAL LEISHMANIASIS

ASRAT BEZUNEH

A THESIS SUBMITTED TO THE CELLULAR, MICROBIAL AND MOLECULAR BIOLOGY PROGRAMME UNIT IN PARTIAL FULLFILMENT FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOMEDICAL SCIENCES.

Addis Ababa University

Addis Ababa, Ethiopia

June, 2012

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

FACULTY OF LIFE SCSIENCE

NAME ASRAT BEZUNEH BEKELE

FACULTY LIFE SCIENCES

DEPARTMENT CELLULAR, MICROBIAL AND MOLECULAR BIOLOGY PROGRAMME UNIT (BIOMEDICAL SCIENCES)

ADVISOR PROFESSOR ASRAT HAILU

TITLE: LABORATORY BASED COMPARATIVE EVALUATION OF THE PERFORMANCE OF rk28 AND rk39 TESTS AND DAT IN THE DIAGNOSIS OF VISCERAL LEISHMANIASIS.

Addis Ababa University

School of Graduates Studies

This is to certify that the thesis prepared by Asrat Bezuneh entitled: *Laboratory based comparative evaluation of the performance of rk28 and rk39 rapid tests and DAT in the diagnosis of Visceral Leishmaniasis and submitted* in partial fulfillment for the Degree of Masters of Science(Biomedical Science) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the examining committee:

Examiner_____Signature_____Date_____

Examiner_____Signature_____Date_____

Advisor_____Signature_____Date_____

Chairperson_____Signature_____Date_____

Acknowledgments

First and foremost I want to extend my heart-felt thanks and appreciation to Professor Asrat Hailu, my advisor and old time friend who has guided and supported me through this project. He never lost confidence in me, in all walks in my life since I had known him some thirty years back.

I also want to acknowledge the Biology Department, (now Faculty of Life Sciences), its former heads Drs.Kifle Dagne and Dawit Abate and the postgraduate programme, for their help and support they have given me the opportunity to continue my education. I also thank Dr. Abebe Getahun for his advice and encouragement.

My deepest gratitude and thanks goes to Professor Hashim Ghalib for his consistent advice and encouragement and also the Infectious Disease Research Institute (IDRI), Seattle USA, who have provided me with all the rk28 test kits.

I am highly indebted to all my colleagues and friends at the Department of Microbiology, Immunology and Parasitology at the Faculty of Medicine for their assistance and help.

I am very gratefull to my family, Haregewoine Asfaw (my wife), Abel and Mahlet (my son and daughter) for their patience and full support without which it would have been more difficult.

Finally I want to thank GOD for pulling me through Life.

Table of Contents

LIST OF TABLE

LIST OF FIGURES

List of Abbreviations

AIDS: Acquired Immune Deficiency Syndrome

AVL: Active Visceral Leishmaniasis

CAT: Category

CI: Confidence interval

CL: Cutaneous Leishmaniasis

DAT: Direct agglutination test

DCL: Diffuse Cutaneous Leishmaniasis

DNA: Deoxyribonucleic acid

DPP: Dual Path Platform

EC: Endemic control

ELISA: Enzyme linked immunoserpent assay

EMT: Ease Med Trend

HC: Healthy control

HIV: Human immunodeficiency virus

ICT: Immuno chromatographic test

IFA: Immuno fluorescent antibody

IVDU: Intravenous drug user

LCL: Localized Cutaneous Leishmaniasis

MCL: Muco Cutaneous Leishmaniasis

MOH: Ministry of Health

NA: not applicable

NNN: Novy-Macneal-Nicolle

NPV: negative predictive value

Neg: negative

OD: Other disease

PKDL: Post Kalazar Dermal Leishmaniasis

Pos: positive

PPV: positive predictive value

RDT: Rapid diagnostic test

rk39: Recombinant antigen 39

rK28: Recombinant antigen 28

TDR: Tropical Disease Reasearch

VL: Visceral Leishmaniasis

WHO: World Health Organization

Abstract

Visceral Leishmaniasis (VL) caused by *L.donovani* is endemic and a major health problem in Ethiopia with 4000-7000 cases annually.

The reference standard for VL diagnosis is parasitological which is highly specific but with varying sensitivity. The procedure is invasive, risky and not suitable for field use. Serological techniques like the DAT and rk39 rapid diagnostic tests (RDTs) have been developed and are in use in different VL diagnostic. The DAT has good sensitivity and specificity, but is not ideal for field work while the performance of the rk39 RDTs tend to show homogeneity in endemic regions and heterogeneity between regions.

This study evaluated the performance of three rk28 based rapid diagnostic test prototypes (Inbioss+, DPP and EMT), two market available rk39 rapid diagnostic tests (Inbioss and Diamed) and FD-DAT and compared the results. The evaluation was carried out using stored sera from VL patients (n=95), healthy controls (n=35) endemic control (n=41), other disease (n=35), cutaneous leishmaniasis (n=35).

The results show that the three prototypes rk28 rapid tests provided sensitivities of 97.9%, 95.8% and 98.9% compared to two rk39 rapid tests and DAT with sensitivity of 96.8%, 92.6% and 91.6% respectively. Specificity results were 82.9% (Inbioss+), 94.6% (DPP), 80.2% (EMT), 98.2% (DiaMed & Inbioss), and 97.3% (DAT), control panel comprising of healthy non endemic control, healthy endemic controls, and other diseases. The DPP test has optimal combination of sensitivity and specificity, 95.8% and 94.6%, than the other tests. Also the DPP is easy to perform and interpret, with lesser steps and convenient format to use in a field setting.

The findings in this study indicate that the DPP test has a great potential as a VL serodiagnostic test simplifying VL disease confirmation mainly at the tertiary health care setting.

Key words: Visceral Leishmaniasis, RDT, rk28, rk39, DAT, Ethiopia

1. **INTRODUCTION**

Leishmaniasis is a vector-borne disease caused by obligate intracellular protozoa that belong to the genus Leishmania and transmitted by the bite of phlebotomine female sand fly (Herwaldt, 1999). The Leishmaniasis can be divided into three major clinical syndromes: cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL) or kala-azar (WHO, 1990).

Most forms of the disease are transmissible only from animals (zoonosis), but some can spread between humans (anthroponosis). About 21 of 30 species that infect mammals cause human infection (Table1). The different species are morphologically indistinguishable but they can be differentiated by isoenzyme analysis, DNA sequence analysis, or monoclonal antibodies.

Table 1: Leishmania found in humans (Source: WHO 2010)

	L.(Leishmania)	*L.(Leishmania)*	*L.(Viannia)*	*L.(Viannia)*
Old World	*L.donovani*	*L.major*		
	L.Infantum	*L.tropica*		
		L.killicki		
		L.aethiopica		
		L.infantum		
New World	*L.infantum*	*L. infantum*	*L .braziliensis*	*L. braziliensis*
		L.mexicana	*L .guyanensis*	*L .panamensis*
		L .pifanoi[a]	*L .panamensis*	
		L.venezuelensis	*L .shawi*	
		L .garnhami[a]	*L .naiffi*	
		L .amazonensis	*L. lainsoni*	
			L .lindenbergi	
			L .peruviana	
			L. colombienshis[b]	
Principal tropism	Viscerotropic	Dermotropic	Dermotropic	Mucotropic

[a] Species position is under discussion

[b] Taxonomic position under discussion

A total of about 30 species in Phlebotomus genus (old world) and Lytzomiya genus (new world) have been identified as vectors. Sandflies are relatively weak, noiseless fliers (fig.1): they rest in dark moist places and are typically more active in evening and night time hours. Other modes of transmission are congenital and parenteral (i.e. by blood transfusion needle sharing and laboratory accident) (Herwaldt, 1999).

Figure 1: Female sandfly (Source: http://honey & poisson.blogspot.com.)

Leishmaniasis is primarily a zoonotic disease in which wild and domestic animals such as the fox, jackal, rodents and wolves serve as reservoir hosts. Other animals in the surrounding areas can become infected and these are referred to as secondary or incidental hosts. Of all the potential animal hosts, domestic dogs by far play the most important role in harboring and transmitting the disease to humans due to the close association between humans and dogs as pets (WHO, 1990; Arias *et al*., 1996).

In anthroponotic VL due to *L.d donovani* such as in India, Sudan and Ethiopia man is

the principal reservoir host. Asymptomatic carriers and post kalazar dermal leishmaniasis (PKDL) patients are a particular source of infection for sandflies (WHO, 1990).

Leishmaniasis is endemic in 98 countries or territories, with more than 350 million people at risk. An estimated incidence of 2 million new cases per year, (0.5million of visceral leishmaniasis and 1.5 million of cutaneous leishmaniasis), occur (WHO, 2010). The leishmaniasis is widely dispersed, with transmission to humans on five continents, but the human disease burden is concentrated mainly in a few major foci (fig.2).

Figure 2. Worldwide distribution of Leishmaniasis (Source:http://www.who.int/ctd/html/leis.html)

In several regions, there has been and still is a clear and worrying increase in the number of cases. According to Desjeux (2004), increase can mainly be attributed to behavioral and environmental changes, including the development of new settlements, intrusion into primary forest, deforestation, massive migration from rural to urban

areas, fast and unplanned urbanization, and the building of dams and new irrigation schemes. However, individual risk factor such as malnutrition and immunosuppression owing to HIV-co infection also have important role (Desjeux and Alvar, 2003).

Since the global epidemic of HIV infection began, there has been a steady rise in the annual number of reported new cases of VL associated with the virus (fig3). *Leishmania*/HIV co-infection is emerging as a new and worrying disease, particularly in south–western Europe (Desjeux, 1998). In Spain, the country reporting the highest incidence of the co-infection, 68% of the cases are intravenous-drug users (Desjeux and Alvar, 2003). In France, Italy, Portugal and Spain, the outbreak of HIV infection has changed the epidemiology of VL, from a disease pre-dominantly found in children to one more common in adults (WHO, 1999). Currently with the introduction of HAART (highly active anti-retroviral therapy), the condition is significantly improving and is becoming manageable in the western world.

Figure 3: Countries reporting Leishmaniasis/HIV co-infection.(Source:http://www.who.int/ctd html. leis)

Co-infection with HIV intensifies the burden of visceral and cutaneous leishmaniasis by causing severe forms that are more difficult to manage. As of March 2010, *Leishmania*-

HIV co-infection had been reported in 35 countries (WHO, 2010). According to a WHO coordinated monitoring system involving 28 institutions worldwide, the number of new cases has declined in Europe since the end of the 1990s, mainly due to access to highly active antiretroviral therapy. In other parts of the world, however, where there is limited access to such treatment, the prevalence is steadily rising, especially in northern Ethiopia, where the rate of HIV infection increased from 19% of people with visceral leishmaniasis in 1998–1999 to 34% in 2006–2007.

HIV co-infection may re-activate latent leishmanial infections, increase the level of transmission by phlebotomine sandflies, and facilitate artificial transmission of *Leishmania*, via the sharing of contaminated syringes and needles, from one intravenous-drug user (IVDU) to another (Molina, et al.,2003).

Leishmaniasis is one of the most important vectors borne disease in Ethiopia and presents many examples in, and challenges for disease ecology and epidemiology. *L.donovani, L.aethiopica, L.major and L.tropica* are the species causing Leishmaniasis in Ethiopia (Hailu & Frommel, 1993). Of these four species, *L.donovani* (causing visceral leishmaniasis) and *L.aethiopica* (causing cutaneous leishmaniasis) are known to be of significant clinical and public health importance. Even though accurate figures are lacking, unofficial estimates show that at least 20,000-50,000 cases of CL and 4,000 cases of VL occur annually. (Hailu, 2008, Hailu et al., 2009).

The phlebotomine species that are known or implicated to transmit the different forms of the disease in Ethiopia include: *Phlebotomus martini* and *P.celiae* (Gebremichael and Lane, 1996), *P. orientalis* (Hailu et.al.1995*), P.pediffer* and *P.longipes* (Ashford et. al. 1973a), *P.duboscqi* (Gebre-Michael et.al., 1993). Other potential vector species, (eg. *P.alexandri, P.saevus*) are known to exist in the endemic areas and some of these

species have been found naturally infected with yet unidentified *Leishmania* species (Balkew et al., 1999). While CL is known to be a zoonosis, there is a lack of information about the primary source of VL infection. However man-to-man transmission contributes to the lion's share of VL burden in affected communities (Hailu, 2008).

In view of the complexity and enormous difficulty of the overall aspects of Leishmaniasis, the primary goals for clinical management are to prevent death from visceral Leishmaniasis and morbidity from cutaneous and mucosal leishmaniasis, which is also challenging due to the apparently innumerable possible combinations of different leishmanial syndromes, species, and geographical areas of acquisition of infection, each combination varying by clinical presentation, ease of diagnosis, natural history and response to therapy (Herwaldt, 1999).

VL shares similar clinical presentations (prolonged fever, weight loss, and splenomegally) with other tropical diseases such as malaria, disseminated tuberculosis, or enteric fever. Therefore, laboratory testing is necessary to confirm the diagnosis of VL (Chappuis,et. al., 2005). Also diagnostics tests for VL need to be highly sensitive and specific because of the fatal evolution of the disease without adequate treatment and the serious toxicity of antimonials (Sundar, et. al., 2000).

VL tests must also be cheap and easy to perform since the disease occurs in poor and remote rural communities with limited access to referral hospitals. The development of diagnostic tests for improved case management of VL has been rated as one of the most needed among the infectious diseases prevalent in the developing world (Mahey, et. al., 2004).

Rapid methods for diagnosis and species identification are needed as are therapies, prophylactics, and control measures that are effective, safe, and affordable and easily administered.

2. LITERATURE REVIEW

2.1 Brief History

Visceral leishamniasis was first described in 1903, by Leishman and Donovan. Both of these physicians separately but simultaneously demonstrated parasites in stained smears from the spleen of patients suffering from a malaria-like illness, which became known as visceral leishmaniasis and its causative agent was named *Leishmania donovani.* This is not to say that leishmaniasis did not exist before 1903, on the contrary. Archibadi in 1922 described an epidemic of Kala-azar which occurred in the Garo hills of Assam in Saudi Arabia as far back as 1870. Cunningham recorded a similar disease that occurred in 1885, caused by a parasite which was later named *Leishmania tropica,* the causative agent of cutaneous leishmaniasis. Nicolle in 1908 reported that mammals including dogs could act as reservoir hosts for the *leishmania* parasite. Swaminath *et al.,* in 1942, proved using human volunteers that the *leishmania* parasite could be transmitted by the phlebotomus sandflies.

2.2 Epidemiology

Visceral Leishmaniasis also known as kala-azar, black fever, and Dumdum fever is a disease caused by *Leishmania donovani* in India and eastern Africa and by *Leishmania infantun/chagasi* in the Mediterranean basin, western Africa and Latin America (Fig4). It is the most severe form of leishmaniasis.

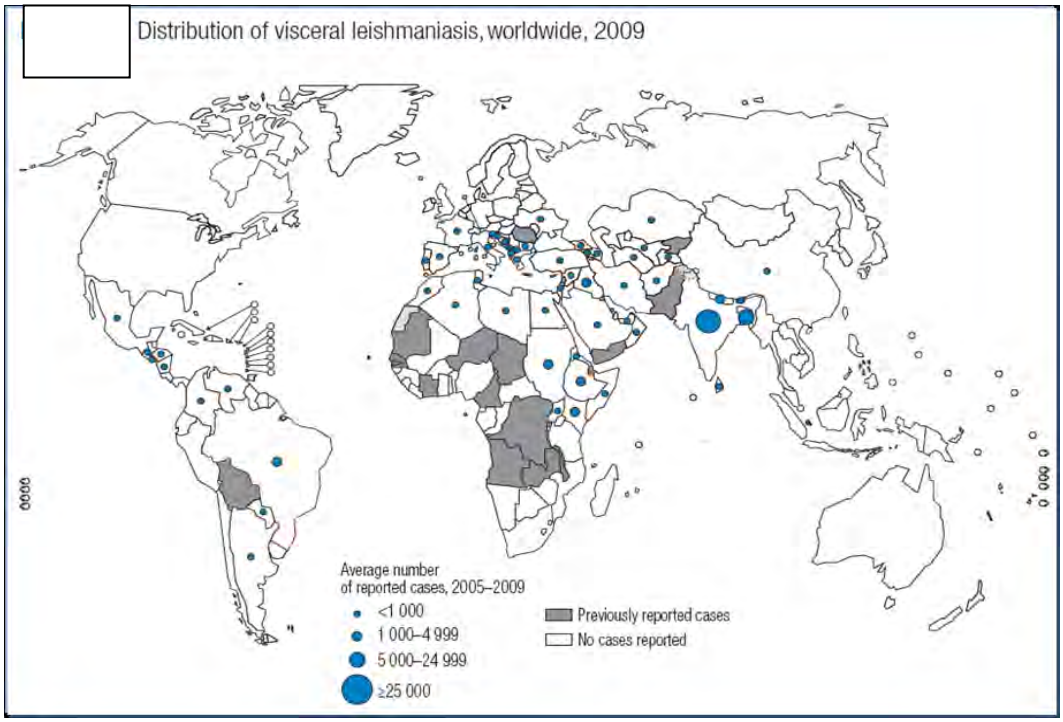

Figure 4. Distribution of visceral leishmaniasis worldwide Source:http://www.who.int/2010 report NTD

In contrast to cutaneous and mucocutaneous leishmaniasis in which the protozoan parasite is histologically localized, visceral disease is caused by parasite growth within the reticuloendothelial cells throughout the body. During the active stage of the disease, patients have anergy to leishmanial antigen as indicated by a negative delayed-hypersensitivity skin test. Yet at the same time, they have intense polyclonal B-cell activation, including high levels of anti-leishmanial antibodies (Boelaret et al., 2000).

VL cases tend to occur sporadically in rural areas where the organisms are endemic, but large epidemics have been associated with famines, mass migrations, and civil disturbances. Malnutrition can suppress cell-mediated immune responses and appears to predispose to the development of progressive visceral lesihmaniasis in some persons.

Anthroponotic *L donovani* transmissions in a relatively small but heavily populated area spanning northeastern India, southeastern Nepal and central Bangladesh accounts for more

than two thirds of all cases of visceral leishmaniasis in the world. The East African *L.donovani* focus, also with an important component of anthroponotic transmission, is the second largest focus of visceral leishmaniasis, with the highest incidence in Ethiopia and the Sudan. The other two important foci both caused by zoonotic *L.infantum* transmission are the Mediterranean Basin, the Middle East and western Asia and the New World. It has been estimated that more than 90% of the burden of visceral leishmaniasis is concentrated in Bangladesh, Brazil, Ethiopia, India, Nepal and the Sudan (WHO, 2010).

2.2.1 Visceral Leishmaniasis in Ethiopia

VL is a serious disease and in Ethiopia is often progressive, with the death rate exceeding 90% within six months if treatment is not instituted early (Habte-Gabr 1981). VL prevails in low lying (<1500 m) arid and semi-arid areas of the country with characteristics focal variation in magnitudes of endemicity (fig.5). A number of factors are believed to determine variations in levels of endemicity. Among these, factors relating to the parasite, the vector and the human hosts involved are determinants of the degree of the level of endemicity (Hailu et al., 1996). There is an association between rainfall patterns and the incidence of VL in Ethiopia (Ayele & Ali 1984), probably as a result of seasonal increase in the vector population and increased activity in the transmission areas during the rainy season.

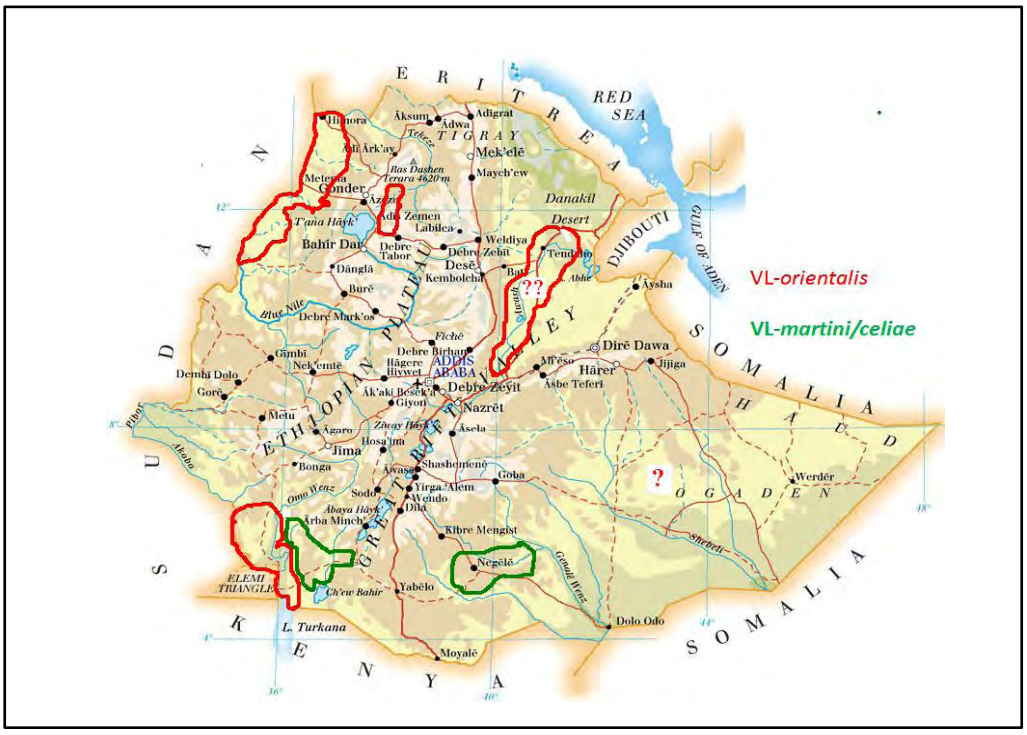

Figure 5: Foci of visceral eishmaniasis and its vectors in Ethiopia Source: Epidemiology and ecology of health and disease in Ethiopia (2006) with slight modifications.

VL in Ethiopia is endemic and at times reaches epidemic proportions, when it often also affects migrant workers and soldiers as well as the productive age group of the resident population of the endemic areas. A large proportion of the fertile Ethiopian lowlands are at risk and also with the advent of HIV epidemic, HIV and Leishmania co-infection have emerged as a major public health problem. The two infections are known to interact and affect the clinical and epidemiological pattern of VL (WHO 1998). With the spread of HIV to rural and sub-urban areas, leishmaniasis has become a threat to the well being of many communities. The spread of HIV in developing tropical countries like Ethiopia is believed to worsen the health impact of endemic tropical diseases, increasing the overall morbidity and mortality (Hailu et al., 2006). Ethiopia now has the second largest number of VL cases of any country in sub-Saharan Africa (after Sudan), with an estimated 4000-7000 people needing treatment for the

disease each year (Hailu et al., 2006). VL is therefore of considerable social, economic and public health importance.

The first evidence of the endemicity of VL in Ethiopia came from Cole et al., (1942), who described 31 cases in a military Battalion travelling in southwestern Ethiopia, northern Kenya and southeastern Sudan. Anderson (1943), in a report of 136 cases in a brigade operating in northern Kenya, claimed that 11 cases had been acquired on the Moyale-Addis Ababa road, just inside Ethiopia: eight further cases were reported in troops who passed through an unspecified area near the southern border.

Subsequent active and passive case finding, leishmania skin test (LST) and entomological surveys provided evidence on the occurrence of VL in the southern, southwestern, northern, northwestern, eastern and southwestern regions of the country.

Ashford et al., (1973 b), reporting the results of the investigations in Belessa (south eastern Gonder), found six recorded cases of VL of which the two cases were confirmed to have acquired their infections in Belessa. The vector *P.orientalis* was abundant in the area and bit man voraciously according to the report.

Fuller et al., (1976), conducting survey using LST in north western Ethiopia (Setit-Humera), found 30% LST positivity and only one active case of kala-azar from some 1200 individuals examined in the survey.

In a survey conducted in south west Ethiopia on 2723 individuals by Fuller et al., (1979), using LST, physical examination and also formal gel test and spleen puncture on suspected cases of kala-azar found two individuals with VL (16 year old Hammer boy and 17 year old Dassenech male).

Ayele and Ali (1984), doing active and passive case finding survey between January 1982 and January 1983 diagnosed 50 cases of VL among 1,184 patients examined in different parts of Ethiopia. Diagnosis was confirmed by smear and culture in 10 cases, by culture alone in 1 case and by smear alone in 39 cases.

Lindtjorn and OLfason (1983), reported the first 29 cases of VL from the Segen and Weyto Valleys.

In the 1990's the result of epidemiological studies in the Aba-Roba focus in Konso district (southern region of Ethiopia) established the endemicity of VL (Ali and Ashford, 1993a, b, 1994), in a unique ecology associated with the eroded termite hills that provide a suitable habitat for the sandfly vectors *P.martini* and *P.celiae* (Gebre Michael and Lane, 1996). The highest incidence has been recorded in the Aba Roba area (WHO, 1990). During the eight year study leading up to 1990, 142 cases of VL were reported in the villages close to the Segen river valley. It was found that 58% of the affected were children below the age of five years with the lowest risk groups being males above the age of 39 years and females above 24 years, also children below the age of 5 years. The reason for this reduced risk is not clear; however it is probably due to acquired immunity in the adult population (Ali and Ashford 1994). However this does not explain the reduced risk in the children younger than 5 years of age.

Hailu et al., (1996), conducting LST and utilizing sero-epidemiological technique in mass screening of VL, in eight localities in south and south western Ethiopia, detected 4 case of VL.

Since the case of VL in Ethiopia was documented in 1942 in the southern parts of the country, the disease has spread to become endemic in the Segen, Woito, Gelana river valleys and the, Metema and Humera plains in northwest Ethiopia. Other than the

lowlands of the Segen, Weyeto and lower Omo valleys, VL occurs in southern Ethiopia such as Moyale and Wadera and the Dawa Valley. These localities are known to be endemic for VL, albeit sporadically (Hailu et.al. 2006)

VL in Ethiopia and in East Africa in general is caused by *L.donovani* senso lato. In this region, the disease occurs in two distinct ecologies based on *P.martini/celiae* or *P.orientalis* as vectors; the former species are associated with eroded-termite hills in southern Ethiopia, Kenya and southern Sudan. On the other hand, *P. orientalis*is associated with areas of Acacia Balanites woodland Savannah (Hailu et al., 1995).

2.3 Biology and Life Cycle

Leishmania live as extracellular, flagellated promastigotes, (Fig. 6a), in the guts of female phlebotomus sandflies. The parasite stages in sandflies vary from rounded or stumpy forms to elongated, highly motile, metacyclic promastiogotes. Most are in the range of 15-26µm in length and 2-3µm in width. The flagellum extends from the anterior pole. Promastigotes grow at ambient temperatures ranging from 22^{o}C to 26^{o}C. (Pearson & Sousa,1996)

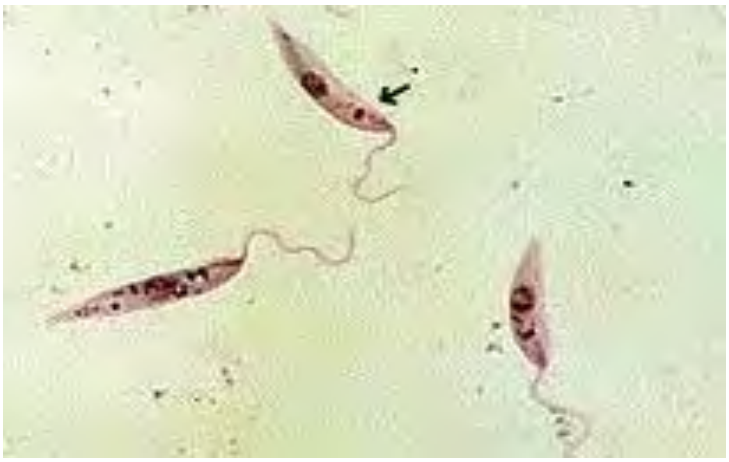

Figure 6a. *L.donovani* Promastigotes (Source:www.msu.edu/zol/316/jpg)

Leishmania exist within the mononuclear phagocytes of mammals as oval, intracellular amastigotes that are 2-3μm in diameter (Fig. 6b) The amastigotes have a relatively large, eccentrically located nucleus and a bar shaped specialized mithochondrial structure, the kinetoplast that contains extranuclear DNA in the form of catenated mini- and maxi circles. Amastigotes are adapted to mammalian body temperature and the acid environment of the macrophage phagolysosome where they reside (Pearson, & Sousa 1996).

.

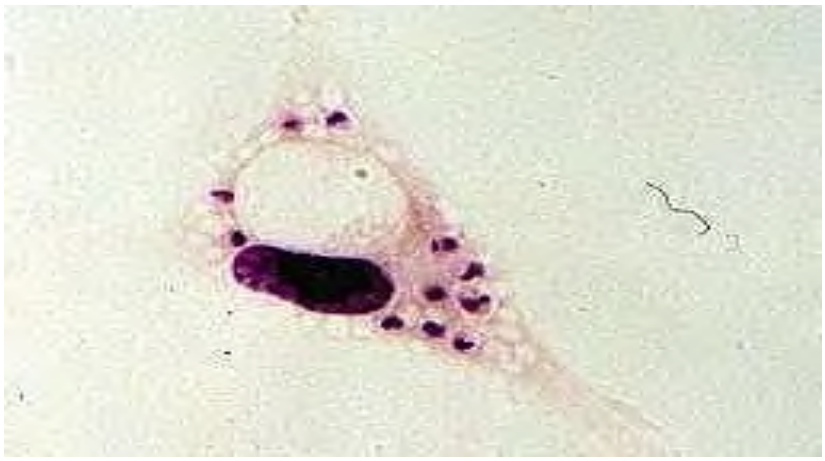

Figure 6b. *L. donovani* amastigotes inside macrophage (Source: www.msu.edu/zol/326/idonprom.gpg)

The sandfly vector becomes infected when feeding on the blood of an infected individual or an animal reservoir host. The *Leishmania* parasites live in the macrophages as round, non-motile amastigotes (3-7 micrometers in diameter). The macrophages are ingested by the fly during the blood-meal and the amastigotes are released into the stomach of insect. Almost immediately the amastigotes transform in to the motile, elongated (10-20 micrometers), flagellate promastigote form (Fig.7). The promastigotes then migrate to the alimentary tract of the fly, where they live extracellularly and multiply by binary fission. Four to five days after feeding the promastigotes move forward to the oesophagus and the salivary glands of the insect. When the sandlfy next feeds on a mammalian host, its proboscis pierces the skin and saliva

containing anti-coagulant is injected into the wound to prevent the blood from clotting. The *leishmania* promastigotes are transferred to the host along with the saliva. Once in the host, the promastigotes are taken up by the macrophages where they rapidly revert to the amastigote form. The *leishmania* are able to resist the microbiocidal action of the acid hydrolases released from the lysozymes and so survive and multiply inside the macrophages, eventually leading to the lysis of the macrophages. The released amastigotes are taken up by additional macrophages and so the cycle continues. Ultimately all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow in VL (Pearson & Sousa, 1995).

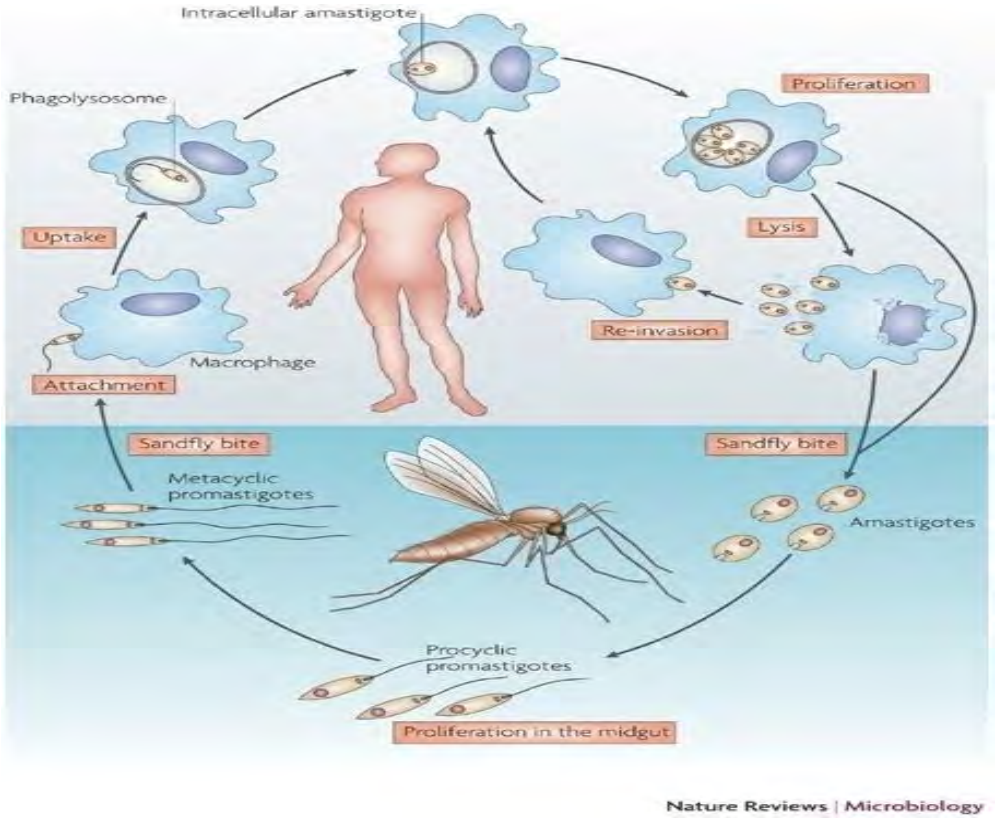

Figure 7. Life cycle of visceral Leishmaniasis Source:(www.nature.com/nrmicro/ suppl.Nov 2007).

2.4 Clinical Manifestations

VL encompasses a broad range of manifestations of infection. Infection remains asymptomatic or subclinical in many cases or can follow an acute, sub acute, or chronic course (Herwaldt, 1999). Classical VL or kala-azar is characterized by fever, malaise, cough, weight loss, hepatomegaly and splenomegaly, lymphoadenopathy, pancytopenia, and hypergammaglobulinimea. Thrombocytopenia may cause uncontrollable epistaxis or bleeding from other sites (Guerin, et al., 2002). On physical examination, the spleen, which is enlarged to a greater extent than the liver is typically appreciated 5-15cm below the left coastal margin: however, it should be noted that splenomegally may be absent in 5% of cases (Hashim,et al.,1994). Although ocular complications are rare they sometimes manifest as retinal hemarroges, keratitis, and central retinal vein thrombosis to name a few. Hemoglobin concentration of 5-9g/dl, WBC counts of 2000-2004/m^3 and platelet counts of 100,000-200,000/m^3 are typical, although lower, more abnormal values can be observed in cases of more extensive disease (Berman, 1997).

The differential diagnosis of VL is broad when patients present with subacute or chronic infection. VL must be differentiated from histoplasmosis, lymphoma, and other myleoproliferative disorders, milliary tuberculosis, brucellosis, hepatosplenic schistosomiasis, subacute bacterial endocarditis, infectious mononucleosis, or prolonged salmonella bacteremia .Massive splenomegally like that observed in patients with VL, is also seen in patients with topical splenomegally syndrome that is associated with chronic malaria. Acute VL must be differentiated from malaria, typhoid fever, acute Chagas' disease, acute schistosomiasis, amebic liver abscess, and a number of bacterial and viral pathogens that cause febrile disease (Pearson & Sousa, 1996).

VL is a well-recognized opportunistic disease in persons with AIDS. They can present in the classical manner with fever, weight loss and organomegaly but atypical presentations are also common (Russo, et.al, 2003). Splenomegally may be absent. Amastigotes have been found in macrophages in the lungs and pleural effusions as well as in the oral mucosae, esophagus, stomach, small intestine and skin. In several cases, amastigotes were found in the bone marrow of HIV-infected persons who presented with aplastic anemia. Asymptomatic leishmanial infections have also been reported in patients with concurrent infections (Russo, et al., 2003).

Viscerotropic leishmaniasis caused by *L. tropica*, which is thought typically to be dermatropic, was parasitologically confirmed in 12 U.S. soldiers who participated in Operation Desert Storm in the Persian Gulf in the early 1990s. The affected persons had light parasite burdens and nonspecific manifestations of visceral infection (e.g., fatigue, fever, and gastrointestinal symptoms) (Magil, et al., 1993).

Post kala-azar Dermal Leishmaniasis (PKDL) develops after therapy in a subset of persons with VL in Africa and India. It is characterized by macular, papular or nodular lesions on the face, trunk or extremities that may be mistaken for leprosy. Amastigotes are present in macrophages in the lesions. In East Africa PKDL appears shortly after VL symptoms subside (0-6months), in India the interval is 1 to 2 years, with up to 30 years being reported in exceptional cases (Zijlstra et al., 1995). In Sudan and India, the main loci of PKDL, 50% and 5-10% of VL treated patients respectively; develop PKDL (Zijlstra, et al. 2003).

2.5 Diagnosis

Diagnostic tests have a crucial role in the management of patients and in the control of infectious diseases, their role or uses can include some or all of the following: patient management, screening for asymptomatic infections, surveillance, including verification of elimination, epidemiological studies and detection of infections with markers or drug resistance. (Peeling et.al, 2008.).

Clinical features of VL can be easily mistaken for other febrile illnesses such as malaria and enteric fever. Reliable laboratory methods become mandatory for accurate diagnosis. Early case detection followed by adequate treatment is central to the control of VL. (Boelaert, et al., 2000, Davies et al., 2003, Srivastava. et al., 2011).

2.5.1 Parasitological Diagnosis (microscopy and culture)

The demonstration of parasites in relevant tissue such as spleen, bone marrow, lymph nodes, liver or the buffy coat of peripheral blood is commonly used and it is the most accurate (specific) method available for diagnosis of VL.(Hailu. et.al.,2005).

Normally the diagnosis is based on finding amastigotes in monocytes or macrophages (seldom are they found free in the tissues except in HIV positive patients). The analysis is done using a special logarithmic scale ranging from 0 (no parasite in 1000 microscopic fields) to +6 (more than 100 parasites per field). The two most commonly used tissues are bone marrow and spleen. The sensitivity of this approach using bone marrow is about 60-85% and in spleen is more than 95% making direct detection of parasites as the most powerful technique and the spleen the most sensitive tissues to detect Leishmania (Santarem and da Silva 2007). However, bone marrow and splenic aspiration are invasive, painful and risky techniques, and also the use of microscopy in the diagnosis of VL, like all microscopic procedures suffers from variability of

detecting sensitivity and the inevitable need for an expert microscopist (Santarem and da Silva, 2007).

Leishmania strains can be isolated and maintained as promastigotes in artificial media. The culture media used may be biphasic, (Novy-McNeal, Nicolle (NNN) medium and Tobias medium), for conversion of amastigotes into promastigotes, while monophasic medium (Schneider's insect medium, M199, or Grace's medium) is preferred for amplifying parasite number (Srivastava et al., 2011.). But the culture technique, too, suffers from the same deficiencies as the microscopic technique, and the tedious, time consuming nature and the high cost are prohibitive and thus, except in dedicated research laboratories, it is seldom used for clinical diagnosis.

In the above mentioned techniques, the correct identification of the parasite requires trained personnel and skilled observer. These special characteristics of this sort of approach to diagnosis makes it unfit for field use as it is time consuming and requires expert personnel.

2.5.2 Serological Diagnosis

Serological methods are non invasive, and are comparatively more suited for diagnosing VL in endemic regions.

Several antibody detection-tests have been developed for field diagnosis of VL, but all have two major limitations. First, though serum antibody levels decrease after successful treatment they remain detectable after several years after cure, therefore VL relapses cannot be diagnosed by serology. Second, a significant number of people living in endemic areas with no history of VL are positive for anti-leishmanial antibodies owing to asymptomatic infection (Chappuis et al.,2007).The sero-prevalence in healthy populations varies from <10% in low to moderate endemic areas to >30 in

high transmission foci or in house hold contacts .Antibody-detection tests must therefore always be used in combination with a standardized clinical case definition for VL diagnosis. Conventional serological methods such as gel diffusion immunoelectrophoresis, complement fixation test, indirect haemagglutination test and counter-current immunoelectrophoresis have limited diagnostic accuracy/or feature for field use. Indirect fluorescence test (IFA) tests showed acceptable estimates for sensitivity and specificity (77%-100%) but the needs for a fluorescence microscope restricts their use to reference laboratories (Boelaret, et al., 2007).

Enzyme linked immunoserpentassay (ELISA) has been used in the serodiagnosis of VL. Sensitivity and specificity depends upon the antigen used. Most promising results are shown by antigen rk39 with sensitivity and specificity of 100% and 96% respectively. The antibody titer to this antigen directly correlates with active disease and have potential in monitoring chemotherapy and in predicting the clinical relapse (Kumar et al., 2001). In addition rk39 ELISA has a diagnostic and prognostic utility in HIV infected patients (Houghton et.al, 1998). Due to the requirements of skilled personnel, sophisticated equipment and electricity, Elisa is not used in the endemic regions for the diagnosis of VL.

Immunoblotting test, is more sensitive than IFAT and ELISA, and provides detailed antibody responses to various leishmanial antigens, but it is expensive, time consuming, and requires considerable skill and is therefore sparingly used in the diagnosis of VL. (Srivastava et al., 2011).

Two antibody detection tests have been extensively evaluated for field use: the DAT using freeze dried antigen (Fig.8a) and the rk39 immunochromatographictest (ICT)

(Fig.8b), generally referred to as 'rk39 RDT', (rapid diagnostic test), (WHO/TDR, 2008).

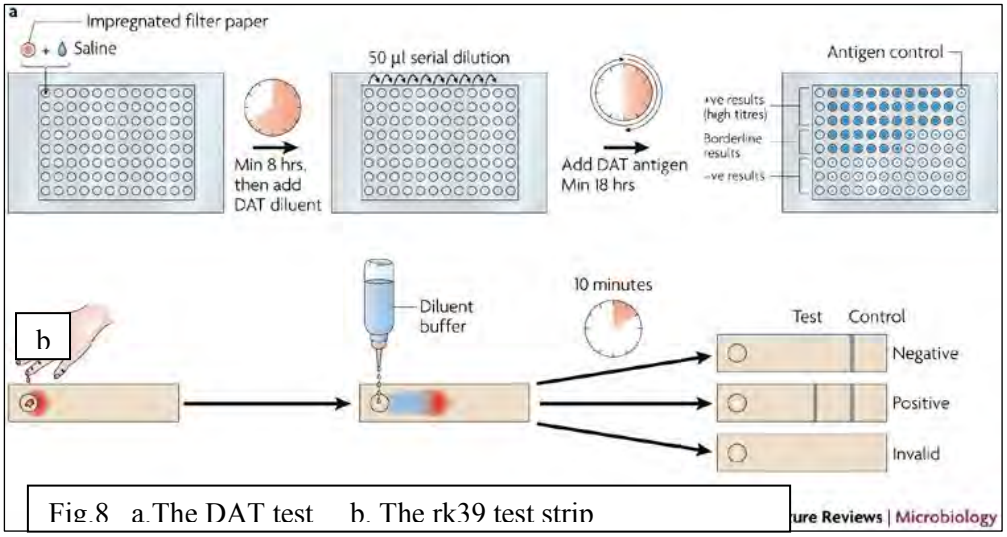

Figure 8. Basic procedure of a) DAT and (b) RDT (rk39 dipstick format) Source: www.nature.com/nrmicro/supplements

In 1986, EL-Harith and his co-workers at the KIT developed the DAT for the detection of anti-leishmanial antibodies in the serum samples from humans and dogs suspected for visceral Leishmaniasis (EL- Harith et al., 1986).The DAT is semi-quantitative test and uses microtitre plates with V-shaped wells in which increasing dilutions of patients serum or blood eluted from filter paper are incubated with comassie brilliant blue stained and killed *L.donovani* promastigotes. If antibodies are present, agglutination is observed after 18 hours (Chappuis et al., 2007).The test gives antibody titers ranging from 1/50 (usually1/100) to 1/102,400 or even higher. The test is the first line diagnostic tool in many countries (EL-Harith et al., 1988). In Ethiopia, DAT is used to confirm diagnosis of VL and initiate treatment in patients that strictly meet the clinical case definition (MOH, 2006). A problem with the DAT is the limited stability of the used aqueous antigen for which cold storage is required (EL-Harith et al., 1988). To circumvent this problem, a freeze dried antigen that remains stable at ambient

temperature (20^{o}C-45^{o}C) compares well with the standard aqueous antigen under laboratory and field conditions has been developed (Meredith et al., 1995, Zijlstra, et al., 1997, Oskam etal., 1999).

A recent meta-analysis of the DAT gave sensitivity and specificity estimates of 94.8% (95%confidence interval (CI) 92.7-96.4) and 97.1% (95% CI 93.9-98.7), respectively (Chappuis et al., 2006).The performance of the DAT was not dependent on the region nor on the Leishmania species **(**Boelaert et. al., 2007**).** However, the major disadvantage of the DAT is the need of multiple pipetting, relative long incubation time, high cost of antigen and limited production facility of quality controlled antigens in two European countries (Schallig et al., 2001).

rk39 ICT is a VL RDT test. A RDT is a simple, test that can be used at all levels of the health care services, it does not require highly skilled laboratory staff and results can be read easily and within 15-20 minutes. Rapid tests expedite the initiation of treatement. (WHO/TDR, 2008).

rk39 is a 39-amino acid repeat that is part of a kinesin-related protein in *Leishmania chagasi* and which is conserved within the *Leishmania donovani* complex. The rk39 ICT is composed of antigen immobilized on a piece of nitrocellulose memberane. Above it we find anti protein A. Half a drop of blood, serum or plasma is placed on the lower part of the strip, after the addition of phosphate buffered saline the mixture flows along the strip by capillarity dragging the conjugate (protein A colloidal gold) that was dried on the strip. Antibodies against the antigen are present in blood/ serum of kala-azar patients and they will bind to the recombinant antigen that is bound to the test strip yielding a pink/purple band. The presence of two bands is the final outcome of a

positive test for the rk39 and the anti-protein band control. (Santarem and da-Silva 2007, WHO/TDR 2008).

A recent meta-analysis of rk39 studies gave sensitivity and specificity estimates of 93.9% (95%confidence interval (CI) 87.7-97.1) and 95.3% (95% CI 88.8-98.1), respectively (Chappuis et al., 2006). The rk39 strip has accurate diagnostic performance in India and Nepal but the test has been shown to be less accurate in East Africa. The disadvantage of rk39 ICT is that a significant proportion of healthy individuals in endemic regions remain positive and for long periods after cure of VL.

Antigen detection tests should be more specific than antibody detection tests as they avoid cross reactivity and can distinguish active from past infections, and is particularly practical in the case of HIV/VL co-infection where deficient antibody production complicates accurate diagnosis of VL (Salam et al.,2011).

A new latex agglutination test (Katex) for detecting leishmanial antigens in the urine of patients with active VL has been introduced by Kalon Biological limited. Katex detects a heat-stable, non-protein, carbohydrate based, disease-specific parasite antigen in the urine of patients with active infections (Sarkari et al., 2002).

In some preliminary studies the test has shown a sensitivity of 68-100% and a specificity of 100 %. However more recent field studies have shown a much wider variation in estimates of sensitivity of (36-100%) and specificity of (64-100%). Apart from its low sensitivity, the limitations of the test are: the urine must be boiled to avoid false-positive reactions and it is difficult to distinguish weakly positive from negative results, which affects the reproducibility of the test (Chappuiset al., 2006, Rijal et al., 2004).

2.5.3 Molecular Diagnosis

A variety of nucleic acid detection methods targeting both DNA and RNA have been developed. The most suitable target for the DNA based diagnosis is kinetoplast DNA mini circle (k-DNA). The Leishmania polymerase chain reaction (PCR) assays using peripheral blood as clinical specimen showed to be a highly efficient non-invasive alternative with sensitivity varying from80-100 % (Singh et al., 2006).

2.6. VL Control

Reservoir and vector control, the use of insecticide impregnated materials and active case detection and treatment are the control strategies for VL control (Chappuis et al.2007).

2.6.1 Reservoir Control

In zoonotic VL, dogs are the main reservoir of *L.infantum*. Screening of dogs and killing of sero-positive animals, has become unacceptable and is increasingly being debated. Treating infected dogs is not an effective strategy as relapses are frequent. In stutdy conducted in Iran the use of delamethrine treated collars, which reduced the risk of infection in dogs by 54% and in children by 45% (Gavgani et al. 2002).

2.6.2 Vector Control

Sandflies are susceptible to the same insecticides as Anopheles mosquitoes. Following the large scale anti-malaria insecticide (dichloro-diphenyl-trichloro ethane, DDT) spraying campaign that were implemented in the 1950s, VL almost completely disappeared from the Indian sub-continent. Unfortunately the disease re-emerged when the spraying campaign was discontinued (Chappuis et al., 2007).

In East Africa VL endemic countries, transmission occurs mainly outside villages during various activities like shepherding and farming. Therefore indoor residual spraying for disease control is unlikely.

2.6.3 Insecticide Impregnated Materials (ITNs).

ITNs could concomitantly prevent VL and vector borne diseases such as malaria. There is limited evidence that bednets provide protection against VL. Depending on the sleeping traditions of the population and the biting habits of the local vector, other insecticide-impregnated materials such as curtains and blankets should be evaluated for use in VL prevention (Chappuis et al., 2007).

2.6.4 Case detection and treatment

Untreated VL patients act as a reservoir for parasites and therefore contribute to disease transmission in anthropomotic VL areas. Early case detection and treatment is considered an essential component of VL control (Desjeux 2004, Boaelaert et al., 2008). The concomitant detection and treatment of PKDL patients is also likely to be beneficial and the feasibility, impact and cost of PKDL management strategy should therefore be properly evaluated.

2.6.5 VL control in Ethiopia

In Ethiopia, knowledge about the transmission of VL is far from being complete. The role of sub-clinical infection of humans, as the source of infection and transmission, PKDL cases, HIV co-infected persons and animal reservoirs in the transmission of VL has yet to be determined (Hailu et al. 2006).

The sandfly vector species in many localities has not been determined. The vectorial status of *P.orientalis*, involvement of other species in the transmission of VL in the

lower Omo valley has not been ruled out, Also the exact role of *P.orientalis* (and possibly *P.alexandri*) in the transmission of VL in north western Ethiopia has yet to be determined (Hailu et al. 2006)

The use of insecticide treated nets might prove useful during epidemics and in areas of high HIV/VL co-infection where man-to-man transmission becomes more important than zoonotic transmission. But their usefulness in endemic settings has been questioned due to, the possibly varying biting and resting behavior of sandflies especially of *P.martini* and *P.orientalis*, both of these species prefer to bite exophagic and rest outdoors, and of the limited evidence that treated bed nets provide. Access to facilities of diagnosis and treatment has to be improved to curb the high mortality rate (Hailu et al. 2006).

3. Objective

3.1 General:

To assess and compare the test performance of different rapid diagnostic tests of VL in stored/archived serum samples originating from VL endemic areas in Ethiopia.

3.2 Specific:

- To determine the specificity, sensitivity and precision of the new RDTs (Inbios plus, DPP® and EMT tests).
- To compare the accuracy of different RDTs used in this study.
- To assess performance of the tests in the diagnosis of VL in HIV-VL co-infected patients.

4. Materials and Methods

4.1 Sample size determination

To estimate the sensitivity (Sn) and specificity (Sp) of the diagnostics tests, the sample size required was calculated using the following formula :

$$n \geq \frac{Z^2_{\alpha}/2*P(1-P)}{e^2}$$

Where Z_{α} = standard score=1.96(using 95% C.I or α=0.05).

P= assumed sensitivity/specificity

e= margin of error

Accordingly, the minimum sample size, assuming sensitivity equals 75%, specificity equals 90%, and e = 10%, required for VL and Non-VL was determined to be 75 and 35. (With 90% SN the sample size would have decreased to 35).

4.2 Serum Samples

In this study, 277serum samples obtained from different subjects were tested. The 242 serum samples were left over samples from previous ethically cleared projects collected and archived at Addis Ababa University, Medical Faculty, Department of Microbiology, Immunology and Parasitology, Leishmaniasis Diagnostics Services. The remaining 35 serum were from blood collected from healthy non-endemic volunteer subjects. The following 6 panels or groups were included:

a) Active VL (AVL) (n=95) versus Healthy controls (HC) (n=35). In the AVL group 13 of the samples are HIV positive, the HIV status of the rest is not known.
b) AVL versus Endemic Controls (EC) (n=41), (identification of asymptomatic infection).

c) AVL versus Other Diseases (Malaria, n=13, Intestinal Schistosomiasis, n=12, Tuberculosis, n=10).

d) AVL versus Cutaneous Leishmaniasis (LCL, n=13; DCL, n=11; MCL, n= 12).

e) AVL versus Post Treatment VL Day 31 (n=35).

f) VL versus HIV/VL (VL, n=82; HIV/VL n=13).

The inclusion criteria for VL serum samples in this study were:

All AVL patients were parasitological positive, previously confirmed by positive smear and/or culture, all had major VL clinical symptoms and were diagnosed as AVL cases.

Panels of negative sera were from 41 endemic controls, confirmed to be DAT negative, from previously collected and archived samples. The 35 healthy control sera were from blood collected from healthy non endemic volunteers, who have never travelled or been in VL endemic area.

For cross reactivity studies, sera of other diseases were used. All sera for other diseases were parasitological and bacteriologically (TB) confirmed cases. The sera for CL diseases were smear and/or culture positive, LCL, DCL, and MCL cases.

No personal identifiers were recorded out as part of this work. Information about the patient's clinical diagnosis was available to use at the time this study was undertaken.

4.3 Diagnostic Tests

Six different tests were employed, which include two rk39 based, three rk28 based RDTs and DAT (Table 2).

Table 2: Tests used for evaluation

Test name	Manufacturer	Lot.No.	Bound Ag
DiaMed-IT LEISH	Diamed-AG	0H0012L	rk39
Kalazar Detect	InBioss International Inc.	NB-138-169	rk39
Kalazar Detect Plus	InBioss International Inc.	Not available	rK28
DPP	Chembio Diagnostics System Inc	DPPHVL050 3111	rk28
EMT	Ease-Medtrend Biotech	Not available	rk28
FD-DAT	KIT Royal Tropical Institute	1008	whole *L.d. promastigotes*

4.3.1 DAT

The DAT was performed using freeze dried antigen (Lot NO. 1008, obtained from KIT Royal Tropical Institute), and essentially as described by EL-Haitth et al., (1988). Briefly serum samples were diluted in NaCl solution 0.85% (wt/vol) containing 0.78% (vol/vol) 2-mercapethanol. To discriminate between agglutination and non-agglutination the use of V-shaped microtitre plates is mandatory (EL-Harith et al., 1986).Two-fold dilution series of the serum samples were made from 1:100 to 1:102,400. Prior to its use the freeze dried antigen was reconstituted in 5ml of normal saline (0.85%wt/vol.Nacl). Reconstituted antigen (50µl)was added to each well of the microtiter plates containing (50µl) of diluted serum. After the samples were incubated overnight at room temperature, the end titer was read as the dilution immediately before the well containing a clear sharp-edged blue spot identical to the negative

control. The cut of titer is set at 1/1600 which is the cut of titer set in Ethiopia (MOH, 2006).

4.3.2 Rapid Tests

In this study five RDTs in different formats (dipstick or cassette format) was used. The respective manufacture's procedures were strictly followed for all the tests. Following is a brief procedure of each test:

4.3.2.1 Diamed-IT leish rk39 RDT

DiaMed-IT leish rk39 RDT,The diagnostic test kit (Lot.No. 0H0012L), was obtained from Diamed AG, 1785 Creisser s/Morat,Switzerland. The test kit is composed of a membrane coated with rk39 antigen in the case of the presence of such antibodies in the sample the antibodies captured by the conjugate react with the specific coated rk39 antigen. The kit is provided with a device containing 2 wells; that of the conjugate well and the wash well. The kit is placed horizontally on a flat surface and labeled. 1 drop of buffer was added to the conjugate well and 4 drops to the wash well. 8-10µl of serum was added to the conjugate well, mixed and allowed to stand for 1 minute. The device was pulled apart and the legs of the dipstick holder inserted into the holes beside the conjugate wells so that the dipstick end reaches the bottom of the conjugate well, after 5-10 minutes the dipstick is transferred to the wash well and let to stand for 10 minutes after which it is clicked back into the clear plastic piece, the reaction read and interpreted i.e. a control line at the top of the strip verifies that the test strip is functional. If this is the only line that appears the test is considered negative; two lines are positive for VL. The dipstick slide was kept for future reference.

4.3.2.2 Kala-azar Detect™ Rapid Test (rk39-Inbioss) Kala-azar Detect™ Rapid Test, (rk39 dip stick), was obtained from Inbioss International Inc., Seattle, WA, USA, (Lot.No.NB-138-169). Briefly the procedures involved loading 20µl serum onto the test strip and placing it into a well of a 96 well microtiter U shaped plate. Next 2-3 drops of the chase buffer solution provided with the test kit was added, finally the test result was read after 10 minutes and recorded qualitatively as positive and negative reactions depending on the presence or absence of a line in the test area of the strip. The test was invalid and repeated if no control line appeared.

4.3.2.3 Kala-azar Detect Plus Rapid Test (rk28-Inbioss +)

Kala-azar Detect Plus Rapid Test, (rk28 dipstick), is produced by Inbioss International Inc., Seattle, WA, USA. 3 drops of the conjugate solution, followed by 5 µl of serum sample is added to a well of a 96 well microtiter U shaped plate and mixed by pipetting several times, after 1 minute the rapid test is placed in the well incubated for 5 minutes, at which time the solution travels visibly up the membrane. The dipstick is transferred to a clean well and 7 drops of the chase solution added to the well, result was read and interpreted after 20 minutes.

4.3.2.4 DPP® Leishmania Rapid Assay (rK28-DPP)

The DPP® Leishmania Rapid Assay (rK28-DPP), produced by Chembio Diagnostic Systems Inc. Medford, NY USA (Lot No. DPPHVL0503111), was used in this experiment. The test device which is in cassette form is removed from its pouch and labeled, 5µl of the serum sample was pipetted into the center of the round sample +buffer well of the device. The running buffer bottle was held vertically over the sample + buffer well and 2 drops of buffer slowly added to the well. After 5 minutes 4

drops of Leishmania running buffer was added to the buffer well and test result read after 15-20 minutes

4.3.2.5 Ease Med Trend (rK28-EMT)

The Ease Med Trend (rK28-EMT) is produced by ease Medtrend Shangai China. The test device is in cassette form. To run the test, initially 2 drops of protein A colloidal gold conjugate was added onto the fiber glass pad, the gold conjugate will run into the membrane, 4 µl of serum sample was added onto the membrane right above the tape onto the running stream of colloidal gold conjugate and was read and interpreted after 10 minutes.

4.4 Test/control panel and categories for comparison

All the sera of patients in the different groups were tested with all the VL tests in five different control panel categories (Tables 3-5).

Table 3: VL tests and number of sera tested in the different patient/control groups.

Groups	DAT	Diamed	Rk39 Inbioss	K28 Inbioss	DPP	EMT
VL	82	82	82	82	82	82
HC	35	35	35	35	35	35
EC	41	41	41	41	41	41
OD	35	35	35	35	35	35
CL	36	36	36	36	36	36
PT	35	35	35	35	35	35
HIV/VL	13	13	13	13	13	13
Total	277	277	277	277	277	277

Table 4: Non-VL control panel classified

Category	Control panel used & (serum sample)	
1	HC,EC,CL,OD	(n = 147)
2	HC, EC, OD	(n = 112)
3	HC	(n= 35)
4	EC	(n=41)
5	HC,EC	(n=76)

Table 5: Elements of the 2x2 table (VL vs category 1 to 5).

Groups	Panel and (serum sample)
VL	AVL (n=95)
Category1	HC,EC,CLOD (n =147)
Category2	HC,EC,OD (n =112)
Category3	HC (n =35)
Category4	EC (n =41)
Category5	HC/EC(n=76)

4.5. Statistical Analysis

4.5.1 Calculation of specificity, sensitivity, PPV, NPV The diagnostic performance of each test was calculated using the classical 2X2 contingency table (Table 6).

Table: 6 *2x2* table to evaluate test performance

Test Under evaluation	Reference test		Total
	Positive	Negative	
Positive	a(TP)	b(FP)	a+b
Negative	c(FN)	d(TN)	c+d
Total	a+c	b+d	

a=true positive, b=false positive, c=false negative, d=true negative
(Source: www.nature.com/reviews.micro 2007)

Test sensitivity= TP/TP+FN = a/a+c

Test specificity= TN/FN+TN = d/c+d

PPV= TP/TP+FP =a/a+b

NPV= TN/FN+TN =d/c+d

Data was analyzed using SPSS for windows (SPSS 15 Chicago, IL 2006) and EpiInfo (EpiInfo version 3.4 2007 CDC) statistical programmes.

4.5.2 Measurement of agreement between tests, Kappa

Kappa, the measurement of aggrement between tests, was calculated using SPSS for windows (SPSS 15 Chicago, IL 2006).

4.6 Ethical Consideration

This study was reviewed and approved by the Ethical Clearance Committee of the Faculty of Life Sciences, Addis Ababa University.

5. RESULTS

5.1 Demographic

A total of 277serum samples, (218 males (78.7 %) and 59 females (21.7%), from previously collected subjects and archived at Leishaniasis Research and Diagnostic Laboratory was used in this study (Table 7).

Table 7: Age and Sex Distribution

AGE CATEGORY	MALE (%)	FEMALE(%)	BOTH SEXES (%) TOTAL
4-13	49	17	23.8
14-23	71	15	31.0
24-33	48	11	21.2
34-43	31	8	14.1
44-53	8	6	5.1
54-63	5	2	2.5
64	6	0	2.1
T0TAL	**218(78.7)**	**59(21.7)**	**100%**

5.2 Serology Results

The sensitivity and specificity for all the tests was calculated in five categories where in each category different control panels were used to see the effect especially on the specificity of the tests.

5.2.1 DAT: The DAT had a sensitivity of 91.6% (95%CI=84.1-96.2). The specificity ranged from 89.8% - 100% according to the control panel used (Table 9). The highest specificity was found to be in categories 3 where the control panel consisted of only healthy non endemic controls. The lowest specificity was in category 1, where the control panel consisted of sera from healthy controls, endemic controls, other diseases and cutaneous leismaniasis subjects. 10 out of 36 CL positive sera cross reacted with the test. The cross reaction was with DCL (7/11) and the MCL (3/12) sera. There was no cross reaction with the LCL sera. Also 3 out of 35 sera from subjects with other diseases cross reacted, all the 3 cases being from the TB group. No cross reaction was

observed with malaria and schistosomiasis positive sera. The DAT tested positive 93.9% of the VL, 76.9% of the HIV/VL and 100% of the PT cases (Table 8).

Table 8. Sero-positivity of different sera of patients and test panel under study

No pos/No test %Pos	DAT	DIAMD	INBIOS	INBIOS+	DPP	EMT
VL=No pos/No test %Pos	87/2 93.3	80/82 97.6	77/82 93.9	81/82 98.8	80/82 97.6	81/82 98.8
HIV/VL=No pos/No test %Pos	10/3 76.9	12/13 92.3	11/13 84.6	12/13 92.3	11/13 84.6	13/13 100
HC= No pos/No test. %Pos	0/35 0	0/35 0	0/35 0	2/35 5.7	0/35 0	0/35 0
EC= No pos/No test. %Pos	0/41 0	2/41 4.9	2/41 4.9	8/41 19.5	3/41 7.3	8/4 19.5
CL= No pos/No test. %Pos	10/6 27.8	29/36 80.6	20/36 55.6	31/36 86.1	21/36 58.3	26/36 72.2
LCL= No pos/No test. %Pos	0/13 NA	10/13 NA	7/13 NA	11/13 NA	7/13 NA	8/13 NA
DCL = No pos/No. test. %Pos	7/11 NA	9/11 NA	6/11 NA	10/11 NA	8/11 NA	9/11 NA
MCL= No pos/No. test. %Pos	3/12 NA	10/12 NA	7/12 NA	11/12 NA	6/12 NA	9/12 NA
PT=No pos/No. test. %Pos	35/5 100	35/35 100	34/35 97.1	35/35 100	34/35 97.1	35/35 100
OD= No pos/No test %Pos	3/35 9.4	0/35 0	0/35 0	9/35 25.5	3/35 8.6	14/35 40
Malaria= No pos/No test %Pos	0/13 NA	0/13 NA	0/13 NA	6/13 NA	0/13 NA	8/13 NA
Schisto = No pos/No test %Pos	0/12 NA	0/12 NA	0/12 NA	3/12 NA	3/12 NA	6/12 NA
Pulm.Tb.= No pos/No test %Pos	3/10 NA	0/10 NA	0/10 NA	0/10 NA	0/10 NA	0/10 NA

Table 9. Sensitivity, specificity, PPV, NPV of DAT based on different sets of control panel (Categories 1-5)

Control panel	DAT Results	VL	Non-VL	Total	Se, Sp, PPV, NPV [95% CI]
Category1 (HC,EC,CL, & OD)	Pos	87	13	100	Se = 91.6% [84.1, 96.3] Sp = 89.8% [83.7, 94.1] PPV = 85.3% [76.9, 91.5] NPV = 94.3% [89.1, 97.5]
	Neg	8	134	142	
	Total	95	144	242	
Category2 (HC, EC & OD)	Pos	87	3	90	Se = 91.6% [84.1, 96.3] Sp = 97.3% [92.3, 99.4] PPV = 96.7% [90.6, 99.3] NPV = 93.1% [86.9, 96.9]
	Neg	8	108	116	
	Total	95	111	206	
Category3 (HC)	Pos	87	0	87	Se = 91.6% [84.1, 96] Sp = 100% [90.0,100] NPV = 94.3%[66.6,91.6] PPV = 100%[95.9,100]
	Neg	8	35	43	
	Total	95	35	130	
Category4 (EC)	Pos	87	0	87	Se = 91.6% [84.1, 96.3 Sp = 100.0% [91.4,100.0] PPV = 100.0% [95.9,100.0] NPV = 83.7% [70.3, 92.7]
	Neg	8	41	49	
	Total	95	41	136	
Category5 (HC & EC)	Pos	87	0	87	Se = 91.6% [84.1, 96.3] Sp = 100.0% [95.3, 100.0] PPV = 100.0% [95.6, 100.0] NPV = 90.5% [82.1, 86.0]
	Neg	8	76	84	
	Total	95	76	171	

5.2.2 rk39 DiaMed: The sensitivity of rk39 Diamed was found to be 96.8% (95%CI,91.1-99.3).The specificity ranged from 78.9 % - 100% .The lowest specificity 78.9% was in category 1,where HC, EC, CL & OD, were used as the negative control panel, while the highest specificity was in category 3, (Table 10), where HC only was used as the negative control panel. 29 out of the 36, (80.6%), CL positive sera tested positive. Also the rk39 Diamed tested positive, 97.6% of the VL, 92.3% of the HIV/VL, and 100% of the PT .From the negative control sera (HC and EC), and other diseases (OD) none tested positive. (Table 8).

Table 10. Sensitivity, specificity, PPV, NPV of DiaMed based on different sets of control panel (Categories 1-5)

Control panel	DiaMed Results	VL	Non-VL	Total	Se, Sp, PPV, NPV [95% CI]
Category1 (HC,EC,CL, & OD)	Pos	92	31	123	Se = 96.8% [91.1,99.3] Sp = 78.9% [71.4-85.2] PPV = 74.8% [66.2,82.1] NPV = 97.5% [92.8,99.5]
	Neg	3	116	119	
	Total	95	147	242	
Category2 (HC,EC, & OD)	Pos	92	2	94	Se = 96.8% [91.1,99.3] Sp = 98.2% [93.6,99.8] PPV = 97.9% [92.2,99.7] NPV = 97.3% [92.4,99.4]
	Neg	3	109	112	
	Total	95	111	206	
Category3 (HC)	Pos	92	0	92	Se = 96.8% [91.1,99.3] Sp = 100% [90.0,100.0] NPV= 92.1%[78.6,98.3] PPV= 100[96.1-100]
	Neg	3	35	38	
	Total	95	35	130	
Category4 (EC)	Pos	92	2	94	Se = 96.8% [91.1,99.3] Sp =95.1% [83.5,99.4] PPV = 97.9% [92.5,99,7] NPV = 92.9% [80.5,98.5]
	Neg	3	39	42	
	Total	95	41	136	
Category5 (HC& EC)	Pos	92	2	94	Se = 96.8 % [91.1,99.3] Sp =97.4 % [90.8-99.7] PPV = 97.9% [92.5,99.7] NPV =96.1 % [89.0-99.2]
	Neg	3	74	77	
	Total	95	76	171	

5.2.3 rk39 Inbioss: The sensitivity of rk39 Inbioss was found to be 92.6% (95%CI,88.4-97.0), the specificity ranged from 85%-100% . The highest specificity was in category 3 and lowest in category 1 (Table 11).

The test cross reacted with 20 out of the 32 CL sera. No cross reaction was observed in the other disease group. While 93.9% of the VL, 84.6% of the HIV/VL, and 97.1% of the PT tested positive. From the negative control sera positive reaction was observed in EC 4.9% while all HC sera tested negative. (Table 8).

Table : 11 Sensitivity, specificity, PPV, NPV of Inbioss based on different sets of control panel (Categories 1-5).

Control panel	Inbioss Results	VL	Non-VL	Total	Se, Sp, PPV, NPV [95% CI]
Category1 (HC,EC,CL, & OD)	Pos	88	22	110	Se = 92.6% [85.4,97.0] Sp = 85.0% [78.2-90.4] PPV = 80.0% [71.3-87.0] NPV = 94.7% [89.4,97.8]
	Neg	7	125	132	
	Total	95	147	242	
Category2 (HC,EC, & OD)	Pos	88	2	90	Se = 92.6% [85.4,97.0] Sp = 98.2% [93.6,99.8] PPV = 97.9% [92.2,99.7] NPV = 93.8% [92.5,99.7]
	Neg	7	109	106	
	Total	95	111	196	
Category3 (HC)	Pos	88	0	88	Se = 92.6% [85.4-96.9] Sp = 100% [90.0,100.0] PPV = 100% [95.9-100] NPV = 83.3[68.6-93]
	Neg	7	35	42	
	Total	95	35	130	
Category4 (EC)	Pos	88	2	90	Se = 92.6% [85.4-96.9] Sp = 95.1% [83.5,99.4] PPV = 97.9% [92.2,99,7] NPV = 84.8% [71.1,93.7]
	Neg	7	39	46	
	Total	95	41	136	
Category5 (HC & EC)	Pos	88	2	90	Se = 92.6 % [85.4-96.9] Sp = 97.4 % [90.8-99.7] PPV = 97.9% [92.5,99.7] NPV = 91.2 % [83.0-96.5]
	Neg	7	74	81	
	Total	95	76	171	

5.2.4 rk28 Inbioss+: The rk28 based Inbioss+, has a sensitivity of 97.9 (95%C,92.6-99.7) and specificity ranging from 65.9% -97.9% (Table 12). The highest specificity being in the HC control panel and lowest in category1. From the 36 CL confirmed sera 31 were positive .In the OD control panel 9 out of the 36 sera cross reacted, no cross reaction was observed in the TB group. The rk28 tested positive, 98.8%of the VL, missing only one VL case, 92.3% of the HIV/VL, and 100% of the PT. Positive reaction was observed in the negative controls, 5.7% HC, 19.5% EC (Table 8).

Table: 12 Sensitivity, specificity, PPV, NPV of Inbioss+ based on different sets of control panel (Categories 1-5)

Control panel	Inbioss + Results	VL	Non-VL	Total	Se, Sp, PPV, NPV [95% CI]
Category1 (HC,EC,CL, & OD)	Pos	93	50	143	Se = 97.9%[92.6,99.7] Sp = 65.9% [57.7,73.6] PPV = 65.0% [56.6, 72.0] NPV = 97.9% [92.9,99.8]
	Neg	2	97	99	
	Total	95	147	242	
Category2 (HC,EC, & OD)	Pos	93	19	112	Se = 97.9%[92.6,99.7] Sp =82.9 % [74.6,89.4] PPV =83.0 % [74.8,,89.5] NPV = 97.9% [92.5,99.7]
	Neg	2	92	94	
	Total	95	111	206	
Category3 (HC)	Pos	93	2	95	Se = 97.9%[92.6,99.7] Sp = 94.3% [80.8,99.3] PPV = 97.9%[92.6,99.7] NPV =94.3%[80.8,99.3]
	Neg	2	33	35	
	Total	95	35	130	
Category4 (EC)	Pos	93	8	101	Se = 97.9%[92.6,99.7] Sp = 80.5% [65.1-91.2]] PPV =92.1 % [84.9-96.5] NPV =94.3 % [80.8-99.3]
	Neg	2	33	35	
	Total	95	41	136	
Category5 (HC & EC)	Pos	93	10	103	Se = 97.9%[92.6,99.7] Sp =86.8 % [77.1,93.5] PPV =90.3 % [82.9,95.3] NPV = 97.1% [87.8,99.6]
	Neg	2	66	68	
	Total	95	76	171	

5.2.5 DPP: The sensitivity of DPP was found to be 95.8 (95%CI= 89.6-98.8), and specificity ranging from 81.6%-100% in the different categories (Table13). The highest like the other tests was in category 3(HC) (Table14c) and lowest in category 1. From the 36 CL positive sera 21 tested positive, the cross reaction was in all the CL types (LCL, DCL, MCL). In the OD group the test cross reacted with only 3 schistosmiasis cases, no cross reaction with malaria and TB cases (Table 8).

The percentage positivity for the VL, HIV/VL, and PT panel was 97.6%, 92.3% and 97.1% respectively. From the negative control sera, EC tested 7.3% positive while HC had no positive reaction (Table 8).

Table: 13 Sensitivity, specificity, PPV, NPV of DPP based on different sets of control panel (Categories 1-5)

Control panel	DPP	VL	Non-VL	Total	Se, Sp, PPV, NPV [95% CI]
Category1 (HC,EC,CL, & OD)	Pos	91	27	118	Se = 97.9%[92.6,99.7] Sp = 81.6% [74.4,87.5] PPV= 77.1% [68.5, 84.4] NPV= 96.8% [92.0,99.1]
	Neg	4	120	124	
	Total	95	147	242	
Category2 (HC,EC & OD)	Pos	91	6	97	Se = 97.9%[92.6,99.7] Sp = 94.6 % [88.6,97.9] PPV = 93.8 % [87.0,97.7] NP = 96.3% [90.9,98.9]
	Neg	4	105	109	
	Total	95	111	206	
Category3 (HC)	Pos	91	0	91	Se = 97.9%[92.6,99.7] Sp = 100.0% [90,0,100.0] PPV = 100%[96.0,100.0] NPV= 89.7%[87.8,97.1]
	Neg	4	35	39	
	Total	95	35	130	
Category4 (EC)	Pos	91	3	94	Se = 97.9%[92.6,99.7] Sp = 92.7% [80.1,98.5] PPV = 96.8 % [90.9,99.3] NPV = 90.5 % [77.4-97.3]
	Neg	4	38	42	
	Total	95	41	136	
Category5 (HC& EC)	Pos	91	3	94	Se = 97.9%[92.6,99.7] Sp = 96.1 % [89.9,99.2] PPV = 96.8 % [90.0,99.3] NPV= 94.8% [87.2,98.6]
	Neg	4	73	77	
	Total	95	76	171	

5.2.6 EMT: The sensitivity of EMT was found to be 98.9% (95%CI, 94.3-99.9).The specificity from67.4% -100% (Table 14). The highest specificity was in category 3 (HC) and the lowest in category 1.EMT cross reacted with 26 out of the 36 CL positive sera (Tables9&10). In the OD group the test cross reacted with the malaria and schistosomiasis positive sera, no cross reaction with the malaria positive sera . EMT tested pos, 98.8% of the VL,100% of the HIV/VL, 100% of the PT. HC group had no positive reaction while EC tested 19.5% positive (Table 8)

Table: 14 Sensitivity, specificity, PPV, NPV of EMT based on different sets of control panel (Categories 1-5)

Control panel	EMT results	VL	Non-VL	Total	Se, Sp, PPV, NPV [95% CI]
Category1 (HC,EC,CL, & OD)	Pos	94	48	142	Se = 97.9%[92.6,99.7] Sp = 67.4% [59.1, 74.9] PPV =66.2% [57.8, 73.9] NPV = 99.0% [94.6, 99.9]
	Neg	`1	99	100	
	Total	95	147	242	
Category2 (HC,EC & OD)	Pos	94	22	116	Se = 97.9%[92.6,99.7] Sp = 80.2% [71.5, 87.1] PPV = 81.0% [72.2, 87.7] NPV = 98.9% [93.9,99.9]
	Neg	1	89	90	
	Total	95	111	206	
Category3 (HC)	Pos	94	0	94	Se = 97.9%[92.6,99.7] Sp = 100.0% [90.0,100.0] Npv =97.2%[85.5,99.9] PPV =100%[96.1,100]
	Neg	1	35	36	
	Total	95	35	130	
Category4 (EC)	Pos	94	8	102	Se = 97.9%[92.6,99.7] Sp = 80.5% [65.1,91.1] PPV = 92.2% [85.1,96.6] NPV = 97.0% [84.7, 99.9]
	Neg	1	33	34	
	Total	95	41	136	
Category5 (HC & EC)	Pos	94	8	102	Se = 97.9%[92.6,99.7] Sp = 89.5% [80.4, 95.3] PPV = 92.2% [85.1, 96.6] NPV = 98.6% [92.2, 89.9]
	Neg	1	68	69	
	Total	95	76	171	

*Summarized results of all the 2x2 tables of the different VL tests are found in the annex part.

5.3 Comparisons of performance of agreement

Comparing the sensitivity and specificity of the different VL tests employed shows that the control panel that is used affects the specificity of the tests. The specificity is lowest when the control panel comprises HC, EC, CL, and OD, while specificity is high when the control panel is HC only (Table15).

Table 16 shows the kappa function between the six VL diagnostic tests used in these studies the kappa coefficient agreement quantifies reproducibility, correction for agreement expected by chance. Kappa values of one represent perfect agreement.

Table: 15 Comparison of sensitivity and specificity in the different control groups

TEST		CAT1	CAT 2	CAT 3	CAT 4	CAT 5
DAT	%Sen.	91.6	91.6	91.6	91.6	91.6
	%Spec.	89.8	97.3	100	100	100
DIAMED	%Sen.	96.8	96.8	96.8	96.8	96.8
	%Spec.	78.9	98.2	100	95.1	97.4
INBIOSS	%Sen.	92.6	92.6	92.6	92.6	92.6
	%Spec.	85	98.2	100	95.1	97.4
INBIOSS+	%Sen.	97.9	97.9	97.9	97.9	97.9
	%Spec.	65.9	82.9	94.3	80.5	86.8
DPP	%Sen.	95.8	95.8	95.8	95.8	95.8
	%Spec.	81.6	94.6	100	92.7	96.1
EMT	%Sen.	98.9	98.9	98.9	98.9	98.9
	%Spec.	67.4	80.2	100	80.5	89.5

Table 16: kappa, measure of agreement between the six tests (DAT, DiaMed, Inbioss, Inbioss+,DPP and EMT)

DAT					
0.734	DIAMED				
0.748	0.884	INBIOSS			
0.606	0.804	0.722	INBIOSS+		
0.734	0.868	0.841	0.762	DPP	
0.613	0.752	0.744	0.788	0.77	EMT

6. Discussion

6.1 rk28 based rdts

Based on Category 2, from the rk28 based RDTs the highest sensitivity was of EMT (98.9%) followed by Inbioss+ (97.9%) and DPP (95.8%). The specificity is highest in the DPP test (94.6%) followed by 82.9% and 80.2% of Inbioss+ and EMT respectively. The sensitivity estimates of the DPP and EMT is in line with the work of Pattabhi et.al. (2010), who reported 96 % (DPP) and 98 % (EMT), in parasitological positive sera of VL patients from Sudan and Bangladesh respectively. While the specificity estimate of the DPP,(94.6%), is slightly lower compared to pattabhi et.al. (2010). who reported 100% specificity using 20 EC,20 malaria and 18 pulmonary tuberculosis confirmed payients as the negative control panel. The specificity estimates of EMT of 80.2% in this study is low when compared to 92.5% of pattabhi et.al. (2010), who used EC sera with no history of VL as the control panel. The differences in specificities between the two studies could be explained by the type of control panel used. When both sensitivity and specificity estimates are taken into account optimal combination of sensitivity and specificity was found out to be in the DPP test.

In last two categories, category 4 and 5 where the control panel was EC and EC/HC respectively, cross reactivity was observed in all the tests, with the highest being in Inbioss+ and EMT. DPP having the lesser cross reactivity in both category 4 & 5, with specificity of 92.7% and 96.1% respectively. In both categories (4 & 5), the EC sera reacted with the different RDTs. This which could be due to the different antigens employed in the rk28 test formats, (the rk28 comprises of k26, k39 and k9 antigens), These multiple antigens could pick circulating antibodies in the healthy endemic

control sera. As proposed by Pattabhi et al. (2010), it should be further investigated to address the question whether rk28 could be used as an indicator of disease progression.

In the percentage positivity results (Table 8), all the rk28 RDTs tested positive the sera of post treated subjects, except DPP which missed one sample (Table 8), this confirms that anti-leishmania antibodies persist long after treatment or cure (Hailu,1990),and also shows the drawback of antibody based serological tests in that they cannot differentiate between present and past infections.

Also all the CL, OD and EC sera cross reacted with all the tests .The highest cross reactivity observed in the CL group. Indicating that there is high cross reaction of the rk28 tests with antibodies against L.aethiopica .

The 13 HIV/VL samples from the AVL group were analyzed separately with all the tests. All the tests showed high percentage positivity, with EMT picking all the 13 cases. Due to the inadequate number of samples tested it is difficult to give any conclusions and further study on a larger sample is needed

6.2 rk39 based rdts

The two rk39 based RDTs (Inbioss and DiaMed) evaluated in this study are commercially available and have been employed in the diagnosis of VL in different VL endemic regions, with high sensitivity and specificity in the Indian sub-continent and lower sensitivity and specificity in the eastern Africa VL endemic regions (Boelaert et al 2008).

In our study we found DiaMed to have a sensitivity of 98.6% compared to Inbioss 92.6%, and both tests having the similar specificites in categories 2, 3, 4, & 5.

Comparing the percentage positivity between the two tests in the different panels, both have very similar results except in the CL panel where DiaMed cross reacted with 29/36, (80.6% positivity), while Inbioss cross reacted with 20/36 (55.6% positivity), of the CL sera.

A meta analysis of the diagnostic performance of the DAT and rk39 RDTs (Inbioss), showed sensitivity of 93.9% and specificity of 90.6% (Chappuis, 2006) for the rk39 in HIV negative parasitologically confirmed VL cases. Diro et al (2007) in Ethiopia showed a low sensitivity of 71.1% for rk39 (Inbioss) (active case detection). Data on 38 passively recruited patients in Ethiopia showed a sensitivity of 75.4% and specificity of 70% for rk39 (Inbioss) (Boelaert et al., 2008)

Canvate et.al, (2011), assessing the performance characteristics of four serological tests in a new epidemic site in Ethiopia found out sensitivity estimates of 94.3% and 91.4% for Inbioss and Diamed respectively and specificity estimates of 98.5% and 94%. Even though direct comparisons between our results and the results of other could be difficult to make, some of the reasons being, test format employed, study design, type of control used. Our results show better performance of both rk39 from previous work done in Ethiopia.

6.3 DAT

The DAT since its development in the 1980s has been in use in the diagnosis of VL in various endemic areas, including Ethiopia, and has been evaluated extensively. In the present study the sensitivity and specificity were 91.6% and 97.3% respectively at a cut of titer of 1:1600, the cut of titer previously determined with the FD-antigen (Meredith et al., 1995) and also recommended by the MOH to be used in Ethiopia (MOH, 2008).

Earlier studies indicating a sensitivity of 100% was frequently reported (Harith et al., 1988, Abdelhammed et al., 1989, Hailu, 1990). Evaluating the DAT on stored sera, at a cut of titer 1600, and similar conditions to this work Oskam et al., 1999 reported sensitivity of 82% which is low compared to our result. The DAT result in this study corroborate with the results of (Olivera et al., 2009), (Canavate et al., 2011), (De Assis et al., 2011) who showed sensitivity ranging from 90%-93.4% and specificity ranging from 96.9%-98.5%.

The slightly lower sensitivity than expected in this study could be due to the presence of samples from patients with recent infections from which it is known that antibody levels may be low, or by batch to batch variation of antigen (Oskam et al., 1999).

Finally the Kappa function shows strong agreement between the DiaMed and Inbioss, and DiaMed and DPP tests, Kappa 0.884 and 0.868 respectively. The weakest agreement was between DAT and EMT tests, Kappa 0.613.

8. Conclusion and Recommendations

All the VL tests evaluated have sensitivity values above 90% (91.6%-98.9%), lowest being of the DAT and highest being of EMT. Specificity values were greater than 80% (80.2%-98.2%) lowest being of EMT and highest of both the rk39 test formats. But when both sensitivity and specificity are taken into account, DiaMed, Inbioss and DPP performed better the other tests in our study.

When ease of use, equipments required, number of steps, band intensity, sensitivity and specificity are all taken into account, the performance of DPP test format is better when compared with the other. This may make the DPP test format preferable to use in primary health care and field setting after further studies in the field and actual setting are carried out.

The importance of VL diagnosis in Ethiopia, where VL is endemic in different remote parts of the country and where it affects mainly the poor population, and because HIV/VL co-infection is on the rise, should be given high priority in order to effectively control the disease.

The knowledge of the performance of the test to be employed is very important to accurately diagnose the disease which in turn helps in case management, avoid unnecessary medication and help to design control strategies.

Future work mainly on the performance of the rk28 based RDTs in passive and active case finding settings in VL endemic areas should be carried out and also the performance of the tests in HIV/VL co-infected patients should be done on a larger number of samples.

8. References

1. Abdelhammeed AA, Harith AE, Abdelrahim IM (1989). Potential of a direct agglutination test (DAT) for serological diagnosis of visceral Leishmaniasis in a known endemic area in Sudan. *Trop Med Paras 40: 470-471*.
2. Ali A and Ashford RW (1993a). Visceral Leishmaniasis in Ethiopia. I. Cross sectional Leishmania skin test in an endemic locality. *Ann Trop Med Paras 87:157-161.*
3. Ali A and Ashford RW (1993b). Visceral Leishmaniasis in Ethiopia. II. Annual leishmanin transformation in a population. Is positive leishmanin reaction a life-long phenomenon*? Ann.Trop Med Paras 87:163-167.*
4. Ali A and Ashford RW (1994). Visceral Leishmaniasis in Ethiopia. III. The magnitude and annual incidence of infection, as measured by serology in an endemic area. *Ann.Trop Med Paras 88:43-47.*
5. Andersen TF (1943). Kala-azar in the east African forces.*East Afr Med J 20:172-175.*
6. Arias JR, Monteiro PS, and Zicker F(1996). The reemergence of visceral Leishmaniasis in Brazil. *Emerg Infect Dis2:145-146*
7. Ashford RW, Bray MA, and Foster WA (1973).The epidemiology of cutaneous leishmaniasis in Ethiopia. *Trans R Soc Trop Med Hyg 67:568-601.*
8. Ashford RW, Hutchinson MP, and Bray RS (1973). Kala-azar in Ethiopia: epidemiological studies in a highland valley. *Eth Med J 11:259-264.*
9. Ayele T and Ali A (1984). The distribution of visceral Leishmaniasis in Ethiopia. *Am.J Trop.Med Hyg 33:(4) 548-552.*
10. Balkew M, Hailu A, Berhe N, and Gemetchu T(1999). Leishmaniasis in the Lower Omo plains, in south-western Ethiopia: The sandfly fauna. *Eth Med J: 37:31-40.*

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

12. Boelaert M, Bhattacharya S, Chappuis S, EL Safi HS, Hailu A, Mondal D, Rijal S, Syndar S, Wassuna M, Peeling WP (2007). Evaluation of rapid diagnostic tests: visceral leishmaniasis. *Nat Rev Micro S30-S39.*

13. Boelaret M, Criel B, Leeuwenburg,J, van Damme, Le Ray D and Van der Stuyft (2000). Visceral leishmanisis control: A public health perspective. *Trans R Soc Trop Med& Hyg 94:465-471.*

14. Boelaert M, El-Safi S, Hailu A, Mukhtar M, Rijal S, Sundar S, Wassuna M, Assefa A, Mbui J, Menten J, Desjeux P, Peeling RW (2008). Diagnostic tests for Kalaazar: a multi center study of the freeze dried DAT, rk 39srtip test and Katex in east Africa and the Indian sub-continent.*Trans R Soc Trop Med&Hyg.102:32-40.*

15. Canavate C, Herrero M, Nieto J, Cruz I, Chiccharro C, Aparichio P, Mulugeta A, Argaw D, Blackstock AJ, Alvar J, Bern C (2011). Evaluation of two rk39 dipstick tests, direct agglutination test and indirect fluorescent antibody test for the diagnosis of visceral Leishmaniasis in a new epidemic site in highland Ethiopia. *Am J Trop Med Hyg 84 :(1) 102-106.*

16. Chappuis F, Muller Y, Ngumifack A, Rwakimari JB, Coufignal S, Boelaret M, Cavailler P, Loutan L, and Poola P (2005). Diagnostics accuracy of two rk39 antigen based dipsticks and the formal gel test for rapid diagnosis of Visceral leishmnaiasis in Northern Uganda. *J Clin Microbiol5973-5977*.

17. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, and Boelaer M (2007) Visceral Leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Micro 5:873-881*.

18. Chapuis F, Rijal, S, Jhk UK, Desjeux P, Karaki, BMS, Koirala S, Louton L, and Boaelaert M (2006). Field validity, reproducibility and feasibility of diagnostic tests for visceral Leishmaniasis in rural Nepal. *Trop Med Int Health 11: 31-40*.
19. Chapuis F, Rijal, S, Soto A, Menten J, and Boelaert M (2006). A meta analysis of the diagnostic performance of the direct agglutination test and rk39 dipstick for visceral Leishmaniasis. *BMJdoi:10.1136/bmj.38917.503056.7C* (1 August 2006).
20. Cole ACE, Cosgrove PC and Robinson, G (1942). A preliminary report of an outbreak of kala-azar in a battalion of Kings African Rifles. *Trans R Soc Trop Med &Hyg 36:25-45.*
21. Davies CR, Kaye P, Croft, SL and Sundar S (2003). Lesihmaniasis new approach to disease control. *BMJ. 326:377-82*.
22. Diro E, Techane Y, Tefera T, Assefa Y, Kebede T, Genetu A, Kebede Y, Tesfaye A, Erghico B, Gebre-Yohannes Assefawosen, Mengistu G, Enghers H, Assefa A, Desjeux P, Boaelaert M, Hailu A. (2007). Field evaluation of FD DAT, rk39 dipstick and KATEX (urine latex agglutination) for diagnosis of visceral Leishmaniasis in northwest Ethiopia. *Trans R Soc Trop Med & Hyg. 101:908-914.*
23. Desjeux P. and Alvar J. (2003). Leishmania/HIV co-infections: Epidemiology in Europe. *Annals of Trop Med and Paras97(Suppl.1); 3-15.*
24. Desjeux P. Leishmaniasis. (2004) *Nat Rev Micro2: 692-693*.
25. Desjeux P. Leishmaniasis and HIV in Gridlock (1998). *Document who/ctd/leish/98.9 who Geneva.*
26. El-Harith A, kolka AHJ, Leewenburg HJ., Muigai R, Huigen E, Jelsma T, and Kajer PA (1988). Improvement of a direct agglutination test for field diagnosis of visceral Leishmaniasis. *J clin Microbiol 26:1321-25.*

27. EL-Harith AE, Kolk AHJ, Kajer PA, Leewenburg HJ., Muigai R, Klugu S, and Larrman JJ (1986). A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral Leishmaniasis. *Trans R Soc Trop Med Hyg 80:583-87.*
28. Fuller GK, Lemma A, Haile T, and Gemeda N (1979) Kala-azar in Ethiopia: Survey of south west Ethiopia. The leishmanin skin test and epidemiological studies. *Ann Trop Med Paras 73: (5) 417-431.*
29. Fuller GK, Lemma A, Haile T. and Atwood CL (1976). Kala-azar in Ethiopia. I: Leishmanin skin test in Setit Humera, a Kala-azar endemic area in northwestern Ethiopia. *Ann Trop Med Paras 70: (2) 147-163*.
30. Gavgani AS, Hodjati MH, Mohite H, and Davies CR (2002). Effect of insecticide impregnated dog collars on incidence of zoonotic visceral Leishmaniasis in Iranian Children: a match clustered-randomized trial. *Lancet 360:374-379.*
31. Gebre-Michael T, and Lane RP (1996).The roles of *Phlebotomus martini and P.celiae* (Diptera: Phlebotominae) as vectors of visceral leismaniasis in the Aba-Roba focus southern Ethiopia. *Med Vet Ent10:53-62.*
32. Gebre-Michael T, Pratlong F, and Lane RP (1993). Phlebotomus *(Phlebotomus duboscqi)* (Diptera: Phlebotominae), naturally infected with Leishmania major in southern Ethiopia*Trans R Soc Trop Med Hyg 87:10-11.*
33. Guerin PJ, Olliaro P, Sundar S, Boelaret M. Croft SL, Desjeux P, Wasunna MK and Bryceson ADM. (2002).Visceral Leishmaniasis:current status of controldiagnosis,and treatment, and a proposed research agenda. *Lancet Infec Dis 2(8):494-501*
34. Habte-Gabr E. (1981). Visceral Leishmaniasis (Kala-azar): Clinical features. In *Leishmaniasis in Ethiopia* (eds. Ayele T. Habte-Gabr E. Belehu A.) Institute of Pathobiology Addis Ababa University,Addis Ababa, 41-48.

35. Hailu A, (1990). Pre-and post-treatment antibody levels in visceral leishmaniasis. *Trans R Soc Med Hyg 84:673-75.*

36. Hailu A. (2008). Visceral Leishmaniasis in south-west Ethiopia. Profiling human immune response in a clinic-epidemiological setting*. Ph.D. Dissertion*. Amsterdam .

37. Hailu A, Balkew M, Berhe N, Meredith SEQ, and Gemetchu T. (1995). Is *Phlebotomus (Larroussius) orientalis* a vector of visceral Leishmaniasis in south-west Ethiopia? *Acta Tropica, 60: 15-20.*

38. Hailu A & Frommel D. (1993). Leishmaniasis in Ethiopia. *In The Ecology of Health and Disease in Ethiopia*(eds. Kloos H & Zein ZA.) pp.375-388 Boulder, CO: Westview press.

39. Hailu A, Berhe N, Sisay Z, Abraham I, and Medhin G (1996). Seroepidemiological and Leishmania skin test surveyof visceral Leishmaniasis in south and southwest Ethiopia. *Eth Med J 34:11-23.*

40. Hailu A, Gebre-Michael T, Berhe N and Balkew M. (2006). Lesihmaniasis. In *Epidemiology and ecology of health and disease in Ethiopia.* (eds. Berhane Y, Haile-Mariam, D and Kloos H.) 1st. edition.

41. Hailu A, Grammiccia M and Kager PA (2009). Visceral Leishmaniasis in Aba Roba, south western Ethiopia: prevalence and incidence of active and sub-clinical infections. *Ann Trop Med Paras103, 8:659-670.*

42. Hailu A, Musa AM, Royce C, and Wassuna M (2005). Visceral Leishmaniasis: New health tools are needed. *PLoS Med (2)7:e211.*

43. Hashim FA, Ali MS, Satti M., eL-Hassan AM, Ghalib HW, El-Safi S., and El- Hag IM (1994). An outbreak of acute kala-azar in a nomadic tribe in western Sudan, feature of the disease in a previously non-immune population. *Trans R Soc Trop Med Hyg 88:431-432.*

44. Herwaldt BL. (1999) Leishmaniasis. *Lancet.354:1191-9*

45. Houghton RI, Peterscu M, Benson DR, Skeiky YA, Scalone A, Badaro R, Reed SG, Gradoni L (1998). A cloned antigen (recombinant k39) of *Leishmania chagasi*diagnostic for visceral Leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy.*J Infect Dis 177: 1339-44.*

46. Kumar R, Pai K, Pathak. K and Syndar S (2001). Enzyme linked immunosorbent assay for recombinant k39 antigen in diagnosis and prognosis of Indian visceral Leishmaniasis. *Clin Diagn Lab Immuno 8:1220-4.*

47. Lindtjorn, B and Olfasson, J. (1983). Kala-azar in the Segen and Woytu valleys, south-west Ethiopia.*Eth Med J 21:35-42.*

48. Magil AJ, Grogl M, Gasser RA, Sun W, Oster CN (1993). Visceral infection caused by *Leishmania tropica* in veterans of Operations Desert Storm. *N Eng J Med 328:1383-1387.*

49. Mahey D, Peeling RW, Ustianowsk A, and Perkin MD (2004).Tropical infectious diseases: diagnostics for the developing world. *Nat Rev Microbiol 2:231-240.*

50. Meredith SEQ, Kroon NCM, Sondorp E, Seaman J, Goris MGA, van Ingen CW, Oosting H, Schoone GJ, Terpstra WL, and Oskam L (1995). Leish K.I.T., a stable direct agglutination test based on freeze dried antigen for serodiagnosis diagnosis of visceral leishmaniasis. *J Clin Microbiol 33:1742-45.*

51. MOH (2006). Visceral Leishmaniasis diagnosis and treatment guidelines for health workers in Ethiopia. *Federal Democratic Republic of Ethiopia, Ministry of Health, Disease prevention and control department. 1st edition*

52. Molina R, Gradoni, L and Alvar J (2003). HIV and the transmission of Leishmania. *Annals of Trop Med and Paras97(Suppl.1); S29-S45.*

53. Oskam L, Nieuwenhujs JL, and Hailu A (1999). Evaluation of the direct agglutination (DAT) using freeze-dried antigen for the detection of anti-leishmanial antibodies in stored sera from various patient groups in Ethiopia.*Trans R Soc Trop Med Hyg 93:273-75.*

54. Oliveria A, Pedras MJ, De Assis IE, Rabello A (2009). Improvement of direct agglutination test (DAT) for laboratory diagnosis of visceral Leishmaniasis in Brazil *Trans R Soc Med Hyg 12:1279-81.*

55. Pattabhi S, Whittle J, Mohamath R, El-Safi S, Moulton GG, Guderian JA, Colombara D, Abdoon AS, Mukhtar MM, Mondal D, Esfandiari J, Kumar S, Chun P, Reed SG, Bhatia A (2010). Design development and evaluation of rk28-based point-of care tests for improving rapid diagnosis of visceral Leishmaniasis. *Plos Neg Trop Dis 4(9):e822 e822.doi.10.1371/journal.pntd.000082.*

56. Pearson RD and Sousa SQ **(**1996)**.**Clinical Spectrum of Leishmaniasis.*Clin Infect Dis22:1-13.*

57. Peeling. R.W., Smith. P.G., Bossuyt. P.M.M., (2008). A guidefor diagnostic evaluation. *Nature Reviews Microbiology. S2-S6.Infect.Dis.2:494-501*.

58. Rijal S, Boaelaert M, Regim S, Karaki 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 Intl.Health 9: 724-729.*

59. Russo R, Laguna F, Lopez-Velez R, Medrano FJ, Rosenthal E, Cacopardo B and Nigro L. (2003). Visceral Leishmaniasis in those infected with HIV: clinical aspects and other opportunistic infections. *Ann Trop Med Para 97:S1, 99-105.*

60. Salam MA, Khan Md GM, Mondal D (2011). Urine antigen detection by latex agglutination test for diagnosis and assesement of initial cure of visceral Leishmaniasis. *Trans R Soc Trop Med Hyg 105:269-72.*

61. Santarem N, da Silva AC. (2007). Diagnosis of visceral leishmaniasis. *www.formatex.org/Microbiol/pdf/pages 839-846.l*

62. Sarkari B, Chance M, and Hommel M (2002). Antigenuria in visceral leishmaniasis: detection and partial characeterization of a carbohydrate antigen. *Acta Trop.82:339-48*.

63. Schallig HD, Schoone GJ, Kroon CC, Hailu A, Chappuis F, and Veeken H (2001). Development and application of simple diagnostic tools for visceral leishmaniasis. *Med Microbial Immunol 190:69-71.*

64. Singh RK, Pandey HP, and Sundar S (2006). Visceral leishmaniasis (kala-azar): Challenges ahead *Indian J.Med .Res.121:331-44.*

65. Srivastava P, Dayama A, Mehrotra S, and Syndar S (2011). Diagnosis of Visceral Leishmaniasis. *Trans R Soc Trop Med Hyg 105:1-6.*

66. Sundar S, Moore DK, Singh MK, Singh VP, Sharma S, Makharia A, Kumar PC, and Murray HW (2000). Failure of pentavelent antimony in visceral leishmaniasis in India: Report from the center of the Indian epidemic. *Clin Infect Dis31:1104-1107*.

67. Tália S.M. de Assisa, Alexandre S. da C. Bragaa, Mariana J. Pedrasa, Edward Oliveiraa,Aldina Barralb, Isadora C. de Siqueirab, Carlos HN Costad, Dorcas L. Costad,Thiago A. Holandad, Vítor Y.R. Soaresd, Mauro Biád, Arlene de J. Caldase,Gustavo AS Romerof, Ana Rabelloa (2011). Multi-centric prospective evaluation of rk39 rapid test and the direct agglutination test for the diagnosis of visceral Leishmaniasis in Brazil. *Trans R Soc Trop Med Hyg 105:81-85.*

68. Vanish M, Bhatia A, Reed SG, Chakravarty J and Sundar S (2011).Evaluation of rk28 antigen for serodiagnosis of visceral leishmanisis in India. *Clin. Microbial Infect. 1-5.*

69. WHO (1999). Leishmania/HIV co-infections, south Western Europe, 1990-1998. Retrospective analysis of 965 cases. *Weekly Epidemiological Record 74:365-375*.

70. WHO (2010) Control of Leishmaniasis. Genève *Wld Health Org Tech.Rep.Ser.949*

71. WHO (1990). Control of the Leishmaniasis.*Wld Hlth Org.Tech Rep Ser 793:1*-158.

72. WHO. Leishmania and HIV in gridlock. (1998). Geneva: World Health Organization. *WHO/CTD/LEISH/98.9*

73. WHO/TDR (2008). The use of visceral leishmanisis rapid diagnostic tests.*TDR/World Health Organization Geneva.1-14.*

74. Zijlstra EE, El Hassan AM and Ismael A. (1995) Endemic Kala-azar in easten Sudan. *AM J Trop Med & Hyg 52: 299-305.*

75. Zijlstra EE, Musa AM, Khalil EA, El Hassan IM, El Hassan AM. (2003). Post Kala-azar dermal Leishmaniasis*. Lancet infect Dis 3:87-98.*

76. Zijlstra EE, Osman AF, Holand HW, Oskam L,Ghalib HW, El Hassen AM, Kager PA, and Meredith SE (1997). The direct agglutination test for diagnosis of visceral

77. Leishmaniasis under field condition in the Sudan: comparison of aqueous and freeze dried antigen. *Trans R Soc Trop Med Hyg 91:671-73.*

Annex 1: Sensitivity, specificity, ppv,npv,chi-square & p-value of the different tests based on control panel comprising: HC,EC,CL & OD(category 1).

Category 1(AVL VS HC,EC,CL & OD)						
	SEN. (%) (CI: 95%)	SPEC. (%) (CI: 95%)	PPV (%) (CI: 95%)	NPV (%) (CI: 95%)	CHI SQUARE	P-value
DAT	91.6 (84.1-96.3)	89.8 (83.7-94.1)	85.3 (76.9-91.5)	94,3 (89.1-97.5)	162.91	0.000
rk 39(Diamed)	96.8 (91.1-99.3)	78.9 (71.4-85.2)	74.8 (66.2-82.1)	97.5 (92.8-99.5)	132.45	0.000
rk 39 (Inbioss)	92.6 (88.4-97.0)	85.0 (78.2-90.4)	80.0 (71.3-87.0)	94.7 (89.4-97.8)	140.39	0.000
rK 28(Inbioss+)	97.9 (92.6-99.7)	65.9 (57.7-73.6)	65.0 (56.6-72.8)	97.9 (92.9-99.8)	97.42	0.000
rk28(DPP)	95.8 (89.6-98.8)	81.6 (74.4-87.5)	77.1 (68.5-84.4)	96.8 (92.0-99.1)	138.45	0.000
rk28(EMT)	98.9 (94.3-99.9)	67.4 (59.1-74.9)	66.2 (57.8-73.9)	99 (94.6-99.9)	104.59	0.000

Annex 2: Sensitivity, specificity, ppv,npv,chi-square & p-value of the different tests based on control panel comprising: HC,EC, & OD(category 2).

Category 2(AVL VS HC,EC,& OD)						
	SEN.(%) (CI: 95%)	SPEC.(%) (CI:95%)	PPV(%) (CI:95%)	NPV(%) (CI:95%)	CHI SQUARE	P-value
DAT	91.6 (84.-96.3)	97.3 (92.3-99.4)	96.7 (90.6-99.3)	93.1 (86.9-96.9)	164.36	0.000
rk39(Diamed)	96.8 (91.1-99.3)	98.2 (93.6-99.8)	97.9 (92.5-99.7)	97.3 (92.4-99.4)	186.37	0.000
rk 39(Inbioss)	92.6 (85.4-96.9)	98.2 (93.6-99.8)	97.8 (92.2-99.7)	93.8 (87.9-97.5)	171.66	0.000
rK28(Inbioss+)	97.9 (92.6-99.7)	82.9 (74.6-89.4)	83.0 (74.8-89.5)	97.9 (92.5-99.7)	134.63	0.000
rk28(DPP)	95.8 (89.6-98.8)	94.6 (88.6-97.9)	93.8 (87.0-97.7)	96.3 (90.9-98.9)	167.84	0.000
rk28(EMT)	98.9 (94.3-99.9)	80.2 (71.5-87.1)	81.0 (72.7-87.7)	98.9 (93.9-99.9)	130.28	0.000

Annex 3: Sensitivity, specificity, ppv,npv,chi-square & p-value of the different tests based on control panel comprising: HC (category 3).

Category 3(VL vs HC)						
	SEN.(%) (CI: 95%)	SPEC.(%) (CI95%)	PPV (%) (CI: 95%)	NPV (%) (CI: 95%)	CHI SQUARE	P-value
DAT	91.6 (84.96.3)	100 (90-100)	100 (95.9100)	81.4 (66.6-91.6)	96.9	0.000
rk 39(Diamed)	96.8 (91.99.3)	100 (90-100)	100 (96.1100)	92.1 (78.6-98.3)	115.95	0.000
rk 39 (Inbioss)	92.6 (85.496.)	100 (90-100)	100 (95.9100)	83.3 (68.6-93.0)	100.35	0.000
rK 28(Inbioss+)	97.9 (92.699.)	94.3 (80.893)	97.9 (92.699.)	94.3 (80.8-99.3)	110.46	0.000
rk28(DPP)	95.8 (89.698.)	100 (90-100)	100 (96.0100)	89.7 (75.8-97.1)	111.75	0.000
rk28(EMT)	98.6 (94.399.)	100 (90-100)	100 (96.1100)	97.2 (85.5-99.9)	125.05	0.000

Annex 4: Sensitivity, specificity, ppv,npv,chi-square & p-value of the different tests based on control panel comprising: EC (category 4).

Category 4(VL vs EC)						
	SEN. (%) (CI: 95%)	SPEC. (%) (CI: 95%)	PPV (%) (CI: 95%)	NPV (%) (CI: 95%)	CHI SQUARE	P-value
DAT	91.6 (84.1-96.3)	100 (91.4-100)	100 (95.9-100)	83.7 (70.3-92.7)	104.21	0.000
rk39(Diamed)	96.8 (91.1-99.3)	95.1 (83.5-99.4)	97.9 (92.5-99.7)	92.9 (80.5-98.5)	113.47	0.000
rk 39(Inbioss)	92.6 (85.4-96.9)	95.1 (83.5-99.4)	97.8 (92.2-99.7)	84.8 (71.1-93.7)	98.53	0.000
rK28(Inbioss+)	97.9 (92.6-99.7)	80.5 (65.1-91.2)	92.1 (84.9-96.5)	94.3 (80.8-99.3)	92.06	0.000
rk28(DPP)	95.8 (89.6-98.8)	92.7 (80.1-98.5)	96.8 (90.9-99.3)	90.5 (77.4-97.3)	105.02	0.000
rk28(EMT)	98.6 (94.3-99.8)	80.5 (65.1-91.1)	92.2 (85.1-96.6)	97.0 (84.7-99.9)	96.38	0.000

Annex 5: Sensitivity, specificity, ppv,npv,chi-square & p-value of the different tests based on control panel comprising: HC & EC (category 5).

Category 5 (VL vs HC & EC)						
	SEN. (%) (CI: 95%)	SPEC. (%) (CI: 95%)	PPV (%) (CI: 95%)	NPV (%) (CI: 95%)	CHI SQUARE	P-value
DAT	91.6 (84.1-96.3)	100 (95.3-100)	100 (95.6-100)	90.5 (82.1-86.0)	141.68	0.000
rk39(Diamed)	96.8 (91.1-99.3)	97.4 (90.8-99.7)	97.9 (92.5-99.7)	96.1 (89.0-99.2)	151.39	0.000
rk 39(Inbioss)	92.6 (85.4-96.9)	97.4 (90.8-99.7)	97.8 (92.2-99.7)	91.2 (83.0-96.5)	137.18	0.000
rK28(Inbioss)	97.9 (92.6-99.7)	86.8 (77.1-93.5)	90.3 (82.9-95.3)	97.1 (87.8-99.6)	126.57	0.000
rk28(DPP)	95.8 (89.6-98.8)	96.1 (89.9-99.2)	96.8 (90.0-99.3)	94.8 (87.2-98.6)	143.87	0.000
rk28(EMT)	98.6 (94.3-99.8)	89.5 (80.4-95.3)	92.2 (85.1-96.6)	98.6 (92.2-89.9)	137.15	0.000