SPECIES TYPING OF *LEISHMANIA* ISOLATES FROM CUTANEOUS LESIONS OF PATIENTS IN ETHIOPIA

BY

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"….consider how great things he has done for you."

"Samuel 12:24: From the Bible

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List of abbreviations

ALERT	All Africa Leprosy and Tuberculosis Research, Rehabilitation and Training Center
bp	Base pairs
cDNA	complementary DNA
CL	Cutaneous leishmaniasis
LCL	Localized cutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
GOT1(2)	Glutamate-Oxaloacetate Transaminase
G6PD	Glucose-6- Phosphate Dehydrogenase
GPI	Glucose Phosphate Isomerase
ITS	Intergenic transcribed spacer
kb	Kilo base
kDNA	kinetoplast-DNA
MCL	Mucocutaneous leishmaniasis
MPI	Mannose Phosphate Isomerase
MTT	Methylthiazol tetrazolium
NAD	Nicotin amide adenine dinucleotide
NADP	Nicotin amide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
NNN-medium	Novy-MacNeal-Nicolle medium

NP1(2)	Nucleoside purine phosphate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGD	Phosphogluconate dehydrogenase
PGM	Phosphogluco mutase
PMS	Phenazine methosulfate
RNA	Ribonucleic acid
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecylsulfate
ssu RNA	small subunit RNA
TAE buffer	Tris-Acetate-EDTA buffer
U	Unit
VL	Visceral leishmaniasis
WHO	World Health Organization

Abstract

The various species of *Leishmania* cannot be distinguished morphologically, but the treatment of leishmaniasis depends on the specific causative species. Thus accurate and rapid diagnosis of the specific species of *Leishmania* should be available for effective treatment of patients. Identification of *Leishmania* species in humans, in insect vectors and reservoir hosts should be available before planning for large-scale epidemiological survey, control programs and possible vaccine and/or drug trials.

Therefore, the aim of this work was to establish isoenzyme technique at AHRI as a gold standard for species identification and introduce PCR-methods for species-specific diagnosis of leishmaniasis directly from clinical materials. In this line, cultured parasites from 55 localized cutaneous leishmaniasis (LCL) and 3 diffused cutaneous leishmaniasis (DCL) cases), diagnosed at the All Africa Leprosy and Tuberculosis Research, Rehabilitation and Training Center (ALERT) and from Ochollo village were analyzed. Species typing with isoenzyme electrophoresis and PCR-based techniques showed that, in all the cases, *L. aethiopica* is the aetiologic agent. Moreover, there was no strain difference between those causing LCL and DCL. The comparison of the isoenzyme and the PCR-based species typing techniques confirmed that the intergenic transcribed spacer-1 polymerase chain reaction-restriction fragment length polymorphism technique alone could be used for species typing. The sensitivity and specificity of the technique shows its potential for species specific diagnosis of leishmaniasis from clinical samples avoiding the need for culture. Thus, effective species-specific treatment of patients, which is a requirement in leishmaniasis, could be effected in a timely manner.

Key words: *Leishmania aethiopica*, Isoenzyme, ITS1-PCR-RFLP, ALERT, Ochollo

1. Introduction

1.1. Leishmaniasis

1.1.1. Epidemiology

Human leishmaniasis is a complex disease with many clinical forms. The symptoms range from mild self-healing cutaneous lesions to fatal visceral disease. The distinct forms, of leishmaniasis, cutaneous (CL), visceral (VL), mucocutaneous (MCL) and diffused cutaneous (DCL) are dependent on the *Leishmania* species/strain involved and on the immune response established by the host. There are at least 21 species of *Leishmania* that are known parasites to man (Croan *et al*., 1997).

Globally, leishmaniasis prevails in 88 countries (82% of which are developing countries) in five continents (Fig. 2) (Davies *et al.*, 2003). Leishmaniasis affects 12 million people worldwide and another 350 million people are at risk of infection. About 1-1.5 million new cases of cutaneous (CL) and 500,000 of visceral (VL) leishmaniasis occur per year (Handman, 2001). Cutaneous forms represent 50 - 75% of all new cases. The disease is greatly under reported with only 600,000 officially declared cases annually. It is not even a reportable disease in most endemic countries (http://www.who.int/emc/diseases/ leish/leisdis1.html).

Leishmaniasis has been present in Ethiopia for a long time. The southeastern part of the country along the Ethio-Kenya and Ethio-Sudan border had suffered an epidemic of kala-

azar in 1938-1942 (Ethiop. Med. J. editorial review, 1965). CL was first described in Ethiopia by Martogilio in 1913 and DCL by Destobies, Schaller and Serie, in 1960 (Ashford *et al*., 1973). Broader epidemiological surveys by Ashford *et al*., (1973) established the endemicity of CL and were able to identify the vectors and reservoir hosts for *L. aethiopica*.

VL caused by *L. donovani* is prevalent in the low lands, (below 1500 m above sea level) of Ethiopia. The CL form, predominantly caused by *L. aethiopica,* is wide spread at altitudes between 1500 and 2700 m above sea level all over Ethiopia. Desjeux (1991) reported a case of CL due to *L. tropica* and two cases of CL due to *L. major*, implying that they may contribute to CL cases in Ethiopia. *L. aethiopica* was isolated from squirrels in a VL focus in southeast Ethiopia at an altitude of 1200 m above sea level (Abebe *et al*., 1990). Moreover, *L. aethiopica* and *L. tropica* were isolated from sandflies sympathetically occurring in the Awash valley at an altitude of 1010 m above sea level (Gebre-Michael *et al*., 2004).

A survey in the villages of Ochollo showed a prevalence of *L. aethiopica* induced LCL with active lesions ranging from 3 - 4% in the general population, with a peak prevalence of 8.5% in the 0-10 years age group. It was also shown that 22- 40% of the population had scars attributed to *Leishmania* (Desjeux, 1991 and Mengistu, 1992).

Ali *et al.*, (1995 and 2002) reported 33-40% leishmanin-positivity in middle Awash and the surrounding area. This could suggest that leishmaniasis may be present in this region as a focal disease as in Ochollo, Sabeta, Kutaber and Aleku (Ashford *et al*., 1973), but probably spread over a wide geographical area (Ali *et al*., 2002). However, the significance of skin reactivity as a proxy indicator of actual disease prevalence needs further evaluation.

Even though there is a clear correlation between the causative parasite species and the clinical presentation, the clinical manifestation may some times vary in individuals depending on the specific characteristics of species/strains and on the immunocompetence of the host. Species causing typically CL may visceralize and visceral species may show

dermatotropism. In many endemic areas of the world a few *Leishmania* species causing similar forms of disease are prevalent simultaneously so that a species-specific diagnosis cannot rely on clinical findings alone. Species-specific diagnosis is necessary for adequate treatment and correct interpretation of research findings, such as vaccine trials (Jacobson, 2003 and Belli *et al*., 1998).

The overlap in the distribution of the three CL causing *Leishmania* species (*L. aethiopica*, *L. tropica* and *L. major*) in Ethiopia highlight the need for a broader epidemiological study to determine their geographical distribution and the relative significance of the different species in human leishmaniasis.

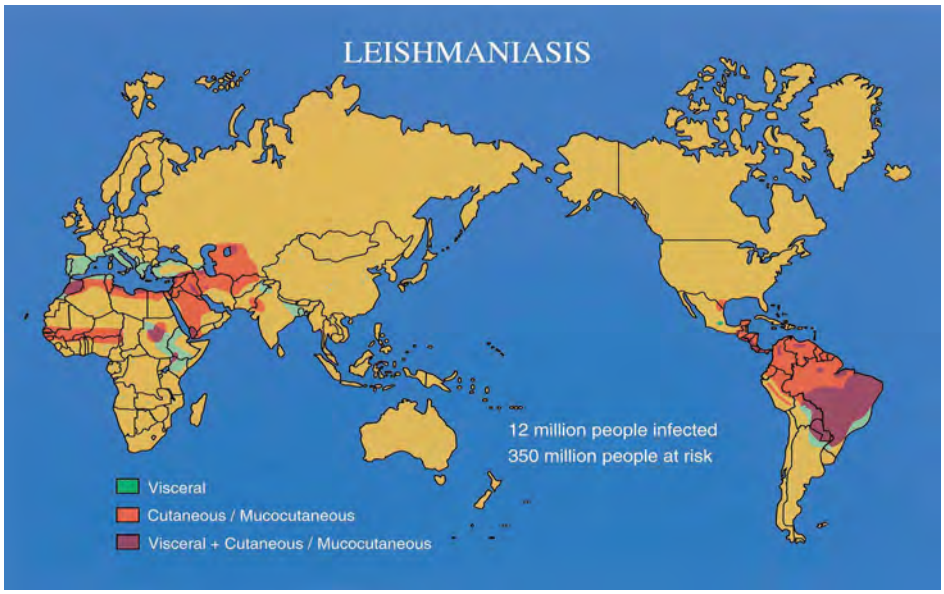


Figure 1: Global distribution of visceral & cutaneous leishmaniasis (From Handman, 2001)

1.2. Clinical forms of leishmaniasis

1.2.1. Cutaneous leishmaniasis (CL)

In CL, the parasite is confined to the skin. The disease manifests with sores or ulcers on exposed parts of the body such as the face, hand, forehead and lower limbs. Lesions start as an itchy erythematous papule at the site of the sandfly bite, after an incubation period of several weeks to several months. Lesions typically ulcerate with a raised inflammatory outline, and are usually painless unless a secondary bacterial infection is present. A slightly depressed and often hypo-pigmented scar remains. If left to self-cure, life-long immunity is usually acquired against the same species (Nimri *et al*., 2002). CL in the Old World is due to *L. major, L. tropica* and *L. aethiopica*. CL due to *L. tropica* is usually more chronic. The severe form recidivance leishmaniasis is very difficult to treat. In the New World, *L. mexicana* usually produces relatively mild self-limiting lesions, but if the ear is infected (Chiclero's ulcer), it may destruct the cartilage and result in the disfigurement of the ear (Desjeux, 1996 and WHO, 1990). Moreover, unusual CL cases caused by *L. infantum* and *L. chagasi* were also reported by Ponce *et al*., (1991).

1.2.2. Diffused cutaneous leishmaniasis (DCL)

DCL is a chronic form of CL. It is characterized by non-ulcerating nodules over the entire body. It represents a condition of anergy with a failure of cell-mediated immune response (negative Leishmanin test) and an abundance of amastigotes. It tends to be refractory to treatment. In the Old World, it is associated with *L. aethiopica* infection that is mostly seen in Ethiopia. Clinically, it resembles lepromatous leprosy, which often led to misdiagnosis in the past**.** As a result, DCL patients were often kept in leprosaria by mistake (Prince and Fitzherbert, 1965)**.** In the New World, DCL is caused mainly by *L. amazonensis*. DCL is also reported to occur in immunodeficient patients with no species-specific relation (Paredes *et al*., 2003**).**

1.2.3. Mucocutaneous leishmaniasis (MCL)

Also called “Espundia”, MCL produces lesions that can lead to extensive and disfiguring destruction of the mucosa and the cartilage of the nose and pharynx, leading to a severe mutilation of the face. In the New World, it is due to *L. amazonensis*, *L. braziliensis,*

L. panamensis and *L. guyanensis.* In the Old World, it is due to *L. aethiopica.* MCL due to *L. donovani, L. major and L. infantum* have also been reported in immunosuppresed HIV patients (Desjeux, 1996 and Paredes *et al*., 2003).

1.2.4. Visceral leishmaniasis (VL)

VL, also called Kala-azar, is typically caused by *L. donovani* species complex that include *L. donovani* and *L. infantum* in the Old World and *L. chagasi* in the New World. The parasite invades the internal organs such as spleen, liver and bone marrow. VL is characterized by irregular fever, weight loss, hepato-splenomegaly and anemia. It causes large-scale epidemics with high case fatality rates. If left untreated, it has a mortality rate approaching 100%. After recovery, patients may develop chronic post kala-azar dermal leishmaniasis, which usually requires long and extensive treatment (Desjeux, 1996). Rarely, VL can also be caused by *L. tropica* and *L. mexicana* (Magill, *et al*., 1993). In India, *L. tropica* has been isolated from 4 patients suffering from classical kala-azar (Sacks *et a*l., 1995). Visceral infections caused by *L. tropica* have also been reported from veterans of Operation Desert Storm **(**Kreutzer *et. al***.,** 1993).

2. The *Leishmania* parasite.

2.1. Life cycle

The insect vectors and mammals alternatively host *Leishmania* parasites. When a female sandfly takes a blood meal from a *Leishmania* infected mammal, the insect ingests intracellular amastigotes. A blood meal is necessary for the maturation of its eggs. Inside the blood meal amastigotes transform into motile promastigotes. The promastigotes multiply intensively inside the intestine of the sandfly, mainly in the midgut**:** suprapyloric

development of subgenus *Leishmania* or in the hindgut and midgut: peripyloric development of the subgenus *Viannia* (Lainson and Shaw, 1987). Then the parasites migrate to the anterior part of the sandfly gut where they change into free-swimming metacyclic promastigotes; the stage infective to vertebrate hosts. During another blood meal the metacyclic promastigotes are inoculated into a mammalian host. Once inoculated by the sandfly into the vertebrate host, the parasites are engulfed by macrophages and multiply intracellularly by binary fission. The infected cells finally rupture, and the liberated protozoa invade other macrophages or are picked up by vectors from peripheral blood (Sacks and Perkins, 1985). The disease then is disseminated locally or systemically (Fig. 2).

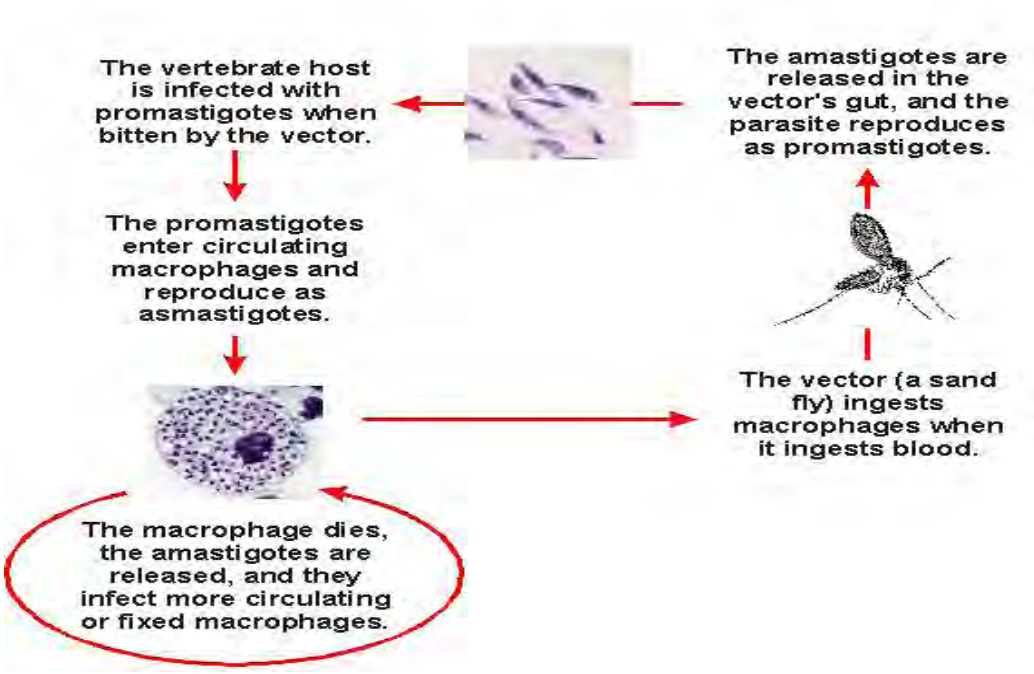


Figure 2: Life cycle of *Leishmania* parasite (From, http://www.biosci.ohio-state.edu/~parasite/copyright.html)

2.2. Vectors and reservoir hosts

The sandflies that are vectors of *Leishmania* parasite are the *phlebotominae* subfamily: the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. The Phlebotomine sandflies are nocturnal with limited migratory habits and as a result leishmaniasis is normally a focalized disease. In tropical and sub-tropical parts of the world, sandflies are active the entire year with small variation in population density (Daba *et al*., 2002).

In Ethiopia, different species of *Phlebotomus* (Table 1) are vectors of *Leishmania* parasites confirmed by demonstration from sandfly gut infection.

Table 1: The vectors of the four *Leishmania* species that are prevalent in Ethiopia.

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

Most *Leishmania* are zoonotic, with mammals such as rodents, canids and hyraxes being the major reservoir hosts in the Old World. Human beings are incidental hosts of infection. In the anthroponotic form, for example CL due to *L. tropica* in North Africa, Middle East and Mediterranean region and VL due to *L. donovani* in India, humans are the sole reservoir hosts (WHO, 1998 and Jacobson, 2003).

In Ethiopia, the reservoir hosts of *L. aethiopica* are the rock hyraxes: *Procarvia habessinica* and *Heterohyrax brucei*. The rodent, *Arvicanthis niloticus*, found infected with *L. major* in the North West and South Western parts of Ethiopia, is the possible reservoir host for *L. major* in the region (Ashford *et al*., 1973 and Desjeux, 1991).

2.3. Molecular biology and the *Leishmania* Genome

As with all the kinetoplastids, *Leishmania* have kinetoplastic and nuclear genomes. A study with pulsed field gel electrophoresis showed that *Leishmania* chromosomes are mainly diploid (Lovannisci *et al*., 1984). Winker *et al* (1996) managed to characterize 36 chromosomes in *L. infantum*, *L. major*, *L. tropica* and *L. aethiopica.* The chromosomes are linear ranging in size from 0.35-3 Mb. Chromosome size variability is characteristic of some *Leishmania* species even between homologous chromosomes (Blaineau *et al*., 1991), which complicates the use of karyotype in taxonomic studies.

The kinetoplastic DNA (kDNA) accounts for 10-20% of the total DNA (Simpson, 1987). It is a network of concatenated circular DNA divided into two classes. The homogenous maxicircles, approximately 25-50 molecules of 20 kb and the heterogeneous minicircles with many copies (~ 10^4) is approximately 0.8 kb. The minicircles vary according to the particular species, but all share conserved 120 bp sequences (Smyth *et al*., 1992).

The *Leishmania* genome contains 30% repetitive sequences (Ivans and Blackwell, 1999). The genes are often found to be organized in tandem arrays or at least two or more copies spread through the genome (Brad, 1989). For example the cystein protease genes are multi-gene families arranged in a single copy or arranged in tandem array. The multi-gene family nature of this gene and the presence of divergence in the CTE terminal of Cys1 of *L. aethiopica*, *L. tropica*, *L. major* and *L. donovani* strains were identified as potential target for molecular diagnosis (Kuru, 2004).

Presence of repetitive sequences and variations in the minicircle formed the bases for DNA based molecular typing techniques. This provides an alternative to alleviate the limitation of identifying the species of *Leishmania* on the bases of morphology. Moreover, due to

their molecular specificity, detection and genetic characterization of *Leishmania* can be accomplished simultaneously (Simpson, 1987 and Smyth *et al*., 1992).

2.4. Classification

Leishmania are protozoa belonging to the order Kinetoplastida, family Trypanosomatidae and genus *Leishmania*. The genus *Leishmania* is subdivided into two sub-genera based on the pattern of development in the sandfly gut: *Leishmania* (Suprapylorian), the development of which is restricted to the anterior portion of the alimentary tract of the sandfly vector and *Viannia* (Peripylorian) which develop in the mid and hind gut of the alimentary tract of the sandfly vector (Lainson and Shaw, 1987), a classification supported by many biochemical and molecular studies (Rioux, 1990, Croan *et al*., 1997 and Cupolillo *et al*., 1998). Various species complexes were individualized within these two sub-genera (Rioux *et al*., 1990).

More recently, Cupolillo *et al*. (2000) proposed the separation of the genus *Leishmania* into two sections: Euleishmania and Paraleishmania. The Euleishmania comprises of the sub-genera *Leishmania* and *Viannia* and the Paraleishmania consists of *L. hertigi, L. herreri, L. deanei, L. colombiensis, L. equatorensis* as well as strains of Endotrypanum. The Paraleishmania is further sub-divided into two, one group formed by parasites of histrycomorph rodents (porcupines) namely *L. hertigi and L. deanei* and the other group formed by the remaining species, which are principally parasites of the Sloth. Strains of Endotrypanum form a polyphyletic group within the paraleishmania.

All *Leishmania* species are morphologically similar, except for minor differences in size. They are dimorphic with the intracellular amastigote form (3-5 mm in diameter), residing within the mononuclear phagocytic cells of the mammalian hosts and the flagellated promastigote (15-30 mm in length, plus the flagella), growing within the intestinal tract of the insect vectors and in culture medium *in vitro* (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. It is a region of the mitochondrion, always found near the basal body at the base of the flagellum (Dedet *et al*., 1999).

2.5. Identification of *Leishmania* species

The identity of the *Leishmania* species involved is a factor that largely determines the clinical manifestation of the disease (Alexander and Russell, 1992) and its response to treatment (Navine *et al.,* 1992). Thus, species identification of *Leishmania* is important for both clinical and epidemiological reasons. *Leishmania* species in the same geographical region may cause similar clinical manifestations and yet the different species require distinct therapeutic approaches. Moreover, the characterization of the *Leishmania* parasite in a given region is critical for documenting the distribution of the prevalent *Leishmania* species and the design of appropriate control measures (Belli *et al*., 1998).

The initial criteria used for identification and classifications were based on extrinsic characteristics such as clinical features, geographical distribution, and behavior in culture, laboratory animals or vectors. The variations produced by these criteria lead to the development of more powerful methods based on the intrinsic character of the parasite, such as immunological, biochemical and molecular methods. Among the techniques currently in use, isoenzyme electrophoresis and DNA based techniques based on different DNA sequences as targets are the most employed for species identification (WHO, 1990 and Cupolillo *et al*., 1998).

2.5.1. Isoenzyme electrophoresis

The electrophoretic analysis of isoenzymes is the gold standard method for species-specific characterization of *Leishmania* (Cupolillo *et al*., 1998). The technique enables examination of a large sample of structural genes, providing genetic evidence to distinguish polymorphism between species from differences within species. It involves separating

soluble enzymes by gel electrophoresis and subsequent visualization of specific enzymes using appropriate staining reaction. Patterns of single or multiple bands are seen on staining. A consistent profile of isoenzyme patterns is found in closely related strains. Isolates with identical banding pattern (alleles) are usually referred to as zymodemes (Cupolillo *et al*., 1998).

Isoenzyme electrophoresis takes advantage of the fact that non-denatured proteins with different net charges migrate at different rates through a gel matrix when electrical current is applied. The characteristic charges of proteins result primarily from the three positively charged amino acids: lysine, arginine and histidine and the two negatively charged amino acids: aspartic acid and glutamic acid. The Isoenzyme represents enzymatically active proteins. In fact, they represent alleles (alloenzymes) generally coded at the same locus (genezyme). If the protein products of two alleles at a particular locus have different charges, their rates of migration are different and both appear as separate bands on a gel (May, 1992). In *Leishmania*, the genezyme usually generates a single isoenzyme. The rarity of heterozygous structures could be explained either by a typical haploidy or a diploidy with elimination of hetrozygotes or repression of one of the alleles. According to the current available evidence, the mode of evolution of *Leishmania* is still considered clonal. Then the zymodeme would represent a true evolutionary unit (Rioux, *et al*., 1990).

Rioux *et al*. (1990) recommended the use of 15 enzyme systems to study the phylogeny and to type a strain of *Leishmania* and give it an international code. However, Perara *et al*., (1998) used 8 enzyme systems (6-PGDH, PGM, GPI, PEP-D, ALAT, ASAT, MPI and NH) and showed that *Leishmania* isolates can be typed to a species level. Kreutzer *et al*., (1997) indicated that most *Leishmania* isolates can be accurately identified using 3 enzyme systems (GPI, MPI and 6-PGDH) from analysis of 20 WHO reference strains and 300 *Leishmania* isolates from New and Old World.

2.5.2. Principle of enzyme visualization

Histochemical stain identifies particular gene products from the many thousands of proteins separated during electrophoresis. The basic principle of enzyme visualization *in situ* is to present an enzyme with a solution containing an enzyme specific substrate. Often, however the primary enzymatic reaction products are colorless and require coupling with visualizing agents to generate a colored product. To demonstrate oxidative enzymes, mostly tetrazolium salts are used. These salts are colorless and water soluble in an oxidized state and turn into deeply colored precipitate, called formazan, when reduced. Transfer of electron from NAD/NADP to a tetrazolium salt requires a redox element such as phenazine methosulfate (PMS) (Fig. 3). To assay a small quantity of oxidative enzymes, more sensitive visualizing agents such as Methylthiazol tetrazolium (MTT) are used**.** Hydrolytic enzymes can be visualized using diazonium salt. These salts react with enzymatically-released naphthol from corresponding esters or glycosides to form a colored insoluble azo dye (Fig. 4). Transferases are mostly visualized by using coupled assay, i.e. one of the reaction products of a transferase reaction is used as a substrate for a coupled dehydrogenase reaction which is made visible. Lyases, isomerases and ligases are also visualized in a coupled reaction as oxido-reductases or hydrolases. The stained banding profile of the enzymes is termed the zymogram **(**Mouren *et al*., 1959).

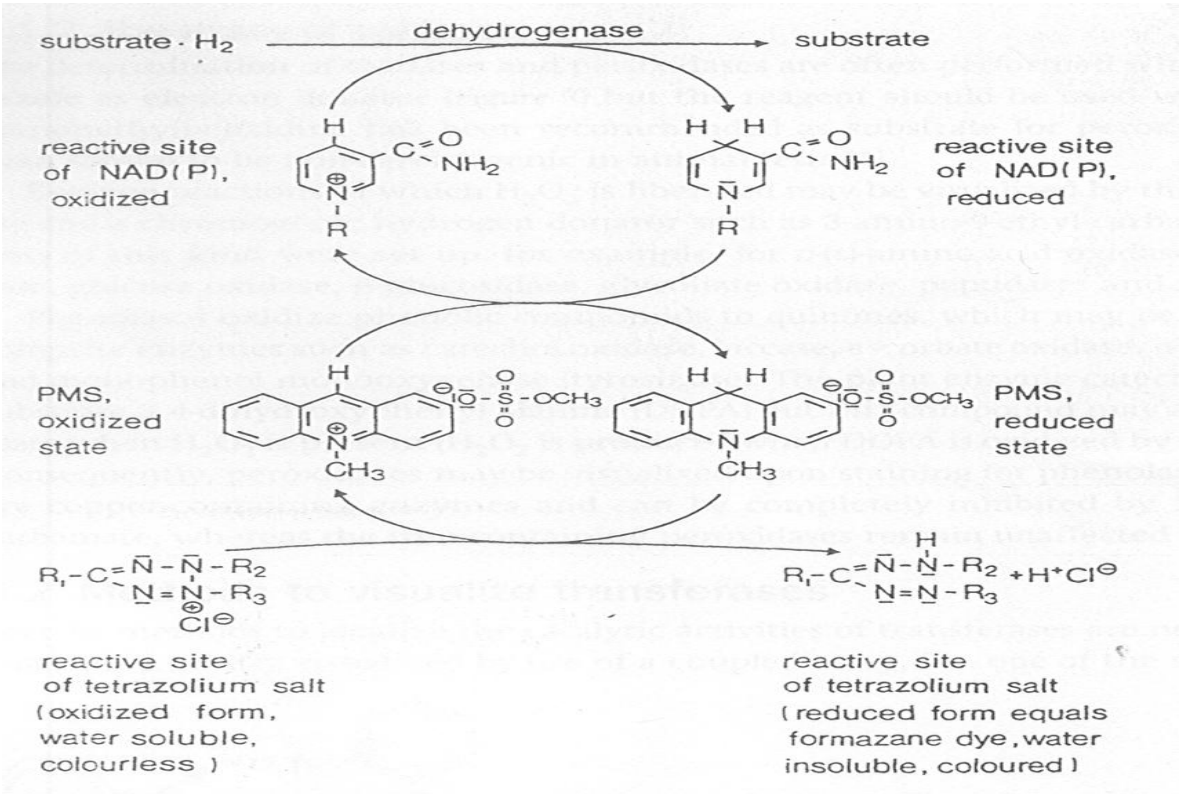


Figure 3. Detection of dehydrogenases using a tetrazolium salt as chromogenic substance and PMS as electron carrier (From Rothe, 2002).

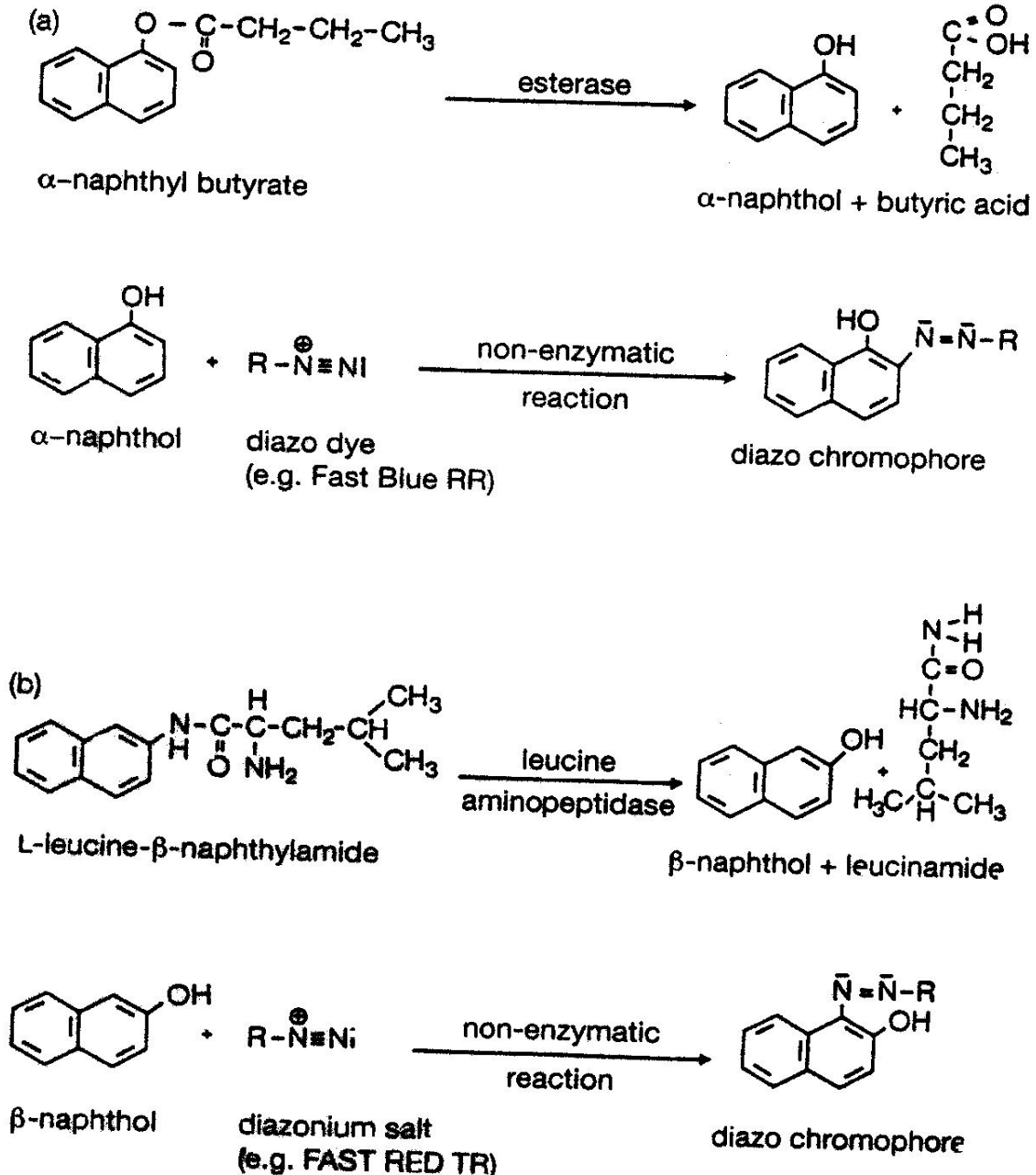


Figure 4: Course of reaction to detect hydrolases by coupling the enzymatically liberated naphthol (From Rothe, 2002).

2.6. DNA based techniques

A major drawback of isoenzyme based typing is that it is labor intensive, time consuming and requires relatively larger number of cultured parasites. A number of molecular markers and PCR-protocols which are less laborious and more powerful for detection and identification of *Leishmania* at different taxonomic levels (genus, complex or species) have become available.

2.6.1. Southern blotting with DNA probes

One of the earliest molecular characterization methods used was Southern blotting using DNA probes. This method involves DNA extraction, gel electrophoresis, Southern blotting and hybridization. DNA probes for *Leishmania* species identification generally target kinetoplast DNA (kDNA) because of its multi-copy nature having variable regions that differ from minicircle classes in the same network (Barker, 1989). Kinetoplast DNA minicircle probes have been developed for *L. major* (Smith *et al*., 1989), *L. aethiopica* (Laskey *et al*., 1991) and *L. infantum* (Gramiccia *et al*., 1992). Probes from different sequences of nuclear DNA have also been described. A complementary DNA (cDNA) probe specific to *L. donovani* complex was developed by Howard *et al*., (1991).

2.6.2. 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. Many PCR-based techniques have been developed for the identification of *Leishmania* species at different taxonomic levels. Depending on the objective, different targets for amplification are chosen. For the purpose of diagnosis, multi-copy sequence repeats are usually selected for their high potential of 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. The specificity can be improved by RFLP analysis of the PCR-amplicon (Schalling and Oskam, 2002).

The conserved 120 bp 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 (ITS1), located between the genes coding for the small subunit (ssu) and the 5.8S rRNA genes combined with RFLP analysis with *HaeIII* and *CfoI* enabled Schonian *et al*. (2000) to differentiate most medically important *Leishmania* species. Van Eys *et al*. (1992) were able to distinguish *L. donovani* from *L. tropica* and *L. major* by digesting the amplicon of ssu rRNA with the restriction enzyme *RsaI.*

3. *LEISHMANIA AETHIOPICA*

L. aethiopica is responsible for CL in the highlands of Ethiopia, western Kenya and eastern Uganda. The CL in Ethiopia predominantly caused by *L. aethiopica* is manifested in three clinical forms. These are similar to what has been described earlier and highlighted here.

LCL is characterized by localized lesions, mostly single, in areas not covered by clothing, such as the face, nose and forearms. It presents as a reddish plaque with irregular borders and shallow ulcer in the center. It may self heal in 8 to 10 months or may persist for several years. Once healed, it is unusual to see a recurrence from scar of LCL due to *L. aethiopica*. However, in *L. aethiopica*-HIV co-infection, recurrence at the site of original lesion was reported. This is potentially a serous implication in areas like Ethiopia where leishmaniasis and HIV co-exist (Berhe *et al*., 1995).

Non-self healing disseminated lesions, covering quite a large area of the face, torso and extremities, characterize DCL. It is often disfiguring and patients often fail to respond to conventional antileishmanial chemotherapy (Laskay, 1991).
MCL spreads to the mucous membrane of the nasopharyngeal area, most likely by direct extension from skin lesions (Hailu and Frommel, 1993).

4. Problem statement

It is very difficult for a clinician to state from examination of early symptoms and geographical criteria that a particular *Leishmania* species is responsible for the particular symptom. Even in the advanced pathology, leishmaniasis can present with a range of symptoms. The proposed use of pentavalent antimonials is dependent on which species of *Leishmania* is present (Blum *et al*., 2004).

An increased leishmanin positive response from 33% - 40% (Ali, 1995 and Ali *et al*., 2002) and an overlap in geographical distribution of *L. aethiopica* and *L. tropica* (Gebre-Michael, 2004) in the low land emphasize the need for a broader epidemiological study. The prevalence of three CL causing *Leishmania* species (*L. aethiopica, L. tropica* and *L.*

major) in Ethiopia with overlapping distribution underlines the need for accurate and rapid diagnosis of the *Leishmania* species before effective treatment and control measures are started (Mengistu *et al*., 1992 and Gebre-Michael *et al*., 2004). The accomplishment of this task depends largely on the accurate identification of the *Leishmania* species in humans, in insect vectors and reservoir hosts. This makes the establishment of species-specific identification capacity an urgent priority.

Though it is generally believed that *L. aethiopica* is the principal aetiologic agent of CL in Ethiopia (Mengistu *et al*., 1992 and Ali *et al*., 2002), its actual proportion among possible species causing cutaneous leishmaniasis is not well documented with typing of isolates from affected individuals.

Thus, the aim of this study was to develop species identification protocols using isoenzyme electrophoresis and to characterize *Leishmania* isolates from cutaneous lesions of patients attending the ALERT Hospital Dermatology Clinic and isolates from an endemic community in Ochollo area.

Since isoenzyme electrophoresis is a labour intensive technique, the study also aimed at testing molecular techniques based on PCR for species-specific diagnosis of leishmaniasis confirmed with isoenzyme electrophoresis as a gold standard. The testing and validation of *L. aethiopica* PCR-based technique on isolates could be applied for direct confirmation of diagnosis on tissue samples eliminating the need for *in vitro* culture. The species identification protocols can be utilized to initiate a broader epidemiological survey, important in the identification and control of vectors and reservoir hosts and to monitor the development of better drugs and/or a vaccine.

5. Hypothesis

- Cutaneous leishmaniasis in patients of Ochollo, an endemic village, or among patients visiting a tertiary referral Hospital in Ethiopia is caused by *L. aethiopica* in more than 90% of the cases.

6. Objectives

6.1. General objective

- Establish methods to identify the *Leishmania* species responsible for cutaneous lesions in Ethiopia

6.2. Specific objectives

1. Determine the species of *Leishmania* isolates from cutaneous lesions of patients living in an endemic area (Ochollo village) and from patients attending a tertiary care dermatology facility (ALERT Hospital, Addis Ababa) using isoenzyme electrophoresis and PCR- based techniques.
2. Test a species-specific PCR-based method to differentiate *L. aethiopica* from other *Leishmania* species.

7. Materials and Methods

7.1. Reference strain

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 an identical manner as the clinical isolates in all procedures.

Table 2: Reference strains used.

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

* DNA samples from University of Calgary, Canada.

7.2. Study population and specimen collection

7.2.1. Ethical consideration

Ethical clearance was obtained from the AHRI/ALERT ethical committee. The regional health bureau and concerned bodies at all level were officially contacted and a support letter was obtained from all levels. After the purpose, procedure and benefit of the study were discussed with the study subjects and a written informed consent was obtained from all participants or parent (guardian) (see attached Amharic version of consent form). Treatment was provided for super infection. Intervention needs improvement and is an ethical concern. Additionally, lack of *Leishmania* control in Ochollo village to date is ethically unacceptable.

7.2.1. Study population

The specimens for the study were obtained from suspected CL patients visiting the ALERT Hospital Dermatology Clinic and from Ochollo Village. ALERT Hospital, in Addis Ababa, is a referral center for patients with dermatological problems. Usually, patients coming to the center are late self-referred or referred after treatment failure from elsewhere in Ethiopia. At the ALERT Dermatology Clinic, patients diagnosed as probable cases of CL by a dermatologist from October 2003 to January 2004.

Ochollo (6^{o} 02' Latitude and 37^{o} 37' 10'' Longitude), southwest Ethiopia, in the Southern Nation Nationalities and Peoples Region, is an endemic focus with a high prevalence of CL (Mengistu *et al*., 1992). Two field trips were made, between May 28-31, 2004 and December 3-9, 2004, and we discussed with the villagers about the objective of the study. They agreed to bring their family members with "Volbo" a vernacular name for CL in the area. Volunteers who presented at the Ochollo Clinic were examined by a clinical research staff from AHRI and suspected CL patients were included.

In all the cases, specimens were collected after getting informed written consent from the patient or patient's parent (guardian).

7.2.2. Preparation of culture media

Novy-MacNeal-Nicolle (NNN) medium with Locke's solution as an overlay was used for the cultivation of all the clinical isolates and the reference strains in the study. NNN medium was prepared as follows: The ingredients of the medium (9.2 g nutrient agar (Difco), 0.6 g D-glucose (anhydrous) and 2.4 g sodium chloride (Sigma)) were weighed, mixed and dissolved in 400 ml of distilled water by boiling in microwave oven with repeated shaking every ten minutes until a clear solution was obtained. It was then autoclaved at 121 ^{o}C for 30 minutes. Sheep blood was aseptically collected with a bottle containing glass beads and shaken to defibrinate. The defibrinated blood was heat-inactivated by keeping it in 37 ^{o}C water bath for 50 minutes and then transferred into 56 ^{o}C water bath for 10 minutes. The autoclaved ingredients (400 ml) and of the heat-inactivated blood (100 ml) were mixed at 50 ^{o}C**.** The mix was dispensed using 8 ml for 50 ml culture flask and 32 ml for 200 ml culture flask and allowed to settle as a slant. The media were stored at 4 ^{o}C until use (Akuffo *et al*., 1987 and Evans, 1990).

The ingredients of 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 the ingredients were weighed, mixed and dissolved in 500 ml of distilled water. The preparation was autoclaved at 121 ^{o}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 filter sterilized and stored ready for use at 4 ^{o}C (Akuffo *et al*., 1987 and Evans, 1989).

7.2.3. Specimen collection

As described by Genetu (2003) and Evans (1989), a trained clinical research nurse took all the specimens at ALERT and Ochollo. The active part of the lesion was selected, properly disinfected with 70% ethanol and allowed to air dry. Skin scrapings and a 4 mm punch biopsy (from patients above 18 years of age) were taken with sterile disposable blade and

punch. The biopsy samples were put in 1 ml Nunc tube in normal saline and stored at -20 ^{o}C until use.

In both ALERT and Ochollo, skin scrapings from the edge of lesions were immediately inoculated in duplicate into NNN media with Locke's solution as an overlay.

Field samples were transported to AHRI in Styrofoam box with tight cover disinfected with 70% alcohol. The cultures were transported under environmental condition. It took 3-4 days to transport samples to AHRI.

At AHRI, cultures were incubated at 26 ^{o}C. The cultures were inspected under inverted microscope (Leitz-wetzlar, Germany) starting on the 4th day of inoculation and in 3-days interval subsequently to monitor the growth of *Leishmania* promastigotes. Contaminated cultures were immediately discarded.

After two weeks, negative cultures were blindly sub-cultured into new NNN media. Those cultures that remained negative after two weeks of sub-culturing were discarded as negative. All positive cultures were sub-cultured by transferring 1 ml of overlay into new NNN media with additional 1ml of Locke's solution added. To mass cultivate the parasites, 1ml of the overlay was seeded into NNN media in duplicates and 5ml and 10 ml Locke's solution was added for 50 ml and 200 ml culture flasks respectively. One flask was used for cryo- preservation and the other was further expanded. Of the expanded culture, 1/3 was used for DNA extraction and the remaining 2/3 used for isoenzyme typing.

7.3. Isoenzyme typing

7.3.1. Harvesting and preparation of whole cell extract

7.3.1.1. Harvesting and cryo-preservation

Cultured *Leishmania* promastigotes were inspected under microscope for purity of the culture and harvested during the exponential growth. The overlay Locke's solution was

transferred into 50 ml tubes and centrifuged at 2060 xg at 4 ºC for 10 minutes. The pelleted promastigotes were then washed by re-suspending in 5 ml of 0.03% saline and phosphate buffered saline (PBS pH, 7.2) consecutively and centrifuged at 2060 xg at 4ºC for 10 minutes. Then, the pellet was re-suspended in 1ml PBS (pH 7.2) if the pellet was small or in 2 ml if it was large, transferred into 1.5 ml eppendorf tube and centrifuged in an eppendorf centrifuge that had been kept in cold room (4 ºC) for 30 minutes. The supernatant was removed and the pellets stored at -70 ºC until used (Genetu, 2003 and Perara, 1998).

7.3.1.2. Preparation of whole-cell extract

Frozen parasites were thawed and whole cell extract for starch gel electrophoresis was prepared by adding 100 µl of lysis reagent (5% Triton-X 100, 0.825g glucose) and homogenizing it with plastic rod until a uniform suspension was obtained. The homogenate was then blotted with Wicks for loading. Samples were kept on ice throughout the preparation of the whole cell extract (Genetu, 2003 and Perara, 1998).

7.3.2. Preparation of gel

A thick (10.25%) starch (Sigma) gel was prepared using the appropriate gel buffer (Table 3) and cooked. The content was shaken continuously. When boiling begun, the heated starch was immediately degassed using a vacuum pump. The degassed hot starch was rapidly poured into the gel mold and was carefully covered with a sheet of glass making sure that no air bubble formed between the glass and the gel. A weight (about 500 g) was put over the cover glass and left overnight to set at room temperature (Genetu, 2003 and Murphy *et al*., 1996).

7.3.3. Loading, electrophoresis and staining

The next morning the cover glass was removed and a comb was inserted at 4 cm towards one end to form wells. A tracking dye (0.25% bromophenol blue) was added onto the teeth of the comb before inserting it into the gel. The whole cell extract blotted with the Wicks was placed vertically into the wells using narrow-tipped forceps (Murphy *et al*., 1996 and Rothe, 2002).

The electrophoretic tank was filled with the appropriate buffer (Table 3) for the particular enzyme. In alkaline solutions most proteins are negatively charged and travel towards the anode. Thus, the loaded gel was placed in the electrophoretic tank orienting the wells towards the cathode. A transparent thin plastic sheet and a sheet of glass were placed over the loaded gel just above the wells to insulate against electrical short circuits. A sponge cloth was used to connect the buffer and the gel and held in position by putting a sheet of glass over it. The electrophoresis was done for 4 hours at 100 V and 65 mA at 4 ^{o}C (Murphy *et al*., 1996 and Rothe, 2002) by putting ice-packs on lid of the electrophoretic tank.

After electrophoresis, the Wicks were removed, the gel sliced with a nylon string into three and the corner of each slice notched to identify the order of loading (Murphy *et al*., 1996 and Rothe, 2002). Each slice was stained for different enzyme systems according to the specific enzyme (Table 3).

Table 3: Enzymes tested in the study and the conditions used

Enzyme	Buffer system	Staining buffer	Coenzyme	Linking enzyme	Substrate	Activator	Visualizing Method
GPI (EC 5.3.1.9)	TAE pH 7.4	Tris HCl 0.2 M pH 8.0	0.5 ml 1% NADP 1ml 1% NAD	16U G6PD	10 mg Fructose -6-phosphate	1ml 0.5M $MgCl_2$. 6H2O	0.5ml PMS 1%, 1ml NBT 1%, 5ml Agarose 2%
MPI (EC 5.3.1.8)	TC pH 8.6	Tris HCl 0.2 M pH 8.0	1ml 1% NADP	33U G6PD, 50U PGI	10mg Mannose-6-Phosphate	1ml 0.5M MgCl2. 6H2O	1ml PMS 1%, 1ml NBT 1%, 5ml Agarose 2%
PGD (EC 1.1.1.44)	TAE pH 7.4	Tris HCl 0.2 M pH 8.0	1ml 1% NADP		6-Phospho-gluconic acid	1ml 0.5M $MgCl_2$. 6H2O	1ml PMS 1%, 1ml NBT 1%, 5ml Agarose 2%
G6PD (EC 1.1.1.49)	TAE pH 7.4	Tris HCL 0.2 M pH 8.0	1ml 1% NADP		10mg D-Glucose -6-Phosphate	EDTA 10mg	0.5ml PMS 1%, 1ml NBT 1%, 5ml Agarose 2%
GOT1 (EC 2.6.1.1)	TAE pH 7.4	Tris HCl 0.2 M pH 8.0			0.1g ∝-Ketoguterate, 0.2g Aspartic acid		0.3% Fast blue BB salt
PGM (EC 2.7.5.1)	TAE pH 7.4	Tris HCl 0.2 M pH 8.0	0.5 ml 1% NADP 1ml 1% NAD	16U G6PD	0.3g Glucose-1-Phosphate	1ml 0.5M MgCl2. 6H2O	0.5ml PMS 1%, 1ml NBT 1%, 5ml Agarose 2%
NP1 (EC 2.4.2.1) NP2 (EC 2.4.2.*)	TC pH 8.6	Tris HCl 0.2 M pH 8.0		Xantine - oxidase 2U	10mg Inosine		1ml PMS 1%, 1ml NBT 1%, 5ml Agarose2%

From, Perara *et al*., (1998) and Kreutzer *et al*., (1997)

EC= Enzyme commission number, * Specific number not assigned

7.4. DNA based techniques

7.4.1. DNA extraction from cultured promastigotes

DNA was extracted from cultured promastigotes by the phenol chloroform extraction method using the modified protocol of Paramchuk *et al*. (1997). Parasites were harvested at late logarithmic stage. The cells were centrifuged for 10 minutes at 2060 xg and washed three times with PBS (pH 7.2), transferred into a 1.5 ml eppendorf tube and lysed with 500 µl of lysis buffer (1% SDS, 50 mM EDTA pH 8.0, 10 mM, Tris-HCl pH 8.3). RNase A (Pharmacia) was added to a final concentration of 100 µg/ml (5 µl of a 10 mg/ml solution) and the samples were incubated at 37 ° C for one hour. Proteinase K (GIBCO) (5 µl of a 10 mg/ml solution) was added to a final concentration of 100 µg/ml and incubated overnight in a 42 ^{o}C water bath. Then, 300 µl of a 25:24:1 mix of phenol- chloroform-isoamyl alcohol (Sigma) was added to each sample and vortexed for 5 seconds. After centrifugation at 1200 xg for 5 minutes at room temperature, the upper phase was carefully removed and transferred to new 1.5 ml eppendorf tubes. Three hundred and fifty micro liters (0.7X volumes) isopropanol was added to the samples and kept -20 ^{o}C for 30 minutes. Subsequently, the samples were centrifuged at 1200 xg for 15 minutes. The isopropanol was removed and ice-cold 70% ethanol (1 ml) 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 in 37 ºC oven. The DNA samples were then resuspended in 20 µl 1X TE buffer and heated for 10 minutes in a 68 ºC water bath to dissolve. Fifty-fold dilutions of the DNA were used to determine concentration spectro-metrically at absorption of 260 nm and dilutions of 1 µg/µl were prepared. DNA samples were stored at -20 ° C until processed further.

7.4.2. DNA extraction from biopsy samples

The phenol chloroform method (van Sooligen and Hermans, 2001) was used to extract DNA from biopsy samples. Each tissue sample was thawed from -20 ºC, transferred into a glass homogenizer and homogenized in 1 ml RPMI 1640. The homogenate was transferred into 1.5 ml eppendorf tubes and centrifuged at 1200 xg for 5 minutes in an eppendorf centrifuge. The supernatant was discarded and 500 µl of 1X TE buffer was added to the pellet, vortexed and incubated in boiling water for 15 minutes. Seventy micro liters of 10% sodium dodecyl sulfate (SDS) and 6 µl of 10 mg/ml proteinase K (GIBCO) were added, vortexed and incubated in a 65 ºC water bath for 10 minutes. One hundred micro liter of sodium chloride (5M) was added to the samples and vortexed. CTAB/NaCl (80µl) pre-heated for 10 minutes in 65 ºC water bath was added to the samples and vortexed until it turned milky white, and then incubated at 65 ºC in a water bath for 10 minutes. A chloroform and isoamyl alcohol mix (chloroform/isoamyl alcohol 24:1) was added in equal volumes to each sample and vortexed for 10 seconds. The samples were centrifuged at 1200 xg for 5 minutes and the upper phase was transferred to a new tube. Isopropanol (0.6X volume) was added to the samples and incubated at -20 ° C for 30 minutes. After 15 minutes of centrifugation at 1200 xg, the supernatant was carefully removed until about

50µl remained; spun for additional 5 minutes, and the remaining supernatant was removed without touching the pellet. The DNA pellets were washed with ice-cold 70% ethanol (800µl). After 5 minutes of centrifugation, the ethanol was removed in two steps, first until 50 µl remained, and then spun again for few minutes and the remaining fluid was removed carefully. The DNA samples were dried in a 37 ºC oven and resuspended in 20µl 1X TE buffer, heated for 10 minutes in a 68 ºC water bath to dissolve. A 50X dilution of the DNA samples in 1X TE buffer was quantified spectro-photometrically. The DNA samples were adjusted to 1µg/µl and stored at -20 ºC until use.

7.5. PCR

7.5.1. PCR conditions

The volume of each PCR reaction was 25 µl for the primers T2 / B4 and 13A/13B and 50 µl for the primer pairs LISTR/L5.8S. Hot start master mix (QIAGEN GmbH, Germany) was used in all PCR reactions. All components were first pipetted into a 1.5 ml tube, well mixed and then aliquoted to PCR tubes. In all the cases a mix without template DNA was used as PCR internal control. The cycling condition used is indicated in Table 4. The numbers of PCR-cycles used were 35 for the primer pairs LISTR/L5.8S and 13A/13B, 40 for the primer pair T2/B4, and 30 cycles for Laefa/Laera with initial denaturizing for 5 minute and 10 minute final extension in automated thermocycler (Thermohybaid, UK).

The PCR products were visualized in 1.8% agarose (Sigma) gels with 0.5 µg/ml ethidium bromide. Four micro liters of each PCR product were loaded with 1µl of 5x loading buffer (0.25% Bromophenol blue**,** 0**.**25% Xylene cyanol FF, 50% Glycerol) on the gel. Three

micro liters of 0.1µg/µl molecular size markers (20µl of 1Kbp ladder, 20µl sterile distilled water and 60µl of 5X loading buffer) were loaded as controls. Electrophoretic separation was performed at 100 V and 50 mA for about 90 minutes. Pictures were taken using a UV trans-illuminator (in Epi-chemi II darkroom, Upland, USA, with a camera attached to it and a computer).

Table 4: PCR Conditions.

Primer pairs	Primer sequence	PCR condition			Reference
		Denaturation	Annealing	Elongation	
13A 13B	5'-GTGGGGGAGGGGCGTTCT-3' 5'-ATTTTCCACCAACCCCAGTT-3'	94 °C for 1min	50 °C for 1 min	72 °C for 1 min	Rodgers *et al*., 1990
LITSR L5.8S	5'- CTGGATCATTTTCCGATG - 3' 5'- AAGTGCGATAAGTGGTA-3'	95 °C for 40 Sec	53 °C for 30 Sec	72 °C for 1 min.	Schonian *et al*., 2000,
T2 B4	5'-CGGCTTCGCACCATGGCGGTG-3' 5'-ACATCCCTGCCCACATACGC-3'	96 °C for 30 Sec	56 °C for 45 Sec.	72 °C for 1 min.	Marfurt *et al.,* 2003
Laefa Laera	5'-CTACCCCTACGTGTCCACCTTT3' 5'-AGCACGCCGTGGTTCAGCTGTTT-3'	94 °C for 1 min	70 °C for 1 min	72 °C for 1 min	Kuru, 2004

7.5.2. Genus specific PCR

The Primers 13A and 13B (Rodgers *et al*., 1990) amplify a sequence of 120 bp of the kDNA minicircles conserved in all minicircle classes of genus *Leishmania.* This primer pairs were used for genus specific screening and to test the efficiency of DNA extraction. Due to the small size of the sequence and its presence in all minicircles the PCR with primers 13A/13B is highly sensitive and therefore suitable for screening at this taxonomic level (Rodriguez *et al*., 1994). *L. chagasi* DNA, kindly provided by Prof. Lashitew Gedamu (University of Calgary, Canada) was used as positive control and mtb DNA as negative control. In the screening of biopsy DNA samples, normal human skin DNA extracted and stored at AHRI for PCR control, was also included as negative control. The DNA was obtained from a healthy skin of a surgical amputation at ALERT with informed consent.

7.5.3. Species Typing

Amplification of the ITS1 and repetitive genomic DNA in combination with RFLP analysis of the PCR-product was used for species identification. The LISTR/L5.8S primer pair was used to amplify DNA samples from biopsy and cultured promastigote. In the case of biopsy, normal human skin and *M. leprae* DNA were included as control.

The amplification of the ITS1 with the primer pair LISTR/L5.8S produces about 350 bp sized PCR-product for all *Leishmania* species (Schonian *et al*., 2003). The primer pairs T2 and B4 designed by Minodier *et al*., (1997) targeting a repetitive genomic DNA amplify 250 bp fragments from Old World *Leishmania* species.

For species identification the ITS1-amplicon was digested with the restriction enzymes *HaeIII* and *HhaI.* The reaction mix contained 17µl PCR product, 2µl of 10X buffer of the respective enzyme and 1µl (10 U) of the enzyme of the buffer. The mixture was vortexed and incubated for 2 hours at 37 ºC (Schonian *et al*., 2003). Seventeen micro liter of the T2/B4 amplicon was treated with *HaeIII* in the same way as the ITS-1 amplicon except that, the incubation period was 3 hours (Minodier *et al*., 1997).

The restriction fragments were analyzed in 2% agarose (Sigma) with 0.5µg/ml ethidium bromide. Ten micro liters of restriction fragments were loaded with 2.5 µl of loading

buffer on the gel. Three micro liters (0.1µg/µl) molecular size markers were loaded as controls. Electrophoretic separation was performed at 100 V and 50 mA for about 90 minutes. Pictures were taken in the same way as the PCR products.

The PCR-RFLP analysis of ITS1 (El Tai *et al*., 2000 and Schonian, 2003) was used for species identification of *Leishmania* isolates and biopsy specimen. The RFLP analysis of T2/B4 amplicon *(*Minodier *et al.,* 1997) was used to type *Leishmania* isolates.

7.6. Development of species-specific PCR

To reduce the material, labor and time required for species specific diagnosis of *Leishmania* isolates an attempt was made to develop a species specific direct PCR method. The species specific primers were designed from the coding region of cysteine protease (Cys1) genes (Kuru, 2004). The *L. aethiopica* specific primer, Laefa/Laera was optimized for its PCR cycling condition and tested for its specificity (Table 4).

8. Data management

Pre-tested questionnaires (see appendix II) were used to collect the clinico-epidemiological data. Data were entered in duplicate in the data management unit of AHRI. Analysis was based on comparison of proportions and similarity of amplification or isoenzyme pattern of zymograms (Fig. 7, 8 and 9-12).

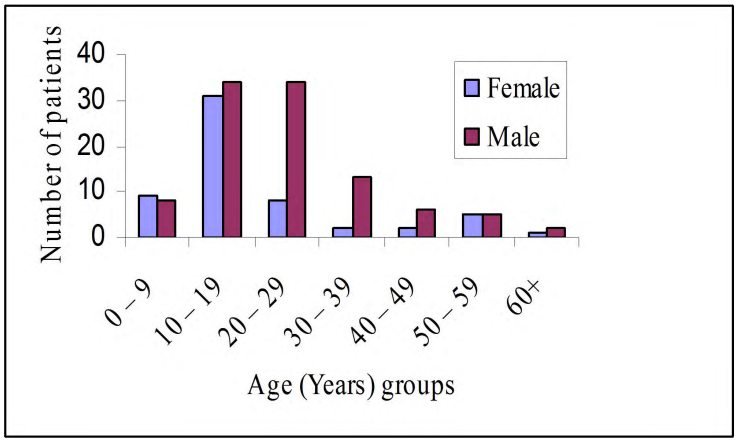
9. Results and Discussions:

9.1. Results

9.1.1. Study population and isolation of *Leishmania* strain

A total of 195 (120 male and 75 female) subjects were involved in the study. Of these 160 were sampled from ALERT Hospital Dermatology Clinic and 35 were recruited from Ochollo village. The culture positivity rate was 41.5%, contamination of cultures was observed in 13 of the 81 positive cultures. A total of 58 culture positive samples were processed in the study. The clinico-epidemiological data of patients used in the study is shown in Appendix 3. At ALERT Hospital Dermatology Clinic, higher number of cases (64%) was recorded in males compared to 36% in females (Fig. 5A). The male to female ratio was almost equal (16:19) at Ochollo. The mean age of patient and the duration of lesion at the two study sites had a significant difference (Figure 5 A and B). The age of patients enrolled at Ochollo ranged from 1 to 28 with a mode 6 years (median, 6 year), which is from 6 to 73 with a mode age of 15 (median, 19 year) for those recruited at the ALERT Hospital Dermatology Clinic. The average duration of the lesion at diagnosis, excluding DCL cases, was 9 months for those at Ochollo and 15 months for those recruited at ALERT Hospital Dermatology Clinic (Appendix, 3).

A.



B.

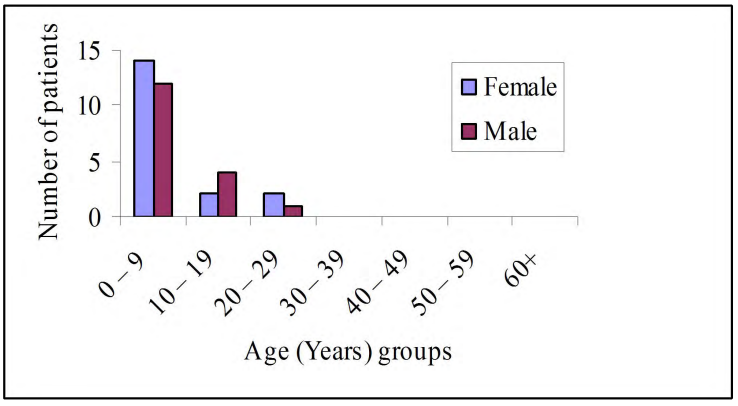
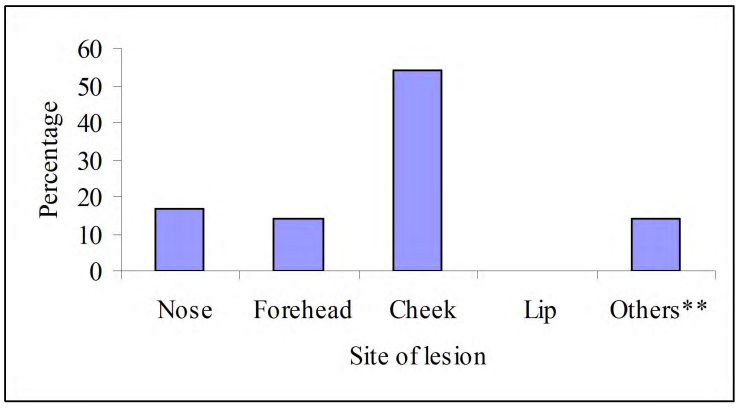


Figure 5: Age and sex distribution of clinically diagnosed CL patients enrolled in the study: A. ALERT Hospital and B. Ochollo village.

Among 160 patients, recruited at the ALERT Hospital Dermatology Clinic, with LCL 122 had single lesions (76.3%), 26 had two (16.3%) and only 12 (7.5%) had more than two lesions. Almost all patients at Ochollo had single lesion. In both cases the most common sites of occurrence, in order of frequency were cheeks, nose, forehead, lips (Ear for Ochollo**),** arms, limbs, eyelid and neck (Figure 6 A and B).

A.



B.

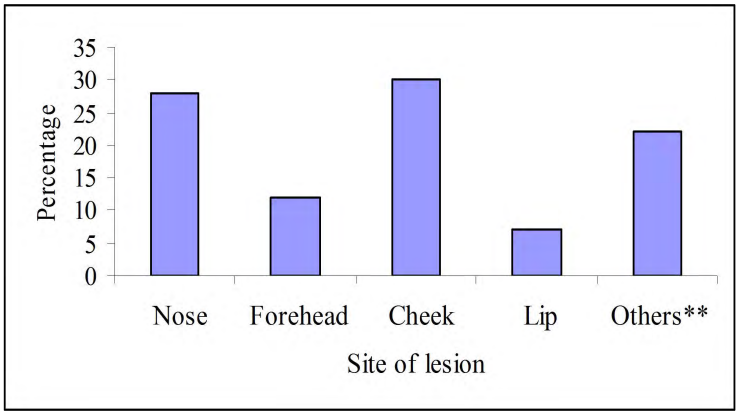


Figure 6: Percentage distribution of CL lesions by site in the study subjects:
A. Ochollo village and B. ALERT Hospital.
NB. ** Others = Leg, Arm, Eye lid, Neck, Chin and Ear; Multiple lesions are counted for individual sites involved.

9.1. 2. Result of isoenzyme analysis

Isoenzyme electrophoresis is the gold standard method for species identification of *Leishmania* (WHO, 1990). We carried out starch gel electrophoresis for the eight enzyme systems, GPI, PGM, PGD, G6PD, GOT1, NP1, NP2 and MPI that allow a good separation of all reference strains (Rioux, 1990). All the 21 isolates typed, with the enzymes: GPI, PGM, PGD, G6PD and GOT1 showed isoenzyme pattern similar to *L. aethiopica* (MHOM/ET/72/L100) and distinguished *L. aethiopica* (MHOM/ET/72/L100) from other

reference strains. The enzymes G6PD and PGM gave two bands for both the clinical isolates and *L. aethiopica* (MHOM/ET/72/L100). The enzymes GPI, PGD GOT1 gave a single band for *L. aethiopica* (MHOM/ET/72/L100) and clinical isolates. Except for GOT1, *L. donovani* (MHOM/IN/80/DD8) and *L. infantum* (MHOM/FR/LEM-75) showed similar isoenzyme pattern. The zymograms are shown in Figure 7 and 8.

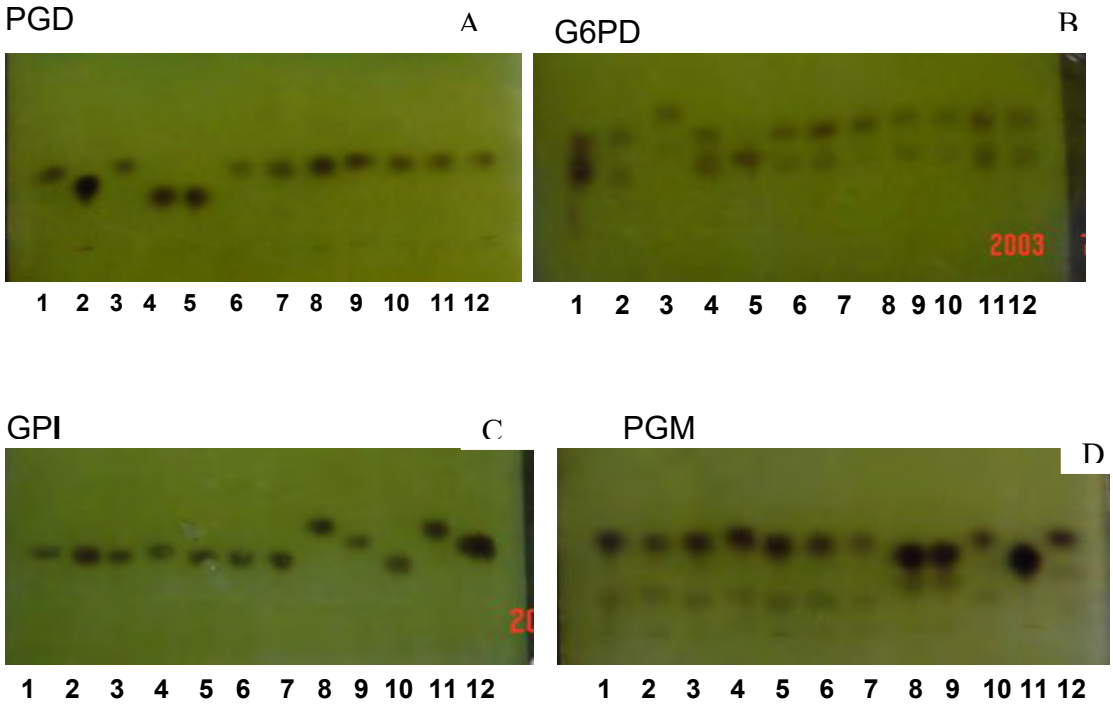


Figure 7: Photographs of Zymogram showing the banding pattern produced for our clinical isolates and the five reference strains. (A). 1= *L. major*, 2= *L. infantum*, 3= *L. aethiopica*, 4= *L. tropica*, 5= *L. donovani,* 6-12= Clinical isolates. (B). 1= *L. infantum*, 2= *L. aethiopica*, 3= *L. tropica*, 4= *L. donovani,* 5= *L. major*, 6-12= clinical isolates. (C; D). 1-7= Clinical isolates, 8= *L. donovani*, 9= *L. tropica*, 10. *L*= *aethiopica*, 11. *L*= *infantum*, 12= *L. major***.**

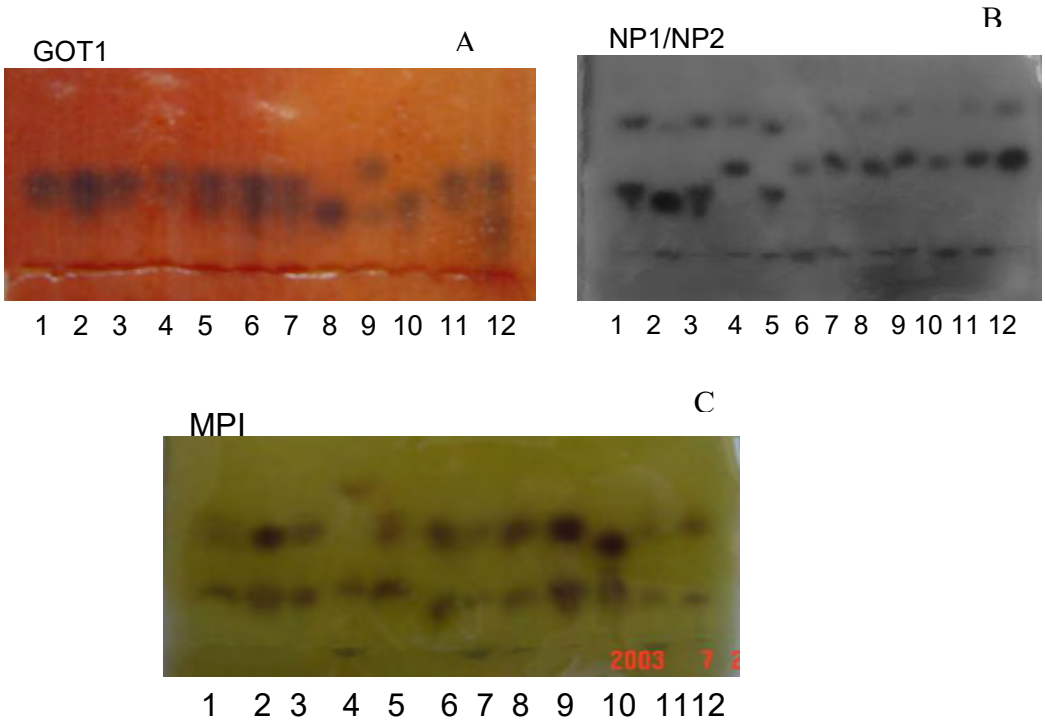


Figure 8: Photograph of zymograms for the five reference strains and our clinical isolates. (A). 1-7= Clinical isolates, 8= *L. major*, 9= *L. tropica*, *10= L. donovani*, 11= *L. aethiopica*, 12= *L. infantum,* (B). 1= *L. donovani*, 2= *L. major*, 3= *L. infantum*, 4= *L. aethiopica*, 5= *L. tropica*, 7-12= Clinical isolates, (C). 1= *L. tropica*, 2= *L. infantum*, 3= *L. donovani*, 4=*L. major*, 5= *L. aethiopica*, 6-12= Clinical isolates

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

The genus specific primer pair 13A/13B was introduced as a screening method in our study. It has been successfully employed on both biopsy and promastigote DNA samples. The high sensitivity of the PCR with primers 13A/13B had been demonstrated in previous studies (Rodriguez *et al*., 1994 and Belli *et al*.1998). We amplified the conserved 120 bp size miniexon kDNA from promastigote (Fig. 9A) and biopsy (Fig. 9B) DNA samples. In

agreement with previous reports, our results show that it is a good screening method for the presence of *Leishmania* DNA directly from clinical biopsy samples.

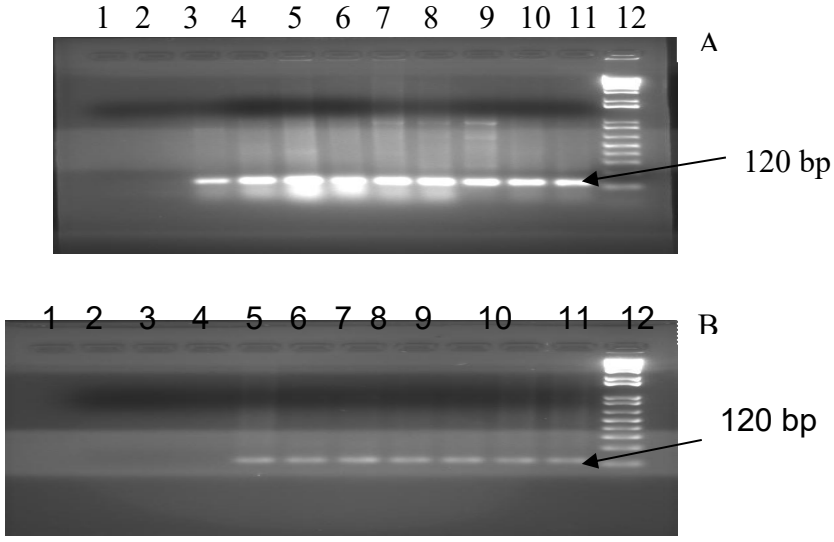


Figure 9: Images of the PCR product amplified with the primer pair 13A/13B after staining with ethidium bromide (A). **From culture promastigote**, 1=Negative control, 2=TB DNA, 3=*L. donovani*, 4=*L. infantum*, 5=*L. aethiopica*, 6= *L. tropica*, 7=*L. major*, 8-11Clinical isolates, 12= 1Kb ladder and (B). **From biopsy**, 1=mTB DNA, 2= Negative control, 3= Normal human skin DNA, 4= *M. leprae* DNA, 5= *L. chagasi*, 6= *L. aethiopica*, 7= *L. donovani*, 8= *L. major*, 9= *L. infantum*, 10-11= Clinical isolates, 12= 1Kbp ladder

9.1.4. PCR amplification results with the T2/B4 primer pair

The T2/B4 primer pair was designed for rapid identification of Old World *Leishmania* species (Rodgers *et al.*, 1990). It targets a repetitive genomic DNA sequence amplifying a 250 bp size product from Old World *Leishmania* species. In our study, the expected 250 bp size was amplified (Fig. 10A) from all the clinical isolates and reference strains (Table, 1). Restriction digestion of the PCR product was made with *HaeIII* for species identification. The clinical isolates and the reference strain *L. aethiopica* gave two bands of 215 bp and

35 bp size. With the reference strains, *L. infantum* produced only one band of about 250 bp size, *L. major* two bands of about 95 and 35 bp size, *L. donovani*, 180 and 70 bp size and *L. tropica* 215 and 35 bp size products (Fig. 10B).The PCR-RFLP analysis could distinguish our clinical isolates from the reference strains, except for *L. tropica* which yielded identical bands as in *L. aethiopica*.

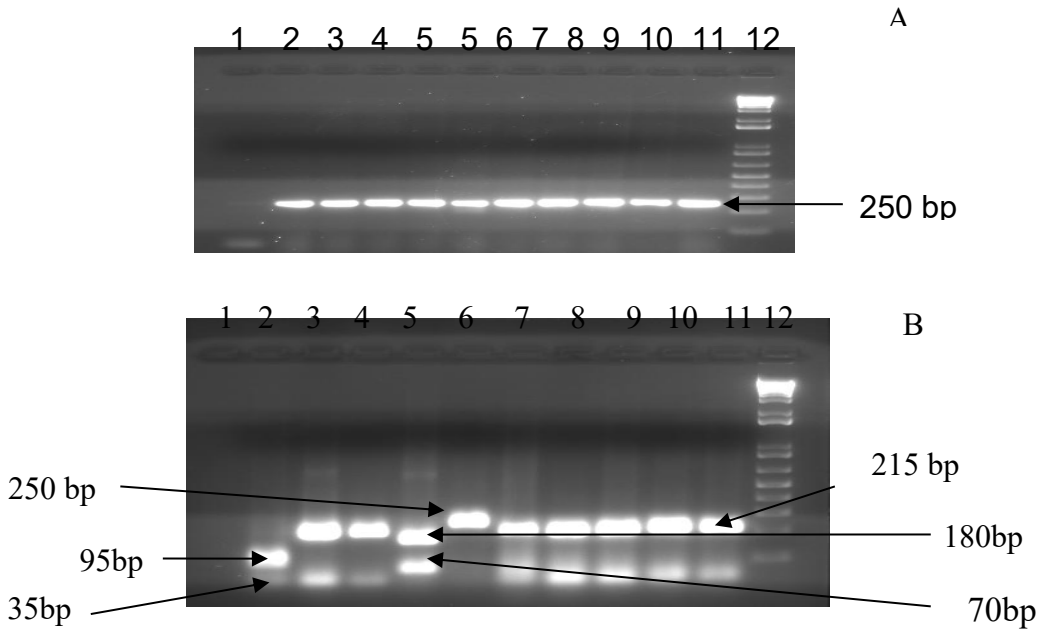


Figure 10: PCR amplification of a repetitive genomic sequence (A), with the primers pair T2/B4 and restriction analysis of the amplicon with the restriction enzyme *HaeIII* (B) after ethidium bromide staining. 1= Negative control, 2= *L. major,* 3= *L. aethiopica,* 4= *L. tropica,* 5= *L. donovani,* 6= *L. infantum* 7-11=Clinical isolates and 12= 1Kbp ladder

9.1.5. PCR amplification results with ITS-1 primers and RFLP-analysis

Amplification of the ITS-1 combined with RFLP-analysis published by Schonian *et al*., (2000) and El Tai *et al*., (2000); was adopted as the standard methodology of species-specific diagnosis by comparing the results of the PCR-RFLP with the result of isoenzyme electrophoresis as a gold standard. The PCR of the ITS-1 with the primer pairs

LISTR/L5.8S gave approximately 350 bp product for all reference strains and clinical isolates both from biopsy and cultured promastigote DNA (Fig. 11A and 12A). The primer did not amplify any product from normal human skin and *M. leprae* DNA (Fig. 12A). Digesting the PCR amplicon with the restriction enzymes *HaeIII* produced three bands of approximately 23**,** 55 and 202 bp size for our clinical isolates, *L. aethiopica* and *L. tropica;* 241, 97 and 78 bp size for *L. infantum;* 186, 78 and 55 bp size for *L. donovani and L. chagasi and* two bands of approximately 204 and 121 bp size for *L. major* (Fig. 11C). Digestion of the PCR amplicon with the restriction enzyme *HhaI* produced a single band of approximately 162 bp size for our clinical isolates and the reference strain of *L. aethiopica* strain. Two bands of approximately 88 and 237 bp size were observed for *L .major. L. infantum*, *L. donovani* and *L. tropica* gave a single 350 bp size band after incubation with *HhaI* showing that it has no restriction site in the ITS1 region of these strains (Fig. 11B). In our study, the ITS-1-PCR-RFLP analysis was applied on both parasite isolates (promastigotes) and biopsy samples. Amplification of the ITS-1 in combination with RFLP analysis gave the same result for both culture isolates and direct biopsy DNA samples (Fig. 12 A, B, and C). The PCR-RFLP analysis with *HaeIII* allowed the differentiation of *L. aethiopica* from all other species, reference strains except for *L. tropica*. The result of the *HhaI* digest differentiated *L. aethiopica* from all the reference strains of the other species.

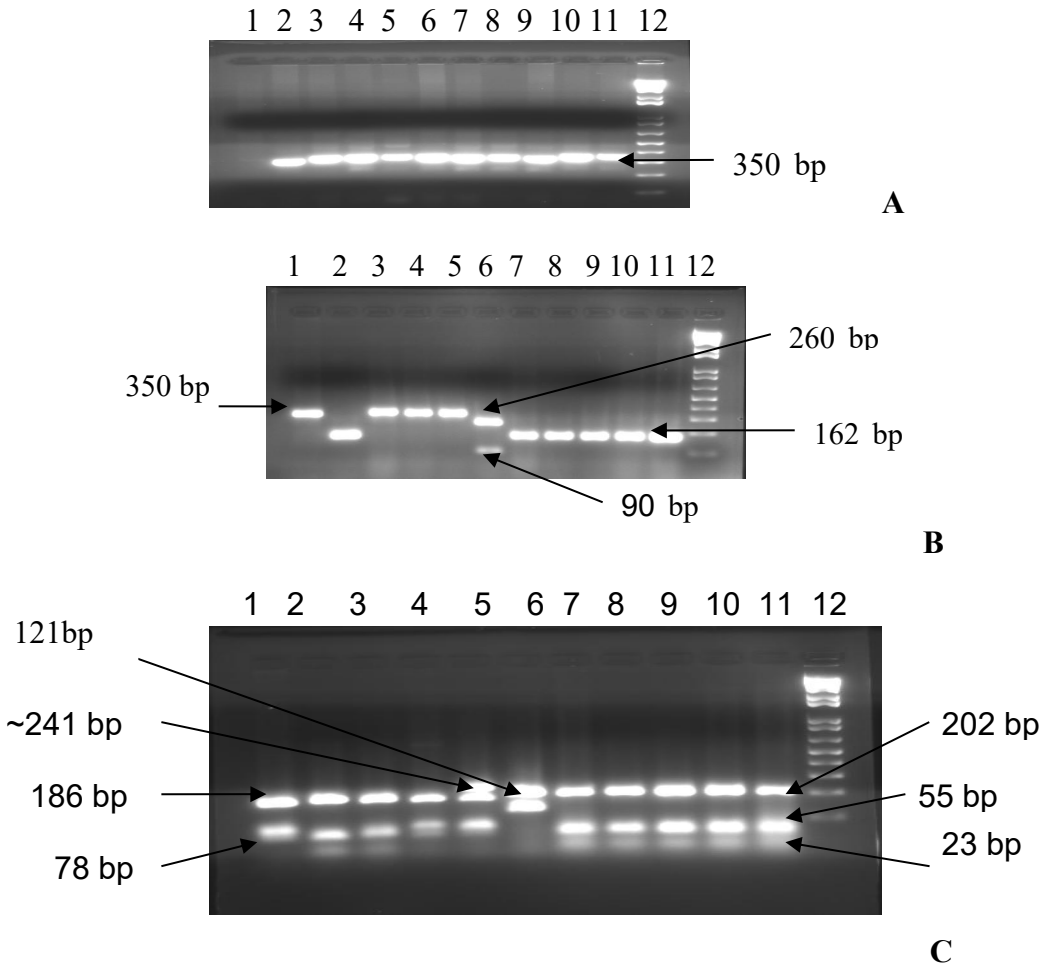


Figure 11: PCR product of ITS-1 from promastigote DNA samples (A), PCR-RFLP of the amplicon with *HhaI* (B) and *HaeIII* (C) after ethidium bromide staining. (A). 1= Negative control, 2=*L. donovani*, 3= *L. tropica*, 4= *L. aethiopica*, 5= *L. major*, 6= *L. infantum*, 7-11= Clinical isolates, (B). 1= *L. tropica*, 2= *L. aethiopica*, 3= *L. chagasi*, 4= *L. donovani*, 5=*L. infantum* 6= *L. major*, 7-11= Clinical isolates and (C). 1= *L. chagasi*, 2= *L. tropica*, 3= *L. aethiopica*, 4= *L. donovani*, 5= *L. infantum*, 6= *L. major*, 7-11= Clinical isolates, 12= 1Kbp ladder

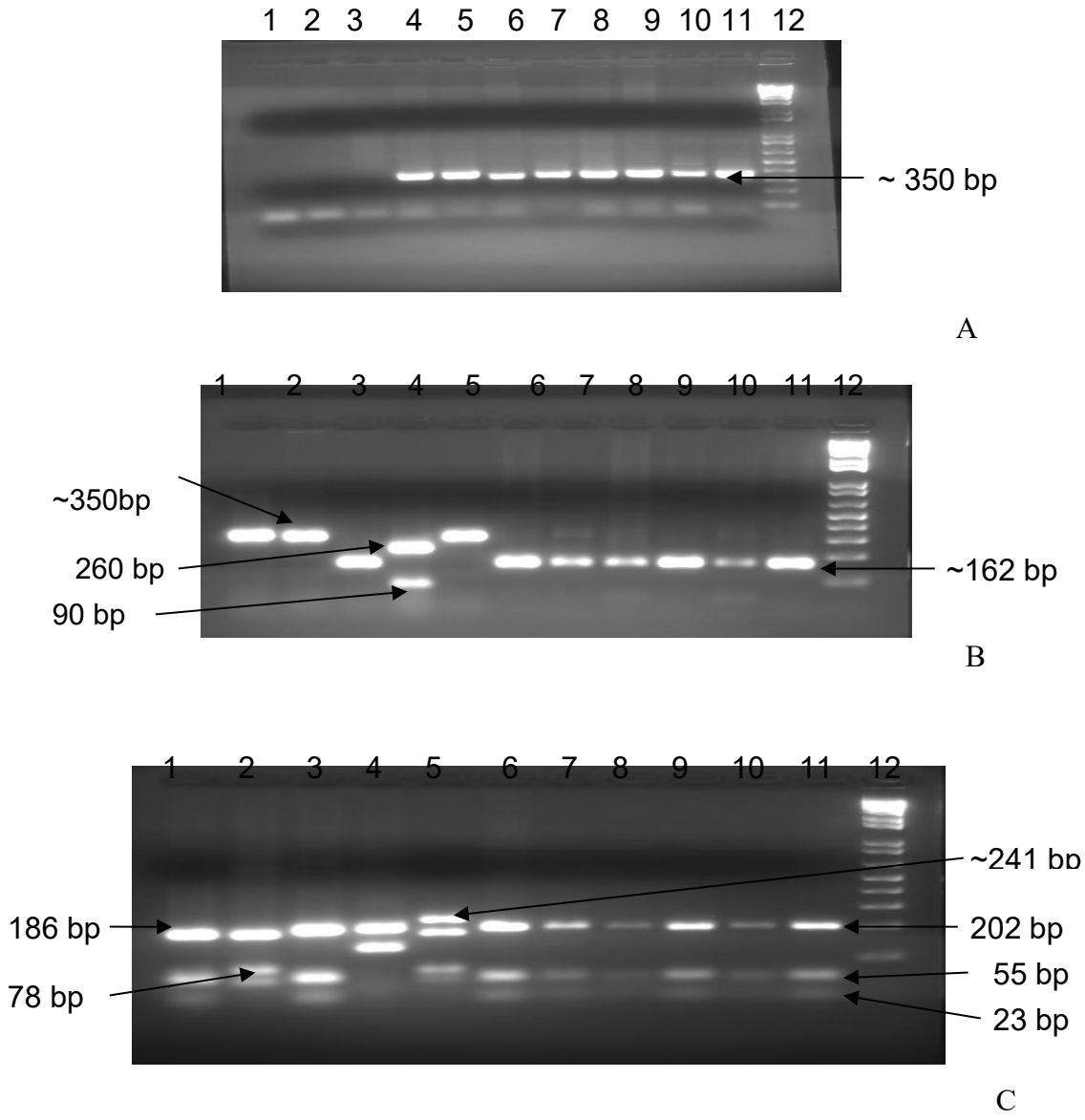


Figure 12: PCR product of ITS-1 from biopsy DNA samples (A) and PCR-RFLP with *HhaI* (B) and *HaeIII* (C) after ethidium bromide staining**.** (A), 1= Negative control, 2= *M. leprae* DNA, 3= Normal human skin DNA, 4= *L. major*, 5= *L. aethiopica*, 6=*L. donovani*, 7= *L. tropica*, 8= *L. infantum*, 9-11= Clinical isolates, (B). 1= *L. tropica*, 2= *L. donovani*, 3= *L. aethiopica*, 4= *L. major*, 5= *L. infantum*, 6-11= Clinical isolates, (C). 1= *L. tropica*, 2= *L. donovani*, 3= *L. aethiopica,* 4= *L. major*, 5= L. *infantum*, 6- 11= Clinical isolates, 12= 1Kbp ladder

9.1.6. Result of species-specific PCR amplification

As shown in figure 13, the primer pair Laefa/Laera amplified 234 bp from *L. aethiopica* (MHOM/ET/72/L100) and *L. tropica* (MHOM/SU/74/K27).

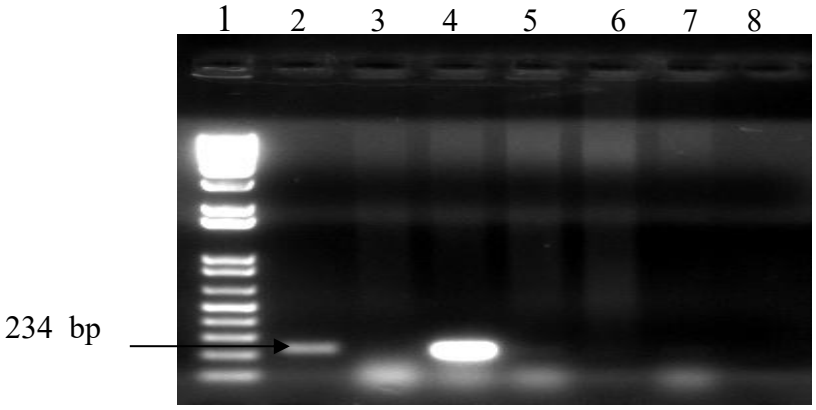


Figure 13. Ethidium bromide stained Laefa/Laera primers PCR product.1= 1Kb ladder, 2= *L. tropica,* 3=*L. major,* 4=*L. aethiopica,* 5=*L. donovani,* 6=*L. chagasi,* 7=*L. infantum* and 8= Non-template control

9.2. Discussion

Prior to the development of biochemical or molecular tools for the characterization of *Leishmania*, diagnosis of leishmaniasis relied on clinical and epidemiological criteria (WHO, 1990, Cupolillo *et al*., 1998). The reports of atypical clinical manifestations such as visceral leishmaniasis caused by the dermotropic species *L. tropica* (Kreutzer *et. al*., 1993 and Sacks *et al*., 1995) and the occurrence of cutaneous manifestations caused by *L. chagasi* and *L. infantum*, which usually cause visceral leishmaniasis (Ponce, *et al*., 1991) and in areas where different species are sympatrically transmitted, have posed a serous emerging challenge to the traditional classification methods. Correct identification of infecting *Leishmania* species causing the disease is important information for the clinician to initiate species-specific therapeutic approaches and design a correct control strategy (Jacobson, 2003 and Belli *et al*., 1998). Moreover, the design of broad epidemiological studies to identify the circulating species of *Leishmania* requires the availability of diagnostic method capable of identifying isolates from reservoir hosts and sandfly vectors (Mengistu *et al,* 1992).

Therefore, the main aim of this work was to validate PCR-based methods for detection and species identification of *Leishmania* isolates. We aimed at establishing isoenzyme electrophoresis technique and compare PCR methods to the gold standard with the main aim of simplifying species specific diagnosis. In that frame work, 58 *Leishmania* isolates from patients who visited the ALERT Hospital Dermatology Clinic and patients from Ochollo village were included in the study, of which 21 isolates were isoenzyme typed and all 58 processed using the PCR-RFLP techniques.

According to the clinico-epidemiological data (Appendix III) our study subjects were from different parts of Ethiopia, hence the clinical isolates obtained from the CL patients might be considered representative of the CL causative species in most parts of the country. The higher number of cases (64%) reported in males compared to females (36%) in all age groups for those recruited from ALERT Hospital could be difference in health care seeking behavior (females less determined), health care access (no money, more household challenges and/or lack of decision making power of the female). It is interesting to note that the males: female ratio was equal at the endemic village compared to the tertiary level referral center (ALERT Hospital) suggesting possible difference in access to health center. This study was not designed however to address the issue. The high number of cases (74.3%) in children (less than 10 years) in Ochollo, community might be explained by the peridomestic transmission and non-immunity. The visit to the village did not identify many adults with lesions and predominance among children was well known (Tsegay, personal communication Ochollo clinic health worker, 2004). Low rate recorded in the age group above 20 years might be explained by development of immunity over time as a result of previous exposure, which is a well-known fact in CL according to previous work by Nimri, *et al*., (2002). The patients that visited the ALERT Dermatology Clinic are referred after treatment frailer or self referred patients that come from different parts of the country. Health care access problems might explain the low proportion of children under 10 visiting a tertiary Hospital facility in Addis Ababa compared to high prevalence in an endemic village in that age group. Moreover, the more distribution of lesion (70.9%) involving the nose, cheek, forehead and lip which has more disfiguring nature might have motivated the socially active younger once to report voluntarily to the center.

Patient's residence and travel history were recorded to determine the location where the infection might have taken place. It is interesting to note that, in 81 of CL patients from Addis Ababa 9 of them reported no travel history out of the city; indicating the possibility of the prevalence of CL in Addis Ababa.

The results from isoenzyme data (Fig. 7 and 8) showed that all the isolates from both study sites were *L. aethiopica*, with similar enzyme pattern as the reference strain *L. aethiopica* (MHOM/ET/72/L100). A study by Sarojini *et al*., (1984) on 104 patients, who visited the ALERT Hospital Dermatology Clinic, typed 29 isolates at the London School of Hygiene and Medicine using 13 enzyme systems all were confirmed as *Leishmania aethiopica*. Thus, our report is in agreement with previous reports, further confirmed that *L. aethiopica* is the principal aetiologic agent of CL in Ethiopia (Mengistu *et al*., 1992 and Ali *et al*., 2002). Though their presence in some parts of the country was reported, absence of *L. major* and *L. tropica* among our clinical isolates might be due to the study design, the limited sample size compared to their possible geographical distribution, the possible focal nature and magnitude of prevalence and/or difference in proportion of overt clinical cases (Gebre-Michael *et al*., 2004) as in case of Awash Valley.

In our study, PCR amplification with the genus specific primer pair 13A/13B was shown to be a sensitive method for both biopsy and isolated promastigote DNA samples. The high sensitivity of this PCR technique had also been shown in a previous study (Harris *et al*., 1998). Moreover, the technique was proven to be an excellent screening method for the presence of *Leishmania* DNA directly in clinical samples (Rodriguez *et al.*, 1994). In this line, PCR with primers 13A/13B was used to screen for the presence of *Leishmania* DNA before using the primer pairs T2/B4 and LISTR/L.58S both on DNA from biopsy and culture promastigote samples. As shown in Fig. 9 (A and B) all culture positive samples were also positive for DNA from biopsy samples suggesting that the primers are sensitive to pick *Leishmania* DNA from clinical samples too.

The result of the new *L. aethiopica* species specific primer designed from the coding region of cysteine (Cys1) genes amplified 234 bp size fragments (Fig. 13) from both *L. aethiopica* and *L. tropica* this could be due to the high homology (90%) in the sequence of Cys1 used to design the primers. It is encouraging that, we know have the full sequence of Cys1 (Sequence not shown) genes to design primers that could specifically amplify *L. aethiopica.*

In order to appraise the validity of the ITS1-PCR-RFLP method, the T2/B4 PCR-RFLP technique was used in addition to isoenzyme electrophoresis. The T2/B4 primers target a repetitive genomic sequence in *Leishmania* genome. As shown in the result, we amplified the expected 250 bp size (Fig. 10 A). A restriction analysis of the amplicon allowed the differentiation of *L. aethiopica* from all reference strains except for *L. tropica*, which is in agreement with previous reports (Minodier *et al*., 1997 and Marfurt *et al*., 2003). The result of this PCR-RFLP (Fig. 10 B) was in complete agreement with the isoenzyme result and the result from ITS1-PCR-RFLP except for *L. tropica*, which could not be identified with this primer system. Therefore, it only served, the requirement as a supportive means of species typing in the adaptation of ITS1-PCR-RFLP technique in our setting.

Amplification of the ITS-1 combined with RFLP-analysis allowed species-specific diagnosis of Old World species and New World complexes (Schonian *et al*., 2000 and El Tai *et al*., 2000). In this study, the ITS-1-PCR-RFLP was applied on both culture promastigote and biopsy DNA. As is reported, the restriction of the PCR-product with the enzyme *HaeIII* allowed the identification of our clinical isolates from the reference strains, except for differentiation from *L. tropica*. Schonian *et al.,* (2003) reported *HaeIII* digest differentiates between *L. aethiopica* and *L. tropica* but the difference between *L. aethiopica* and *L. donovani* was confusing. The other enzyme *HhaI* enabled the differentiation between all reference strains. Our result is in agreement with the result of Schonian *et al*., (2003). The difference in the result of the *HaeIII* digest might be due to a possible minor strain difference in the reference strain, *L. aethiopica* (MHOM/ET94/Gere) itself since this was not detected in our clinical isolates. It was surprising, however, to obtain three bands of 241, 97 and 78 bp size in the *HaeIII* restriction analysis of *L. infantum*. The presence of the unexpected 241 bp size could have been the consequence of partial digestion, but the electrophoresis patterns did not change when longer incubation and higher enzyme concentration were used. It could also be due to deletion or insertion of a nucleotide which changed the restriction site; this can be demonstrated by sequencing the PCR product. The results of the 21 clinical isolates were confirmed by isoenzyme electrophoresis and our isolates were distinctly different from both *L. tropica* and *L. donovani*, which were the references in both studies. Amplification of the ITS-1 in

combination with RFLP analysis direct from clinical samples showed its potential for species-specific diagnosis. This is of great benefit for a country like Ethiopia, where four *Leishmania* species (*L. aethiopica. donovani, L. tropica and L. major*) are prevalent, three of which (*L. aethiopica, L. tropica and L. major*) cause CL which makes empiric diagnosis based on clinical manifestation alone even more difficult. Therefore, the availability of such species-specific diagnostic method from clinical samples avoids the need for culture for species identification providing the information necessary to adequately treat patients. Moreover, the establishment of the isoenzyme technique formed the basis to plan for a broader countrywide prevalence studies. Currently, there are no such prevalence data, which is a requirement to plan for control measures as well as determining the relative importance of the prevalent *Leishmania* species, which is important to plan for vaccine and/or drug development and trial.

10. Conclusions

The results of the ITS1-PCR-RFLP species typing method were full agreement with the isoenzyme analysis, which is the gold standard for *Leishmania* typing. Therefore it can be recommended as a sensitive and specific method for diagnosis of *Leishmania aethiopica* at ALERT Hospital Dermatology Clinic. The method offers an advantage over the currently used techniques (Histopathology, Culture and Microscopy) in species identification and it also rules out the possibility of lepromatus leprosy, which is often confused with *L. aethiopica* caused DCL.

11. Recommendation

1. Develop a direct PCR based method which can specifically detect *L. aethiopica*. This has already been initiated and various primers from Cys1 sequence, being tested. The work will continue (Endalamaw data not included) and new primers designed (Prof. Lashitew Cys1 sequences not included).
2. The recurrence of lesions in *L. aethiopica*-HIV co-infection, the major CL aetiologic agent underlines the need for a less invasive technique for the diagnosis of patients and prognosis in course of treatments. Therefore, optimizing the ITS1-PCR-RFLP technique from cutaneous scrapings in CL patients avoids the need for biopsy, which is more prone to infection and invasive.

12. Future directions

1. Conduct an intervention study in Ochollo area as a model to control cutaneous leishmaniasis in endemic foci of Ethiopia.
2. Plan for a country wide survey of leishmaniasis to map the distribution of leishmaniasis in Ethiopia and determine the magnitude of the problem which is important to design control program.
3. Plan for molecular epidemiology to determine the local variation of the prevalent *Leishmania* species which is important to see if there is variation in the virulence of the local varieties which is important for vaccine and/or drug trial.

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Appendices I: Consent form (የስምምነት ቅጽ)

ተራ ቁጥር _____

የካርድ ቁጥር _____

ስም _____ _____

እኔ ከላይ በስም ተጠቃሹ በቆዳዬ ላይ ያለዉ (Cutaneous leishmaniasis) **ሳይሆን እንደማይቀር ተነግሮኛል። በሽታዬን ለመመርመር ከቆዳዬ ተቆንጥሮ የሚወሰድ ናሙና** (Skin scraping) **እንደሚያስፈልግ ተነግሮኛል። ይህ ናሙና የበሽታዬን ምንነት ለማወቅ የሚያስችል ምርመራ የሚካሄድበት ነዉ። በተጨማሪም ከዚሁ ናሙና የተወሰነዉ ክፍል የበሽታውን አምጪ ጥገኛ ተውሳክ ዝርያ** (*Leishmania* species) **ለመለየት ለሚደረገዉ ጥናት ስራ አላማ እንደሚዉል ተገልጾልኛል። አጥኝዉ የጥናቱን አላማ ና ባጋጣሚ**

ሊከሰቱ የሚችሉትን ጉዳቶች በሚገባ አስረድቶኛል። እኔም ስለጥናቱ በሚገባ ከተረዳሁ በሁዋላ አስፈላጊ ናቸዉ ብዬ ያሰብኩዋቸዉን ጥያቄዎች ጠይቄ አስፈላጊዉን ማብራሪያ አግኝቻሁ። በጥናቱ መረጃ መሰብሰቢያ መጠይቅ ላይ የተያዙት መረጃዎች ሁሉ በሚስጥር የሚያዙ መሆንቸዉን ና የስምምነት ቅጹም በሚስጥር ተይዞ ጥናቱ ከተጠናቀቀ ከሦስት አመት በሁዋላ እንደሚወገድ ተገልጾልኛል።

ስለራሴ የምሰጠዉን መረጃ ያለመስጠት፣ ለጥናቱ ያለመተባበር ና በየተኛዉም ወቅት ከጥናቱ እራሴን የማግለል መብቴ የተጠበቀ መሆኑን የተገለጸልኝ ሲሆን ይህንንም በማድረጌ ምክንያት በአጠቃላይ በሽታይን ለመታከም የሚያግደኝ ነገር የሌለ መሆኑን በሚገባ ተረድቻለሁ። ስለሆለም ለሐኪሙ ና ለተማሪዉ መስማማቴን በፊርማዩ ያረጋገጥኩት አጠቃላይ ሁኔታዉን በመረዳቴ ነዉ። በተጨማሪም አስፈላጊ ሆኖ ሲገኝ የተወሰደዉ ናሙና ወይም የሚገኘዉ ተዉሳክ ዝርያ ለጥናት ወደ ዉጪ ሃገረ ቢላክ ተቃዉሞ የለኝም። እንዲሁም ከጥናቱ የተረፈዉ ናሙና ወደፊት ሌላ የዚሁ ተመሳሳይ የጥናት ተግባር ላይ ቢዉል ፈቃደኛ ነኝ።

ፊርማ _____ ቀን _____

Appendix II: Questionnaire

Date___ ____ _____

I. Patient Code: AHRI number _____ ALERT number _____

II. Patient information:

1. Age_____ Sex _____

2. Period of onset of lesion: Before travel_____ after return_____ If after returns specify the gap_____

3. Clinical form _____

4. Type of lesion: Single_____ Multiple_____

5. Site(s) of lesion: Nose____ Ear___ Lip (Upper/Lower) _____ Forehead_____ If other site(s) specify_____

6. Duration of lesion_____, cutaneous scars: Present_____ Absent_____

7. Is there a change in the nasal and mucous membranes? Yes___ No__

8. Is there lymphadenopathy? Yes___ No____ if yes specify_____

9. Specimens submitted for examination:

   A. Mucosal smear, Yes____ No_____

   B. Biopsy, Yes_____ No_____

   C. Blood, Yes_____ No_____

   D. Lymph node aspirate, Yes____ No_____

10. Type and duration of treatment if any_____

11. Response to treatment_____

12. Complications

   A. Pulmonary_____

   B. Anemia _____

   C. Supper infection _____

Appendix III: Clinico-epidemiological data of samples processed in the study

A. Patients Recruited from Ochollo

S/no	Code	Sex	Age (Years)	Type of lesion	Site of lesion	Duration of lesion (Months)	Travel history
1	Ocho 1	F	13	DCL	All over the body	36	No
2	Ocho 2	M	13	LCL	Eye lid	9	No
3	Ocho 3	F	6	LCL	Nose	6	No
4	Ocho 4	F	3	LCL	Forehead	2	No
5	Ocho 6	M	3	LCL	Left cheek	6	No
6	Ocho 7	F	2	LCL	Chin	5	No
7	Ocho 8	F	4	LCL	Left cheek	3	No

8	Ocho 9	F	10	LCL	Nose	6	No
9	Ocho 11	M	1	LCL	Left cheek	2	No
10	Ocho 12	M	3	LCL	Left cheek	9	No
11	Ocho 13	F	7	LCL	Left hand, Right cheek	7	No
12	Ocho 14	M	3	LCL	Left cheek	2	No
13	Ocho 18	M	2	LCL	Right cheek	3	No
14	Ocho 33	F	4	LCL	Left cheek	9	No
15	Ocho.34	F	3	LCL	Left cheek	3	No
16	Ocho 35	M	6	LCL	Left cheek	7	No
17	Ocho 32	M	13	LCL	Cheek	12	No
18	Ocho 31	F	28	LCL	Nose	12	No
19	Ocho 30	F	20	LCL	Nose	12	No
20	Ocho 29	F	4	LCL	Forehead	2	No
21	Ocho 28	M	2	LCL	Left cheek	12	No
22	Ocho 27	F	11	LCL	Forehead	12	No
23	Ocho 26	M	10	LCL	Left cheek	48	No
24	Ocho 25	F	6	LCL	Nose	11	No
25	Ocho 24	F	8	LCL	Right arm	8	No
26	Ocho 23	M	6	LCL	Right cheek	24	No
27	Ocho 20	M	12	LCL	Right cheek	4	No
28	Ocho 21	F	9	LCL	Both cheeks	7	No
29	Ocho 22	M	9	LCL	Ear	3	No
30	Ocho 19	F	2	LCL	Right cheek	3	No
31	Ocho 17	M	2	LCL	Right cheek	8	No
32	Ocho 16	F	26	LCL	Nose	36	No
33	Ocho 15	M	1	LCL	Right cheek	1	No
34	Ocho 10	M	1	LCL	Forehead	7	No
35	Ocho 5	F	6	LCL	Forehead	5	No

B. Patients recruited from ALERT Hospital Dermatology Clinic

S/no	Code	Sex	Age (Years)	Type of lesion	Site of lesion	Duration of lesion (months)	Travel history
1	1093/02	M	26	LCL	Cheek, Lower lip	2	Magere
2	1185/02	F	7	LCL	Right arm Left cheek	12	Unknown
3	1471/03	F	16	LCL	Left leg	36	Unknown
4	1474/03	F	23	LCL	Left cheek	3	Unknown
5	1475/03	M	12	LCL	Left eye lid	2	Buei
6	1477/03	M	28	LCL	Right cheek	8	Wellega
7	1502/03	M	43	LCL	Nose	4	Unknown
8	1540/03	F	18	LCL	Nose	12	Dembidollo

9	1630/03	M	22	LCL	Face	3	D/Zeyt (Yerer)
10	16/04	M	9	LCL	Right cheek	4	Gelangora
11	112/04	M	35	LCL	Right cheek	24	Sebeta
12	195/04	M	19	LCL	Right cheek	15	No
13	208/04	F	18	LCL	Nose, Forehead, neek	9	Sodo
14	257/04	F	22	LCL	Forehead, Cheek	6	No
15	260/04	M	40	DCL	All over the body	300	Gonder
16	288/04	M	18	LCL	Right eye lid	5	No
17	292/04	F	26	LCL	Fore head, right hand	12	Butajira
18	396/04	M	20	LCL	Forehead	3	Sodo/Buei
19	430/04	M	16	LCL	Left cheek	12	Meta-abo
20	976/04	M	18	LCL	Right cheek	1	Jimma
21	1124/04	F	50	LCL	Right cheek	5	Jiru
22	420/02	M	34	LCL	Lower lip, Right cheek	18	Unknown
23	985/03	F	13	LCL	Nose Left cheek	6	Unknown
24	890/03	M	32	LCL	Nose,Forehead	6	No
25	1047/03	M	45	LCL	Right side of neck	60	Debre-zeyit
26	1114/03	M	22	LCL	Right cheek	24	Mekele
27	1116/03	M	16	LCL	Left of right eye and left cheek	8	Unknown
28	287/04	F	16	LCL	Leg	12	Unknown
29	332/04	M	38	DCL	All over the body	72	No
30	291/04	M	25	LCL	Arm	18	No
31	489/04	M	23	LCL	Nose	7	No
32	799/04	M	15	LCL	Left cheek	18	No
33	800/04	M	15	LCL	Neck	9	Gutu
34	918/04	M	28	LCL	Near left eye	12	No
35	996/04	F	8	LCL	Chin, Rght cheek	72	No
36	1028/04	F	25	LCL	Chin, arm	6	D. libanos
37	1151/04	M	31	MCL	Nose	4	No

38	1152/04	M	18	LCL	Nose	5	Bahir-dar
39	1235/04	M	25	LCL	Forehead	7	No
40	1289/04	F	17	LCL	Right cheek	6	Gonder
41	001/05	M	17	LCL	Leg	6	No
42	986/03	F	16	LCL	Lower lip,chin , left chech	4	Menz
43	998/03	M	15	LCL	Nose	12	Silti
44	1007/03	M	18	LCL	Nose	9	Wolkite
45	1024/03	F	35	LCL	Nose	24	Unknown
46	1048/03	M	19	LCL	Forehead	12	shewa-robit
47	1060/03	M	19	LCL	Right arm	12	Gonder
48	1064/03	M	62	LCL	Nose	8	Unknown
49	1083/03	M	51	LCL	Face	12	Sebeta
50	1111/03	M	20	LCL	Right hand, Right check	6	No
51	1112/03	M	35	LCL	Forehead	6	Unknown
52	1113/03	M	13	LCL	Left cheek	48	Desie
53	1115/03	M	31	LCL	Right hand	8	Unknown
54	1117/03	M	56	LCL	Nose	3	D/zeit
55	1127/03	M	25	LCL	Upper lip	8	Sela-digay
56	1139/03	M	15	LCL	Lower lip	36	No
57	1254/03	M	18	LCL	Forehead	36	No
58	1255/03	M	25	LCL	Ear	24	Tigray
59	1256/03	M	40	LCL	Forehead	24	Sheka
60	1298/03	M	20	LCL	Nose	4	D/libanos
61	1386/03	M	25	LCL	Nose	6	Unknown
62	1387/03	F	55	LCL	Right cheek	6	Arsi
63	1388/03	F	13	LCL	Lower lip	6	Tigray
64	1404/03	F	16	LCL	Nose	12	No
65	1398/03	M	36	LCL	Nose	6	Welkit
66	1411/03	F	58	LCL	Face, Leg	6	No
67	1409/03	M	30	LCL	Nose, chin	4	No
68	1412/03	M	12	LCL	Upper lip	12	No
69	1413/03	M	22	LCL	Nose	24	Unknown
70	1461/03	F	12	LCL	Nose	48	Semen-shewa

71	1476/03	M	10	LCL	Left cheek	6	No
72	1499/03	M	20	LCL	Nose	9	No
73	1501/03	M	25	LCL	Lip	48	Tigray
74	1539/03	M	23	LCL	Cheek	12	Weliso
75	1522/03	M	51	LCL	Left cheek	7	Yerga-chefe
76	1548/03	M	18	LCL	Cheek,Ear, Arm	36	Unknown
77	1553/03	M	26	LCL	Upper lip	7	Bonga
78	1583/03	F	19	LCL	Nose, Forehead	12	Desie
79	1677/03	M	14	LCL	Chin	12	No
80	1678/03	F	9	LCL	Nose	12	No
81	53/04	M	9	LCL	Right cheek	8	No
82	40/04	F	16	LCL	Nose	12	Lemen(Tere)
83	56/04	F	16	LCL	Lower lip, Right cheek	12	Tigray
84	57/04	F	29	LCL	Upper lip	3	Unknown
85	58/04	M	50	LCL	Arm, Chin	4	Wello
86	94/04	M	25	LCL	Face	3	Unknown
87	118/04	M	25	LCL	Arm, Chin	4	No
88	125/04	F	19	LCL	Arm, Face	12	Unknown
89	130/04	M	15	LCL	Left cheek	12	Unknown
90	134/04	F	15	LCL	Upper lip	5	No
91	190/04	M	29	LCL	Right cheek, Ear	2	Merabete
92	191/04	M	33	LCL	Forehead	8	Adigrat
93	192/04	F	15	LCL	Nose	4	Unknown
94	193/04	F	14	LCL	Forehead	12	Adigrat
95	194/04	M	6	LCL	Forehead	12	No
96	196/04	F	55	LCL	Nose	4	Butajira
97	199/04	M	24	LCL	Lower lip	12	No
98	240/04	M	15	LCL	Left cheek	2	No
99	250/04	M	73	LCL	Right cheek	8	Unknown
100	258/04	F	18	LCL	Left cheek, Chin	12	Unknown
101	259/04	M	12	LCL	Leg	6	Sodo
102	275/04	M	5	LCL	Left cheek	9	Shenkora
103	284/04	M	14	LCL	Nose	12	No
104	285/04	F	14	LCL	Nose, Forehead	6	Weliso

105	286/04	M	25	LCL	Forehead	5	No
106	289/04	M	55	LCL	Nose, cheek	5	No
107	290/04	M	23	LCL	Nose, Ear, right cheek	12	No
108	301/04	F	15	LCL	Nose	12	No
109	322/04	F	16	LCL	Nose	4	No
110	329/04	F	27	LCL	Nose	6	No
111	330/04	M	27	LCL	Nose	6	No
112	331/04	F	5	LCL	Forehead	24	No
113	353/04	M	14	LCL	Upper lip	24	No
114	361/04	M	9	LCL	Forehead, Cheek	24	Bulga
115	367/04	M	12	LCL	Upper lip	48	Unknown
116	382/04	M	18	LCL	Face	24	No
117	450/04	M	38	LCL	Face	12	Unknown
118	465/04	F	16	LCL	Nose	24	Unknown
119	489/04	M	23	LCL	Nose	7	No
120	490/04	M	44	LCL	Nose	3	D/libanos
121	550/04	M	20	LCL	Left cheek	24	No
122	563/04	F	40	LCL	Eye lid	3	Unknown
123	573/04	F	10	LCL	Nose	24	No
124	584/04	F	30	LCL	Nose	5	No
125	585/04	M	9	LCL	Right cheek	16	No
126	630/04	F	22	LCL	Arm	12	D/libanos
127	636/04	F	15	LCL	Nose	4	No
128	718/04	M	38	LCL	Right hand	12	No
129	733/04	F	15	LCL	Nose, Lip	3	No
130	734/04	F	10	LCL	Forehead	4	No
131	740/04	F	18	LCL	Forehead	4	Unknown
132	816/04	F	60	LCL	Nose	12	Sodo
133	848/04	F	16	LCL	Nose, Left cheek	16	No
134	865/04	M	19	LCL	Nose	12	No
135	860/04	M	20	LCL	Nose	36	No
136	870/04	M	21	LCL	Left cheek	4	No
137	897/04	F	7	LCL	Right arm	12	No

138	907/04	M	8	LCL	Left cheek	12	No
139	973/04	M	20	LCL	Right cheek	8	No
140	998/04	F	9	LCL	Nose	9	No
141	999/04	F	19	LCL	Nose, Left hand	7	No
142	1001/04	M	14	LCL	Forehead	48	No
143	1004/04	M	15	LCL	Nose, Right cheek	36	No
144	1007/04	F	22	LCL	Left cheek	4	Unknown
145	1008/04	M	24	LCL	Nose	2	No
146	1021/04	M	17	LCL	Right cheek	12	No
147	1025/04	M	25	LCL	Nose	8	Unknown
148	1026/04	F	57	LCL	Nose	12	No
149	1048/04	M	6	LCL	Right cheek	12	No
150	1066/04	M	18	LCL	Right cheek	48	No
151	1067/04	M	25	LCL	Left cheek	24	Tigray
152	1125/04	F	13	LCL	Nose	6	No
153	1148/04	M	15	LCL	Right cheek	12	Unknown
154	1179/04	F	6	LCL	Forehead	12	No
155	1180/04	M	42	LCL	Forehead	12	No
156	1227/04	F	45	LCL	Right cheek	12	No
157	1229/04	F	15	LCL	Right cheek	12	No
158	1239/04	M	30	LCL	Nose	4	Welega & Welkite
159	1257/04	F	15	LCL	Face	36	No
160	1270/04	F	9	DCL	All over the body	24	No

M, Male; F, Female; LCL, Localized cutaneous leishmaniasis; DCL, Diffused cutaneous leishmaniasis; MCL= Mucocutaneous leishmaniasis; AA= Addis Ababa

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 the thesis have been duly acknowledged.

Name of the candidate Endalamaw Gadisa

Signature _____

Place Addis Ababa

Date May, 2005

This thesis has been submitted for examination with my approval as a University advisor,

Name of advisor Dr. Kifle Dagne

Signature _____