ADDIS ABABA UNIVERSITY
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INVESTIGATION OF CUTANEOUS LEISHMANIASIS USING CONVENTIONAL AND MOLECULAR METHODS IN SILTI WOREDA, ETHIOPIA

By

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List of abbreviations

AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency Syndrome
ALERT	All Africa Leprosy and Tuberculosis Research, Rehabilitation and Training Center
AP-PCR	Arbitrary primed polymerase chain reaction
bp	Base pair
CL	Cutaneous leishmaniasis
DAT	Direct agglutination test
DCL	Diffuse cutanaeous leishmaniasis
DIG	Digoxigenin
DNA	Deoxy-ribonucleic acid
dNTPs	Deoxy-nucleotide triphosphates
ECL	Ethiopian cutaneous leishmaniasis
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
HIV	Human Immunodeficiency Virus
IFA	Immunofluorescence assay
im	Intramuscular injection
ITS	Internal transcribed spacer
kDNA	Kinetoplast-DNA
LCL	Localized cutaneous leishmaniasis
LST	Leishmanin skin test
Mb	Mega base
MCL	Muco-cutaneous leishmaniasis
NNN	Novy- McNeal -Nicolle
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PKDL	Post kala-azar dermal leishmaniasis
PVAs	Pentavalent antimonials
RAPD	Randomly amplified polymorphic DNA analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPM	Revolution per minute
RPMI	Roswell Park Memorial Institute
SAP	Streptavidin-alkaline phosphatase
SDS	Sodium dodecyl sulfate
SE	Standard error
SNNPR	Southern Nations Nationalities and Peoples Regional State
SSG	Sodium stibogluconate
ssu rRNA	Small subunit ribosomal RNA
TE	Tris-EDTA
TAE	Tris-Acetate-EDTA buffer
TDR	Tropical Disease Research Program, WHO
U	Unit
VL	Visceral leishmaniasis
WHO	World Health Organization

Abstract

Man-made risk factors for leishmaniasis are increasing while knowledge of risk factors, accurate diagnosis and treatment of leishmaniasis is poor. As a result, deadly epidemics of leishmaniasis occur periodically, but tools for prediction and prevention are lacking. Hence, research is needed to address these constraints. Leishmaniasis in Ethiopia is mainly due to *L. donovani* and *L. aethiopica*, which causes visceral and cutaneous leishmaniasis respectively. Although the exact magnitude of the problem is not known, several surveys have indicated the importance of the diseases as a public health problem. A complete mapping of the diseases remains to be accomplished in view of the increasing number of patients reporting from regions hitherto unknown to be endemic.

Therefore, the main aim of this work is to describe the epidemiology of the disease through identification of the causative agent using molecular epidemiological tools in Silti Woreda. The study was conducted in two Phases: in phase I, house-to-house survey was conducted and in phase II, parasitological identification was done. The treatment response of *L. aethiopica* to liquid nitrogen (cryotherapy) and pentostam was documented as follow up activity. The prevalence of the disease in the area was found to be 4.82% with highest prevalence among age group 10-20 years. Some plants like *Adathoda shimperina* and *Acacia sp*p. and hyraxes and domestic animals were associated with increased risk of cutaneous leishmaniasis. The sole causative agent identified was *L. aethiopica*. The disease was found to be recently introduced in the area. In conclusion, the importance of the risk factors identified in this study should be investigated further and molecular epidemiological studies should be conducted in other areas to map the exact magnitude of the disease in the country. The clinical service in the outbreak site (Silti) needs to be prepared to provide the required care and treatment of patients who will keep coming from the area. Leishmaniasis control program has to be initiated in Ethiopia so as to prevent the disease from expanding.

Keywords/Phrases: **Cutaneous leishmaniasis, Risk-factor, Molecular epidemiology, Prevalence, Treatment,** *L. aethiopica***.**

1. Introduction

1.1. Leishmaniases

The leishmaniases are a group of diseases caused by obligatory and intracellular haemoflagellate protozoan parasites of the genus *Leishmania* (family trypanosomatidae). Human leishmaniasis is a complex disease with many clinical forms, which range from mild self-healing cutaneous lesions to fatal visceral disease.

1.2. Historical background

The *Leishmania* parasite was first discovered and named by Scottish physician Dr. William Boog Leishman. Following Leishman's discovery, an Irish physician Dr. Charles Donovan investigated splenic aspirates from kala-azar patients and confirmed Leishman's discovery. He called the tiny organisms he observed Leishman-Donovan bodies.

Leishmaniasis has been an ancient public health problem in South-West Asia and the Arab World reported from time immemorial as the pharaohs ruled in Egypt and Assyrians in Mesopotamia. It was extensively described by Arab-Islamic scientists like Avicenna (Ibn-Sina, 980-1037 A.D.) who wrote a complete chapter in his prominent book entitled AlkanounFi El Tebb raising the possibility of mosquitoes being involved in the transmission of the disease (Cox, 2002). Cox (2002), described cutaneous leishmaniasis (CL) as a disease endemic in Balk (Afghanistan) and Baghdad. Russell described CL in Aleppo-Syria in 1756 (Klaus *et al*., 1999).

1.3. Clinical forms of leishmaniasis

The disease is polymorphic in its clinical presentation and evolution. Differences in clinical presentations may be due to *Leishmania* species or strains, genetic background of the host, geographical location and immune response (Desjeux, 1996).

1.3.1. Localized cutaneous leishmaniasis (LCL)

The parasite is confined to the skin where it produces one or more shallow healing sores, but occasionally multiple lesions with disfiguring scars creating a life long aesthetic stigma. LCL in the Old World is mostly due to *L. major* and *L. aethiopica*. LCL due to *L. tropica* is usually more chronic. In its severe form, recidivance leishmaniasis is very difficult to treat. It is often long lasting, destructive and disfiguring. In the New World, *L. mexicana* usually produces mild lesions, but in some locations such as the ear's pinna is very difficult to treat.

1.3.2. Diffused cutaneous leishmaniasis (DCL)

Diffused cutaneous leishmaniasis is another chronic form of CL. The infection spreads over large areas of the body. It represents a condition of anergy with a failure of cell-mediated immune response (negative leishmanin test) and an abundance of amastigotes. In the Old World, it is associated with *L. aethiopica* infections and is mostly seen in Ethiopia (Desjeux, 1996). It resembles lepromatous leprosy, which often led to misdiagnosis in the past. As a result, DCL patients were often kept in leprosaria by mistake. In the New World, DCL is caused mainly by *L. amazonensis* (30% of the infections result in DCL). DCL also occurs in immunodeficient patients with no species-specific relation and co-infection with HIV is the most common cause (Gillis *et al.,* 1995; Ramos-Santos *et al*., 2000).

1.3.3. Muco-cutaneous leishmaniasis (Espundia)

Muco-cutaneous leishmaniasis (MCL) spreads to the mucosal membrane, where it causes extensive damage. MCL produces an extensive destruction of oral and pharyngeal cavities with disfiguring lesions, mutilating the face creating a great suffering for life. In the New World, MCL is caused by *L. amazoneansis*, *L. braziliensis*, *L. panamensis* and *L. guyanensis*. In the Old World, it is caused by *L. aethiopica*, (*L. donovani*, *L. major* and *L. infantum* in immuno-compromised patients*)* (Desjeux, 1996; Hepburn, 2000).

1.3.4. Visceral leishmaniasis (VL)

Visceral leishmaniasis (VL, Kala-azar) is a fatal disease if it is left untreated. The parasites colonize the internal organs: spleen, liver, bone marrow and lymph nodes. The most severe form is characterized by irregular fever, loss of weight, splenomegally and / or lymphadenopathy and anemia. VL is caused by *L. donovani* and *L. infantum*. It causes large-scale epidemics with a high fatality rate. After recovery, patients may develop chronic post-kala-azar leishmaniasis, which usually requires long and extensive treatment (El-Hassan *et al*., 2001).

1.3.5. Post kala-azar dermal leishmaniasis (PKDL)

It is a dermatropic form of leishmaniasis developed by part of the ex-VL patients (WHO, 1990), but there are cases without any previous known history of VL (El-Hassan *et al*., 2001). The disease is characterized by the development of macules, papules and nodules, which first appear around the mouth and those which do not heal spontaneously become denser and spread over the entire body (Berman, 1997). The interval between the end of treatment of VL and the onset of PKDL is variable. It may appear during or directly after treatment up to 2 years post treatment (El-Hassan *et al*., 2001). PKDL patients may be important sources of infection in VL transmission (Addy and Nandy, 1992).

1.4. Epidemiology of leishmaniasis

1.4.1. Global epidemiology

Leishmaniases are parasitic infections caused by a range of *Leishmania* parasites supported by a wide range of vectors and reservoirs distributed on all inhabited continents except Australia (Ashford, 2000). However, recently kangaroos in Australia have been reported to be infected by *Leishmania*-like parasites (Rose *et al*., 2004). The disease is endemic in 88 countries, of which 66 are in the Old World and 22 in the New World. From the 88 countries

endemic for leishmaniasis, 72 of them are developing countries. It has been estimated that 12 million people are infected and 350 million people are at risk. There are 500,000 new VL cases each year. Confirmed cases of VL have been reported from 66 countries and 90% of the world's VL burden occurs on the Indian subcontinent (India, Bangladesh and Nepal), East Africa (Sudan, Ethiopia and Kenya) and Brazil (WHO, 1998). There are 1.0-1.5 million cases of CL each year, with 90% of CL cases occurring in 8 countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria, showing that the Middle East is a central focus for CL (Desjeux, 2004).

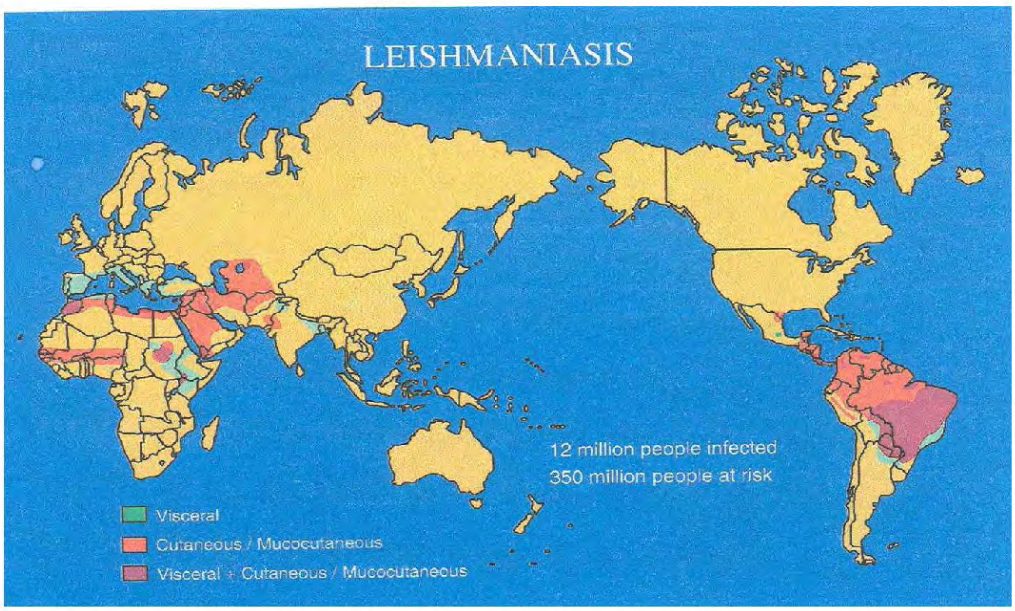


Figure 1: Global distribution of visceral and cutaneous leishmaniasis (From Handman, 2001).

1.4.2. Local epidemiology (Ethiopia)

Ethiopia is one of the endemic areas for leishmaniasis in the world. Both CL and VL are prevalent. Four species of *Leishmania*: *L. donovani* (causing VL), *L. aethiopica, L. major* and *L. tropica* (causing CL) are responsible for human leishmaniasis (Hailu and Frommel, 1993). Visceral leishmaniasis is mainly found in the arid and semi-arid low lands such as the Segen valley around Konso (Ali and Asford 1994; Hailu, *et al.* 1996), Humera and Metema (Mengesha and Abuhay, 1978), and low lands of Afebat, Teseney, and Ghenale river basin, West of Moyale (Ayele and Ali, 1984). The main endemic areas for VL begin in the north-west and extend down along the lowlands of southwest to the Kenya border (WHO, 1981; Hailu and Frommel, 1993). Studies have indicated that the importance of VL in Ethiopia Rift Valley, Awash Valley (northeastern Ethiopia) (Ali, 1997*;* Ali *et al,* 2002).

Cutaneous leishmaniasis in Ethiopia is caused mainly by *L. aethiopica* and occasionally by *L. tropica* and *L. major (*Ashford *et al*., 1973; Gebre-Michael *et al.*, 2004; Hailu *et al*., 2006). The disease is found mainly in high and mid-altitudes ranging between 1,400 to 2,700 meters above sea level (Hailu and Frommel, 1993). However, Gebremichael *et al.* (2004) isolated *L. tropica* from sandflies in low altitude of the Awash Valley (about 900 meters above sea level). From the same area, Hailu *et al.* (2006) isolated and described *L. tropica* from one patient who had lived in the area. Different authors reported the presence of CL in wide areas of Ethiopia such as Kutaber, Ocholo and Aleku (Ashford *et al*. 1973), Deberesina, Sebeta, Tulukuche, Goba and Adigrat (Ayele and Ali, 1984).

1.4.3. Risk factors for the increasing of leishmaniasis in the world

In several regions there is a clear increase in the number of reported cases, which is worrying. For example, the incidence of CL in Brazil increased from 21,800 cases in 1998 to 60,000 in 2003. In the city of Kabul (Afghanistan), among the less than 2 million inhabitants the number of cases of CL increased from 14,200 in 1994 to 67,500 in 2002 and in Syria from 3,900 cases in 1998 to 6, 275 in 2002 (Dujardin, 2006).

Several conditions are responsible for the emergence and the spread of leishmaniases worldwide. The increases have mainly been attributed to three major risk factors (Dujardin, 2006). These are: i) man-made and environmental changes ii) host immune status and iii) treatment failure and drug resistance.

According to the TDR Disease Watch Focus for Leishmaniasis (2000), man-made risk factors are increasing while knowledge of risk factors is poor and health education is lacking. As a result, deadly epidemics of leishmaniasis occur periodically, but tools for prediction and prevention are lacking and research is needed to address these constraints. The resurgence of leishmaniasis has occurred because of deficiencies in the control of the vector (sandfly), absence of a vaccine, and lack of access to medical treatment because of the cost and increasing drug resistance to first-line treatment (WHO, 2000).

1.4.3.1. Man-made and environmental changes

Man-made and natural changes to the environment can lead to alterations in the range and densities of vectors and reservoirs, thereby, increasing human exposure to infected sandflies (Peterson and Shaw, 2003). For instance, new villages in southern Mediterranean countries were developed with inadequate abattoirs that are attractive for semi-domestic and/or stray dogs, a situation that is likely to favor an increase in the population of these animals and, consequently, the sources of *Leishmania infantum* for human infection (Dujardin, 2006).

The development of new settlements, intrusion into primary forest, deforestation, massive migration from rural to urban areas, rapid and unplanned urbanization, the building of dams and new irrigation schemes are primary risk factors for the spread of leishmaniasis. (Peterson and Shaw, 2003).

1.4.3.2. *Leishmania* - HIV co-infection

Leishmania – HIV co-infection has emerged because of the increasing overlap between HIV and *Leishmania*. Cases of co-infection have been reported from 36 countries (Dujardin,

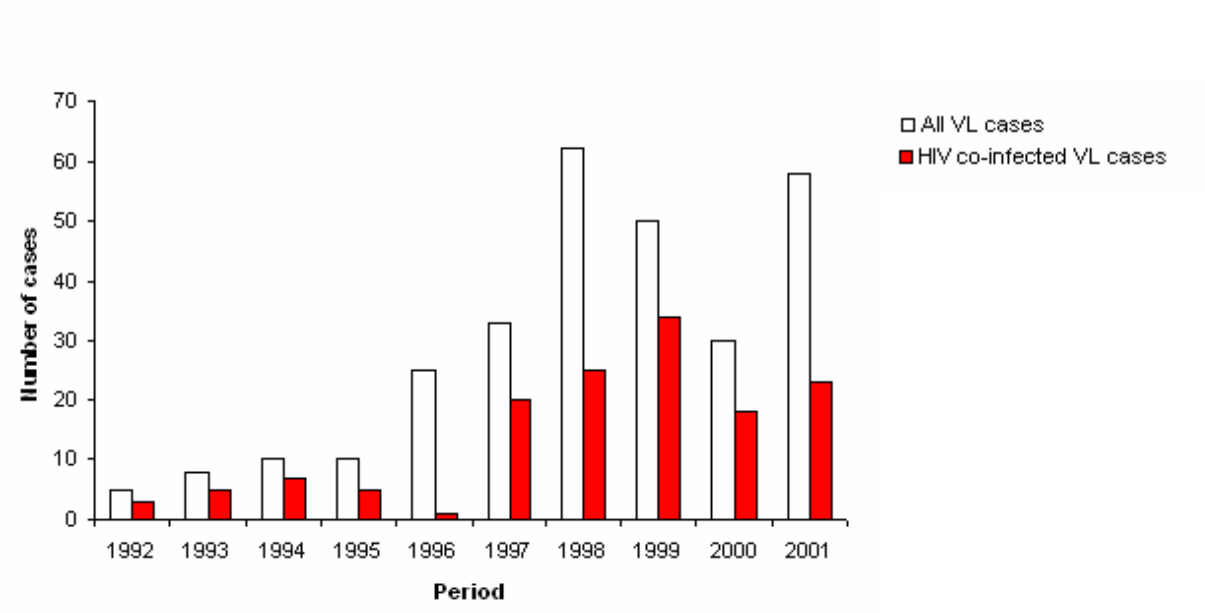
2006) and the co-infection is increasing especially in Southern Europe and Africa (Desjeux, 2001). Leishmaniasis is spreading in several areas of the world as a result of the rapidly spreading epidemic of AIDS. The immune deficiency has led to increased susceptibility to infections, including leishmaniasis. Co-infection with HIV has led to the spread of leishmaniasis, typically a rural disease, into urban areas (Cruz *et al*., 2006).

In patients infected with HIV, leishmaniasis accelerates the onset of AIDS by cumulative immunosuppression and by stimulating the replication of the virus (Dujardin, 2006). It may also change asymptomatic *Leishmania* infections into symptomatic ones. Sharing of needles by intravenous drug users can spread not only HIV but also leishmaniasis (Desjeux, 2001).

Cases of *Leishmania*-HIV co-infections are being reported more frequently in various parts of the world and it is anticipated that the number of co-infections will rise and be no longer restricted to endemic areas. For example, in West Africa where there is no official surveillance system, *Leishmania*-HIV co-infection cases have been reported from Cameroon, Guinea Bissau, Mali, Senegal and Ghana (Cruz *et al*., 2006).

Figure 2: Global distribution of *Leishmania* and *Leishmania*-HIV co-infection (Cruz *et al*., 2006)

In 1995 seven cases of VL with HIV co-infection was reported for the first time in Africa from Ethiopia (Berhe *et al*., 1995). The number of such cases has now increased substantially and the proportion of HIV co-infection in hospitalized VL patients remained high during the ten-year period (Figure 3). The overall pattern of HIV co-infection among 291 VL patients hospitalized in Addis Ababa in the ten-year period between 1992 and 2001 has shown that 141 (48.5%) were HIV co-infected. Two CL-HIV co-infection were reported in Ethiopia by Berhe *et al*. (1995). Other three co-infection (2 from ATERT and 1 from Silti) were diagnosed (personal communication).



Source: Ministry of Health (2007), unpublished data

Figure 3: Number of VL cases with and without HIV co-infection hospitalized in Addis Ababa from 1992-2001

1.4.3.3. Treatment failure and drug resistance

Chemotherapy is crucial for reducing the burden of leishmaniasis and the population of reservoirs of antrhroponotic leishmaniasis. Antimonials are the first line drugs for all clinical forms of leishmaniasis, but the increasing failure of antimony (SbV) based regimes is a major concern. For example, in India, 60 % of VL patients did not respond to SbV based regimes

(Sundar *et al.*, 2000).

1.5. The *Leishmania* parasite

1.5.1. The parasite and Life cycle

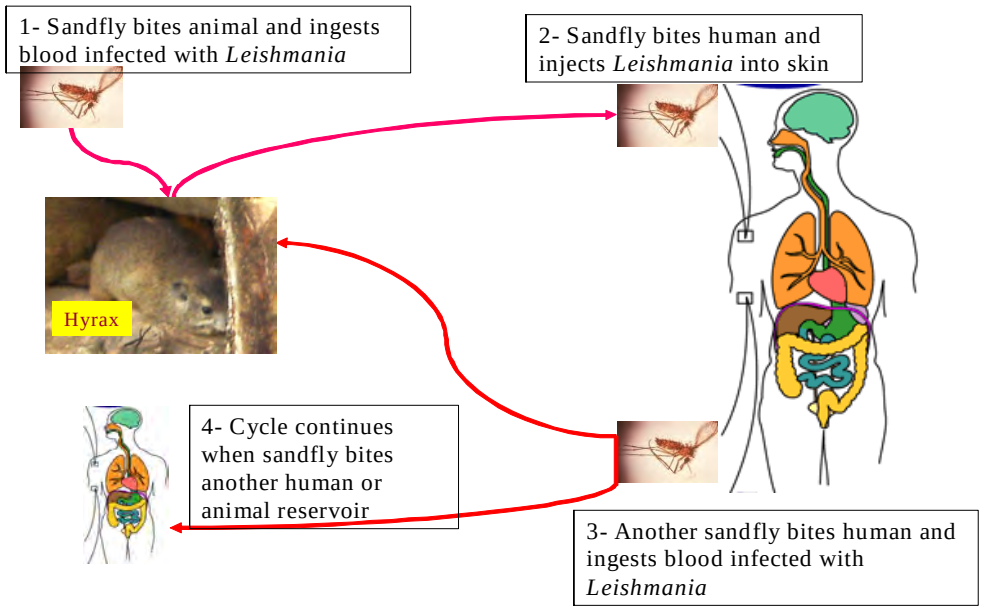
Leishmania are dimorphic parasitic protozoa of human and animals presenting two principal morphological forms that are found alternately as flagellated, motile extra-cellular promastigote in the alimentary tract of Phlebotomine sandflies (invertebrate host) and in culture medium, or as obligate intracellular aflagellate amastigotes in the phagolysosomes of mammalian (vertebrate) host macrophages. The amastigote stage is a round or oval body about 2 to 6 µm in diameter containing a nucleus, a kinetoplast, and an internal flagellum. The promastigote has a long and slender body (about 15-30µm by 2-3µm), with a central nucleus, a kinetoplast and a long, free, anterior flagellum (Dedet *et al.*, 1999).

The parasite contains two prominent organelles, the nucleus and the kinetoplast. The kinetoplast is found in all protozoa of the order kinetoplastidae (eg. *Leishmania*, *Trypanosoma*, *Crithidia*). It is a rod-shaped mitochondrial structure consisting of a DNA network of about 10,000 minicircles and about 50 maxicircles, the kinetoplast-DNA (kDNA). The function of the kinetoplast has not been clear until recently. It was found that maxicircles encode for mitochondrial ribosomal RNAs. The minicircles play a role in the editing process of these mRNAs (Shlomai, 1994).

During their life cycle, the promastigotes multiply intensively inside the intestinal tract of the sandfly, mainly in the midgut (section suprapylaria of the subgenus *Leishmania*) or in the hind and midgut (section peripylaria) of the sub genus *Viania* (Dedet *et al.*, 1999; Hommel, 1999). In both groups, the parasites migrate to the anterior part of the sandfly mid-gut where they change into metacyclic promastigotes, the stage of *Leishmania* infective to the vertebrate host. The transmission from sandfly to the vertebrate host is described as follows: The female sandfly picks up infected cells from the skin with their blood meal. The amastigotes are released in the mid-gut of the insect, transform to the procyclic stage and start multiplying actively without penetrating the hemocoele. After a few days, numerous

procyclics invade the gut of the insect. Then the elongated procyclic promastigote attaches to the mid-gut epithelium by inserting their long flagella between the microvilli that line the mid-gut. They migrate to the cardiac valve, where they transform into short, spherical, non-dividing promastigotes. Then the parasites are released from the mid-gut and penetrate the pharynx (proboscis) as metacyclic promastigotes, also termed paramastigote. From the proboscis, the metacyclic promastigotes are introduced to the new mammalian host during the bite (Dedet *et al.*, 1999).

Metacyclic promastigotes enter the skin of the vertebrate host when the infected sandfly takes its blood meal. It may inoculate 10-200 promastigotes into the dermis. Within the macrophages and related cell types, they rapidly transform into amastigotes, remain within the phagocytic vacuole, where they develop and multiply. At some stage, this infected cell, which may harbor up to 20 or more amastigotes, bursts and releases free amastigotes, which infect other cells. Infected macrophages move from the skin to other tissues, infecting the spleen, liver and bone marrow in case of VL (Hommel, 1999). The survival of amastigotes in vertebrate host cells is the result of several factors related to the cell itself, namely, a decrease in the production of oxidative and nitrogen derivatives triggered by the presence of the parasite and the amastigote's ability to resist lysosomal hydrolyses, a property probably related to surface glycol-inositol-phospholipids (Dedet *et al.*, 1999).



Source:http://www.biosci.ohio-state.edu/~parasite/lifecycles/leishmania_lifecycle.html (accessed in 2006); the hyrax picture was taken from Silti, Ethiopia, (2006)

Figure 4: The life cycle of *Leishmania*

1.5.2. Vector and reservoir host

The sandflies that are vectors of *Leishmania* parasite are the Phlebotominae sub-family: the Genus *Phlebotomus* in the Old world and *Lutzomya* in the New world. The Phlebotomine sandflies are nocturnal with limited migratory habits and as a result, leishmaniasis is a focalized disease. In tropical and subtropical parts of the world, sandflies are active through out the entire year with a small variation in population density (Daba *et al.,* 2002). They are small (2–3 mm), hairy, midge-like insects, with long slender legs and rather short mouth parts. They are not strong fliers and usually stay within a 300 meters radius of their breeding

sites. In cities, they breed in refuse piles, in cracks in the walls, and foundations of buildings and fences. In desert areas, they breed within the burrows of rodents (Killick-Kendrick, 1999; Klaus *et al.*, 1999).

Most leishmaniases are zoonotic (transmitted to humans from animal, reservoir hosts) and humans become infected only when accidentally exposed to the transmitting sandflies. However, in the anthroponotic form (those transmitted from human to human through the sandfly vector), humans are probably the sole reservoir host (WHO, 1996). The reservoir of *L. infantum* and *L. chagasi* is usually dog, but in several Old World and New World foxes, rats, opossum or raccoon dogs may also act as reservoirs in some areas (WHO, 1981). The reservoir of *L. donovani* is mainly human (Hommel, 1999), although other species are incriminated in Africa e.g. *Arvicanthis niloticus* in Sudan (El-Hassan *et al.*, 1995). Domestic dog is incriminated as a reservoir host of American cutaneous leishmaniasis caused by *L. braziliensis*, *L. panamensis* and *L. peruviana* (Reithinger and Davies, 1999). Gerbils (*Psammomys obesus*) have been implicated as a reservoirs host of *L. major* in different Asian countries (Elbihari and El-Hassan, 1987; Rioux *et al.*, 1990; El-Sibae *et al.*, 1993). *Psammomys obesus* is also suspected as a reservoir host of *L. major* in Egypt (Morsy *et al.*, 1996).

In Ethiopia, the reservoir hosts of *L. aethiopica* are rock hyraxes: *Procavia capensis* and *Hetrohyrax brucei*. The rodent, *Arvicanthis niloticus*, found infected with *L. major* in North West and South West parts of Ethiopia is a possible reservoir host for *L. major* in the region (Ashford *et al*., 1973; Desjeux, 1991). In Ethiopia, different species of *Phlebotomu*s (Table1) are vectors of *Leishmania* parasites, confirmed by demonstration from sandfly gut infection.

1.5.3. The genome of *Leishmania*

Molecular studies have shown that the genome of *Leishmania* is about 35Mb in size and is distributed over 36 chromosomes, which range in size from 0.35Mb to about 3 Mb (Wincker *et al*., 1996). The 36 chromosomes are conserved among the Old World species of *Leishmania*, while the New World *Leishmania* species *L. mexicana* complex and

L. braziliensis complex have 35 and 34 chromosomes respectively (Britto *et al.*, 1998). Size polymorphisms of chromosomes are common in *Leishmania* such that each strain of a given species has a distinctive molecular karyotype (Lightall and Gianni, 1992). The chromosome size variation ranges from 10-20 % between different strains and within strains of the same species (Wincker *et al.*, 1996; Britto *et al*., 1998).

The *Leishmania* genome is distributed within two organelles: in the nucleus as chromosomal and episomal DNA accounting for greater than 80% of the genome and in the kinetoplast as extrachromosomal circular DNA molecules called kinetoplast DNA, consisting of 10-30 % of the total cellular DNA. The kDNA is organized into a network of several thousand interlocked DNA circles containing of several thousands small minicircles and a few larger maxicircles (Englund *et al*., 1996).

1.6 Diagnostic methods for leishmaniasis

The diagnosis of leishmaniasis can be made on the basis of clinical and epidemiological data but has to be confirmed by the demonstration of the parasite to avoid potential misdiagnosis (Marfurt *et al.*, 2003). Because of the differences among the *Leishmania* species in level of virulence and in responses to various chemotherapeutic regimens, correct identification is essential in order to determine the clinical prognosis and prescribe appropriate species-specific therapeutic regimens (Berman, 1987). Hence, it is important to identify the species of *Leishmania* for both clinical and epidemiological reasons. Fore example, *Leishmania* species of different complexes found in the same geographic region can cause cutaneous lesions of similar appearance, yet the different complexes require distinct therapeutic approaches (Berman, 1987). The epidemiologic information furnished by the identification of parasites in a given region is critical for the design of appropriate control measures.

Leishmania organisms have been classified as different species primarily on the basis of clinical, biological, geographical and epidemiological criteria. During the last decade a number of methods have become available for the differentiation of *Leishmania* isolates. Some of these methods are reviewed in the following sections.

1.6.1 Classical methods

1.6.1.1 Direct methods

The classical methods used for the direct detection of the parasite include the visualization of the amastigotes by microscopic examination of Giemsa–stained smears or in histological sections and *in vitro* culture of the parasite.

1.6.1.1.1 Microscopy

Although microscopic examination is rapid, cheap and easy to perform, it lacks sensitivity due to the generally low number of parasites found in tissue samples (Weigle *et al.,* 1987). Furthermore, using microscopic examination, species-specific diagnosis cannot be achieved. Biopsies have shown a greater sensitivity than smears for the diagnosis of CL **(**Rodriguez *et al.,* 1994). Andresen *et al*. (1996) identified amastigotes in 76% of histological sections, but only in 55% of smears taken from CL lesions.

1.6.1.1.2. Culture

In vitro cultures are obtained from aspirates, biopsies or from skin scrapings from suspected patients. Culture techniques are more sensitive than microscopy, but the sensitivity of culture is variable and the differences are based on various factors as for example, the viability of collected parasites, the strain and the media used, the presence of super infection and the expertise of the investigator. In addition, culture is more prone to contamination. Different success rates have been reported ranging between 4 % in Nicaragua (Belli *et al.,* 1999) and 95 % in Switzerland (Grimm *et al*., 1996). Mostly a sensitivity of 40% -50 % was obtained on average (Rodriguez *et al.,* 1994).

1.6.1.2. Indirect methods

An indirect method of diagnosing leishmaniasis is based on the analysis of specific immune response. Leishmanin skin test (LST), also called the Montenegro skin test detects specific cutaneous delayed-type hypersensitivity in individuals who had previous contact with the parasite, but it cannot distinguish between current and past infection.

Several serological tests are now used for detecting anti-leishmanial antibodies with different sensitivity (Harith, *et al.,* 1989; Ashford, *et al.,* 1993). Serological diagnostic techniques present drawbacks that include the cross reactivity of leishmanial antigens with antibodies induced by other kinetoplastids such as *Trypanosoma cruz*i (Badaro *et al*., 1986) as well as poor sensitivity due to low antibody titer which is characteristic of cutaneous leishmaniasis. Species-specific identification cannot be achieved using indirect methods.

1.6.2. Modern Methods

1.6.2.1. Isoenzyme analysis

The isoenzyme technique still remains the 'gold standard' for *Leishmania* taxonomy and its principal advantage is to provide a stable marker for clusters of geographical isolates within each given species (Hommel, 1999). However, it is restricted in that it assays the genotype indirectly, so that nucleotide substitutions that do not change the amino acid composition remain undetected. Changes in the amino acid composition that do not change the electrophoretic mobility may also be not observed. Furthermore, isoenzyme analysis is slow, laborious, and expensive, requiring cultivation of the isolates and a large number of parasites ($5 \times 10^9 - 1 \times 10^{10}$) to estimate the profiles of 10-20 different enzymes (Andresen *et al.*, 1996; Noyes *et al.,* 1996). Recently the isoenzyme method has been introduced to AHRI and successfully applied for species typing (Genetu *et al.,* 2006; Gadisa *et al*., 2007).

1.6.2.2. DNA Based techniques

1.6.2.2.1. Southern Blotting using DNA probes

Efforts have been made to develop nucleic acid hybridization methods for *Leishmania* identification. Before the introduction of PCR, nucleic acid Southern blotting, using radio labeled probes for hybridization was one of the most sensitive detection methods. This method involves DNA extraction, gel electrophoresis, Southern blotting and hybridization. DNA probes for *Leishmania* species identification generally target kDNA because of its multi-copy nature having variable regions that differ from mini-circle classes in the same network (Barker, 1989). kDNA mini-circle probes have been developed for *L. aethiopica* (Laskay *et al.,* 1991), *L. major* (Smith *et al.,* 1989) and *L. infantum* (Gramiccia *et al*., 1992).

1.6.2.2.2. Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) based methods

PCR is the amplification of a known specific nucleic acid sequence using oligonucleotide primers that specifically bind to the DNA flanking the region of interest. Amplification is achieved by using a heat stable DNA polymerase.

In many studies, PCR was found to be more sensitive than microscopy (Osman *et al*., 1997a), and it has been proved more sensitive (100%) when compared to serology (63%) (Ashford *et al.*, 1995). So far, several different PCR approaches have been developed for the diagnosis of leishmaniasis. Depending on the objective, different targets for amplification are chosen. For diagnosis, multi-copy sequence repeats are usually selected, implying the potential for high sensitivity. For this purpose, the sequences need to be highly conserved either within the genus or within the species, depending on the specific aim. For genetic characterization of individual strains, more variable regions are selected, which usually do not appear in high copy numbers and are therefore less sensitive targets. Sensitivity and specificity can be enhanced by the hybridization of PCR products.

The specificity can also be improved by restriction fragment length polymorphism (RFLP) analysis of the PCR-amplicon (Schalling and Oskam, 2002; Gadisa *et al*., 2007).

The conserved 120bp kDNA shared by all *Leishmania* species was used by Rodgers *et al.* (1990) to develop a genus specific PCR-assay. The assay enabled them to identify *Leishmania* at the genus level. The non-transcribed spacer region, which is distinct in length and sequence among different *Leishmania* species, has been used by Ramos *et al.* (1996) to design primers and amplify the mini-exon intergenic region and repeats that differentiated between *L. braziliensis, L. donovani* and *L. mexicana, L. major* and *L. aethiopica* species.

Amplification of the internal transcribed spacer (ITS-1), located between the genes coding for the small subunit (ssu) and the 5.8S rRNA genes, combined with RFLP analysis with *Hae III* and *Hha I* enabled one to differentiate most medically important *Leishmania* species (Schonian *et al.*, 2000; Genetu *et al.* 2006; Gadisa *et al*.; 2007) successfully identified old

world *Leishmania* at the species level by digesting the ITS-1- PCR amplicon using *Hae III* and *Hha I*.

1.7. Treatment Modalities of Cutaneous Leishmaniasis

The management of CL provides a real challenge. Fortunately, its control is usually hampered by ignorance of its true prevalence (WHO, 1984). Most CL lesions are self-limiting and may heal spontaneously within a short period. In spite of this, treatment of CL is justified in a variety of cases including early lesions, multiple lesions, lesions involving cosmetically sensitive sites, mucosal lesions, disseminated lesions and patients with significant immunosuppression (Kubba and Gindan, 1989). In addition, the psychological impact of CL cannot be ignored. Unfortunately, to date, there is no safe, simple and effective treatment for CL and the pentavalent antimony compounds, "the best drugs of a bad bunch," still remain the mainstay of treatment in the majority of cases since 50 years ago (Kubba and Gindan, 1989). In spite of many new therapeutic studies, treatment of CL is to some extent still empirical. The assessment of the efficacy of any therapeutic agent in a self-healing disease such as CL is very difficult. Cure rates have varied from 0 to 100% in different areas of the world (Stratigos, 1980).

1.7.1. Antimony Compounds

Kikuth and Schmidt (1937), first reported the anti-leishmanial activity of solustibosan (sodium stibogluconate), a pentavalent antimony compound. Durand *et al*. (1946) were the first to test pentavalent antimony, N-methylglucamine antimoniate (glucantime) in man against leishmaniasis. Today, these two pentavalent antimonials (PVAs), sodium stibo-gluconate (Pentostam,® Wellcome Foundation, UK) and meglumine antimoniate (Glucantime,® Specia, France), are the most widely used leishmanicides. Although it is usually reported that both have similar efficacy and toxicity, in relation to their pentavalent antimony content, sodium stibogluconate (SSG) solution (Pentostam ®) contains about 10% antimony (100 mg Sb/ml) whereas meglumine antimoniate (MA) solution (Glucantime®) contains about 8.5% antimony (85 mg Sb/ml).

The WHO recommendation on the dosage of PVAs for CL is 20 mg Sb/kg body weight per day to a maximum of 600 mg intramuscular injection (im) daily for 20-30 days (WHO, 1990). The course can be repeated in resistant cases after a rest period of 14 days. Voller *et al.* (1963) reported that SSG could inhibit glucose uptake by promastigotes of *L. tropica.* Berman *et al. (*1985) were able to show that SSG decreases DNA, RNA and protein synthesis in a dose-dependent manner in SSG-treated *L. mexicana.* In addition, both the anaerobic and aerobic glucose oxidations are inhibited, resulting in a reduction in ATP and GTP production in the amastigotes exposed to SSG.

There are various reports on the clinical efficacy of PVAs. When given in adequate dosage and duration, PVAs have been reported to be effective in VL in different parts of the world (Navin *et al.*, 1992). They also reported that the efficacy of PVAs ranged from 70% to 100% in CL when given daily in a dose of 10-30 mg Sb/kg for 15 days. It is reported that all patients who were given a daily dose of 20 mg/kg with no upper limit on daily dose were cured from acute CL (Ballou *et al.*, 1987). In Saudi Arabia, the positive response of CL to PVAs varies from 50% to 100% (Ghosh, 1979). There are also various reports of treatment failure with PVAs in CL (Marsden *et. al.*, 1985; Oster *et. al*, 1985).

Antimonials are contraindicated in pregnancy and in patients with significant renal, hepatic or cardiac diseases. The first signs of toxicity are myalgia, joint stiffness, malaise, anorexia and bradycardia. Hepatotoxicity, hemolytic anemia, nephro-toxicity, pancreatitis and anaphylaxis are rare occurrences (Chulay *et al.*, 1985). Other drugs, which are used in the treatment of CL include: Imidazole compounds, Pentamidine, Amphotericin B, allopurinol, rifampicin, dapsone, chloroquine, and nifurtimox (WHO, 2000).

1.7.2. Cryotherapy

This treatment requires repeated topical applications of liquid nitrogen. The freezing time is about 15 to 20 seconds per application, repeated two or three times at short intervals, resulting in a total of 30 to 60 seconds of freeze time (Gurei e*t al.*, 2000). Afterward, there is some edema and blistering of the lesion for 2 to 3 days, followed by crusting and formation of an eschar. Lesions are treated in one to three sessions over 3 to 8 weeks. Rahmatolah *et al.*

(2006) reported that the efficacy of cryotherapy was found to be 67.8% for CL. They also reported that the efficacy of liquid nitrogen increased to 100% for small lesions less than 1cm diameter. In the same study, it was reported that the efficacy of cryotherapy is higher for small and younger (less than three months) than large and older lesions. When liquid nitrogen is applied to the CL lesion, at approximately -40°C (-40°F) or less, intracellular lethal-ice crystals begin to form that will tear apart almost any cell. If ice forms only outside the cell, osmosis, the movement of a solution through the membrane of a cell, causes the cell to shrink as it gives up water to replace the water that has turned to ice. Then, as the area thaws, water rushes into the shrunken cell and causes it to burst. This process kills *Leishmania* parasites localized in the area. When ice is formed within cells, the blood supply to the cells is believed to be choked off and as a result cells will die. Once the cells are destroyed, components of the immune system, primarily the white blood cells, clear out the dead tissue. There is some evidence to suggest that this procedure also stimulates the immune system to attack remaining parasitic cells.

1.8. Control measures

As a rule of thumb, control measures target on the interruption of the transmission cycle. There is no single method that can be used for all situations and one method may be successful in one place, but not in another. Moreover, control measures should always be revised and evaluated. In addition, cost effectiveness has to be considered before adopting a certain method. For efficient control, the ecology and epidemiology of the disease have to be understood (differences depending on species and local ecology). Depending on the circumstances (e.g. zoonotic or anthroponotic transmission), the control of either the reservoir or the vector is advisable. The human link in the transmission cycle can be controlled by taking personal precautions (Ashford, 1996; Klaus *et al*., 1999).

1.8.1. Prevention of human infections

Since sandflies are active only at night, inhabitants of endemic areas or travelers in endemic areas can reduce the risk of exposure to sandfly bites by long sleeve clothing during evening hours, application of insect repellents and usage of bed nets. Fine mesh screens for windows are advisable as well. In the case of anthroponotic leishmaniasis, early case detection and treatment are the most important control measures. Despite various attempts to develop vaccine, no efficient immunization is yet available against leishmaniasis (Eisenberger and Jaffe, 1997). There are attempts to develop *Leishmania* vaccine, but no definite results have been obtained yet (Modabber *et al.*, 1998; Handman, 2001; Valenzuela *et al*., 2001; Coler and Reed, 2005).

1.8.2. Vector control

The sandfly vector has continuously been a target for control measures. This included the destruction of breeding sites by removing garbage and debris left near houses and by covering cracks in buildings. In addition, spraying of residual insecticide inside houses and outside under windows were used. Plants like *Bougainvillea glabra* were shown to decrease the risk for leishmaniasis by reducing the life span of sandflies (Schlein *et al*., 2001). Impregnated bed nets with various insecticides such as deltamethrin were applied as control measures with significant reduction in CL incidence rate (Alten *et al*., 2003).

1.8.3. Control of the reservoir

One effective measure for the control of zoonotic CL is a deep ploughing of the area around houses, in order to destroy the rodent burrows. It has been shown in a study in Brazil that the removal of infected dogs led to a lower incidence, but did not eradicate the disease (Ashford *et al.,* 1999). Vaccination of dogs might be an alternative approach for the future in such cases (Tesh, 1995). Another control measure has been presented by Killick-Kendrick *et al.,* (1997) who found that deltamethrin impregnated collars protect dogs very efficiently from sandfly bites and the biting activity was reduced by 96%.

1.9. Cutaneous leishmaniasis in Ethiopia

1.9.1. Historical background of CL in Ethiopia

CL was first described in Ethiopia by an Italian epidemiologist Martogilo in 1913 (Ashford *et al*, 1973). Since then many scientists from different areas studied the distribution of CL in Ethiopia in some focal areas. CL is known by different vernacular name in different localities of Ethiopia such as: "Volbo" in Ocholo, "Finchoftu" in central Shoa, "Kunchir" in Gojam, Gonder and parts of Wollo, "Giziwa" in Tigray, "Chewie" in Sodo "Simbira halkani" in Wollega and "Shahegne" in north Shewa (Lemma *et al.*, 1969; Ashford *et al.*, 1973; Ayele and Ali, 1984; Gemechu, 1990; Hailu and Frommel, 1993).

1.9.2. Prevalence of cutaneous leishmaniasis in Ethiopia

Ethiopian cutaneous leishmaniasis (ECL) manifests as localized (LCL), mucocutaneous (MCL) or diffuse (DCL) skin lesions (Bryceson, 1969; Lemma *et al*., 1969; Ashford *et al*., 1973; Sarojini *et al*, 1984). While LCL may self heal after mild or severe skin lesions, persistent LCL, MCL, and DCL lesions are difficult to treat and can lead to non-healing and disfiguring skin and mucosal lesions. Presently, no drugs are available to cure ECL completely (WHO, 1984). Thus, the disease remains to be a major challenge to the affected communities in Ethiopia.

The overall prevalence of cutaneous leishmaniasis in Ethiopia is not well studied. However, CL is a widespread tropical infectious disease with a major focus in Ethiopia affecting thousands of people in the highlands. Belehu, (1980) estimated that the disease affects almost 0.5% of the total population. The prevalence of CL has been documented by authors from results of field surveys in endemic focal areas and from hospitals as self-reported cases. Bryceon and Nichol (1966), conducted field survey in West Wellega, Dambi Dollo, and found a total of 14 active CL cases and 12 with old scars. Lemma *et al.* (1969) conducted an epidemiological survey of CL in 3 highland areas and in the lake region of the Rift valley and found that leishmanin skin test positivity ranged from 22.5% to 44.2% in Dessie and

Karakore towns. Similarly, they found that leishmanin skin positivity was 6.7% and 5% in school children in Aleku and Shashemane towns respectively. Ashford *et al.* (1973) reported that the prevalence rate of active infection was between 5.5% and 40% per 1,000 population in the former Shewa Region. Ali *et al.,* (2002) reported that the prevalence of leishmanin skin positivity in Awash valley was about 40%.

Wilkins, (1972) conducted an epidemiological study in Sebeta, 25 km south west of Addis Ababa and found that the prevalence of CL with active infection was 0.9% (9/1,000). Similarly, Ashford *et al.* (1973) conducted an epidemiological survey of CL in the highland areas over the central plateau in Kutaber (Wollo), Aleku (Wellega), and Ocholo (Arbaminch) and came up with 9/1,000 prevalence of active infection of CL in Aleku and Kutaber, but higher prevalence in Ocholo (107/1,000) active lesions and 34.4% scars.

Mengistu *et al*. (1992) conducted a survey in Ocholo and found that the prevalence of active CL cases was 3.6% and 34.3% scars. In this study, they reported that the prevalence of active lesions was higher (8.5%) in the age group of 0-10 years old. Desta (1982) reported that 11 active lesions of CL in Adigrat Hospital. Similarly, 4 histopathologically proven cases in Sirba areas of Blue Nile Valley were reported (Sarojini *et al*, 1984). Nine DCL patients were recorded as self-reported cases to ALERT Hospital (Sarojini *et al.,* 1984; Hailu and Frommel, 1993). The disease is a major cause of outpatient visits at ALERT Hospital in Addis Ababa with the patients being among those who suffer from severe and mutilating skin lesions (Sarojini *et al*, 1984).

However, a complete mapping of the disease remains to be accomplished in view of the increasing number of patients reporting from places hitherto unknown to be endemic, including Addis Ababa. Recently, the presence of CL in Silti woreda, 150kms, south of Addis Ababa was reported by Endalamaw Gadisa (personal communication). This indicates the need for epidemiological surveillance of the disease.

1.9.3. Reservoir Hosts and vectors of CL in Ethiopia

CL-causing *Leishmania* in Ethiopia is naturally harbored by rock hyraxes (*Procavia capensis* and *Heterohyrax brucei*) and transmitted by two members of the subgenus *Larroussius*: *P. longipes* and *P. pedifer* (Lemma *et al*., 1969; Foster, 1972; Ashford *et al*., 1973; Bray, 1983). The disease is characterized by various ecotypes in which diverse species of the vectors and animal reservoirs may be involved. The disease as well as the sandfly vectors is widespread over much of the highlands of Ethiopia, mainly between 1,700 and 2,700 meters above sea level. (Lemma *et al*., 1969; Ashford *et al*., 1973; Lindtjorn, 1981).

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Table 1: The Leishmania parasite and the vectors in Ethiopia.

Leishmania species	Vectors	Reference
L. aethiopica	*P. longipes*	Ashford *et al.,* (1973)
	P.pedifer	Ashford *et al.,* (1973)
	P. sergenti	Gebre-Michael *et al.,* (2004)
L. tropica	*P. sergenti*	Gebre-Michael *et al.,* (2004)
	P. saevus	Gebre-Michael *et al.,* (2004)
L. major	*P. dubosqui*	Gebre-Michael *et al.,* (1993)
L. donovani	*P. martini, P. celiae*	Gebre-Michael and Lane, (1996)
	P.orientalis	Hailu *et al*., (1995)

1.10. Statement of the problem

Cutaneous leishmaniasis is one of the widespread tropical infectious diseases. It affects thousands of people in Ethiopia. However, the magnitude of the disease in the country is not well documented. A complete mapping of the disease remains to be accomplished in view of the increasing number of patients reporting from places hitherto unknown to be endemic, including Addis Ababa (the Capital city of Ethiopia). Recently, the presence of CL in Silte zone, 150 km south of Addis Ababa has been reported. This indicates the need for epidemiological surveillance of the disease in Silti, in particular, and in the country in general. Furthermore, for proper treatment, prevention and control of the disease, the species circulating in the area has to be identified since species-specific treatment is needed (Blum *et al.*, 2004).

Therefore, the aim of this study was to describe the magnitude of the disease in Silti Woreda with possible risk factors and identifying the causative agent using classical and molecular techniques.

2. Hypothesis and objectives

2.1. Hypothesis

The causative agent of cutaneous leishmaniasis in the study area belongs to a single species (*L. aethiopica*) and the disease is endemic with a prevalence of 3.0 %.

2.2. Objectives

2.2.1. General objective

To describe the epidemiology of cutaneous leishmaniasis in Silti Woreda

2.2.2. Specific objectives

1. To describe the prevalence of the disease in the area
2. To investigate possible determinants of the disease
3. To determine the prevailing *Leishmania* species in the area
4. To validate *L. aethiopica*-specific primers for diagnosis of clinical samples
5. To assess the treatment outcome to cryotherapy and pentostam

3. Material and Methods

3.1. Study area and population

Silte Zone is one of the administrative zones of the Southern Peoples Nations and Nationalities Regional State (SNNPR) with a population of close to one million. Silti Woreda is one of the ten woredas of the zone with an area of 850 km^2. The Woreda is divided into 54 administrative kebeles (lowest administrative body). Highland (> 2,000 meters above sea level, a.s.l.) comprises about 20% of the total area whereas 53% of the landscape is mid-altitude (1,500-2,000 a.s.l.) and 27% of the land is lowland (< 1,500 a.s.l.). The population of the woreda was close to 200,000 during the study period (2006/7) (Silti woreda Health Bureau, 2006).

The climate of the region is tropical with an estimated average annual rainfall of 780 mm. The highest rainfall (>1,000 mm) is obtained during July- August months. The daily minimum mean temperature is 20^{O}C in the rainy season and 16^{O}C in the dry season. The corresponding maximum temperature is 27^{O}C and 23^{O}C respectively. The natural vegetation of the area is bushy and shrubby type and the main indigenous trees in the area are *Cordia africana*, *Juniperus procera* and *Shiferena abyssinica (*Personal communication with the Head Woreda Agricultural Bureau, 2006).

During this study period, 2006/7 the health coverage of the Woreda was 62%. There are one Health Center and 8 Clinics (two private clinics) and 13 Health Posts in the Woreda. Malaria, diarrheal diseases and tuberculosis are the leading health problem in the Woreda (Woreda Health Bureau report, 2007).

This study was conducted in three kebeles, namely: Kibet (the town of the woreda), Woliya Sidist and Boze (the surrounding rural kebeles). The population of each kebele during the study period was 5,291, 6,029 and 5,723 respectively.

The Christian Children Fund (CCF) reported to AHRI a sudden appearance of unusual skin lesions among residents of Kibet and Woliya Sidist in 2005. Cutaneous leishmaniasis was diagnosed by Dr. Selamawit Ejigu and Sr. Genet Amare (ALERT) and parasitologicallly confirmed at AHRI (by Endalamaw Gadisa., personal communication).

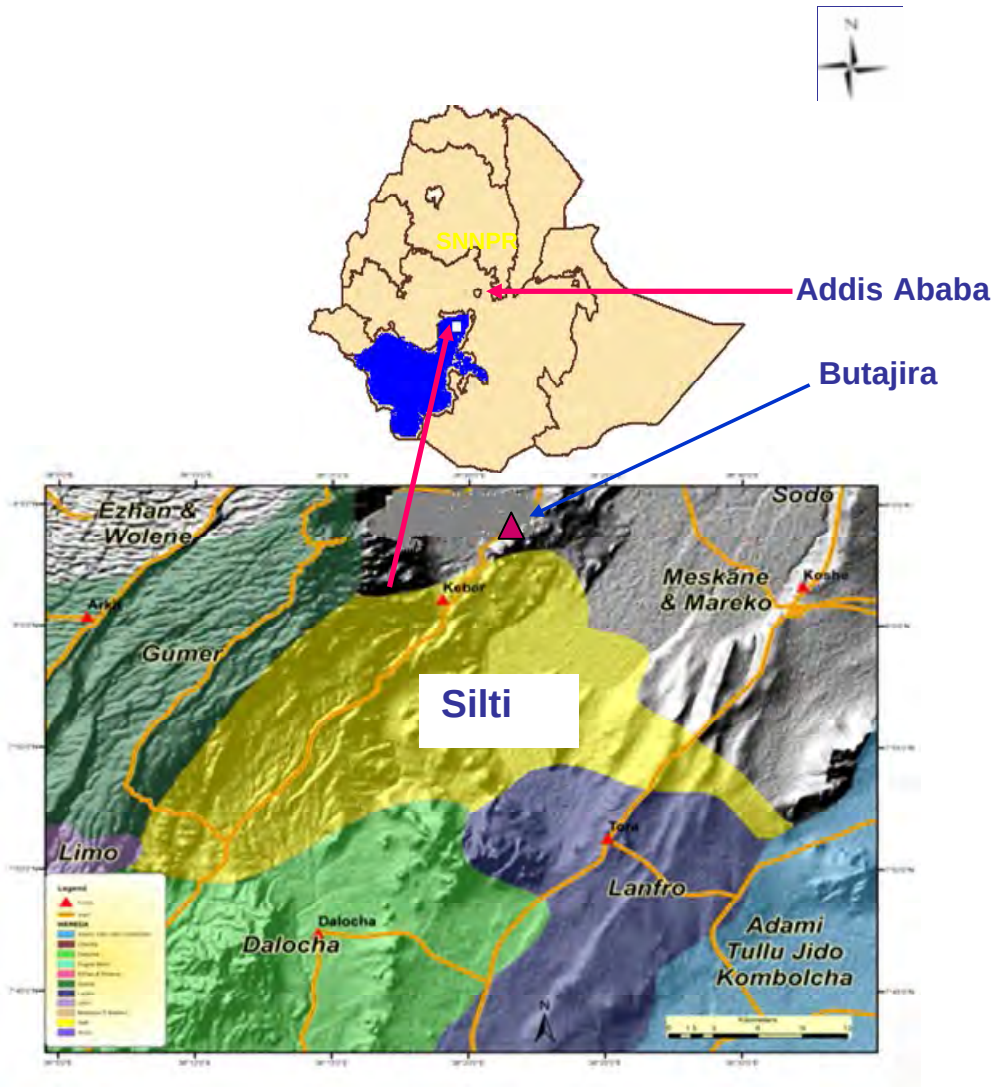

Figure 5: Map of the study area (Silti)

3.2. Study design

The study design for prevalence survey was cross sectional study and unequal non-randomized design for treatment response assessment.

3.3. Source population and study population

The sourse population of the study were all residents of Kibet Twon, Woliya 6 kebele and Boze kebele. The study population included all randomly selected households and individuals within the selected households.

3.4. Sample size

For the house-to-house survey sample size was determined after the formation of clusters. The sample size of each cluster was determined by assuming that in each clusters the prevalence of cutaneous leishmaniasis is the same (3%) with the marginal error of 2% and 95% confidence interval. The following single population proportion formula was applied to obtain the actual sample size with 10 % contingency.

$$n = \frac{(Z_{\alpha\,1/2})^2 * (pq)}{d^2}$$

Where: d= Level of marginal error (2%)

Z = confidence interval (95%)

P = prevalence of CL (3%), q = 1-p

$$n = \frac{(1.96)^2\,(0.03)\,(0.97)}{(0.02)^2}$$ + 10 % of n = 307 house holds

In each kebele, 100 households (50 house holds/cluster) were contacted.

For species identification, samples were taken from clinically suspected subjects for cutaneous leishmaniasis. The sample size was calculated assuming that 95 % of the prevailing species in the study area belong to *L. aethiopica* with 5 % marginal error and 95% level of confidence interval using single population proportion formula:

$$n = \frac{(Z_{\alpha 1/2})^2 * (pq)}{d^2}$$

Based on this formula the sample size calculated was 73.

3.5. Sampling techniques

For house-to-house survey, house number list for the Kibet kebele (Town) was obtained from the Kebele administrative office and 100 households were selected for this particular study randomly using random table from the lists obtained. For the rural kebeles (Boze and Woliya 6), we took every fourth house for our study since there is no house numbers assigned.

For species identification, from a total of 92 samples 73 samples were selected using simple random and used for identification using molecular tools.

3.6. Inclusion and Exclusion criteria

For the house-to-house prevalence study, all individuals of a selected household were eligible. However, for sample collection (skin scrapings and biopsy), only individuals with suspected cutaneous leishmaniasis were considered with other dermatological diseases (such as skin TB and scabies) excluded by qualified health professionals based on clinical criteria. Samples were taken only from those who gave informed consent / assent (see appendix1). Biopsy samples were taken from participants aged five years and above. Children below age 5 were excluded from undergoing the invasive procedures for the purposes of this study. Study subjects whose lesion was contaminated by secondary infection were excluded from giving skin scrapings because of the expected risk of culture contamination. Those who

developed the lesion on their lips, nose or ears were excluded from biopsy to avoid possible risk of cosmetic problems later.

3.7. Data collection

3.7.1. Preliminary survey

Before the study sites and kebeles were selected, a group of experts, which included principal investigator and senior scientists (Advisors), traveled to the zone. Discussions were held with the Zonal Health Bureau to get a generalized overview about the disease and to facilitate the study. According to health profiles of the Zone, the risk area for leishmaniasis was the Silti Woreda. The Silti Woreda Health Bureau and the Kibet Health Center were contacted. Although the Health Bureau knew of the presence of the disease, there was no documented case history. After contacting the woreda administration and explaining the objective of the study, a site visit was made to Kibet Town and the surroundings where the disease had been seen over the last few years. Hyraxes were observed in the banks of the 'kerati' stream, which bisects the Town. People with active lesions were noted in the area. The population size of each kebele of the Woreda was documented. After the preliminary observations, three adjacent kebeles were selected systematically as described below, one from the town and the other two from the surrounding rural kebeles.

3.7.2. Questionnaire administration

A pre-test survey was conducted to test the structured questionnaires prepared for both households and household individuals (all individuals within a given household). Based on the results of the pilot study, each kebele was clustered into two. The clusters were made to fit the administrative division to get representative samples. Accordingly, a total of six clusters were made.

Three types of questionnaires were prepared for household head, individual within a given household and suspected coetaneous leishmaniasis patients separately (Appendix1). For household head demographic and socioeconomic data were filled by directly interviewing the

household head and environmental data was filled by assessing the presence and absence of each variables. For individuals within a given household one questionnaire was filled for each individual by interviewing the individual (parents or guardians for children) and by clinical assessment for the presence and absence of suspected scars and lesions for leishmaniasis. A separate questionnaire was prepared for CL patients and filled by interviewing the patients and by clinical assessment at the Kibet Health Center.

Based on the results of the pilot study, questionnaires were re-corrected and administered to the participants.

3.7.3. House-to- house survey

Before the actual survey commenced, a team was formed which included: the principal investigator, one senior nurse from Kibet health center, one expert from woreda health bureau (Vector borne diseases control expert) and one guide/tracer who speaks the local language and knows each kebele very well. After the formation of the team, discussions were held for one day on the prepared questionnaires to create a common understanding of procedures within the team. Clinico-Pathological assessment was conducted on the randomly selected households of the sampling areas (kebeles).

All household leaders were asked about the environmental factors that may influence exposure to the parasite. These factors included the presence of hyrax in the area, presence of pits around house, vegetation type in the compound, waste disposal system and the general condition of the house (Appendix 2). We also asked whether the study subjects were involved in any activities that may increase exposure to sandfly. These activities included working in the field near the ‘Kerati stream; in Silte language meaning bank (ditches along the bank of the stream) where hyraxes are expected to live, early morning travel to the banks and number of hours /minutes they spend at the ‘kerati’. We also took geographical coordinates of each households, altitudes and distance of each households included for the study using GPS12 (Grahams town, 12 channel, South Africa). Indigenous knowledge of the study subjects about the disease and means of prevention were documented. General information on awareness or knowledge and attitudes of study subjects towards the disease

were recorded. Physical examination was made by a health professional for the presence of any lesions and scars on each interviewed individual. Suspected individuals for CL were requested to come to Kibet health center to give sample for laboratory confirmation. All study subjects, whose samples were confirmed to be *Leishmania,* were put under treatment free of charge. Patients whose result were negative for leishmaniasis were referred to hospitals discussing with the Kibet Health Center for appropriate diagnosis and treatment

3.7.4. Patient data sheets

Data of each consenting study patient were collected in a pre-tested questionnaire administered directly to the patients. The questionnaire included demographic, clinical, diagnostic, and epidemiological questions. The questionnaires were designed to be user friendly with a computerized input interface using Access-based Epi-Info 2005 and SPSS version 13.0.

3.7.5. Clinical sample collection

Two types of clinical samples, skin scrapings and biopsy were collected for parasitological identification. Before taking samples from each individual, clinically suspected patients were informed about the study and only those who agreed to give informed consent were included. In addition, some basic informative data such as educational background, occupation, travel history, treatment for leishmaniasis if any, and duration of stay in the study area were obtained using a pre-tested case record form.

A trained clinical research nurse took the specimens from the consented suspected study subjects. The active part of the lesion was identified, disinfected properly with 70% ethanol and allowed to dry. Skin scrapings were taken with sterile disposable blade after cleaning the area with 70% ethanol. Three and four mm punch biopsy tissue specimens were taken from patients from the face and other affected areas, respectively. The skin scrapings were inoculated immediately onto Novy-MacNeal-Nicolle (NNN) medium in duplicates. The biopsy samples were divided into two equal parts and one part was put in 1ml Nunc tube in normal saline for DNA extraction and the other part placed in a 1ml Nunc tube containing 10% formalin for histopathological examination. All samples were transported to AHRI

laboratory at room temperature. Biopsy samples for histopathology examination were put at room temperature whereas those put in saline solution for DNA extraction were kept at -20 ^{0}C until used (Laskay *et al.*, 1991; Van Sooligen and Hermans, 2001).

3.7.6. Culture media preparation

NNN medium with Locke's solution as an overlay was used for the cultivation of all clinical isolates and the reference strains used in this study. NNN medium was prepared as follows (Genetu *et al*., 2006): the ingredients of the medium (9.2g nutrient agar (Difco), 0.6g D-glucose (anhydrous) and 2.4g sodium chloride (sigma) were weighed, mixed and dissolved in 400ml of distilled water by heating a microwave oven with continuous shaking every 5 minutes until a clear solution was obtained. It was then autoclaved at 121^{0}C for 30 minutes. Sheep blood was aseptically collected with a bottle containing glass beads and shaken to defibrinate. The defibrinated blood was heat-activated by keeping it in a 37^{0}C water bath for 1hr and then transferred into a 56^{0}C water bath for 20 minutes to heat-inactivate the complement. The autoclaved ingredients (400ml) and the heat-inactivated blood (100ml) were mixed at 50^{0}C. Eight ml of the mix was dispensed into a 50ml culture flask and 32ml of the mix to 200ml culture flask using pipette and allowed to settle as slant. The media was then stored at 4^{0}C until used (Genetu *et al*., 2006; Gadisa *et al.*, 2007).

The ingredients of the Locke's solution (all from Sigma) are: Sodium Chloride 4.5g, Potassium Chloride 0.2g, Calcium Chloride 0.1g, Sodium Bicarbonate 0.1g, and D-glucose (anhydrous) 1.25g. To prepare the solution, all ingredients were weighed, mixed and dissolved in 500 ml of distilled water. The preparation was then autoclaved at 121^{0}C for 30 minutes. Penicillin- streptomycin (100IU/ml-100µg/ml) and L-Glutamine (0.29 mg/ml) were added to the autoclaved Locke 's solution and filter-sterilized using 0.2µm pore size diameter syringe filter and stored ready for use at 4^{0}C (Genetu *et al*., 2006; Gadisa *et al.*, 2007).

3.7.7. Parasite culture

In order to culture promastigotes, the skin scrapings of the specimen were inoculated in duplicate into NNN medium with Locke's solution as an overlay immediately at the study sites. The cultures were then incubated at 24^0C. Cultures were inspected under inverted microscope (Leitz-Wetzlar, Germany) starting on the 4^{th} day of inoculation and in three days interval afterwards to monitor the growth of *Leishmania* promastigotes. Contaminated samples were discarded immediately (Evans, 1987).

After two weeks, negative cultures were sub-cultured into new NNN media and those cultures that remained negative after two weeks of sub-culturing were recorded as negative and discarded (Genetu *et al.,* 2006). All positive cultures were sub-cultured in a liquid medium containing RPMI 1640 medium (Sigma), 10 % heat inactivated bovine calf serum (Sigma), 2mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO BRI, Scotland) and grown to stationary phase as required for subsequent experiments (Nimiri, 2002). The isolates were divided into two parts. One part was used for species identification using PCR and the other was preserved in liquid nitrogen for future use.

3.7.8. Reference strains

The reference strains used as positive controls in this study (Table 2) were kindly provided by the WHO Collaborating Center for Leishmaniasis, National Center for Microbiology, Institute de Salud Carlos III, Madrid Spain. The reference strains were treated in identical manner as the clinical isolates in all procedures.

Table2. References Strains

Species	International Code	
L.aethiopca	Strain	MHOM/ET/72/L100
L.donovani	Strain	MHOM/IN/80/DD8
L.chagasi *	DNA	MHOM/BR/00/1669
L.infantum	Strain	MHOM/FR/LEM-75
L.tropica	Strain	MHOM/SU/74/K27
L.major	Strain	MHOM/ SU/73/5-ASKH

* DNA samples from University of Calgary, Canada

3.7.9. DNA extraction

3.7.9.1. DNA extraction from cultured samples

Cultured promastigotes of *Leishmania* were harvested during late exponential growth phase. The genomic DNA was isolated from the promastigotes by the phenol-chloroform and ethanol precipitation method (Sambrook and Russell, 2000). Promastigotes were pelleted by centrifuging (Beckman coulter, Allgera 6R centrifuge, USA) at 3,000 rpm for 15 minutes and the pellet was washed twice in PBS at 3,000 rpm for 10 minutes and transferred to 1.5 ml eppendorf tube. Parasites were lysed with 300µl of lysis buffer (10mM Tris-Cl, pH 8.3, 50 mM EDTA, 1% SDS). The lysate was then incubated with 20µg RNase (Pharmacia) to final concentration of 10 µl /ml at 37^0C for 1 hr to destroy contaminating RNA and further incubated overnight with 20µg of Proteinase K (GIBCO) to final concentration of 10µl/ml) at 42^0C to remove proteins that would interfere with further analysis of DNA. Then, 300µl a 25:24:1 mix of phenol: chloroform: isoamyl alcohol (Sigma) was added to each sample and vortexed for 5 seconds. After centrifugation at 12,000xg for 5 minutes at room temperature, the upper phase was carefully removed and transferred to 1.5 ml eppendorf tubes. Isopropanol was added at 350µl volume to each sample and kept at -20^OC for 30 minutes. Subsequently, the samples were centrifuged at 1,2000xg for 15 minutes. The isopropanol

was removed and ice-cold 70% ethanol (1ml) was added. After 5 minutes of incubation at -20 ^{O}C, the samples were centrifuged for 5 minutes. The ethanol was removed and the DNA pellets were dried at room temperature. The dried pellet were then re-suspended in 20µl DNase/RNase free 1X TE buffer (pH = 8.0) and heated for 10 minutes in a 68^{0}C water bath to dissolve the pellet. Fifty-fold dilutions of DNA were used to determine the concentration spectrometrically at absorption of 260nm and dilutions of 1µg/µl were prepared. The DNA samples were stored at -20 ^{O}C until processed further.

3.7.9.2. DNA extraction from biopsy samples

To extract DNA from biopsy samples the phenol-chloroform and ethanol precipitation method developed by Van Sooligen and Hermans, (2001) was used.

Tissue sample was transferred into a glass homogenizer and homogenized in 1ml RPMI-1640. The homogenate was transferred into 1.5 eppendorf tubes and centrifuged at 12,000xg for 5 minutes in an eppendorf centrifuge. The supernatant was discarded and 500µl of 1 x TE buffer was added to the pellet, vortexed and incubated in a boiling water bath for 15 minutes. Then, 70µl of 10% SDS and 6µl of 10 mg/ml Proteinase K (GIBCO) was added, vortexed, and incubated in a 65 ^{0}C water bath for 10 min. One hundred micro liters of 5M NaCl was added, vortexed and then 80 µl of a preheated CTAB/NaCl for 10 minutes in 65^{0}C water bath was added to the samples and vortexed until it turned to milky white and then incubated at 65 ^{0}C in water bath for 10min. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and vortexed for 10 seconds and centrifuged in a micro-centrifuge at 12,000xg for 5 min. The upper phase was colleted in a sterile 1.5 ml eppendorf tubes and 0.7X volume of Isopropanol was added, kept at -20 ^{O}C for 30 minutes and centrifuged for 15 minutes at 12,000xg. The supernatant was discarded in two steps: first, the supernatant was removed until 500 µl solutions was left and centrifuged for 5 minutes and then the remaining supernatant was removed. The pellet was washed in 1ml ice-cold 70% ethanol, kept at -20 ^{O}C for 5 minutes and then, centrifuged for 5 minutes. The ethanol was discarded and the pellet was dried at room temperature. The dried pellet was then, re-suspended in 20-µl DNase/RNase free 1X TE buffer and dissolved by heating for 10 minutes in a 68^{0}C water bath. Fifty-fold dilutions of DNA were used to determine the concentration spectro-

metrically at absorption of 260nm and dilutions of 1µg/µl were prepared. The DNA samples were stored at -20 ^{O}C until processed further.

3.7.10. Polymerase Chain Reaction (PCR)

3.7.10.1. PCR conditions

All PCR assays were optimized with regard to denaturation, annealing and elongation temperatures (Table 3). The volume of each PCR reaction was 25µl for 13A/13B and Laer3Lash/ Laef3Lash primers and 50µl for the primers LITSR/ L5.8S. Hot start master mix (QIAGEN GmbH Germany) was used in all PCR reactions. In all cases a mix without template DNA was used as PCR internal control. The cycling conditions used are indicated in Table 3. The numbers of PCR-cycles used were 35, 40 and 32 for LITSR/ L5.8S, 13A/13B and Laer3Lash/ Laef3Lash respectively.

The PCR products were visualized in 1.8% agarose (Sigma) gel with 0.5 µg/ml ethidium bromide. Four micro liters of each PCR product was loaded with 1µl of 5x-loading buffers (0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 50% Glycerol) on the gel. Three micro liters of 0.1µg/µl molecular size markers was loaded as a marker. An electrophoresis separation was performed at 100V and 50mA for 90 minutes. Pictures were taken using a UV trans-illuminator (UVP-imager) (In Epi- chemi II, darkroom, Upland, with camera attached to it and a computer).

Table 3: PCR conditions

Primer Name	Primer sequence	PCR condition			Source
		Denaturation	Annealing	Elongation	
LISTR L5.8S	5'-CTG GAT CAT TTT CCG ATG-3' 5'-AAG TGC GAT AAG TGG TA-3'	95^0C for 40sec	53^0C for 30 sec	72^0C for 1min	Schonian *et al.*, (2000)
13A 13B	5'- GTG GGG GAG GGG CGT TCT-3' 5'- ATT TTC CAC CAA CCC CAG TT-3'	94^0C for 1 min	50^0C for 1 min	72^0C for 1min	Rodgers *et al.*, (1990)
Lae3rLash Lae3fLash	5'-CTG TCC GTG CTC GGG CCA GGC GCA-3' 5'-AGC TCC TTC ATG TCC TAC CAC G	94^0C for 1 min	67.8^0C for 1 min	72^0C for 1min	Kuru, (2004)

3.7.10.2. Genus level specific PCR

For this study part genus specific primer (13A and 13B) was used. This primer pair amplifies a sequence of 120bp of the kDNA minicircle of *Leishmania*, which are conserved in all minicircle classes of the genus *Leishmania* (Rodgers *et al*., 1990). This method was used as a screening method to test the presence of *Leishmania* DNA directly in the clinical biopsy samples.

3.7.10.3. Identification of the species using ITS1-PCR- RFLP method

Species identification was done according to Schonian *et al.* (2000). The internal transcribed spacer (ITS-1) in the ribosomal operon was amplified with primers LITR/L5.8S and followed by restriction digestion with enzymes *Hae III* and *Hha I* for species identification. The ITS-1-amplicon was digested with the restriction enzymes *Hae III* and *Hha I.* The reaction mix contained 17µl PCR product, 2 µl of 10x buffer of the respective enzyme and 1 µl (10 U) of each enzyme separately.

The reaction mixture was vortexed and incubated for 2 hrs at 37^0C (Schonian *et al.*, 2003). The restriction fragments were analyzed in 2 % agarose (Sigma) in the presence of 0.5µg/ml-ethidium bromide along with molecular size markers for band size estimation. Ten µl of sample was mixed with 2.5µl of 5x-loading buffers and analyzed on the gel. Four micro liters (0.1µg/µl) molecular size marker was loaded to estimate size of DNA fragments. Electrophoretic separation was performed at 100 V and 50 mA for 90 minutes and pictures were taken and used for further analyses.

3.7.11. Histopathology

Before processing the skin biopsy, the fixative was removed by dehydration with upgraded ethanol at four stages (70 %, 80 %, 95 % and 100 % ethanol). After dehydrating, the tissue was subjected to xylene to remove the alcohol until the tissue became white translucent. Xylene was removed from the tissue and the tissue was impregnated by liquid paraffin in water bath at 58^0C. The impregnated tissue was put in cold water to solidify the paraffin and prepare blocks. The block was then sectioned into 5µm using Microtome. The sections were soaked in warm water, picked and attached to microscope slides. The slides were incubated at 65^0C for 1 hour to melt the paraffin and stained using Mayer's hematoxylin progressive staining method. The slides were then examined with 400 X and in an oil immersion (1000x) magnification for the presence of *Leishmania* parasites.

3.7.12. Validation of the *L. aethiopica* specific primers

One primer pair, which has been designed from *Leishmania aethiopica* cysteine protease (Kuru, 2004) sequence (Laef3Lash and Laer3Lash), was tested for their specificity to identify *L. aethiopica*. Biopsy samples were taken from suspected patients, DNA was isolated and then subjected to PCR using the above primers under the conditions specified in Table 3. Reference strains and clinical isolates were amplified with Lae3rLash/Laef3Lash primers. The amplification was carried out using an initial 5-minute denaturation step at 94^0C followed by 32 cycles at 94^0C for 1 minute, annealing at 67.8^0C for 1 minute, elongation at 72^0C for 1 minute and final extension step of 10 minutes at 72^0C. The results were compared with reference strains, which were used as positive controls.

3.7.13. Treatment response and clinical outcomes

3.7.13. Treatment response

All clinically confirmed cutaneous leishmaniasis cases were treated free of charge either with pentostam or with liquid nitrogen based on the decision of the treating physician.

Patients were assigned to one of the treatment options non-randomly into two unequal groups. Grouping was done to treat the patients based on their clinical grounds and not for treatment comparison. However, data on treatment response were collected while patients were under treatment, and documented as a follow up activity.

Although cryotherapy has been used in some referral hospitals such as ALERT for the treatment of cutaneous lesihmaniasis in Ethiopia, information on the treatment response of cutaneous leishmaniasis to cryotherapy is lacking. Hence, we tried to collect some preliminary information, which may help for future investigation.

Systemic treatment with pentostam was given for patients with mucocutaneous lesion and multiple lesions with super infection. Sodium stibogluconate (Pentostam®), which contains

10 % antimony (100 mg Sb/mL), was given intramuscularly at a dose of 20 mg Sb/kg body weight per day for 30 consecutive days (Navin *et al*., 1992).

Liquid nitrogen (cryotherapy) was given for patients with single, young and small sized lesions. Treatment was performed in an outpatient basis. The surface of the lesion was cleaned with an antiseptic solution (70% ethanol). Freezing was achieved using liquid nitrogen as the cryogen. Liquid nitrogen (−196°C) was applied 3-4 times to the lesion with 1–2 mm margin of healthy skin with a cotton applicator. The freezing time was approximately 10–30 seconds with a thawing interval of 20 seconds.

Post cryotherapy care included daily cleansing with an antiseptic solution and topical application of an antibiotic cream (for patients with super infection). The patients returned for evaluation in 2 weeks and additional cycles were performed if residual disease was clinically evident. Follow-up was carried out at 1, 3 and 6 months after the last treatment session. Physical change of the treated area was recorded. Compliance was documented by asking patients who were under treatment.

3.7.13.2. Clinical outcomes

Outcomes were categorized (Bermu *et al.*, 2006) as:

1. **Initial cure**: ulcers showing complete scarring and disappearance of inflammatory signs within 3 months of completing treatment, or nodular lesions showing flattening and absence of infiltration or other signs of inflammation within same period
2. **Unresponsiveness**: no or incomplete scarring of lesion(s) and/or persistence of inflammatory signs 3 months after completing treatment, or either worsening of existing lesion(s) or appearance of new lesion(s) within 3 months of completing treatment
3. **Relapse**: reappearance of an ulcer, nodule and/or local signs of inflammation after initial cure.
4. **Treatment failure**: Any patient showing unresponsiveness or a relapse
5. **Definite cures**: showing initial cure and no relapse at least within 6 months of the end of treatment

3.8. Data entry and analysis

3.8.1. Data entry and cleaning

All laboratory and field data were recorded on a logbook during the study period. Each question was properly coded and key was prepared for each code. All data were double entered into an Excel spreadsheet, cleaned, verified using STATA version 8.2 and made ready for analysis.

3.8.2. Data analysis

Descriptive analysis: Descriptive analysis which included mean, standard deviation, frequency, standard error of the mean, proportions, percentages as well as descriptive graphs and tables were used with respect to the given variables such as age, sex, educational status, family size, distance of house from nearby 'kerati', number of CL cases, number of CL treated and cured, and other similar variables.

Single variable (univariate analysis): - Explanatory variables were individually cross tabulated with the outcome variable and statistical significance assessed using chi-square, Fisher's exact test or student t-test (for continuous variables). Odd ratio (OR) and 95% confidences interval (CI) were calculated to determine the strength of the association.

Multivariate analysis: - Explanatory variables significantly associated ($p \leq 0.05$) with the outcome variable in univariate analysis were included in a multivariate logistic regressions analysis using the program packages SPSS (SPSS Inc, Chicago, Illinois, USA, version 13.0 and STATA version 8.0) to detect confounding effects and to evaluate the relative influence of the different co-variates on the probability of developing the disease (outcome variable).

Added stepwise forward conditional logistic regression analysis: - Added stepwise forward conditional logistic regression analysis was carried out to determine the most predictor variable (s) affecting the out come variable to predict and model the relationship.

3.9. Ethical considerations

This study was part of the *Leishmania* Research Program at Armauer Hansen Research Institute (AHRI) and the protocol of the study was approved by the Department of Biology, Science Faculty (Addis Ababa University) and by the AHRI/ALERT Ethical Review Committee. Support letters had been obtained from the SNNPR Health Bureau, Silte Zone Health Bureau and Silti Woreda Health Bureau. Informed consent was obtained from all adults who participated in the study. For children of less than 12 years of age, the informed consent was obtained from their parents or guardians. An assent was obtained from 12-17 year old children in addition to the consent obtained from their parents or guardians. For all suspected CL cases, laboratory diagnosis and treatment were given free of charge and all results were kept confidentially (appendix 1). Only samples from consenting participants were used for the study.

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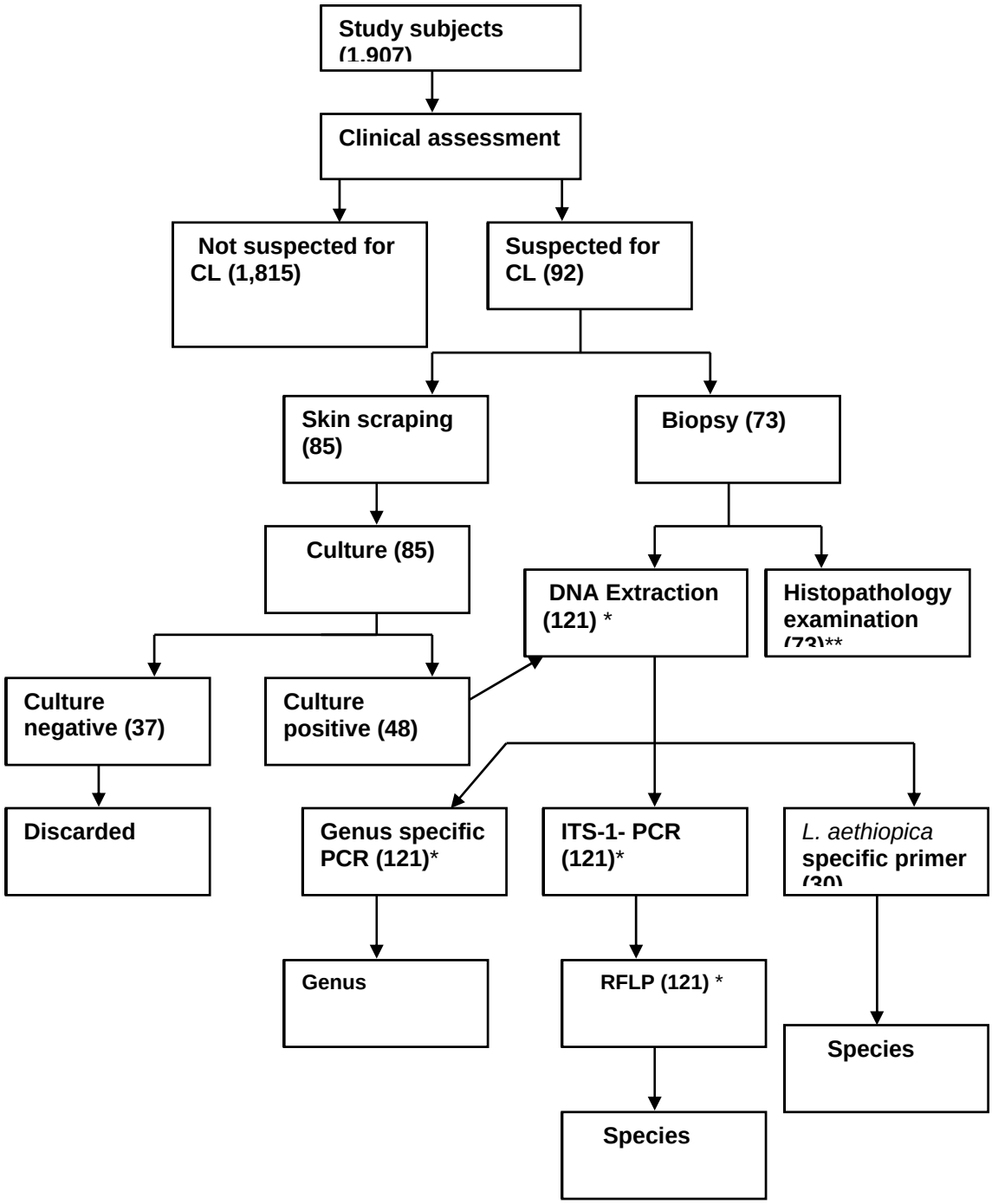


* = the number indicates the number of samples not number of CL cases

** = 60 biopsy samples were positive (Table 9)

Figure 6: Field and laboratory chart for diagnosis and species typing

4. Results

4.1. Descriptive analysis

We studied the population living in three kebeles of Silti woreda (Kibet, Woliya 6 and Boze). A total of 300 households and 1,907 individuals (all individuals within a given household) agreed to participate in the study, all were included and clinical assessment was done for all individuals. The mean age of the participants was 19.4 years $\pm$ 0.39 standard error (SE) (range: 2 weeks - 86 years). The proportion of male to female was 50.8% (969/1907) to 49.2% (938/1907) (Table 4A).

Table 4: (A) Prevalence of CL cases in the study area (n= 1,907), (B). Distribution of CL cases by kebele (n=92) in Silti Woreda in 2006/7

A.

Kebele	Total sample population	Total sample population by sex and keble		CL prevalence in each kebeles
		Male	Female	
Kibet	566 (29.7 %)	267 (14.0%)	299 (15.7%)	59 (10.42%)
Woliya 6	701 (36.8%)	358 (18.8%)	343 (18.0%)	28 (3.99%)
Boze	640 (33. 6%)	344 (18.0%)	296 (15.5%)	5 (0.78 %)
Total	**1907 (100%)**	**969 (50.8%)***	**938 (49.2 %)***	**92 (4.82%)**

*= non significant ($p \geq 0.05$)

B.

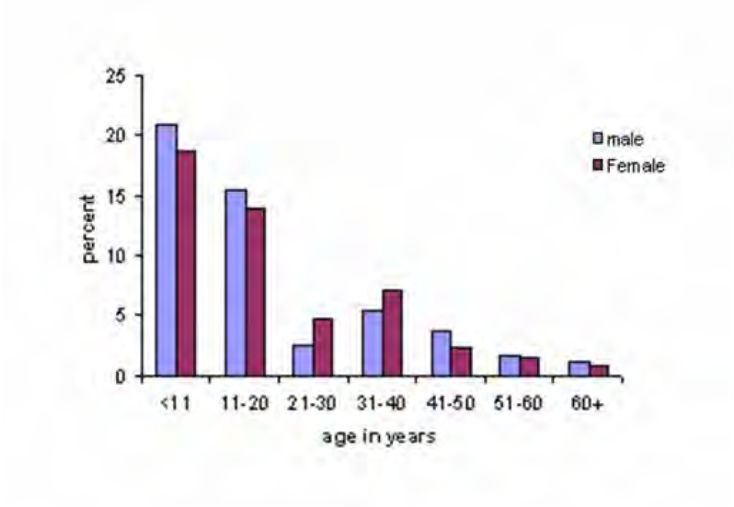
Kebeles	Male	Female	Total CL distribution by kebele
Kibet	31 (33.3%)	28 (30.4%)	59 (64.1%)**
Woliya 6	15 (16.3%)	3 (14.1%)	28 (30.4%)**
Boze	2 (2.2%)	3 (3.3%)	5 (5.4%)**
Total	**48 (52.2 %)**	**44 (47.8%)**	**92 (100%)**

**= Significant ($p \leq 0.05$)

The total prevalence of active CL lesion in the study population was 4.8% (92/1,907) (Table 4A). The mean age for the CL cases was 17.88 years ± 1.49 SE. The minimum age and maximum age was 2 years and 70 years respectively. Although CL cases were slightly more in males, 52.2% (48/92) than in females, 47.8% (44/92) the difference was not statistically significant ($p > 0.05$) (Table 4B).

We analyzed the distribution of CL cases of the three kebeles. The highest 10.42% (59/566) CL cases were from Kebet, which is the main town of Silti woreda, followed by Woliya 6 3.99% (28/701), which is the second highest and the least number of cases 0.78% (5/640) was from Boze kebele (Table 4A).

A.



B.

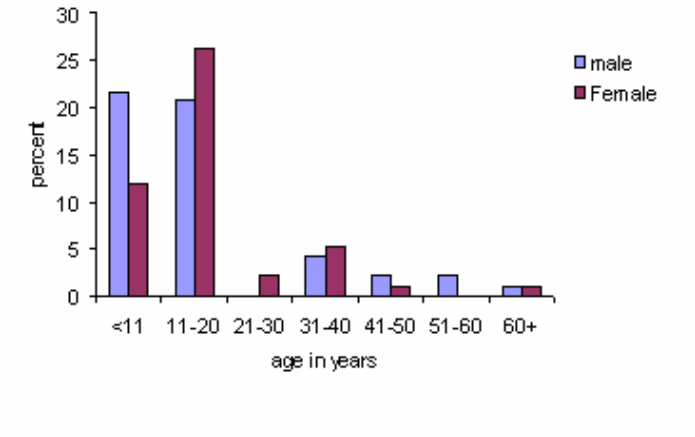


Figure 7: Age and sex distribution of study subjects: (A) All study population in the tree Kebles and (B) CL cases in Silti Woreda in 2006/7

Analysis of the age profile showed a significant difference in the age distribution of the CL cases compared with the general study population. Among the CL cases, most, 46.7% (43/92), were in the age group 11- 20 years followed by 33.7% (31/92) in the age group 0 to 10 years and the result was statistically significant ($p < 0.0001$) (Figure 7B).

Multiple mean comparisons were done for each age group and tested if there is any significance mean difference between the means of CL cases. The mean of CL cases of the age group 11 to 20 years was significantly different from the means of the other age groups (P= 0.002). There were no significant difference CL cases among the age groups of 0-10, 31-40 and 51-60 years. We also tried to calculate age specific prevalence and the age group of 11-20 was found to have the highest age specific prevalence followed by the age groups above 60 and 0 to 10 (Figure 8).

Figure 8: Age specific CL prevalence in Siltii Woreda in 2006/7

Table 5: Clinical characteristics of confirmed cutaneous leishmaniasis lesions among patients diagnosed in the Silti woreda, 2006/7: lesion number, duration of lesions and location of lesion (n=92)

Confirmed cutaneous leishmaniasis	**Male**	**Female**	Total
CL type *			
CL (cutaneous)	31 (42.5%)	28 (38.4%)	59 (80.9%)
MCL (mucocutaneous)	7 (9.6%)	7 (9.6%)	14 (19.2%)
Lesion number **			
1	19 (20.7%)	24 (26.1%)	43 (46.7%)
2	20 (21.7%)	13 (14.1%)	33 (35 .9%)
3 or more	9 (9.8%)	7 (7.6%)	16 (17.4%)
Total	**48 (52.2 %)**	**44 (47.8%)**	**92 (100%)**
Duration of lesion ***			
Less than four months	6 (6.5%)	7(7.6 %)	13 (14.1%)
Four to six months	4 (4.3%)	6 (6.5%)	10 (10.9%)
Six months to one year	22 (23.9%)	19 (20.7%)	41 (44.6%)
Above one year	16 (17.4%)	12 (13.0%)	28 (30.4%)
Total	**48 (52.2 %)**	**44 (47.8%)**	**92 (100%)**
Location of lesion **			
Cheek	27 (29.3%)	24 (26.1%)	51 (55.4%)
Ear	2 (2.2%)	0	2 (2.2%)
Lips	2 (2.2 %)	4 (4.3%)	6 (6.5%)
Leg	1 (1.1%)	0	1 (1.1%)
Hand	4 (4.3%)	7 (7.6%)	11 (12.0%)
Chin	8 (8.7%)	3 (3.3%)	11 (12.0%)
Nose	9 (9.8%)	3 (3.3%)	12 (13.0%)
Neck	2 (2.2%)	0	2 (2.2 %)
Other	0	1 (1.1%)	1 (1.1%)

* Confirmed by histopathology, **= obtained by clinical assessment, ***= obtained by interviewing the patient

Among the CL cases confirmed clinically by histopathology, 19.2 % (14 /73) were found to be mucocutaneous type (Table 5).

Most of the patients, 46.7% (46/92) had single lesions and only 17.4% (16/92) developed three or more lesions (Table 5). There was no statistically significant difference in the number of lesions between males and females ($p > 0.05$).

The duration of lesion was determined by directly interviewing the patients and most of them, 75 % (69/92), had lesion that had developed 6 or more months before (Table 5).

The location of the lesion was assessed physically by observation and more than half of the CL cases, 55.4% (51/92), developed lesion on either or both sides of their cheeks. Next to the cheek, lesions were common on the nose (Table 5).

Different types of lesions were observed, such as nodules with or without crust, nodules with osseous crust, violaceous infiltrative plaque with vegetative nodule, erysipelas like plaques, verrucous annular plaques, and impetigous or eczema-like lesions.

Table 6: Distribution of healed scars over body surfaces in Silti Woreda in 2006/7

Location of healed leishmaniasis scars	Male	Female	Total
Cheek	0	1 (16.7%)	1 (16.67%)
Lip	0	1(16.67%)	1(16.67%)
Hand	0	1(16.67%)	1(16.67%)
Nose	1(16.37%)	0	1(16.67%)
Neck	0	1(16.67%)	1(16.67%)
Forehead	0	1(16.67%)	1(16.67%)
Total	**1(16.37%)**	**5 (83.33%)**	**6(100.00%)**

Healed CL suggestive scars were assessed during the survey by interviewing the study subjects and direct physical observation. The presence of scar was registered in 0.31% (6/1,907) of the participants (Table 6). Four of them were treated at ALERT Hospital and the remaining two were treated traditionally at Buie recently.

4.2. Environmental and host factor analysis

We tried to assess whether some environmental factors could contribute for the introduction of CL in the study area involving 300 households and 1,907 individuals. On univariate analysis, some environmental and host factors were found to be associated with the development of CL (Table 7). These included the distance of the banks of the stream ('Kerati') from the participants' house (OR=11.687), presence of *Adathoda shimperina* and *Acacia spp.* in the compound (OR=16.163 and 2.446 respectively), the presence of hyrax near the participants' house (OR=36.328), use of impregnated bed nets (OR=0.349), educational status of the head of the household (OR=4.066) and having domestic animals (OR= 4.707).

The presence of hyraxes, *Adathoda shimperina,* domestic animals and *Acacia spp*. were found to be associated with increased prevalence of CL whereas, use of bed net was associated with low prevalence. The probability of having CL was found to increase as distance to the ('Kerati') in which hyraxes are observed to live become closer to the participant's house. In families where the head of the household is literate, the prevalence of CL was low. Host factors such as sex, age, and family size, presence of latrine, presence of pits near house and waste disposal system were also tested in the analysis. Age was associated with the risk of the development of CL. No correlation with CL was found for latrine, waste disposal site, presence or absence of pit near house, family size and gender.

A Multivariate analysis was carried out to detect confounding effects and to evaluate the relative influence of these different covariates on the probability of developing CL (Table 7). In multivariate analysis, the presence of hyrax near the house (OR=14.079) and *Adathoda shimperina* (OR= 4.416) were associated with an increased prevalence of having CL. Living

at a distance of less than 300 meters from the 'Kerati' was also found to be associated with increased prevalence of CL (OR=1.762).

Table 7: Univariate and multivariate analysis of environmental and host factor with adjusted odds ratio

	Univariate		Multivariate	
	P	OR (95% CI)	P	OR (95% CI)
Host factors				
Age (in years)				
≥20	-	1		1
<20	0.016*	1.901 (1.125-3.213)	0.412	0.616 (0.193-1.964)
Sex				
Female	-	1		
Male	0.873	0.966 (0.636-1.469)		
Family size (in numbers)				
<6	-	1		
≥6	0.335	1.375 (0.701-2.698)		
Educational status of the head of the household				
Literate	-	1		1
Illiterate	0.000*	4.066(2.045-8.083)	0.238	0.604(0.262-1.394)
Trees and shrubs				
Acacia spp.				
Absent		1		1
Present	0.014*	2.446 (1.203-4.975)	0.226	0.572 (0.231-1.414)
Adathoda shimperina.				
Absent		1		1
Present	0.000*	16.163 (7.009-37-271)	0.003*	4.416 (1.647-11.839)
Persia americana				
Absent		1		
Present	0.635	1.192(0.578-2.460)		
Eucalyptus spp.				
Absent		1		
Present	0.870	0.947 (0.489-1.831)		
Croton macrostachyus				
Absent		1		
Present	0.360	1.41 (0.719-2.769)		
Catha edulis				
Absent		1		
Present	0. 310	0.571(0.593-1.697)		

Coffee arabica				
Absent		1		
Present	0.695	1.166(0.540-2.517)		
Insete Ventriosum				
Absent		1		
Present	0.550	1.228 (0.625-2.413)		
Animals				
Hyraxes near house				
Absent		1		1
Present	0.000 *	36.328 (10.885-121.248)	0.005 *	14.079(3.288 -60.296
Domestic animals				
Absent		1		1
Present	0.000 *	4.707 (2.348-9.435)	0.777	1.148(0.442-2.981)
House condition				
Wall				
Not cracked	-	1		
Cracked	0.593	1.364(0.467-4.2629)		
Holes formed	0.446	1.674(0.445-6.294)		
Other factors				
'kerati' (distance in meters)				
≥300 meters	-	1		1
< 300 meters	0.000*	11.687(5.154-26.501)	0.03*	1.762(2.600-5.179)
Pit near house				
Present		1		
Absent	0.330	0.562 (0.176-1.795)		
Latrine				
Present	-	1		
Absent	0.464	1.745(0.393-7.748)		
Waste disposal near house				
No		1		
Yes	0.802	0.820(0.173-3.880)		
Damping waste				
Closed		1		
Open	0.372	0.679(0.291-1.587)		
Bed nets				
Absent		1		1
Present	0.002*	0.349 (0.197-0.679)	0.320	0.656(0.286-1.506)

* Significant (p value ≤ 0.05)

Table 8: Coefficient and goodness of fit of logistic binary model predicting presence and absence of CL in three kebeles on the basis of observed data of disease and environmental and host factor variables; (A) Percentage of correct predictions for the presence of CL in at least one of the members in the households, (B) Variables in the equation, (C) Model with terms removed

A

Observed CL	Predicted CL		Correct prediction in %
	Absent	present	
Absent	247	10	96.1
Present	19	24	55.8
Overall			90.3

B.

Variable	B	SE	wald	df	sig	Exp (B)
Hyraxes	3.155	0.631	25.012	1	0.000	23.443
Adathoda shiperina	1.643	0.458	12.856	1	0.000	5.171
Constant	-4.178	0.583	51.362	1	0.000	0.015

df= degree of freedom, SE=standard error, sig= significance

C.

Terms removed	Model Log likelihood	Change in -2 log likelihood *	df**	Sign of the change
Hyrax	-101.586	42.576	1	0.000
Adathoda shiperina	-87.086	13.575	1	0.000

* = Binary logistic regression, ** = degree of freedom

We subjected the data from the environmental and host factors to stepwise-added conditional logistic regression analysis to find out the best predictor(s) for the absence and presence of CL in the study area during the study period (2007). The presence of hyrax near house and *Adathoda shimperina* in the compound and are the best predictors for the presence and absence of CL in the area. The relationship may be modeled as follows (Table 8 B):

Probability of the presence CL in a given household = $1/ (1 + e^{-z})$.

Where:

Z = -4.178 + 3.155 (presence of hyrax) +1.643 (presence of *Adathoda shimperina* in the compound).

Although none of the 10 negative households were correctly predicted by the model to be free of infection and none of the 19 positive households were correctly predicted to be positive for CL infection, 90.3 % overall accuracy of the model in predicting the probability of the presence and absence of CL infection in the population was achieved (Table 8 A).

At each step of computation the change in -2 log likelihood is larger for hyrax compared to that of the *Adathoda shimperina* (Table 8C). This indicates that the variable hyrax contributes more to the model compared to the variable *Adathoda shimperina.*

4.3. Parasitological identification

4. 3.1. Diagnosis

From 85 cultured samples, 56.47% (48/85) were culture positive and 27.06 % (23/85) were culture negative. The remaining 16.47 % (14/85) were contaminated. Histopathological examination was performed in 73 cases. As indicated in Table 9, 82.19% (60/73) was found to be positive for the presence of amastigotes in tissue sections and macrophages. Of the histopathologically positive samples, 19.2 % (14/73) were mucocutaneous type and the rest were cutaneous type.

Of the total of 71 biopsy samples tested with PCR, 83.1 % (59/71) were positive for the presence of *Leishmania* amastigote DNA in the sample. From 48 total culture positive samples 89.6% (43/48) were positive in PCR (Table 9).

Table 9: Parasitological identification clinical samples from Silti Woreda in 2006/7

Diagnostic method	**Total samples tested**	**Positive samples (%)**
Culture	85	56.5% (48/85)
Histopathology *	73	82.2% (60/73)
PCR *	71	83.1 % (59/71)
PCR**	48	89.6% (43/48)

* =From biopsy samples, ** = from culture positive samples

4.3.2. Analysis of the histopathological patterns

The epidermal findings were non-specific histological changes including ulcerations, acanthosis, spongiosis, epidermal atrophy and basal cell degeneration. The leishmaniasis specific histopathological changes were seen on the reticular, papillary dermis and peri-adenexal areas, and included:

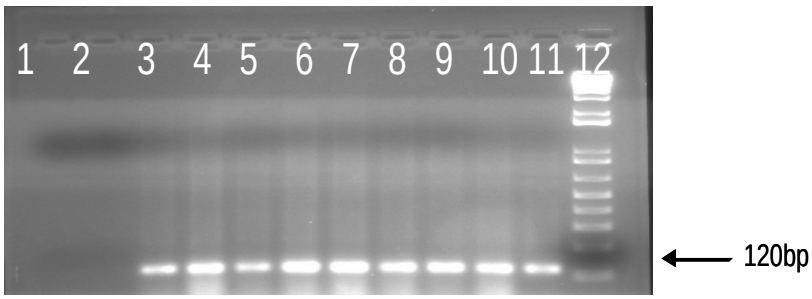
1. Diffuse infiltration by *Leishmania* parasite laden macrophages
2. Sheets, and aggregates of lymphocytes, eipthelioid cell aggregates and *Leishmania* parasite laden macrophages
3. Infiltration by predominantly aggregates of epithelioid cells and very few scattered *Leishmania* parasite laden macrophages.

Only few parasites were seen outside macrophages and no differences in the histopathological patterns were seen among the cutaneous and muco-cutaneous lesions.

4.3.3. PCR amplification with the genus specific kDNA-primers 13A/13B

The genus specific primer pair 13A/13B (Schonian *et al.*, 2000) was introduced as a screening method in our study. We amplified the conserved 120 bp size miniexon kDNA from promastigotes (culture) and amastigotes (biopsy) DNA samples (Figure 9). The amplification has been successfully applied on both biopsy and culture samples. The results obtained are in agreement with previous reports (Rodriguez *et al.*, 1994; Genetu *et al.*, 2006; Gadisa *et al.*, 2007).

A.



B.

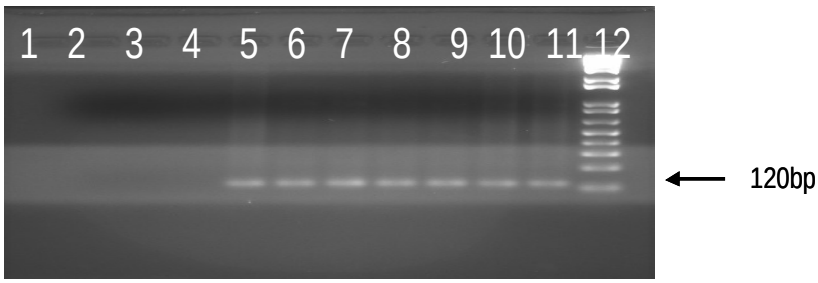


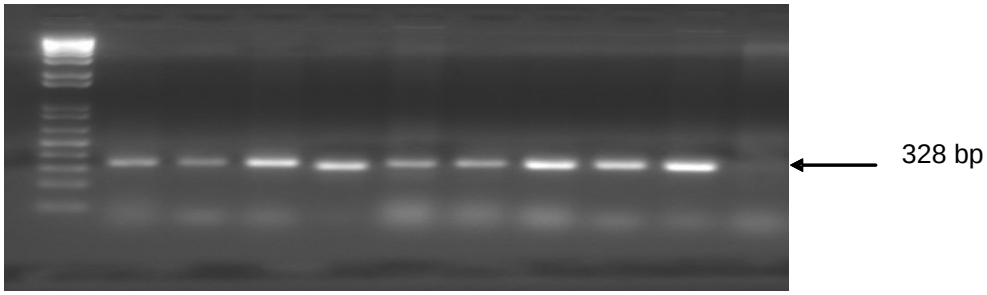
Figure 9: Images of PCR product amplified with primer pair 13A/13B after staining with ethidium bromide; (A) from **culture promastigotes**: 1= Negative control (TE), 2= Mtb DNA, 3= *L. infantum*, 4 = *L. donovani*, 5= *L. tropica*, 6=*L. major*, 7=*L. aethiopica*, 8-11 Clinical samples, 12= 1kb ladder.

(B) from **skin biopsy**: 1=Negative control (TE), 2=Mtb DNA, 3= normal human skin DNA, 4=*M .leprae* DNA, 5= *L. donovani*, 6= *L. tropica*, 7= *L. infantum*, 8=*L .major*, 9=*L. aethiopica*, 10-11 Clinical samples 12= 1kb ladder

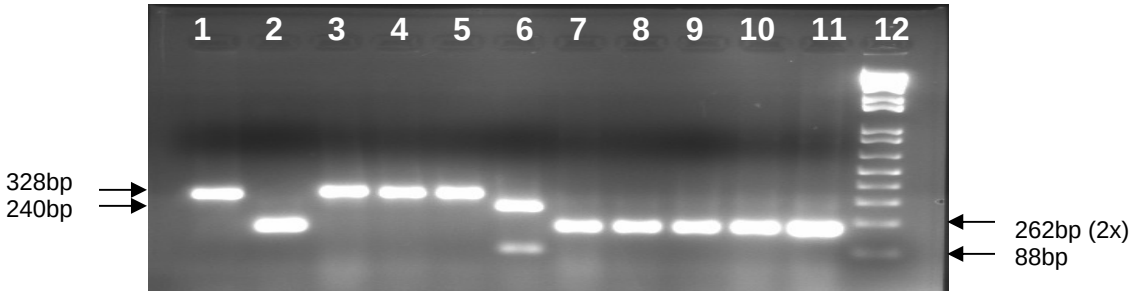
4.3.4. PCR amplifications with the ITS1 primer pair (LITSR/L5.8S) and RFLP analysis

The PCR of the ITS-1 with primer pairs LITSR/L5.8S gave approximately 328 bp product for all reference strains and clinical isolates both from skin biopsy and cultured promastigotes DNA (Figure 10A and 11A). When the ITS-1 PCR product was digested by an enzyme *Hha I, L. aethiopica* reference strain and our clinical isolates produced a band approximately 162 bp size (Figure 10B and 11B) on the gel which is according to the sequence information from gene bank the 162 bp is actually a superimposition of 164 bp and 162 bp bands (accession no AJ000311). Two bands of about 88 bp and 240 bp sizes were produced for *L. major* where as, *L. infantum, L. donovan*i, *L.chagasi* and *L. tropica* gave single band size of 328 bp (Figure 10B and 11B) showing that there is no restriction site in the ITS-1 region for these strains. When the ITS-1 PCR product was digested by *Hae III, L. aethopica* reference strains, *L. tropica* and clinical samples produced three bands of size 202 bp, 55 bp and 23 bp (Figure 12). The clinical isolates (biopsy and culture samples) produced identical bands with *L. aethiopica* reference strain. Similar result was obtained by Schonian *et al.*, (2003) and Gadisa *et al*., (2007).

A*



B*

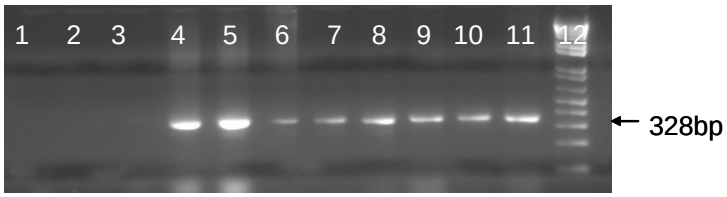


* = culture (DNA from promastigotes)

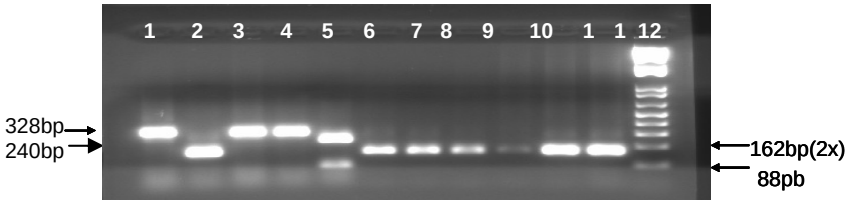
Figure 10: (A). PCR products of ITS-1 from promastigote DNA samples, 1= 1kb ladder, 2=*L. donovani*, 3=*L. tropica*, 4= *L.infantum*. 5= *L. major*, 6= *L.aethiopica*, 7-10 Clinical isolates, 11= negative contro(TE).

(B). PCR-ITS1-RFLP of the amplicon with *Hha I* from promastigote DNA, 1= *L. donovani*, 2= *L. ethiopica, 3=L. infantum, 4=L.tropica*,5= *L. chagasi* 6=*L. major*, 7- 11 Clinical isolates, *12*= 1kb ladder

A **



B**



** = Biopsy (DNA from amastigotes)

Figure 11: (A). PCR products of ITS-1 from skin biopsy DNA samples: 1= Negative control (TE), 2=Mtb DNA, 3= Human skin DNA, 4=*L. donovani*, 5=*L. infuntum*, 6= *L. tropica*, 7= *L. major*, 8= *L. aethiopica*, 9-11 Clinical isolates, 12=1kb ladder

(B). PCR- RFLP of the ITS1-amplicon with *Hha I* from skin biopsy DNA samples, 1= *L. donovani* 2= *L. aethiopica*, 3= *L. tropica* ,4= *L .infantum*, 5= *L. major* 6-11= clinical isolates, 12=1 kb ladder

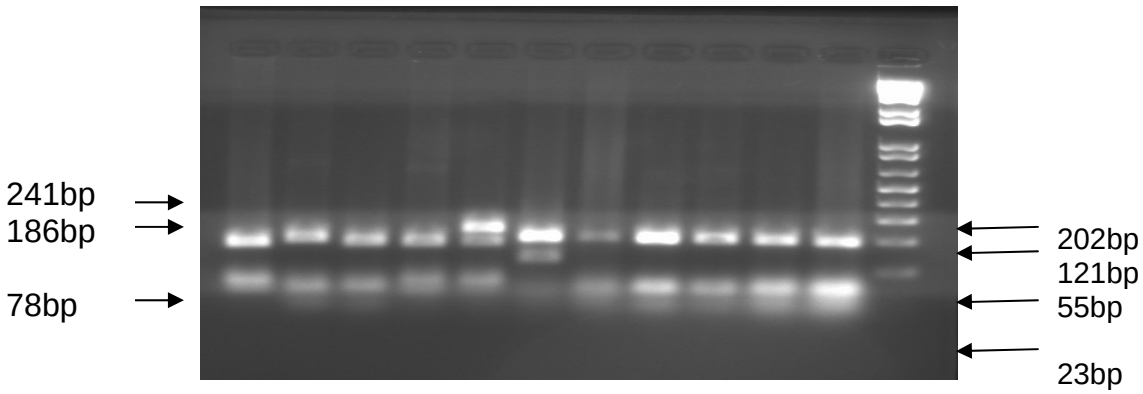


Figure 12: PCR-ITS1-RFLP of the amplicon with *Hae III*, 1= *L. chagasi*, 2=*L.tropica*, 3=*L.aethiopica*, 4= *L. donovani*, 5=*L.infantum*, 6=*L. major*, 7-11=Clinical isolates, 12=1kb ladder

4.3.5. Results of *L. aethiopica* specific PCR amplification

The primer pair amplified about 256 bp from *Leishmania aethiopica* (MHOM/ET/72/L100) and biopsy samples. The primers did not amplify the DNA samples from other strains (Figure 13).

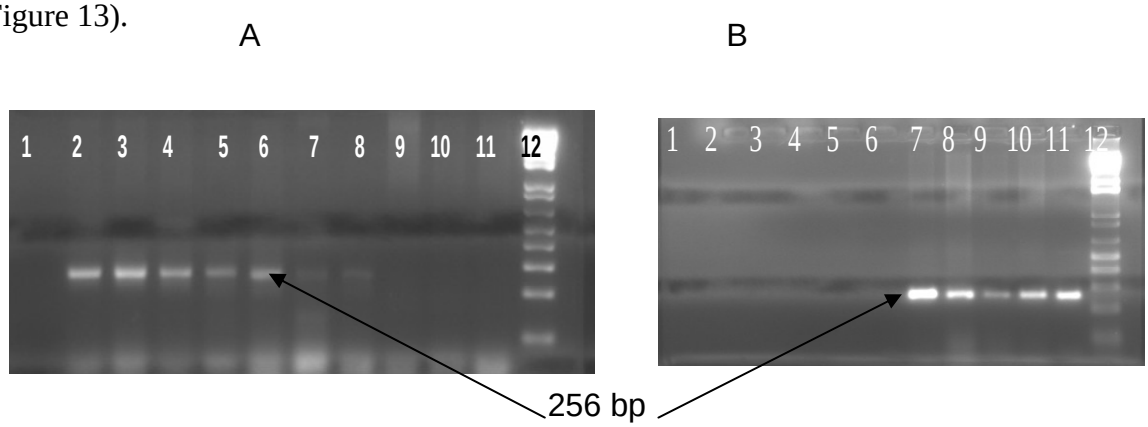


Figure 13: PCR amplification result with Lae3rLash/Lae3fLash primers: (A) Culture; 1=Negative control, 2-5 Clinical samples, 6=*L. aethiopica*, *7=L .tropica*, 8=*L. major*, 9= *L. infantum*, 10=*L .chagasi*, 11=*L. donovani*, 12=1kb ladder (B) Biopsy; 1=Negative control, 2= *L. infantum* ,3= *L.donovani*, 4= *L.tropica* 5= *L. major*, 6 =*L. chagasi*, *7= L. aethiopica*, 8-11 =Clinical samples, 12=1kb ladder

4.4. Treatment response

4.4.1. Response of cryotherapeutic treatment

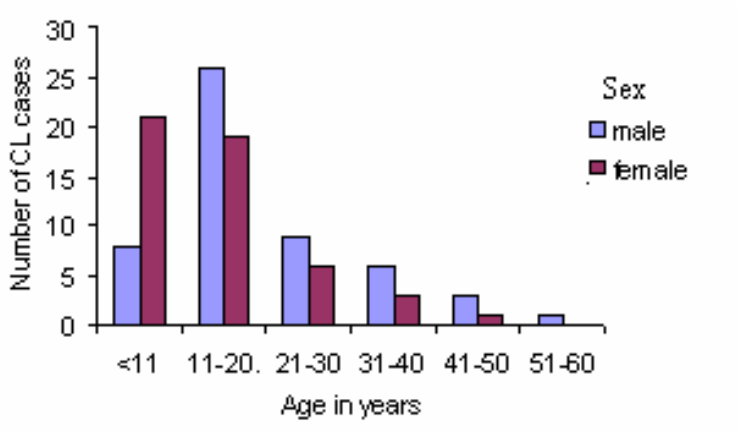
Patient treatment was carried out not for the purpose of experimental trial, but for the care of the consenting patients who participated in the house-to-house survey according to the protocol developed prior to the study. During the house-to-house survey, only 92 CL cases were clinically confirmed. Here we treated additional patients who came from the study area, but who were not included in the sample survey. We continued treating additional clinically confirmed CL cases at the end of the study because there was as yet no alternative source of care for the patients. We have started negotiating with the local and regional health authorities regarding the care for CL cases in the area.

Of the 103 patients, 51.5 % (53/103) males and 48.5% (50/103) females with a mean age ± SE of 18.37 years ± 1.15 were treated with liquid nitrogen (Table 10B). The mean ± SE duration of the lesions before the onset of treatment with cryotherapy was 8.46 months ± 0.66 SE. There were no significant difference ($P \geq 0.05$) between males and females with respect to age and duration of lesion. However, when individuals are considered, there was a significant age difference between them ($P = 0.017$). On average, liquid nitrogen was applied for 6.43 times ± 0.326 SE with minimum and maximum of 3 times and 13 times respectively per person until healing was achieved (Table 10A).

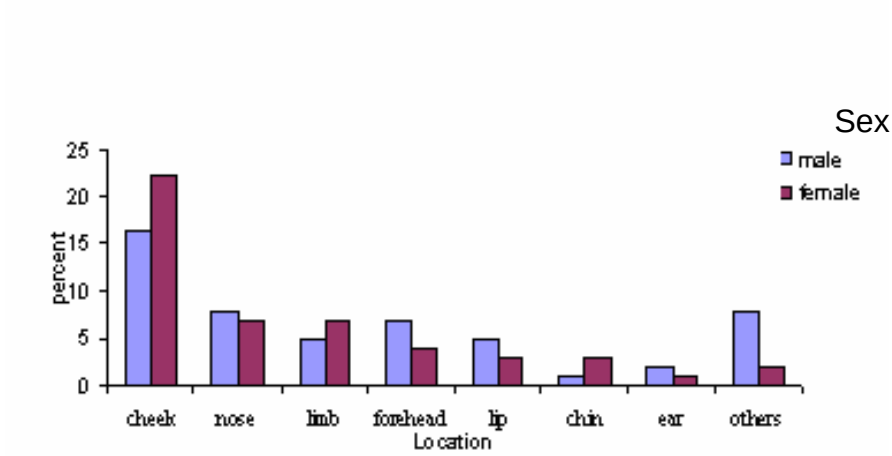
The majority of patients who were treated with cryotherapy had lesions located on the cheek 38.8% (40/103) and nose 14.6% (15/103) (Figure 14B). Of the total 103 CL patients put on cryotherapy treatment, 45.7% (45/103) of them were in the age group of 11-20 years and 28.1 % (29/103) were in the age group of 0-10 years (Figure 14A). From the total patients put on this treatment, the number of dropouts was 13.6% (14/103). The number of male dropouts was almost two times (9:5) of female dropouts. Six patients 5.8% (10/103) did not respond to the cryotherapeutic treatment and were cured when treated with pentostam using conventional dose (20mg/kg per day for 30 consecutive days).

Follow up was done after the completion of treatment for 1 month, 3 months and 6 months. From 103 patients included in this treatment, 83 of them were completely cured giving a 93.3% cure rate for cryotherapy. Minor adverse events were recorded by observation and asking the patients under treatment. The major adverse events were blister formation immediately after the application of liquid nitrogen, erythema, burning sensation, and scarring in a few patients.

A



B



Others include neck, eye, head region, and their combination

Figure.14: Study subjects treated with cryotherapy: (A) Age of study subjects by sex (B) lesion distribution by sex in Silti Woreda in 2006/7

Table10. Cryotherapy treatment in Silti Woreda in 2006/7: (A) duration of lesion (months) versus liquid nitrogen application (repeats), (B) treatment response to cryotherapy

A.

Number of applications	Sex	Duration of lesion before treatment (months)					
		≤3 months	4-6 months	7-9 months	10-12 months	>12 months	Total
1-3 times	Male	7 (6.8%)	3 (2.9%)	0	3 (2.9%)	1 (0.97%)	14 (13.6%)
	Female	1 (0.97%)	2 (1.9%)	1 (0.97%)	2 (1.9%)	0	6 (5.8%)
4-6 times	Male	8 (7.8%)	9 (8.7%)	1 (1.9%)	2 (1.9%)	0	20 (19.4%)
	Female	7 (6.8%)	1 (0.97%)	3 (2.9%)	1(0.97%)	2 (1.9%)	14 (13.6%)
7-9 times	Male	2 (1.9%)	1 (0.97%)	0	4 (3.9%)	2 (1.9%)	9 (8.7%)
	Female	1 (0.97%)	8 (7.8%)	4 (3.9%)	4 (3.9%)	1 (0.97%)	18 (17.5%)
> 9 times	Male	2 (1.9%)	2 (1.9%)	1 (1.9%)	3 (2.9%)	2 (1.9%)	10 (9.7%)
	Female	0	0	1 (0.97%)	4 (3.9%)	7 (6.8%)	12 (11.7%)
Total		**28 (27.2%)**	**26 (25.2%)**	**11 (10.7%)**	**23 (22.3%)**	**15 (14.6%)**	**103 (100%)**

B.

Sex	Treated	Cured	Treatment failure	Dropout	Remark
Male	53 (58.5%)	41 (39.8%)	3 (2.9%)	9 (8.7%)	If dropouts excluded, cure rate will be 93.3%
Female	50 (48.5%)	42 (40.850	3 (2.9%)	5 (4.9%)	
Total	**103 (100%)**	**83 (80.6%)**	**6 (5.8%)**	**14 (13.6%)**	

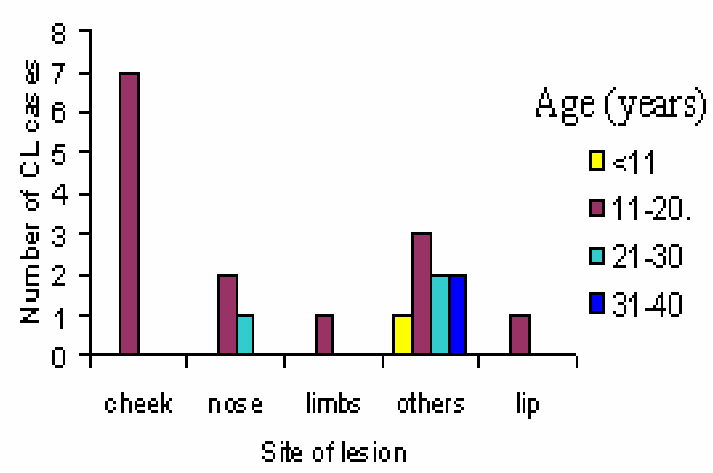
4.4. 2. Treatment with sodium stibogluconate (SSG)

A total of 20 subjects (70% male and 30 % female) with mean age of 19.55 years ±1.64 SE were put on treatment with pentostam on conventional dose of 20mg SSG/kg body weight for 30 consecutive days. Most of the patients 70.0% (14/20) who were treated with pentostam were in the age group of 11-20 years (Figure 15A).

Of the 20 patients treated with pentostam, 60% (12/20) had four or more lesions and from these 45% (9/20) were in the age group of 11-20 years old (Figure 15A). From the total patients who received pentostam, 85.0% (17/20) were cured, 10.0% (2/20) were unresponsive and 5.0% (1/20) was dropout (Table 11). If the dropout is excluded the cure rate of pentostam will be 89.5%. Patients who were not cured by pentostam treatment were successfully treated with liquid nitrogen.

A

B



Others include combinations of limb, cheek, neck, forehead, nose and chin

Figure 15: Study subjects treated with pentostam in Silti Woreda in 2006/7;

(A) Age of study participants who received pentostam treatment versus number of lesions, (B) Site of lesion defined by age

Table11: Treatment response of patients to pentostam in Silti Woreda in 2006/7

Sex	Treated (total)	Cured	Dropout	Resistance	Total
Male	14 (70.0%)	13 (65.0%)	0	1 (1.0%)	14 (70.0%)
Female	6 (30.0%)	4 (20.0%)	1 (5.0%)	1 (5.0%)	6(30.0%)
Total	20 (100 %)	17 (85.0%) *	1 (5.0%)	2 (10.0%)	20 (100%)

*If dropouts excluded, the treatment response of pentostam will be 89.5%

5. Discussion

In this study, a molecular epidemiological approach was combined with classical methods to determine the prevalence, etiologic agent/s and determinants of cutaneous leishmaniasis in the Silti Woreda. We applied molecular diagnostic tools in combination with the classical methods (culture and histology) and classical epidemiology for the identification of the circulating *Leishmania* species in Silti woreda as the best means to attain our objectives.

The distribution and incidence of CL is greatly influenced by environmental factors affecting the population of vectors, reservoirs, and human hosts. Although this notion has long been realized, and despite the expansion of the information on effects of environmental factors on sandfly vectors of leishmaniasis, little attempt has been made to the determinants that could be related to CL in Ethiopia. Assessment of determinants would allow managers and health professionals to define the extent of the problem and the rational use of interventions, where it is most likely to succeed.

In this study, the analysis depended mainly on field results (assessment of the determinants) and parasitological identification using both traditional and molecular techniques.

A total of 1,907 individuals participated in our study. The male to female ratio was almost equal (Table 4A). The average family size was six (range between 1-12). The prevalence of CL cases was higher in Kibet, than in Woliya 6 and Boze (table 4B). The prevalence of CL varied significantly between each Kebele and this difference could be associated with environmental risk factors. The habitat of hyraxes, the 'Kerati', bisects the Kibet town into two and extends to Woliya 6 kebele and does not touch Boze. Since Kibet is a town, the population density is higher than that of Woliya 6, which is a rural kebele. Hence, more houses are constructed near the 'Kerati' in Kibet than in Woliya 6. As a result more people are exposed to the bites of sandflies at Kibet than in Woliya 6. Moreover, more people stay outside house in town than in rural areas during the nighttime owing to the availability of electricity and recreational places like restaurants and hotels.

In Boze kebele, we found leishmaniasis cases near the 'Fincha' River, which separates the Boze and Yetiker kebeles, in which a few hyraxes were observed. The low number of CL cases in Boze is probably due to the fact that a few houses were constructed near the river ('Fincha') or possibly, hyraxes moved to the area only recently.

The overall prevalence of active CL during the study period was 4.82 %, which is higher than the previous reports in the endemic focus of Ocholo, which was 3.0 % (Ashford *et al*., 1973) (Figure 16).

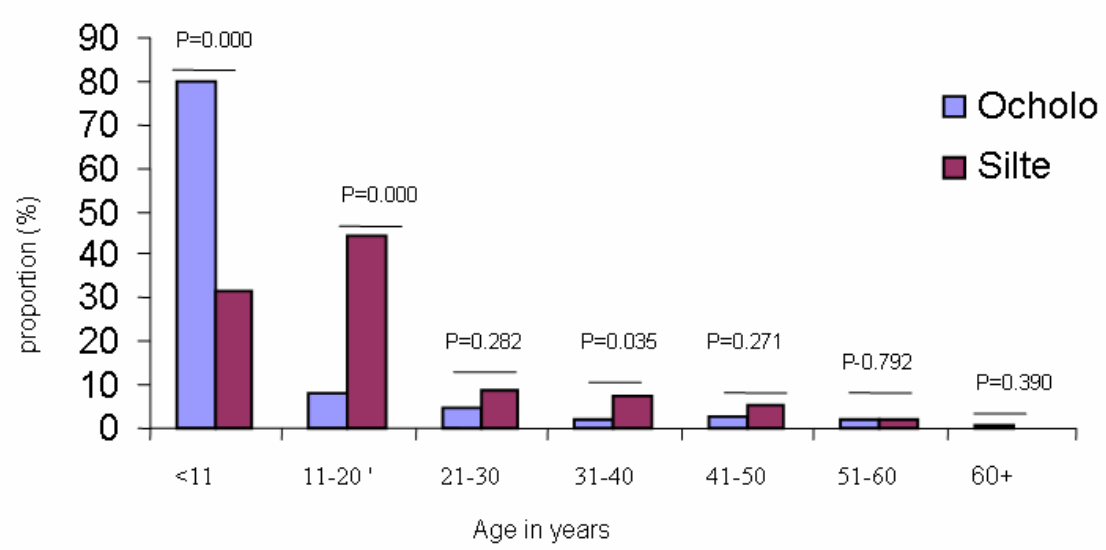


Figure 16: Comparison of CL cases with previous report by Mengistu *et al*., (1992) in endemic area (Ocholo) with the current result (Silti) across age groups

Mengistu *et al*. (1992) conducted similar study in Ocholo. The methods used for house-to - house survey by these authors are similar to the methods we adopted for this study to describe the prevalence of CL in Silti woreda. . Hence, we did comparison of CL cases across age groups considering Ocholo as a model of endemic area for CL.

We compared the proportion of CL cases in Silti with the previous reports of the CL cases in an endemic area, Ocholo, (Mengistu *et al*., 1992) across age groups (Figure 16). The proportion of CL cases in the age group of 0-10 years old in Ocholo was about 80% whereas in Silti it was only 31.5% and the proportion was significantly different (p=0.000) (Figure 16). The proportion of CL cases in the age group of 11-20 years old was 8.3 % and 44.6% in Ocholo and Silti respectively (Figure 16), which is again significantly different (p=0.000).

Unlike in endemic areas like Ocholo, more CL cases were found in an age group of 10-20 in Silti (Figure 6 and figure 14). In endemic areas, the prevalence of CL is highest among children of age group 0-10 (Ashford *et al*., 1973: Mengistu *et al*., 1992; Al-Jawabreh *et al*, 2003). Adults will have protective immunity since they will be exposed to CL in early childhood.

In this study, we asked the head of the households and elderly people in the area their opinions about the time of the first appearance of the disease and their estimated time since the disease occurred in the area. The average time from their responses was calculated to be 3 years. Moreover, we asked them if the disease has any vernacular (local) name and it was found that the disease does not have a local name unlike in the neighboring areas like Kella and Buei who called 'Chawie' in the local language. They simply call it 'Tul Zemen' 'Yezemenu kusil'in Amharic which means the recent lesion and this is an indication that the disease has probably appeared in the area recently. To supplement our results, we tried to collect information from the health records, for the last 7 years, at the health center and we couldn't find even a single documented leishmaniasis case.

We also tried to assess the prevalence of scars suggestive of healed CL through clinical assessment and observations in markets, meetings, assembly points (mosques) and schools. Only very few people (Table 6) have scars and these people were treated recently at either ALERT hospital or using traditional herbs at Buie town (Sodo woreda). Hence, our findings are conclusive enough to say that CL in Silti Woreda seems recent introduced.

CL erupted in the Silti probably hyraxes moved to the area following the banks of a stream due to ecological changes such as deforestation. However, this hypothesis needs further investigation.

Of the total number of individuals who had skin lesions, 89.1 % (82/92) had the lesions on their face, mostly on the check or the nose (Table 5). The face is the exposed body part which is easily accessible to sandfly bites. In addition, the softness of the skin on the face makes it a preferable site of feeding for the sandfly (Al-Jawabreh *et al*., 2003).

Although the determinants of *Leishmania* infection such as the parasite, the vector, the reservoir, the host factors and the environment are multifactorial, we tested for some selected demographic, host and environmental factors. The demographic factors, age and sex have been reported as source of variation in exposure to CL. Host behavioral/ cultural factors were thought to make males more exposed than females and lack of immunity to make children more at risk of disease than adults (Al-Jawabreh *et al*., 2003).

Attempts were made to see if there is any significant difference between male and female CL cases. We found that, although male CL cases were more frequent than females (48:44 ratio), (Table 4B) the difference is not statistically significant and both groups had the same risk of infection. The age group 10-20 years had relatively higher risk of infection and this is probably attributed to host behavior. The youth at this age group usually would like to spend outdoors in the evening by forming peer groups. The youth at this age group are also actively involved in fetching water, firewood collection, keeping crops from wild animals (the case of Woliya sadist) and other similar activities, which may increase their chance of being bitten by sandflies.

Our results show that the presence of hyrax near a house, the presence of *Adathada shimperina* and *Acacia spp*. plants in the compound, the distance of the hyrax habitat, the distance of 'kerati',from the participants' house and presence of domestic animals (mainly cows) close to study participants' house were independently associated with the prevalence of CL. It is possible that these variables influence the populations of the sandfly vectors and/or the reservoir hosts of *L. aethiopica* in the area by affecting other microclimatic factors in the area (such as Ecophysiology of host parasite interaction).

Distances from the banks of the stream (habitat of hyrax in the study area) were correlated with the presence and absence of CL. We tried to divide the distance of the 'kerati' from study participants ' houses into two taking the average flight range of sandfly into account. Both in univariate and multivariate analysis, the adjusted odds ratio suggested that individuals living closer to the banks of the stream (≤300 meters) are at relatively higher risk than those living farther away from the banks of the stream (more than 300 meters).

Hyraxes, the reservoir host of *L. aethiopica*, were found to live in the banks of the stream which bisects the Kibet town and extends to Woliya 6 Kebele. We observed the presence of sandflies in the burrows where hyraxes live. Sandflies primarily feed on reservoir hosts. They are poor flyers, usually fly quite low and will remain in the vicinity of their breeding ground. Hence, the probability of humans being bitten by sandflies is considerably affected by the distance of the reservoir host (or its dwelling place) from their house (Al-Jawabreh *et al*, 2003).

A univariate analysis of epidemiological data showed that the presence of *Adathada shimperina* 'Sensel' in Amharic and 'Tumuga' in Silte and *Acacia spp*. in the compound are significantly associated with CL. Sandflies, as they are nocturnal, may use *Acacia* and *Adathada shimperina* for resting during the daytime and as a source (Bucheton *et al.,* 2002) of nutrition. Moreover, these plants may allow some flowering forages that could be used as a source of nutrition for sandflies (Daba, *et al.,* 2002). Humans come into contact with the sandflies because of the proximity of these plants to homes and backyards. In multivariate analysis and stepwise-added conditional logistic regression analysis, *Adathada shimperina* appeared in the model and it is highly associated with the presence of CL in the area. Hence, detailed investigation is required to better understand the association between the two

The presence of domestic animals, mostly of cows, in the households or compounds was associated with an increasing prevalence for CL (table 7). The presence of cows probably has a positive effect on the density of sandflies around houses, as cow dung provides a rich environment for the development of sandfly pre-imaginal stages, drawing the vectors into closer association with the humans and increasing the risk of their being bitten. Cows may also act as a bait-factor attracting sandflies. Positive correlation of disease and the presence

of domestic animals have been shown in some studies (Southgate, 1962; Bucheton *et al.*, 2002). However, the nature of the relationship between disease transmission and domestic animals is complex (Bern *et al.*, 2000).

The host factors assessment showed that educational status of the head of the household was significantly related to the prevalence of CL. The univariate analysis indicated that when the head of the household is literate the probability of the presence CL in the family is low. However, according to our finding it is not possibly direct, but involves a number of related confounders that have to be considered; better economic strength leading to a cleaner yard, better housing, etc (Al-Jawabreh *et al*, 2003).

In univariate analysis, having bed nets is associated with lower probability of acquiring CL and this presumably, as they are less exposed to the sandfly vectors during its active hours. The effect is probably because they are possibly killed due to the impregnation of the bed nets.

The diagnosis of leishmaniasis requires the identification of the strain (species), especially in the muco-cutaneous forms of the disease, whose clinical characteristics alone do not allow clinicians to establish prognosis. The morphologies of various *Leishmania* species in different approaches of microscopic examinations such as histology and stained smears are very similar and the methods cannot be used to differentiate the various species.

In this study, we used ITS-1- PCR-RFLP to identify the circulating species of *Leishmania* in the study area. The ITS-1- PCR-RFLP results showed that all our clinical isolates belong to *L. aethiopica* indicating that the causative agent in Silti is *L. aethiopica*. Previous studies in different parts of the country reported that *L. aethiopica* is the principal causative agent of CL and our current findings are in agreement with these previous reports (Ashford *et al*., 1973; Gebre-Michael *et al.*, 2004; Hailu *et al*., 2006 and Gadisa *et al*., 2007). This establishes however that *L. aethiopica* is possibly breaking out in new foci and spreading, establishing new endemic sites.

As to the treatment response, many of the commonly used drugs are less effective against *L. aethiopica (*WHO, 1990). Localized cutaneous leishmaniasis is usally unresponsive to

systemic treatment with antimonials at conventional doses (WHO, 1990). Pentamidine is effective for treatment of LCL infection but its high toxicity makes it unacceptable for routine uses (Bryceson, 1970). Studies have been conducted to identify the best drug against LCL from safety and economic points of view. Itraconazole was tried for the treatment of *L. aethiopica* on DCL and LCL patients (Akuffo, 1990) but it was not better than placebo tablets.

Although treatment of *L. aethiopica* is being done with various drugs, the efficacy of antileishmanial drugs is controversial. Physicians may be confused when selecting an appropriate treatment option for *L. aethiopica*. Pentostam, the most universally used drug for treatment of leishmaniasis, is not as effective against *L. aethiopica* (WHO, 1990) as in infections with other species. On the other hand, data on the response of *L. aethiopica* to treatment with pentostam is scarce, in spite of the fact that the drug has been used in the country for years. Treatment of *L. aethiopica* with liquid nitrogen is common in some places such as ALERT. However, its efficacy has not been properly documented.

In the present study, while we were treating patients, we tried to assess and document the efficacy of pentostam and external application of liquid nitrogen and generated preliminary data, which will be valuable for further studies. In our preliminary study, we found that the clinical efficacy of liquid nitrogen and pentostam was 93.3 % and 89.5% respectively. Similar results (85.4 %) were obtained on treatment of *L. tropica* with pentostam in Saudi Arabia (Allhawja *et al*., 1997) and 84 % in Syria on *L. major* (Ghosh. 1979) although the designs and methods adopted are quite different as in our cases it is a follow up activity not a clinical trail.

Although for multiple and old lesions, treatment with liquid nitrogen takes a relatively longer time than for single and young lesions, both types responded to cryotherapy. Hence, liquid nitrogen can be used as one of the potential treatment options in the future in such cases for developing countries especially where there is shortage or total lack of drugs. Where there is risk of failure or toxicity, external application of liquid nitrogen may be a practical and safer alternative depending on the size, site and clinical features of the lesion.

6. Conclusions and recommendations

We established that Silti is a newly identified endemic focus of CL due to *L. aethiopica* which appeared in the last three years and is associated with presence of hyraxes along the banks of a stream bisecting the Kibet Town where the highest proportion of residents is affected. It appears that certain plants favor transmission of CL and that cow dung favors its spread.

The disease is new to the area and has recently introduced. Factors favoring transmission should be investigated further.

Pentavalent antimonials could be used as the drug of choice for treatment of lesions involving multiple and disseminated lesions of *L. aethiopica*. For simple lesions, which are few in number and with short duration, cryotherapy is effective. Moreover, the application of cryotherapy is simple, cost effective (compared to pentostam) and safe. However, the clinical service in the affected area, Silti, needs to be prepared to provide the required care and treatment of patients who will keep coming from the area.

The population is not aware of the disease and needs to be educated to undertake control measures, such as environmental conservation to minimize the formation of small gorges due to soil erosion that serve as a home of hyraxes, reducing the sandfly population through environmental measures, use of impregnated bed nets, cleaning the backyards, avoiding house construction along the banks of a stream. Minimizing plants such as *Acacia* and *Adathoda shimperina* around house as these points are likely related to sandfly habitat, whereby the sandflies seek out a favorable habitat in that would be supportive of egg laying and survival of immature forms.

The domestic animals are so important for the community and not so directly related as causative, the proposed measures should be formulated in a more practical way such as removing cow dung and drying it up further away from homes, having separate house for cows, composting, etc.

Thus, the socioeconomic and environmental characteristics identified as risk factors of CL in this study could help implementation of control strategies. Proper implementation of existing health awareness programs could help people in Silti areas modify their behavioral patterns by keeping their domestic animals in separate house and minimizing vegetation around houses that supports sandflies. The main emphasis on targeting intervention would be enhancing community awareness in areas exposed to the risk of CL like Silti through dissemination of appropriate information.

Regional governments and local non-governmental organizations should be actively involved in developing awareness programs. Our findings have important practical implications because they suggest that these measures, along with appropriate vector control and improved treatment facilities in the areas, may be particularly effective in reducing the incidence of CL and its transmission of infection by sandfly vectors. Finally, a prospective intervention study should be needed to evaluate the effectiveness of targeting control interventions in the high-risk areas identified by this study.

7. significant contributions of the study

1. The magnitude of cutaneous leishmaniasis in the study area is described and confirmed that it is recently introduced. This should help the zonal and woreda health bureaus to plan their activities and human resources to take actions to minimize the disease burden.

2. Some major potential determinants of the disease have been identified and this will help in planning and executing control programs.

3. The prevailing *Leishmania* species in the area is identified which will help to break the transmission cycle and to choose appropriate treatment options.

4. The study confirms the usefulness of molecular epidemiology approaches using descriptive epidemiology (distribution), analytical epidemiology (risk factor analysis) and molecular biology (species identification) for a complete description of the disease.

5. Baseline data on observations on the use of external application of liquid nitrogen and administration of pentostam in field conditions and success rate with the individual methods are available. These data will help to initiate further studies and to come up with safe, simple and cost effective *L. aethiopica* treatment options in the future.

8. Limitations of the study

1. The study area coverage is restricted to Silti woreda and it does not represent the whole zone (Silte Zone).

2. The treatment response of cryotherapy and pentostam presented in this study are not designed for comparison and it is not a clinical trial. We presented the treatment response of cryotherapy and pentostam as preliminary information.
3. The origins of the outbreak have not been investigated and the possible source of the new focus described.

4. The relationship of this new focus to existing foci in the area has not been mapped and further risk areas identified.

5. The transmission dynamics of the diseases such as the reservoir hosts (animals, rodents etc.), the vectors (the species of sandflies, infected sandflies with *L. aethiopica*) and environmental change are not covered in this study.

9. Future directions

1. Conduct controlled case study to determine the importance of the identified risk factors for the transmission of CL in the study area.

2. Conduct study to determine the transmission dynamics of leishmaniasis in Silti and surrounding areas.

3. Conduct an intervention study in Silti to control the disease and learn from the experience.

4. Detailed study on the response of *L. aethiopica* to cryotheraputic and pentostam treatment.

5. Conducting epidemiological studies in wider coverage in different areas to map the distribution of the disease in Ethiopia, including molecular studies.

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www.biosci.ohio-state.edu/~parasite/lifecycles/leishmania_lifecycle.html

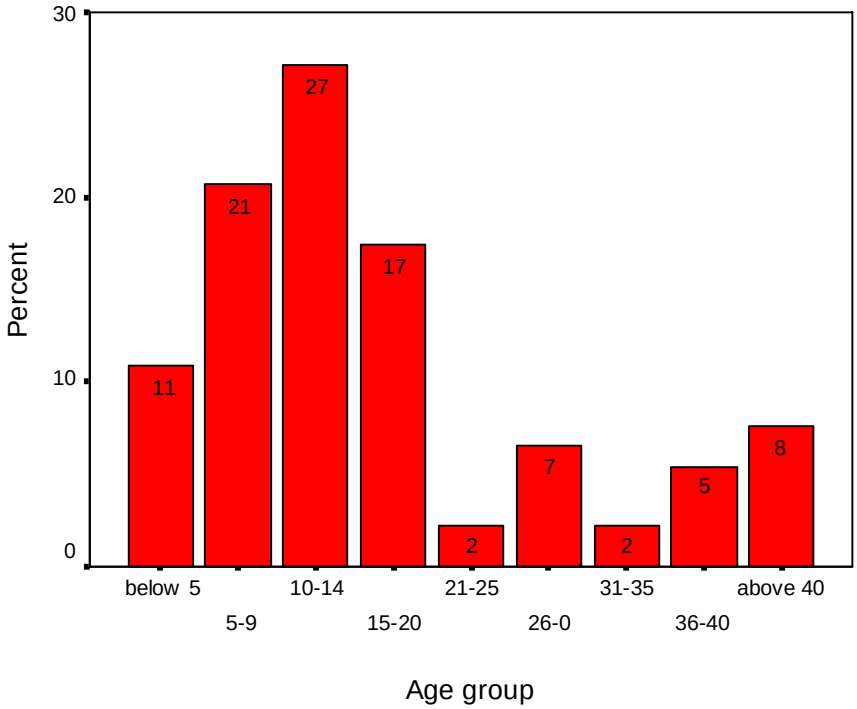
Appendix 1: Socioeconomic and demographic characteristics of the study population

Table 12: Socioeconomic and demographic characteristics of study population in Silti Woreda in 2006

Variables	***Male (%)***	***Female (%)***	Total (%)
Education (N=248) *			
Illiterate	43.5	26.6	70.2
Basic	6.0	2.8	8.9
Primary	9.3	6.9	16.1
Secondary	2.4	1.2	3.6
College or above	1.2	0	1.2
Total	**62.5**	**37.5**	**100**
Occupation (N=273) *			
Farming	50	22.7	72.7
Trading	1.9	6.9	8.8
Keeping cattle	0.4	0.4	0.8
Salary	3.7	2.6	6.3
Manual work	2.2	5.9	8.1
Wood sell	0.4	0	0.4
others	1.8	1.1	2.9
Total	60.4	36.9	100
Ethnicity (n=1907) **			
Silte	48.9	46.9	95.8
Guraghe	0.9	0.9	1.8
Hadiya	0	0.1	0.1
Walayita	0	0.1	0.1
Oromo	0.7	0.8	1.5
Amhara	0.3	0.4	0.7
Total	**50.8**	**49.2**	**100**

* computed for household head ** computed for individuals

A



B

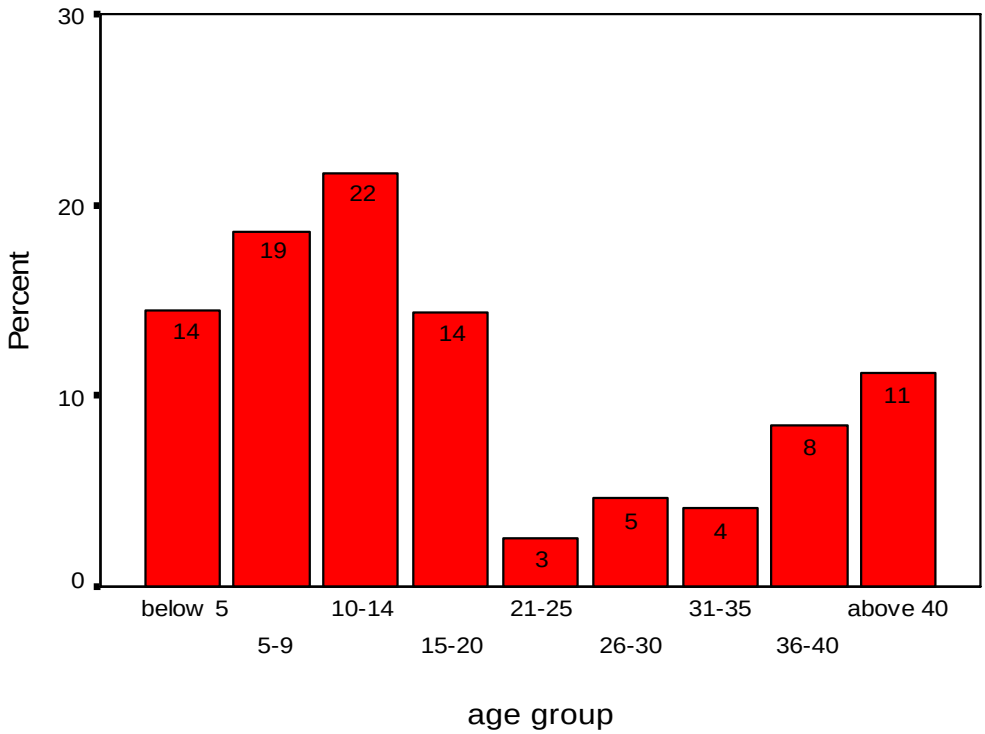


Figure 18: age distribution of (A) study population (B) CL cases

Appendix 2. Information Sheet

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dÃ ንe óክM+ ¾T e} ` e } T ] ' ክõ } ኛ (c=Â`) } S ^ T] ች ከአl] ና ከአÇ=e አuv
¿ ነ? c= l ክ=U ና ó" M+ • ና ክeMÖ?¨ [Ç Hክ=V ችን ነ¬ ::

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Ø ናǦ ¾ ²ÒË ¾S ÖÃp pê S S ቴǦ ÁeðMÒK<:

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Appendix 3. House and house index hold questionnaires

A

Molecular Epidemiology of Cutaneous Leishmaniasis in Silti Woreda, Ethiopia

Armauer Hansen Research Institute (AHRI)

House hold code ☐☐☐

Participant Code ☐☐☐☐

A. Questionnaires to be filled for the <u>head of house holds only</u>

<u>Part I: Basic data</u>

1.Household head name _____

Sex 1= Male ☐ 2= Female ☐ ; age in years ☐☐

I do not know ☐

2.1. Woreda **Silti** Kebele: 1= Woliya 6 ☐ 2= Qibet ☐ 3= Boze ☐

2.2. House number _____

3. Educational status, 1=Illiterate ☐ 2=basic education ☐

3= Primary school ☐ 4= Secondary school ☐

5= College ☐

<u>Part II. Geographical data</u>

4. Geographical coordinates

4.1. Latitude ☐☐ ^{0} (degree) ☐☐ minute (‘)

4.2. Longitude ☐☐ ^{0} (degree) ☐☐ minute (‘)

4.3. Altitude ☐☐☐☐ m. a. s. l

Part III. Socioeconomic Data

5. Family size (number) ☐☐

6. Income source

1= farming	1= yes	☐	2= no	☐
2= trading	1= yes	☐	2= no	☐
3 = keeping cattle	1= yes	☐	2= no	☐
4= salary	1= yes	☐	2= no	☐
5= wood selling	1 = yes	☐	2= no	☐
6= manual work	1 = yes	☐	2= no	☐
7= other	1 = yes	☐	2= no	☐

If other, specify _____

7. If your income source is farming, the location of the farmland is

1= near home ☐

2= near the gorge (Kerati) ☐

3 = both ☐

8. Early morning or night time spending near/at Gorge 1 = yes ☐ 2= no ☐

9. If Q.7 is yes, how long do you spend near/at gorge /kerati ☐☐☐☐

minutes

10. If Q. 8 is yes, why do you spend time near or at gorge/ kerati?

_____

_____

_____

11. Housing condition

11.1. Type of roof 1=. Corrugated iron ☐ 2= Straw ☐

11.2. Type of floor 1= cemented ☐

2= pasted with cow dung/mud ☐

3= covered with straw ☐

4= other ☐

If other, specify _____

11.3. Distance from gorge/ kerati (approximate) ☐☐☐☐ meters

11.4. Gates /windows

1= directly facing (opened) to the gorge ☐

2= opposite to the gorge ☐ 3. No window ☐

11.5. Condition of the wall (filled by the interviewer observation)

1. Cracked ☐ 3. No Crack or holes ☐
2. Holes formed ☐

11.6. If cracked**,** 1= almost all walls ☐

2= some of the walls ☐

3. Only few areas ☐

12. Animals

12.1. Domestic animals kept near/in house 1= yes ☐ 2= no ☐

12.2. Pet animals kept near/in house 1= yes ☐ 2= no ☐

13. Habits

13.1 Pit near house 1= yes ☐ 2= no ☐

13.2. Sitting in the home yard in the evenings 1= yes ☐ 2= no ☐

13.3. If question 13.2.is yes, how often?

1= always ☐ 3= sometimes ☐

2= frequently ☐ 4= rarely ☐

13.4. Sleeping in the home yard 1= yes ☐ 2= no ☐

13.5. Using impregnated bed nets 1= yes ☐ 2= no ☐

13.6 If 13.5 yes, how is the distribution? _____

13.7. If yes (Q.13.5) the source is 1=donation ☐ 2= purchase ☐

13.8. If donation, name of the donor ----------

13.9. Do you sleep your face/hands covered 1= yes ☐ 2= no ☐

13.10. Early morning working in the garden 1= yes ☐ 2= no ☐

14. Area around the house

15.1. Farm present within 300 meters radius (approximate) 1= yes ☐ 2= no ☐

14.2. Damping animal dung near house 1= yes ☐ 2= no ☐

14.3. Presence of animal burrow around house (approximately within 300mtrs.radius)

1= yes ☐ 2= no ☐

14.4. Waste disposal around house 1 = open ☐ 2= closed ☐

14.5. Latrine 1= present ☐ 2= absent ☐

14.6. If Q.14.5 is present, the condition is (filled by interviewer observation)

1= good ☐ 2= Fair ☐ 3= bad ☐

14.7. Types of trees /bushes in the garden/ compound (list the most common once only)

1. _____
2_____
3_____
4 _____
5_____

Part IV Indigenous knowledge of the respondent and general information

15. Can you identify cutaneous leishmaniasis lesion from other lesions?

1= Yes ☐ 2=No ☐

16. If Q.15 is yes, how do you identify CL lesions from other lesions? _____

_____

☐ ☐

17. Do you know the cause for CL lesion? 1= Yes 2=No

18. If Q. 17 yes, what is the source of your information?

1= from school ☐

2= media (Magazine, Radio, TV etc...) ☐

3. from health center and health Education ☐

4. Other ☐

If other, specify _____

19. If, Q. 17 is yes, what do you think the cause for CL? _____

20. Is there any traditional treatment for this disease? 1= Yes ☐ 2=No ☐

2= I do not know ☐

21. If Q.20 is yes, how much does it cost in Birr per person? ☐☐

22. When was the first case of such type of lesion (CL) seen in your family/ neighbor?

☐☐☐☐ Months

23. Does the diseases have local name? 1= Yes ☐ 2=No ☐

24. If Q.23 is yes, the local name for the diseases is _____

25. How is the condition of the forest around the area changing?

1= high degree deforestation ☐

2= medium degree deforestation ☐

3=minimum degree deforestation ☐

4= no deforestation ☐

26. Have you seen hyraxes living near your house? 1= Yes ☐ 2=No ☐

27. If Q.26 is yes, when did you see hyrax for first time in this area? ☐☐☐

Months

28. Do you think that the population of hyrax is increasing in your area?

1= Yes ☐ 2=No ☐

29. Do you think that hyrax moved to the residence area? 1= Yes ☐ 2=No ☐

30. If there are hyraxes near your house, do they come to your compound?

1= Yes ☐ 2=No ☐

31. What do you think is/are the best way(s) to control this disease?

_____

_____

~~Comments for the enumerator~~

_____

_____

_____

Filled by _____ Signature _____ Date _____/_____/____

Checked by _____ Signature _____Date:____/_____/_____

B

Molecular Epidemiology of Cutaneous Leishmaniasis in Silti Woreda, Ethiopia

Armauer Hansen Research Institute (AHRI)

House hold code ☐☐☐

Participant Code ☐☐☐☐

B. Questionnaires to be filled for <u>each of the household members</u>

<u>Part I: Basic Data</u>

1. Name _____

Sex 1= Male ☐ 2= Female ☐ ; age in years ☐☐

2.1 Woreda **<u>Silti</u>** Kebele: 1= Woliya 6 ☐ 2= Qibet ☐ 3= Boze ☐

2.1. House number----------

3. Ethnicity 1= Silti ☐ 5. Kambata ☐

2= Gurage ☐ 6. Oromo ☐

3= Hadiya ☐ 7. Amhara ☐

4= Walayita ☐ 8. Others ☐

If other, specify _____

4. Educational status, 1=Illiterate ☐ 2=basic education ☐

3=Primary school ☐ 4=Secondary school ☐

5. College/above ☐ 6= under school age ☐

Part II Diseases assessment (to be filled based on health professional observation)

4. Lesion (Cutaneous leishmaniasis) 1= present ☐ 2= absent ☐

5. Number of lesion

1=one ☐ 2=two ☐ 3 = three or more ☐

6. Duration of lesion/s since first appeared ☐☐☐ months

7. Location of lesion

7.1. Cheek 1= yes ☐ 2= no ☐

7.2. If yes, 1= left cheek ☐ 2= right cheek ☐ 3= both ☐

7.3. Ear 1= yes ☐ 2= no ☐

7.4. If yes, 1= left ear ☐ 2= right ear ☐ 3= both ☐

7. 5. Lip 1= yes ☐ 2= no ☐

7.6. If Yes, 1= lower lip ☐ 2= upper lip ☐ 3= both ☐

7. 7. Leg 1= yes ☐ 2= no ☐

7.8. If yes, 1= lower limp ☐ 2= upper limp ☐ 3= both ☐

7.9. Hand 1= yes ☐ 2= no ☐

7.10 If yes, 1= lower limp ☐ 2= upper limp ☐ 3= both ☐

7.11. Chin 1= yes ☐ 2= no ☐

7.12. Nose 1= yes ☐ 2= no ☐

7.13. Neck 1= yes ☐ 2= no ☐

7.14. Fore head 1= yes ☐ 2= no ☐

7.15. Other ☐

If other specify_____

8. Suspected leishmaniasis

1= CL ☐ 2= MCL ☐ 3= DCL ☐

9. Scars suggestive of healed leishmaniasis 1= present ☐ 2= absent ☐

10. If cutaneous scar/s present, the location is

10.1. Cheek 1= yes ☐ 2= no ☐

10.2. If yes, 1= left cheek ☐ 2= right cheek ☐ 3= both ☐

10.3. Ear 1= yes ☐ 2= no ☐

10.4. If yes, 1= left ear ☐ 2= right ear ☐ 3= both ☐

10.5. Lip 1= yes ☐ 2= no ☐

10.6. If Yes, 1= lower lip ☐ 2= upper lip ☐ 3= both ☐

10.7. Leg 1= yes ☐ 2= no ☐

10.8. If yes, 1= lower limp ☐ 2= upper limp ☐ 3= both ☐

10.9. Hand 1= yes ☐ 2= no ☐

10.10 If yes, 1= lower limp ☐ 2= upper limp ☐ 3= both ☐

10.11. Chin 1= yes ☐ 2= no ☐

10.12. Nose 1= yes ☐ 2= no ☐

10.13. Neck 1= yes ☐ 2= no ☐

10.14. Fore head 1= yes ☐ 2= no ☐

10.15. Other ☐

If other specify_____

11. Have you been treated for CL? 1= yes ☐ 2=no ☐

12. If yes, where were you treated?

1= Traditional healer's house ☐ 2= at health center/hospital ☐

13. If Q. 12 is yes, how much does the treatment cost in birr per person ☐☐☐

14. Have you faced any lesion that has self-healed? 1= yes ☐ 2=no ☐

15. If Q.14 is yes, was the lesion painful/ itchy? 1= yes ☐ 2=No ☐

16. If Q.14 is yes, was the cause for the lesion physical damage?

1= Yes ☐ 2=No ☐

17. Knowledge of the diseases and suggestion for control (<u>For respondents above 17 years old only)</u>

17.1. Can you identify cutaneous leishmaniasis lesion from other lesions?

1= yes ☐ 2=no ☐

17.2. Do you know the cause of CL lesion? 1= yes ☐ 2=no ☐

17.3. If Q.17.2 is yes, what do you think the cause for CL?

_____

17.4. If Q. 17.2. Yes, what is the source of your information?

1= from school ☐

2= media (Magazine, Radio, TV etc...) ☐

3. from health center and health Education ☐

4. other ☐

If other, specify _____

17.5. When did these diseases first appeared in this area? ☐☐☐ Months

17.6. Does the diseases have local name?

1= yes ☐ 2=no ☐

17.7. If Q.17.6 is yes, what is the local name for the disease? _____

17.8. Is there any mechanism to avoid the diseases?

1= yes ☐ 2=no ☐

17.9. What do you think is/are the best way(s) to prevent this disease?

_____

_____

Comments for the enumerator

_____

_____

_____

_____

Filled by _____ Signature _____ Date _____/_____/____

Checked by _____ Signature _____Date:____/_____/_____

Armauer Hansen Research Institute (AHRI)

Molecular Epidemiology of Cutaneous Leishamaniasis in Silti Woreda, Ethiopia

Patient Code ☐☐☐☐☐ **AHRI Code** ☐☐☐☐ ☐☐

Questionnaires to be filled for the patients (For Clinico-Pathological use only)

Part I: Basic Data

1. Name_____
2. Woreda_**Silti**_ Kebele: 1= Woliya 6 ☐ 2= Qibet ☐ 3= Boze ☐
3. Village (if any) _____
4. Duration of stay in the area (Year) ☐☐
3. Age (year) ☐☐
4. Sex 1= male ☐ 2= female ☐
5. Educational status 1= illiterate ☐ 2= primary school ☐
   3= Secondary school ☐ 4= tertiary school ☐
   5. Under age ☐
6. Marital status 1= Married ☐ 2= single ☐ 3= divorced ☐
   4. Separated ☐ 5= widowed ☐ 6. Under age ☐

Part II. Clinical data

7. Lesion 1= yes ☐ 2=no ☐
8. If yes, 1=single type ☐ 2=Multiple type ☐
9. If Q.7 is yes, duration since lesion occurred (month/s) ☐☐
10a. Site of lesion 1= nose ☐ 2= ear ☐

3= lip ☐ 4= forehead ☐

5. Chin ☐ 6= upper and lower limp ☐

7. Cheek ☐ 8. Other ☐

If, other specify _____ _____

10b. If lip, it is : 1= on the lower lip ☐ 2 = on the upper lip ☐ 3= both ☐

10c. number of lesion 1=one ☐ 2= two ☐ 3 ≥ three

10d. type of lesion ---------- ☐

---

11. Suspected clinical form 1= CL ☐ 2= MCL ☐ 3= DCL ☐

12. Cutaneous scars 1= present ☐ 2 = absent ☐

13a. If scars present, the location is: 1= nose ☐ 2= ear ☐

3= cheek ☐ 4= forehead ☐

5= chin ☐ 6. Other ☐

If, other specify_____.

13b. If lip, 1= on the lower lip ☐ 2 = on the upper lip ☐ 3= both ☐

14. Specimens submitted for examination

a. Culture (skin scrapings) 1=yes ☐ 2 = no ☐

b. Biopsy 1=yes ☐ 2 = no ☐

15. Travel history 1=yes ☐ 2 = no ☐

16. If Q. 15 is yes, period of on set of lesion is 1= before travel ☐ 2= after travel ☐

17. If lesion on set is after return, this happened after what period following travel?

1= less than 1 month ☐ 2= 1- 3 months ☐ 3= 4-6 months ☐

4= after 6 months ☐

18. Have you taken any treatment for Cutaneous L.? 1= yes ☐ 2= no ☐

19. If yes, how long ago (years)? ☐☐

Part III. Socioeconomic Data (Optional)

20 .Family size (Number) ☐☐

21. Income source 1= agriculture ☐ 2= trade ☐

3= civil servant ☐ 4= other ☐

If, other specify_____

22. If your income source is agriculture, the location of the farmland is

1= near home ☐ 2= near forest ☐ 3= both ☐

23a. Early morning or night travel to forest area / caves 1= yes ☐ 2= no ☐

23b. If yes, why do you travel to forest/caves?

_____

24. Is there any cave or gorge near your house? 1 = yes ☐ 2= no ☐

25. If yes, How far the cave/gorge from your house? (Meters) ☐☐☐☐

26. Are there hyraxes near you house? 1= yes ☐ 2= No ☐

Checked by _____ signature _____date_____

Appendix 4. Declaration

I the undersigned declare that this thesis is my original work. It has not been presented for a degree in this or any university and all the source materials used for this thesis have been duly acknowledged.

Name of the candidate Edessa Negera

Signature ----------

Place Addis Ababa

Date -------/-------/----------

This thesis has been submitted for examination with my approval as university advisor.

Name of the advisor Prof. Asrat Hailu

Signature ----------

Place Addis Ababa

Date -------/-------/----------