MOLECULAR CHARACTERIZATION OF SUPEROXIDE DISMUTASE GENE FROM *LEISHMANIA AETHIOPICA*

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Table of Contents

Page

ACKNOWLEDGMENT I

LIST OF TABLES V

LIST OF APPENDICES VI

ABSTRACT IX

1. INTRODUCTION 1

1.1. LEISHMANIASIS 2

1.1.1. The disease *2*

1.1.2. Epidemiology *3*

1.1.3. Life Cycle *4*

1.1.4. Clinical Forms *6*

1.1.5. Diagnosis *8*

1.2. CUTANEOUS LEISHMANIASIS IN ETHIOPIA 9

1.2.1. The Parasite *9*

1.2.2. Geographical Distribution *9*

1.2.3. Vectors and Reservoir Hosts *10*

1.2.4. Clinical Features *11*

1.2.5. Prevention and Control *12*

1.3. LEISHMANIA – MACROPHAGE INTERACTION 13

1.3.1. Leishmanial Invasion and Activation of Macrophages *14*

1.3.2. Macrophage Response Mechanisms *16*

1.3.3. Evasion Strategies of Leishmania *20*

1.4. SUPEROXIDE DISMUTASE 22

1.4.1. Structure of SOD *23*

1.4.2. Types of SOD *24*

1.4.3. Mechanism of Action *25*

1.4.4. Use of SOD in Aerobic Life *26*

1.4.5. Genetics of SOD *28*

2. OBJECTIVES 30

2.1. GENERAL OBJECTIVE 30

2.2. SPECIFIC OBJECTIVES 30

3. MATERIALS AND METHODS 31

3.1. STUDY POPULATION 31

3.2. SPECIMEN COLLECTION 31

3.3. MEDIA PREPARATION 32

3.4. PARASITE CULTURE 32

3.5. REFERENCE STRAINS USED 34

3.6. SPECIES TYPING 34

3.6.1. Isoenzyme Electrophoresis *34*

3.6.2. PCR-RFLP *36*

3.7. CHARACTERIZATION OF SUPEROXIDE DISMUTASE GENE 37

3.7.1. DNA Extraction *37*

3.7.2. Polymerase Chain Reaction *38*

3.7.3. Gel Electrophoresis *39*

3.7.4. DNA Purification *40*

3.7.5. Restriction Digestion of the PCR Product *40*

3.7.6. Sequencing *41*

4. RESULT 42

4.1. STUDY POPULATION 42

4.2. SPECIMEN COLLECTION 42

4.3. SPECIES TYPING 43

4.3.1. Isoenzyme Electrophoresis *43*

4.3.2. PCR-RFLP *44*

4.4. CHARACTERIZATION OF SOD GENES 46

4.4.1. Polymerase Chain Reaction *46*

4.4.2. Restriction Digestion of SOD Genes *47*

4.4.3. Sequencing *49*

4. DISCUSSION 52

CONCLUSION 58

REFERENCES 59

APPENDIX 65

List of Figures

Page

Figure 1. Life Cycle of *Leishmania* 6
Figure 2. Assembly/Activation of the NADPH Oxidase System 18
Figure 3. Synthesis of Nitric Oxide from L-arginine 19
Figure 4. Structure of the Active Site of FeSOD 23
Figure 5. Isoenzyme Electrophoresis of GPI. 43
Figure 6. PCR Product of Amplification of the Repetitive Genomic DNA. 45
Figure 7. Restriction Digestion Pattern of the PCR Product. 46
Figure 8. First PCR Products of SODB1 46
Figure 9. First PCR Products of SODB2 47
Figure 10. Restriction Digestion of SODB1 and SODB2 48
Figure 11. Multiple Amino Acid Sequence Alignment of *Leishmania* Iron SODs 50

List of Tables

Page

Table 1. Clinico-Epidemiological Data of the Study Subjects 42
Table 2. Zymogram of the Different *Leishmania* Strains. 44
Table 3. Comparison of Similarity of LaethFeSODs with SODs of other organisms 51

List of Appendices

Page

Appendix 1. Data Acquisition Format..65

Appendix 2. Multiple Nucleic Acid Alignment of *Leishmania* SODs....................67

List of Abbreviations

AHRI - Armauer Hansen Research Institute

AIDS - Acquired Immunodeficiency Syndrome

ALERT - All African Leprosy Rehabilitation and Training Center

C3 - Complement type-3

CL - Cutaneous Leishmaniasis

CR - Complement Receptor

CuSOD - Copper- Superoxide Dismutase

DCL - Diffuse Cutaneous Leishmaniasis

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic acid

dNTP - Deoxyribonucleotide Triphosphate

EDTA – Ethylenediamine tetra-acetic acid

FCS - Fetal Calf Serum

FeSOD - Iron- Superoxide Dismutase

GP - Glycoprotein

GPI - Glucose Phosphate Isomerase

GFP – Green Florescent Protein

HIV - Human Immunodeficiency Virus

IE – Isoenzyme Electrophoresis

IL - Interleukin

INF-γ - Interferon - Gamma

iNOS - Inducible Nitric Oxide Synthase

LaethFeSOD – *Leishmania aethiopica* iron-containing superoxide dismutase

LcFeSOD – *Leishmania chagasi* iron-containing superoxide dismutase

LC - Langerhan Cell

LCL - Localized Cutaneous Leishmaniasis

LmFeSOD - *Leishmania major* iron-containing superoxide dismutase

LPG - Lipophosphoglycan

MCL - Mucocutaneous Leishmaniasis

MnSOD - Manganese- Superoxide Dismutase

NAD – Nicotinamide Adenine Dinucleotide

NADP - Nicotinamide Adenine Dinucleotide Phosphate

NADPH – Reduced Nicotinamide Adenine Dinucleotide

NBT - Nitro Blue Tetrazolium

NiSOD - Nickel- Superoxide Dismutase

NNN - Novy-MacNeal-Nicolle

ORF - Open Reading Frame

PCIA - Phenol Chloroform Isoamyl Alcohol

PCR - Polymerase Chain Reaction

PKDL - Post Kalazar Dermal Leishmaniasis

PMS - Phenazine Methosulfate

PS - Penicillin-Streptomycin

RFLP - Restriction Fragment Length Polymorphism

RNI - Reactive Nitrogen Intermediate

ROI - Reactive Oxygen Intermediate

RPMI – Roswell Park Memorial Institute

Rxn - Reaction

SDS - Sodium Dodecyl Sulfate

SOD - Superoxide Dismutase

TBE - Tris Borate EDTA

TNF-α - Tumor Necrosis Factor -Alpha

VL - Visceral Leishmaniasis

WHO - World Health Organization

ZnSOD - Zinc- Superoxide Dismutase

ABSTRACT

This study was performed with the aim of characterizing the genes that code for superoxide dismutase in *Leishmania aethiopica*. It involved three main steps: specimen collection and parasite isolation, species typing, and molecular characterization of the SOD genes. A total of 20 suspected cutaneous leishmaniasis patients were enrolled in the study. Clinical and epidemiological information was collected from all the subjects including age, sex, address, duration of the lesion, and site of lesion. Skin slit was taken from the active lesion(s) and was subjected to *in vitro* parasite culture on NNN medium. Promastigote stage of *Leishmania* was then isolated and characterized. Species typing was done on the isolate using isoenzyme electrophoresis and PCR-RFLP. Total genomic DNA was extracted from the promastigotes and then the SOD genes were amplified using polymerase chain reaction. The amplified fragments were then sequenced and the sequence information was analyzed using vector NTI software.

The study subjects came from different parts of the country including Addis Ababa. The mean age of the patients was 25.6 year with a range of 7 to 65 years. Out of 20 skin slit specimens cultured and processed from suspected cutaneous leishmaniasis patients enrolled in the study, five (25%) were found to be positive for motile promastigotes. Isoenzyme electrophoresis and PCR-RFLP results of one isolate (1093/02) confirmed that the isolate is *L. aethiopica*. The SODB genes amplified from promastigotes of *L. aethiopica* were similar in size as the SODB genes of other *Leishmania* species. Nucleotide sequences of LaethFeSODB1 showed 94.9% and 73% identity with LcFeSODB1 and LmFeSODB1 respectively. On the other hand, predicted amino acid sequence comparison indicated that LaethFeSODB1 had 89.8% and 90.8% identity with LcFeSODB1 and LmFeSODB1 respectively. The high degree of amino acid sequence similarity of *L. aethiopica* SODB genes with those of other species has an important implication in the use of new antileishmanial drugs that are targeting SODs of other *Leishmania* species to treat cutaneous leishmaniasis in Ethiopia.

Key words: *Leishmania aethiopica*, superoxide dismutase, gene, Ethiopia

1. INTRODUCTION

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania*. It has a wide range of clinical manifestations, ranging from self-healing cutaneous type to the most severe visceral form that is associated with high morbidity and mortality.

In Ethiopia, visceral and cutaneous leishmaniases are caused mainly by *L. donovani* and *L. aethiopica* respectively. Cutaneous leishmaniasis (CL) ranges from localized self-healing type to the disfiguring mucocutaneous and diffuse cutaneous types.

To date, no effective drug or vaccine is available for Ethiopian cutaneous leishmaniasis. The ones currently in use are not efficacious and/or induce severe complications. Thus, the need to develop a drug or vaccine against this disease is of paramount importance.

In order to escape from the host immune response and survive inside host cells, *Leishmania* utilizes several evasion mechanisms. One among these is the detoxification of oxygen radicals produced by the immune cells. The metalloenzyme, superoxide dismutase (SOD) is used in the first stage of this process. The enzyme converts superoxide to hydrogen peroxide.

Several studies have been performed on the genomics and proteomics of superoxide dismutase in order to understand the role of this enzyme in the survival of *Leishmania* and to test whether it could be a possible target for new antileishmanial drugs. These studies have confirmed that superoxide dismutase plays an important role in host-

parasite relationship in leishmaniasis suggesting the possible use of it as target for intervention.

The whole genome of *L. aethiopica* in general and the SOD genes in particular have not been characterized yet. This, in effect, will limit the development of new drugs or vaccines or the use of the newly emerging ones against CL in Ethiopia. This work, therefore, aimed at characterizing *L. aethiopica* with respect to the genes encoding superoxide dismutase.

1.1. Leishmaniasis

1.1.1. The disease

Leishmaniases are parasitic diseases caused by protozoan parasites belonging to the order *kinetoplastida* and genus *Leishmania*. More than 20 species have been reported as etiologic agent of the diseases (El Hassan, 2001).

The parasite is transmitted by the bite of female sandflies, which are of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. About 30 species of sandflies are proven vectors of leishmaniasis; the usual reservoir hosts include humans and domestic and/or wild animals (El Hassan, 2001).

Most leishmaniases are zoonotic (transmitted to humans from animals), humans being infected only secondarily, but in antroponotic forms (those transmitted from humans to humans), humans are believed to be the unique reservoir. Female sandflies become infected by feeding from reservoir hosts (Desjeux, 1996).

1.1.2. Epidemiology

According to WHO report (2000), leishmaniases are endemic in 88 countries in Africa, Asia, Europe, North America and South America. In these countries, a total of 350 million people live at risk of infection. Of the 88 countries, 16 are developed, 72 are developing and 13 of them are among the least developed. It is believed that 12 million people are affected by leishmaniasis worldwide. Furthermore, 1.5 to 2 million new cases of leishmaniasis are estimated to occur annually. Of this, 500, 000 are visceral leishmaniasis (VL) and 1 to 1.5 million are cutaneous leishmaniasis (CL).

The geographical distribution of leishmaniasis is limited by the distribution of the sandfly, its susceptibility to cold climates, its tendency to take blood from humans or animals only and its capacity to support the internal development of specific species of *Leishmania*.

Since 1993, *Leishmania* endemicity has expanded significantly, accompanied by a sharp increase in the number of recorded cases. It is partly due to rural-urban and agro industrial development projects that bring non-immune urban dwellers into endemic rural areas. Man-made projects with environmental impact, like dams and irrigation systems also contribute to the spread of leishmaniasis (WHO, 2000).

The emergence of HIV/AIDS pandemic has increased the risk of *Leishmania*-infected people developing leishmaniasis. The risk of *Leishmania*/HIV co-infection has increased as a result of changes in the pattern of spread of the two infections.

Leishmaniasis and HIV/AIDS are spreading from the rural and urban areas respectively (WHO, 2000). In certain countries like Brazil, VL is becoming more urbanized and simultaneously in countries of East Africa, such as Ethiopia and Kenya, HIV infection is becoming progressively more common in rural areas (Desjeux, 1996 and WHO, 2000).

1.1.3. Life Cycle

Leishmania is an obligate intracellular parasite. It has digenic life cycle consisting of a uniflagellated promastigote stage, which resides within the alimentary tract of the sandfly vector and the amastigote stage, which multiplies inside phagolysosomes of mammalian macrophages (Mosser and Brittingham, 1997).

As it is shown in Figure-1 below the vectors introduce infective (metacyclic) promastigotes into the subcutaneous tissue of man during blood meal. Then, the promastigotes are quickly taken up mainly by localized tissue phagocytes (macrophages) and also by monocytes and neutrophils brought to the site due to infection. Within macrophages, promastigotes lose their flagellum and transform themselves into nonmotile amastigotes. The amastigotes survive and replicate within the acidic environment of the phagolysosome, eventually lysing the infected cell and freeing themselves to infect nearby cells. When a sandfly acquires a blood meal from an infected host, it acquires either free amastigotes or amastigote- infected mononuclear cells. The amastigotes in the midgut of the sand fly develop into log-phase (procyclic) promastigotes and finally to metacyclic ones ready to infect a new host (Mosser and Brittingham, 1997).

In zoonotic leishmaniasis, wild animals such as rodents and domestic animals such as dogs are important reservoirs of the parasite. Whereas, in anthroponotic forms, humans are believed to be the unique reservoir.

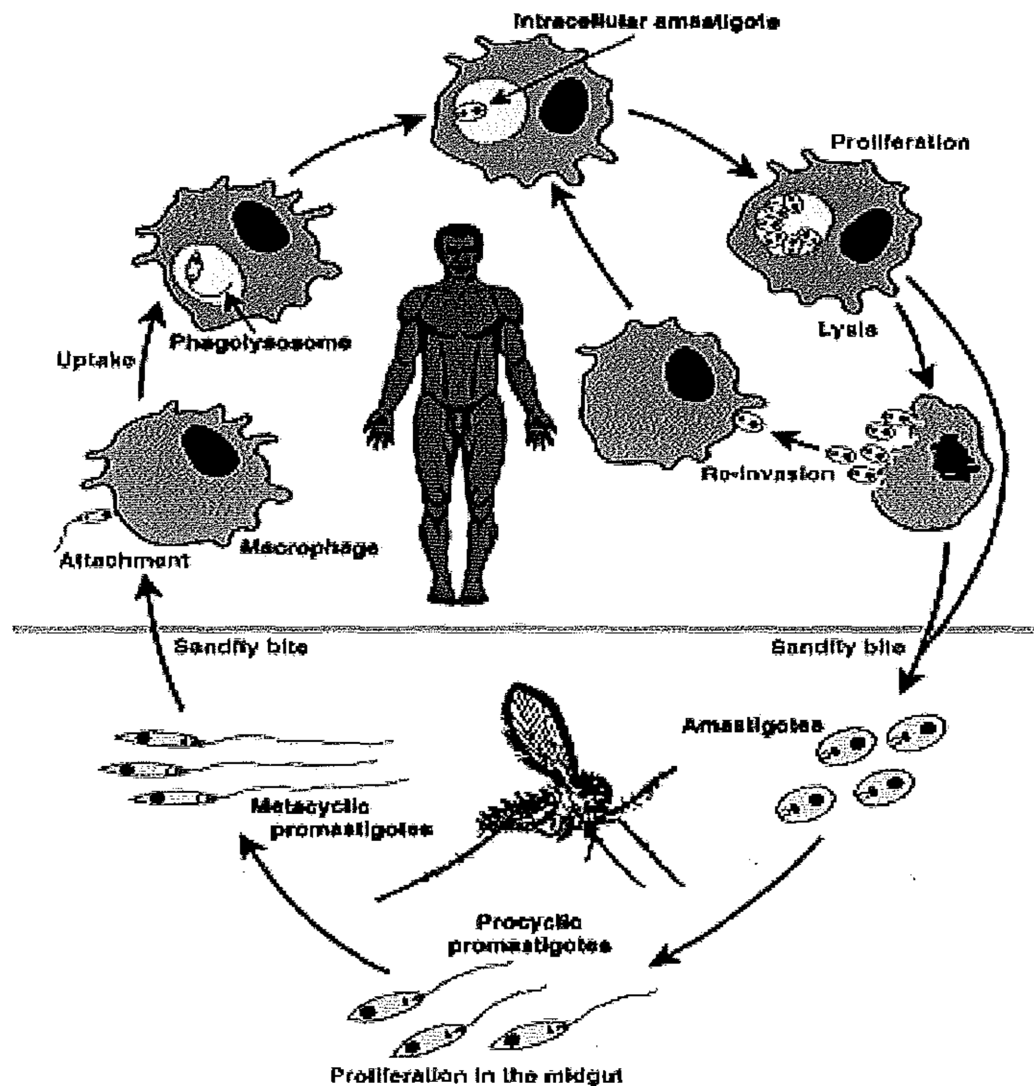


Figure 1. Life Cycle of *Leishmania*
From: Handman (2001)

1.1.4. Clinical Forms

Leishmaniasis presents itself in humans in four major forms with a broad range of clinical manifestations: visceral, localized cutaneous, diffuse cutaneous and mucocutaneous leishmaniases.

Visceral leishmaniasis (VL), also known as Kala azar, is the most severe form of the diseases. If untreated, it has a mortality rate of almost 100%. This type of leishmaniasis is caused by *L. donovani* and *L. infantum*. It is characterized by irregular fever, substantial weight loss, swelling of the spleen and liver and anemia. It causes large-scale epidemics with a high fatality rate. After recovery, patients may develop a chronic CL form called post kala azar dermal leishmaniasis (PKDL), which usually requires a prolonged duration and expensive treatment.

Mucocutaneous leishmaniasis (MCL) produces lesions which can lead to extensive destruction of mucus membranes of the nose, mouth and throat cavities, mutilation of the face, and great suffering for life. It is mostly related to *Leishmania* species of the New World such as *L. braziliensis*, *L. panamensis*, and *L. guyanensis*, but mucosal lesions have been reported in the Old World due to *L. infantum* in immunosuppressed patients and due to *L aethiopica*, *L. donovani*, *L. major*.

Localized cutaneous leishmaniasis (LCL) produces skin ulcers on the exposed part of the body, such as the face, arms and legs, leaving the patient permanently scarred. In the Old World, LCL is mostly due to *L. major* and produces self-healing lesions. It also creates a life long stigma when the lesions are multiple and disabling with disfiguring scars. In *L. major* infection, the early lesion starts as a painless papule that soon becomes a nodule or an ulcer with a flat base and slightly raised margin. Characteristically the nodulo-ulcerative lesion has a volcanic appearance and the major part may be in the subcutaneous tissue. Secondary bacterial and fungal infections are common in ulcerated lesions. Cutaneous leishmaniasis due to *L. tropica*

is usually more chronic. The lesion starts as a nodule that eventually forms a crusted ulcer over a period of 6 months. The healing may take up to 2 years. A less common form of leishmaniasis caused by *L. tropica* is the recidiva or relapsing leishmaniasis in which, following healing of the original cutaneous lesion, there is recurrence of active disease on the edge of the scar. This type of lesion is very difficult to treat, often becoming long-lasting, destructive and disfiguring.

In *L. aethiopica* infection there is usually a single ulcer often situated in the central part of the face. In the New World, *L. mexicana* usually produces relatively benign lesions but some locations such as the ear's pinna are very difficult to treat in general.

Diffuse cutaneous leishmaniasis (DCL), due to *L. aethiopica* and *L. amazonensis*, occurs rarely and is associated with leishmanial antigen- specific defective cell mediated immune response. Its severity is due to disseminated lesions that resemble those of lepromatous leprosy, which never heals spontaneously and often relapse after treatment (Desjeux, 1996 and El Hassan, 2001).

1.1.5. Diagnosis

Diagnosis of leishmaniasis involves clinical investigation of the suspected patient and laboratory identification of the parasite. Cutaneous lesions and for many geographical areas, visceral diseases are often diagnosed clinically with considerable accuracy and reliability. In these areas, the diseases appear as epidemic or endemic and are relatively easily differentiated from similar conditions by local health workers. Diagnosis becomes a major challenge when a case is displaced to a new non-endemic

area or when different signs and symptoms appear because of superinfection with other agents.

Definitive diagnosis is based on demonstration of parasite in direct smear, or in animal or culture inoculation. This can be done on samples taken directly from the lesion (CL and MCL) or lymph node, bone marrow and spleen (VL) (Moncayo *et al*, 1994). Lymph node aspiration is by far the most convenient method for VL. Lymphadenopathy is an important clinical feature of VL in many parts but parasites may also be found in lymph nodes that are not enlarged. Bone marrow aspiration is painful and unlike lymph node or splenic aspiration, is often not preferred by patients. Splenic aspiration is generally accepted as the most sensitive method but must be done with care and according to the recommended method (Moncayo *et al*, 1994).

1.2. CUTANEOUS LEISHMANIASIS IN ETHIOPIA

1.2.1. The Parasite

Cutaneous leishmaniasis in Ethiopia is caused mainly by *L. aethiopica* and less frequently by *L. major* and *L. tropica* (Hailu and Frommel, 1993). The results of isoenzyme electrophoresis have confirmed *L. aethiopica* as a separate species from other leishmanias. Out of 13 enzymes tested using starch - gel electrophoresis, only one enzyme pattern was held in common with the *L. tropica* reference strain enzyme profile, and none with *L. major* reference strain (Le Blancq *et al*, 1986).

1.2.2. Geographical Distribution

In Ethiopia, cutaneous leishmaniasis caused by *L. aethiopica* is endemic and widespread at altitudes between 1,400 and 2,700m above sea level (Hailu and

Frommel, 1993). Prevalence rates of active infection between 5.5 and 40 per 1,000 population was reported from villages in the former Shewa, Wollo and Gamo Gofa regions, with the highest rates in Ocholo village in Gamo Gofa (Ashford, *et al*, 1973; Hailu and Frommel, 1993).

According to Mengistu *et al* (1992), the overall prevalence of LCL in Ocholo was 3.6 -4.0%. In this community, scar of LCL was present in 34.3% of the residents and leishmanin skin test was positive in 54% of 120 school children without signs of the disease. The age distribution confirmed that 80% of LCL cases with active infection fall in age group 0 to 10 years. Both sexes were found to be equally affected. According to hospital reports of Sarojini *et al* (1984), out of 104 CL patients, 59.6% were between ages of 10 and 29 years.

High prevalence rates and clustering of cases in some households and neighborhoods in Ocholo have been attributed to the proximity of hyrax colonies and resting sites of *Phlebotomus pedifer* to human dwellings (Ashford, *et al*, 1973; Hailu and Frommel, 1993).

1.2.3. Vectors and Reservoir Hosts

The main vectors of CL in Ethiopia are *Phlebotomus longipes* and *Phlebotomus pedifer* (Hailu and Frommel, 1993). These species of sandflies do not serve as vectors of leishmaniasis anywhere else. Two species of hyraxes, *Procavia habessinica* and *Hetrohyrax brucei*, are the major animal reservoir hosts for *L. aethiopica* (Hailu and Frommel, 1993). These animals serve as the main source of blood meal for the

vectors. The prevalence of *Leishmania* among these hyraxes in Ethiopian was found to be 21%, 23%, and 27% in Ocholo, Kutaber, and Aleku repectively (Ashford, *et al*, 1973). Rock holes, caves and tree holes where hyraxes live are also used as shelters by *P. longipes* and *P. pedifer*. Infection rates in man vary from place to place depending primarily on the proximity of hyrax colonies and vector habitats to human dwellings. Human activities such as land use, and environmental factors, including vegetation type, topography, as well as wind direction and intensity are also implicated. Humans intrude into the zoonotic cycle in various ways to the extent of settling in hyrax colonies (Ashford, *et al*, 1973; Hailu and Frommel, 1993).

It has been shown that isoenzyme electrophoresis patterns of *L. aethiopica* isolated from *Phlebotomus longipes* and *Procavia habessinica* were indistinguishable from those isolated from man (Le Blanq *et al*, 1986).

1.2.4. Clinical Features

In Ethiopia, cutaneous leishmaniasis is manifested in three clinical forms:

1. Localized cutaneous leishmaniasis (LCL)
2. Diffuse cutaneous leishmaniasis (DCL)
3. Mucocutaneous leishmaniasis (MCL)

LCL is characterized by lesions most often single, which are mainly localized in areas uncovered by clothing: the face, nose, ears, and forearms. The lesion presents as a reddish plaque with irregular boarder, and commonly with a shallow ulcer in its center; it tend to self-heal within 8 to 10 months, leaving a hyperpigmented scar, but may persist for several years (Hailu and Frommel, 1993).

MCL caused by *L. aethiopica* involves the mucocutaneous junctions of the nose and/or the mouth, likely by direct extension from skin lesions, differing thus from the metastasizing spread of the organism observed with the New World *Leishmania* species (Hailu and Frommel, 1993).

DCL is one type of manifestation of *Leishmania aethiopica* infection leading to the formation of multiple, raised, cutaneous nodules on the face, torso, and extremities. The disease is often disfiguring, and may confuse with lepromatous leprosy. DCL patients often fail to respond to conventional antileishmanial chemotherapy (Akuffo *et al*, 1990).

1.2.5. Prevention and Control

Prevention and control of CL in Ethiopia relies mainly on the control of the reservoir host and the vector as well as the use of chemotherapy (Hailu and Frommel, 1993). Relatively speaking, the man-hyrax anthropozoonotic leishmaniasis could be prevented by controlling the population of rock hyraxes for hyraxes are colonial animals having poor reproductive performance.

Vector control with chemicals does not seem to be promising because *P. pedifer* and *P. longipes* live in deep rock holes and crevices. They are opportunistic and use many alternatives resting sites, including tree holes and abandoned houses (Hailu and Frommel, 1993).

Chemoprophylactic approach is not feasible for use against highland CL due to *L. aethiopica*. That is because the parasite is resistant to most anti-leishmanial compounds except pentamidine, which is also a drug with major side effects. In addition, no new drug that is effective against *L. aethiopica* is available yet (Hailu and Frommel, 1993; Sarojini *et al*, 1984).

1.3. LEISHMANIA – MACROPHAGE INTERACTION

Leishmania parasitizes man when the parasite enters and multiplies inside macrophages. Here a complex phenomenon occurs that involves two oppositely functioning arms: the pathological effect of the parasite and the defense mechanisms of the host (Bogdan *et al.*, 1996 and Richard *et al.*, 1994).

In order to combat leishmanial infection, the host mobilizes its specific and non-specific immune responses. The non-specific type ranges from the effect of complement to the different effector mechanisms of macrophages. The first line of defense against *Leishmania* is mediated by the complement system. The third component of the complement system (C3) opsonizes the parasite and leads to the formation of a membrane attack complex that in turn leads to channel formation and disruption of the cytoplasmic content and eventually death of the parasite (Pearson and Steingbigel, 1980).

The macrophages also play a major role in the non-specific immunity against *Leishmania*. These cells devise several mechanisms that modify the physical and chemical environment in which the parasite lives. Macrophages produce reactive

oxygen intermediates (ROIs), reactive nitrogen intermediates (RNIs) and several enzymes that exert deleterious effects on the infecting parasite. ROIs such as superoxide, produced by the NADPH-oxidase system in the plasma membrane that forms phagolysosome, oxidatively act on the parasite to impair its functions. RNIs likewise produced by the nitric oxide synthase pathway damage parasite molecules such as enzymes of the central metabolic pathways, DNA and membranes (Robinson and Badway, 1994; Woods *et al*, 1994).

On the other hand, *Leishmania* has developed a variety of ways that enable it escape from the host's defense mechanisms. These include, scavenging oxidative metabolites, and inhibiting their synthesis as well as modulation of the immune system (Bogdan *et al.*, 1996).

1.3.1. Leishmanial Invasion and Activation of Macrophages

In order to successfully parasitize the host cell, *Leishmania* utilizes several mechanisms of attachment and entry. The two best characterized molecules on the surface of the parasite that are used for attachment and subsequent internalization to macrophages are; the glycolipid (lipophosphoglycan) and the glycoprotein (gp63). These molecules are extremely abundant on the surface of promastigotes ($1.25\text{x } 10^6$ and $5\text{x}10^5$ copies per cell respectively) (Talamas-Rohana *et al*, 1990). Lipophosphoglycan (LPG) of *L. major* has multiple, phosphorylated tri- and tetra-saccharide units containing galactose, mannose, glucose and arabinose that effect attachment to the host cell. Gp63 contains a Ser-Arg-Tyr-Asp sequence which mimic the cell attachment sequence Arg-Gly-Asp-Ser (RGDS) of fibronectin. Both LPG and

gp63 are anchored in the membrane by phosphatidylinositol tails (Keller *et al.*, 1995; Russell and Talamas-Rohana, 1989).

Several receptor molecules on the surface of macrophages are involved in mediating the attachment and uptake of *Leishmania.* These include, complement type 1, complement type 3 and mannose/fucose receptors (Richard *et al*, 1994). C3b and iC3b bind to LPG and gp63 on the surface of promastigotes via an ester linkage. These bound complement components will then be attached to the respective receptors on macrophages. LPG binds with CR1 and CR3 via C3b and iC3b, whereas gp63 binds with CR1 via C3b opsonization (Kelleher *et al.*, 1995).

Macrophages are central cells in the interactions of man and *Leishmania.* These cells serve as; a site of replication for amastigote stages of the parasite, antigen presenting cells and effector cells. The effector function is responsible for the recovery of the host from the disease, leishmaniasis.

In order to effect microbicidal activity macrophages are activated by cytokines that are produced by *Leishmania*-specific Th1 cells. According to Crawford *et al* (1994), the activation of macrophage for induction of antimicrobial immunity is influenced by two groups of signals: primary signals such as INF-γ and secondary signals such as TNF-α.

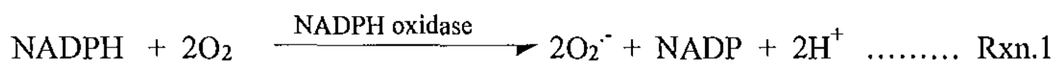
1.3.2. Macrophage Response Mechanisms

The various secretory or phagolysosomal products of macrophages are usually sufficient to neutralize, degrade and eliminate most foreign bodies. However, infectious pathogens like *Leishmania* resist this first line of defense and thrive within the phagolysosome. Upon activation, macrophages produce a variety of chemically reactive substances. The most prominent ones include reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). Two key macrophage enzymes responsible for generating these toxic intermediates, phagocytic (NADPH) oxidase and nitric oxide synthase, are together critically important for host antimicrobial defense.

1.3.2.1. Reactive Oxygen Intermediates

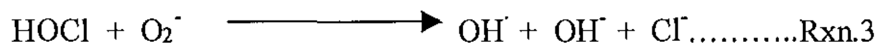
Reactive oxygen intermediates such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^·$) and singlet oxygen ($^1O_2$) are effector molecules used by macrophages for their activity against a variety of intracellular pathogens. Macrophages produce massive amounts of superoxide and hydrogen peroxide during phagocytosis. This is accompanied by a large increase in the consumption of oxygen and has, therefore, been termed as "respiratory burst" (Robinson and Badway, 1994).

Reactions that produce ROIs are summarized as follows:

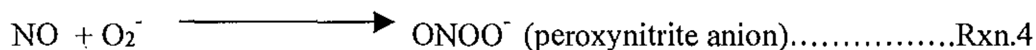


$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidase}} 2O_2^{·-} + NADP + 2H^+$ Rxn.1

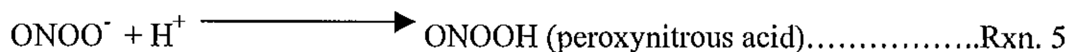
$O_2^- + 2H^+ \longrightarrow H_2O_2$ Rxn.2



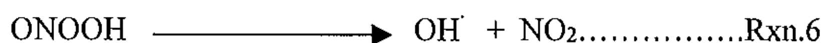
$HOCl + O_2^- \longrightarrow OH^· + OH^- + Cl^-$..........Rxn.3



$NO + O_2^- \longrightarrow ONOO^-$ (peroxynitrite anion)...............Rxn.4



$ONOO^{-} + H^{+} \longrightarrow ONOOH$ (peroxynitrous acid)................Rxn. 5



$ONOOH \longrightarrow OH^{\cdot} + NO_2$...............Rxn.6

Synthesis of ROIs is intiated when NADPH oxidase catalyses the reduction of molecular oxygen to superoxide anion and the concomitant oxidation of NADPH. Superoxide anion is subsequently converted to hydrogen peroxide, which can be converted to hydroxyl ions. The latter two products are potent toxins for many microbes.

The production of ROIs is started by the production of superoxide via Rxn.1. This reaction is catalyzed by the enzyme system, NADPH oxidase. NADPH-oxidase system is a complex that contains several proteins. As it is shown in the Figure-2 below, this oxidase system is dormant and dissociated in unstimulated cells, with the components residing in the membrane (cytochrom b_{558}), cytoplasm (P^{47}, Rac-2) and cytoskeleton (P^{67}) (Robinson and Badway, 1994).

During stimulation of the cells, there is translocation of the cytoplasmic and cytoskeletal subunits to the plasmalemma, where the functional oxidase is assembled (Figure-2b). As the plasmalemma invaginates to produce the phagosome, superoxide formed by the oxidase is transported into the phagosome (Robinson and Badway, 1994).

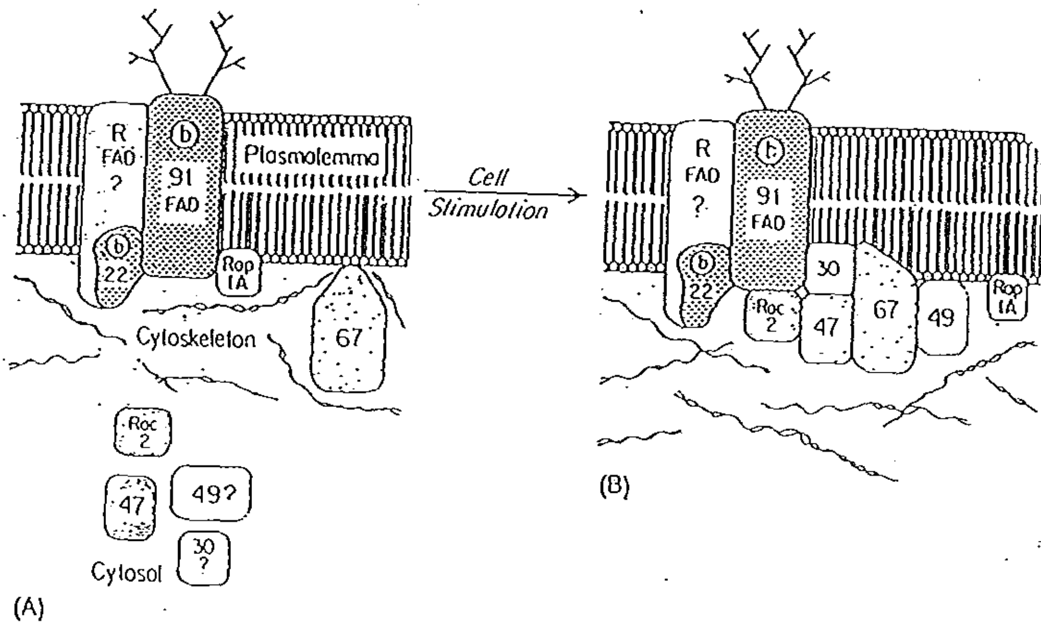


Figure 2. Assembly/Activation of the NADPH Oxidase System
From: Robinson and Badway (1994)

Hydrogen peroxide is produced by the dismutation of superoxide (Rxn2). Hydroxyl radical ($OH^{\cdot}$) can be produced by the reaction of superoxide with either hypochlorous acid (Rxn.3) or nitric oxide (Rxn.4 to 6). In the latter case, hydroxyl radical is generated during the decomposition of peroxynitrous acid (Rxn.6), which is formed by the reaction of nitric oxide (NO) with superoxide followed by protonation (Rxn.4 and 5) (Robinson and Badway, 1994). Hypochlorous acid and nitric oxide are formed in phagocytic cells in the myleoperoxidase and nitric oxide synthase pathways, respectively. Hypochlorous acid can also react with hydrogen peroxide to generate singlet oxygen (Robinson and Badway, 1994).

Reactive oxygen intermediates mediate oxygen-dependent antileishmanial activity of macrophages. As it is pointed out in section 1.3.1 above, the release of ROIs is enhanced by lymphokines (e.g.INF-γ) thus, inducing a higher antileishmanial activity in macrophages.

1.3.2.2. Reactive Nitrogen Intermediates

Reactive nitrogen intermediates (RNIs) include nitric oxide (NO), and derivatives of NO [nitrogen dioxide (NO_2), nitrite (NO_2^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4) and peroxynitrite ($ONOO^-$).

Nitric oxide is produced by oxidation deimination of L-arginine. The reaction is catalysed by the cytokine inducible enzyme nitric oxide synthase (iNOS) and produces L-citruline as a by-product (Woods *et al.*, 1994).

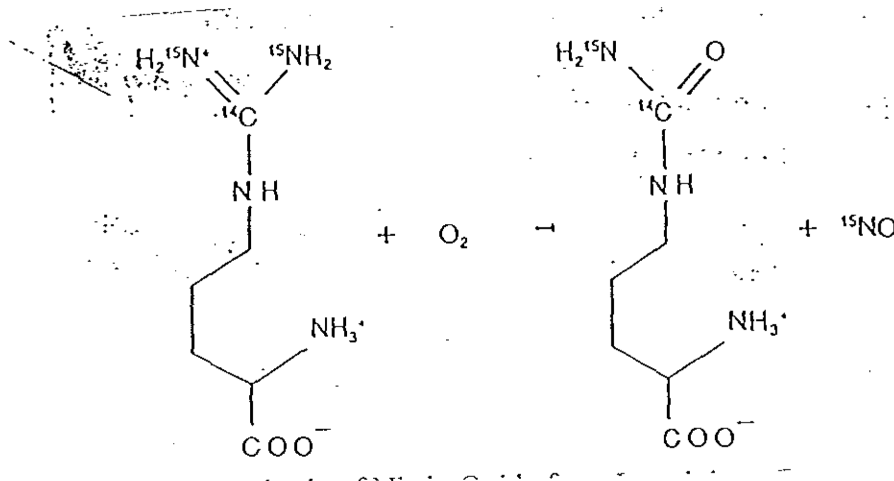


Figure 3. Synthesis of Nitric Oxide from L-arginine.
From: Woods *et al* (1994)

Nitric oxide production by the inducible nitric oxide synthase is stimulated by pro-inflammatory cytokines such as INF-γ, TNF-α, IL-1 and IL-2, as well as by microbial products such as lipopolysaccharide and lipoteichoic acid (Leiw *et al.*, 1990 and Fang, 1997).

Nitric oxide related antimicrobial activity has been demonstrated against a broad range of pathogenic microorganisms. These include: viruses (Epstein-Barr virus), bacteria

(mycobacteria), fungi (*Cryptococcus neoformans*) and parasites (*Leishmania major* and plasmodia) (Fang, 1997 and Woods *et al.*, 1994).

It has been shown by Green *et al* (1990) that microbicidal activity of macrophages against *L. major* is dependent on NO. This activity decreases as the amount of inhibitor of NOS increases in an *in vitro* experiment.

1.3.3. Evasion Strategies of *Leishmania*

Like in other pathogens, host-parasite relationship in *Leishmania* involves the interaction of the parasite with the host's immune system. These interactions determine the fate of the parasite as well as the type and severity of the disease. For example, *L. major, L. aethiopica*, and *L. mexicana* normally cause local cutaneous leishmaniases which are self-healing. In contrast, infection with *L. donovani* usually results in systemic and fatal disease (Kala-azar). However, depending on the parasite and/or host factor(s), the type of disease caused by these species may be changed. That is, *L. major* may cause diffuse cutaneous leishmaniasis or a disease resembling Kala-azar and infection by *L. donovani* could remain asymptomatic (Bogdan *et al.*, 1990).

It has been said that even in the presence of intact immune response of the host, infection by *Leishmania* may result in disease conditions. This is partly because the parasite develops several mechanisms that enable it to escape from the host response. According to Bogdan *et al* (1990) *Leishmania* devises several mechanisms to escape the immune responses imparted by the host both inside and outside macrophages. This include:

1. Inhibition of the oxidative burst
2. Evasion from reactive nitrogen intermediates and
3. Scavenging oxidative metabolites

1.3.3.1. Inhibition of the Oxidative Burst

Once inside macrophages, *Leishmania* downregulates the oxygen-dependent killing mechanisms of activated macrophages. This is performed by acid phosphatase and lipophosphoglycan of the parasite (Bogdan *et al.*, 1990). It has been proposed that leishmanial membrane acid phosphatase may make dephosphorylation of phosphoproteins on the host phagocytic cell surface. This suppresses the production of oxygen metabolites by host cell and helps the parasite survive in the host. Katakura and Kobayashi (1988) has also showed that the membrane- bound phosphatase activity was 2.4-fold higher in virulent strains of *L. donovani* than avirulent ones.

1.3.3.2. Evasion from Reactive Nitrogen Intermediates

As it is explained in section 1.3.2.2, one of the most effective mechanisms used by macrophages to defend against leishmanial infections is by the production of nitric oxide and its derivatives. However, the parasite escapes this action either by inhibiting its synthesis or by parasitizing host cells, which are incapable of producing NO.

Like skin macrophages, epidermal Langerhan cells (LCs) are also infected by metacyclic promastigotes. Langerhans cells initially serve as a safe habitat, transporting the parasite from the infected skin to the draining lymph node. These cells are not only sites of initial survival of the parasite but also are involved in processing

and presenting it to specific T-cells. However, these cells are incapable of producing iNOS. Therefore, *Leishmania* that infect Langerhans cells evade NO-mediated host defense (Blank *et al.*, 1996 and Bogdan *et al.*, 1996).

Lipophosphoglycan-associated kinetoplastid membrane protein-11 downregulates iNOS activity in infected macrophages. This is because at amino acid 45, this protein contains N^G-monomethyl-L-arginine, a molecule that inhibits L-arginine dependent production of NO (Bogdan *et al.*, 1996).

1.3.3.3. Scavenging Oxidative Metabolites

Leishmania counteracts the toxic oxygen intermediates that are produced by macrophages using enzymes and surface molecules that scavenge the toxic substances and neutralize into non-toxic forms. These include superoxide dismutase (SOD), peroxidoxin, and lipophosphoglycan (Bogdan *et al.*, 1990).

Superoxide dismutase detoxifies superoxide anions by converting them to hydrogen peroxide and oxygen. Peroxidoxin neutralizes hydrogen peroxide to water and oxygen. By doing so, these enzymes protect the parasite from oxygen toxicity and death (Paramchuk *et al.*, 1997).

1.4. SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD) (EC 1.15.1.1) is an antioxidant that serves as the primary defense against damage that can be caused by superoxide and its reactive progeny. By

its ability to change superoxide to hydrogen peroxide, the enzyme plays an important role in the survival of parasites inside host cells.

1.4.1. Structure of SOD

Superoxide dismutases are a group of metalloenzymes with metal ion in the active site. Depending on the type, each enzyme is composed of two or four subunits with a molecular size ranging from 32 kDa to 80 kDa. The catalytic function is due to the metal ion in the active site. To date, five different metal ions are known to occur in the SODs. These are iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), and nickel (Ni). It is generally accepted that in all SODs the metal ion catalyzes dismutation of superoxide radical through a cyclic oxidation-reduction mechanism (Fridovich, 1997).

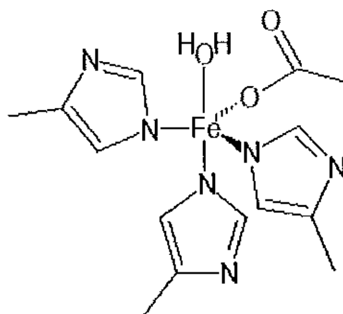


Figure 4. Structure of the Active Site of FeSOD
From: http:/metallo.scripps.edu/PROMISE/FESOD

As it is shown in Figure-4, the iron in the active site of iron-SOD (FeSOD) is pentacoordinate. The metal ion is bound to three conserved His residues, one conserved Asp residue and a water molecule arranged in distorted trigonal bipyramidal geometry (http:/ metallo. scripps. edu / PROMISE/FESOD.html).

1.4.2. Types of SOD

Superoxide dismutase has different forms (types) based on differences in amino acid sequence, type of metal ion in the active site, and cellular location (Buettner, 1998). Four classes of SODs are known, distinguished by the metal prosthetic group: Cu/Zn, Fe, Mn and Ni. Fe- and Mn-SODs constitute a structural family and occur as homodimers or homotetramers.

The manganese-containing superoxide dismutases (MnSODs) are found in prokaryotes and in the matrix of mitochondria. The related iron-containing superoxide dismutases (FeSODs) are found in prokaryotes, protozoan parasites and in a few families of plants. The unrelated copper and zinc superoxide dismutases (Cu, ZnSODs) occur primarily in the cytosol of eukaryotic cells and in chloroplasts but have also been found in a few species of bacteria (Fridovich, 1989, Ismail *et al*, 1997 and Tannich *et al*, 1991). Cu/Zn-SOD is a dimer; each subunit has a molecular weight of 16kDa and contains one ion each of Cu and Zn. An entirely new SOD, with nickel at its active site, has been found in *Streptomyces*. This is homotetrameric enzyme whose subunit weight is 13kDa. It bears no obvious sequence homology to known Mn-SODs or Fe-SODs (Fridovich, 1997).

Structural studies have indicated that the iron and manganese enzymes have similar primary, secondary and tertiary structures but bear no resemblance to the copper/zinc enzyme (Parker and Blake, 1988). The marked homology between prokaryotic and mitochondrial MnSODs are in accord with an endosymbiotic origin for these

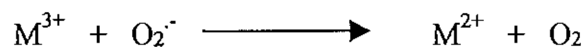
organelles. All these SODs catalyze the same process and do so with comparable efficiency.

Most MnSODs and FeSODs are active only with the native metal at the active site. Yet the SODs from some bacteria like *Bacteriodes fragilis* appear to be active with either manganese or iron at their active sites. This is consistent with a common origin FeSOD and MnSOD, as are their very similar conformations deduced by X-ray crystallography. This similarity in sequence and conformation explains the formation, in aerobic *Escherichia coli*, of a hybrid SOD which contains one subunit each of MnSOD and FeSOD, in addition to the homodimeric MnSOD and FeSOD.

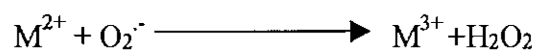
Unlike higher eukaryotes, protozoans like *Entamoeba histolytica*, *Trypanosoma cruzi* and *Leishmania chagasi* possess FeSOD rather than Mn- or Cu/Zn-SOD (Tannich *et al*, 1991, Ismail *et al*, 1997 and Paramchuk *et al*, 1997). Comparison of the *L chagasi* SODs deduced amino acid sequences with MnSOD and FeSOD amino acid sequences of other microorganisms has revealed that they have higher homology to, and complete conservation of, invariant residues found in iron-containing SODs.

1.4.3. Mechanism of Action

Superoxide dismutases are antioxidant metalloenzymes catalyzing the redox disproportionation (dismutation) of superoxide radical, $O^{\cdot-}_2$. The metal ion (M) in the enzyme does this through a cyclic oxidation-reduction mechanism:



$$M^{3+} + O_2^{\cdot-} \longrightarrow M^{2+} + O_2$$



$$M^{2+} + O_2^{\cdot-} \longrightarrow M^{3+} + H_2O_2$$

Among the targets susceptible to direct oxidation by superoxide radical are those dehydratases that contain [4Fe-4S] clusters. These enzymes, which include dihydroxy acid dehydratase, aconitase, 6-phosphogluconate-dehydratase, and fumarase A and B, react with superoxide with rate constants of $\sim 10^7 M^{-1} S^{-1}$. Univalent oxidation of the [4Fe-4S] clusters by $O_2^{\cdot -}$ leads to loss of iron., leaving [3Fe-4S] clusters and an inactive form of the enzymes. The enzymes can subsequently be reactivated by reductive reconstitution. Both inactivation and reactivation are ongoing processes in aerobic cells and their balances determines the fractional level of activity of these enzymes (Benov and Fridovich, 1998; http:/ metallo.Scripps.edu/PROMISE/ FESOD). By scavenging the superoxide radical, SODs prevent not only these enzymes but also DNA and cell membranes from damage caused by the oxygen radicals.

1.4.4. Use of SOD in Aerobic Life

Superoxide dismutases are enzymes responsible in defense against toxic ROIs that are produced in an aerobic life. The importance of these enzymes has been clarified by the phenotypic deficits of mutants defective in their production and by the complementing effects of homologous or heterologous SODs. These demonstrations have been achieved in bacteria, *Leishmania*, yeast, *Drosophila*, nematodes, *Neurospora*, and even in mice.

The consequences of a lack of both the constitutive FeSOD (SODB) and the inducible MnSOD (SODA) in *E.coli* induces oxygen dependent decrease in growth rate, hypersensitivity towards redox cycling compounds such as paraquat and quinones, and an increase in the rate of spontaneous mutagenesis (Fridovich, 1997).

Support for free radical theory of senescence was provided by the shortened life span of *Drosophila* with a mutational defect in Cu, ZnSOD. These flies were also hypersensitive towards paraquat and were sterile. Mice lacking Cu, ZnSOD appeared normal while young but were less able to recover from axonal injury and could not successfully reproduce. They also exhibited a shortened life span. Lack of MnSOD imposed more serious consequences. These animals lived only a week or two and exhibited faulty mitochondrial activities in several tissues, especially the heart (Fridovich, 1997).

It has been shown that FeSODs of *Shigella flexneri* and *Nocardia asteroids* play an important role in protection against phagocytic leukocytes (Beaman and Beaman, 1990 and Franzon *et al*, 1990). *In vitro* incubation of *Nocardia astroides* with murine monoclonal antibodies specific for nocardial SOD decreased the survival of the bacteria in the lung and the liver of mice which had received anti-SOD antibody treated nocardiae. Pesci and his colleagues (1994) also demonstrated the role of FeSOD in the intracellular survival of *Campylobacter jejuni*. They found out that strains lacking SOD showed 12-fold decrease in survival within INT407 cell lines than the parent strains.

The molecular study of Paramchuk and his colleagues (1997) on *L. chagasi* demonstrated the importance of SOD to protect the parasite from free radical damage. Transfectants (*L. chagasi* which was made mutant to SOD gene and then received a plasmid vector containing *Leishmania* SOD gene by transformation) overexpressing *L.*

chagasi SOD genes yielded a higher resistance to toxic oxygen compounds as compared to wild type parasites or transformants that received the vector without the SOD gene. Moreover, the level of expression of SOD genes in stationary phase promastigotes was found to be 1.5-fold higher than the level in logarithmic phase promastigotes. The activity of SOD obtained from whole cell extract of amastigotes and stationary phase promastigotes was also higher than the one obtained from log-phase promastigotes.

1.4.5. Genetics of SOD

Genetic studies of *L. chagasi* and *L. tropica* have shown that FeSODA and FeSODB are encoded by two genes that are located on different chromosomes (Ghosh *et al*, 2003 and Paramchuk *et al*, 1997). Pulsed field gel electrophoresis of the total genomic DNA of L. *chagasi* has indicated that FeSODA shows localization to a single band at a lower molecular weight chromosome of ~582kb, whereas, L.c SODB is localized to a single higher molecular weight chromosome of ~920kb (Paramchuk *et al*, 1997). In addition, SOD-A has been found to be a single copy gene whereas, SOD-B is multicopy gene. The two genes differ from each other in the 3'nucleotide sequence, particularily in the presence of a 3'-terminal extension in the SOD-A gene (Ghosh *et al*, 2003 and Paramchuk *et al*, 1997).

Comparison of the homology of FeSOD-A and FeSOD-B of *L. chagasi* with phylogenetically diverse organisms has shown that the two *L. chagasi* SODs have less identity (37%) than each enzyme separately with the other organisms. According to the authors, this could be due to the fact that *L. chagasi* FeSOD-A contains a 31 amino

acid extension at the amino terminus, which is absent in *L. chagasi* FeSOD-B. In addition to this difference in the N-terminus extension, there are several amino acid differences between the two L.c. FeSODs. L.c. FeSOD-B showed a closer identity to the iron-containing SODs of *Entamoeba histolytica*, *Escherichia coli*, and *Coxellia burnetti* (52, 54, and 56%, respectively) as compared to L.c. FeSODA (43, 48, and 45%, respectively). Comparing the L.c. FeSODs with Mn-containing SODs, no MnSOD displayed greater than 39% identity. Cross-species comparison of the deduced amino acid sequences from *L. chagasi* and *T. cruzi* resulted in higher identity scores than intra-species comparison. L.c. FeSOD-A and T.c FeSOD-A show a 55% identity, whereas, L.c.FeSODB and T.c. FeSODB have 62% identity (Paramchuk *et al*, 1997).

2. OBJECTIVES

2.1. General Objective

To identify and characterize superoxide dismutase gene from *Leishmania aethiopica.*

2.2. Specific Objectives

1. To identify SOD gene in *L. aethiopica.*

2. To compare the degree of homology of *L. aethiopica* SOD gene with SOD genes of other *Leishmania* spp.

3. MATERIALS AND METHODS

3.1. Study Population

The materials for the study were obtained from suspected cutaneous leishmaniasis patients attending dermatology clinic of ALERT hospital, Addis Ababa. The patients were referred to the hospital from different parts of the country. Physical examination was done by a dermatologist as part of the routine work at the hospital. Meanwhile, clinico-epidemiological data were collected in a structured questionnaire. These included, the patient's age, sex, residence, duration of lesion, type of lesion (single or multiple), site of lesion, as well as type and duration of previous treatment (if any). The patients were then sent to leishmaniasis laboratory for diagnosis. Specimens were collected from patients who gave written informed consent.

The proposal for this study was reviewed for scientific merit and ethics by the AHRI/ALERT and the national ethical clearance committees. The study was initiated after the proposal received approval from both committees.

3.2. Specimen Collection

Skin slit was made from the lesion following standard procedures. In brief, an active part of the lesion was selected and thoroughly cleaned with 70% ethanol and air-dried. The skin over the area to be sampled was pinched firmly between thumb and forefinger and a shallow slit about 1mm deep was made. The cut edge of the slit was scraped from below upward using the sharp edge of a sterile scalpel blade. The pressure on the sides of the slit was released and a sterile dressing was applied to the cut surface immediately. In order to stop bleeding, the wound was tightly held for 1 to

2 minutes. Finally, the fluid from the scratch was immediately inoculated into a biphasic medium, Novy-MacNeal-Nicolle (NNN) medium in duplicate (Evans, 1989).

3.3. Media Preparation

NNN, a blood agar medium, was prepared from 24g Bacto Nutrient agar (Difco), 1.5g D-glucose, 6g sodium chloride dissolved in 1000ml distilled water and 100ml sheep blood. The nutrient agar, glucose and NaCl were dissolved in distilled water by boiling and autoclaved at 121°C for 30 minutes.

Venous blood was collected aseptically from sheep in a bottle containing 2% EDTA. The blood was heat inactivated at 55 ^{O}C in a water bath for 30min and added into the autoclaved nutrient base at around 50 ^{O}C. Eight mililiter of the mix was dispensed into a 50ml culture flask. The solidified culture medium was then stored in a refrigerator ready for use.

Locke's solution was used as an overlay. It constituted 9.2g NaCl, 0.24g $CaCl_2$, 0.15g $NaHCO_3$, 0.42g KCl, and 1.0g D-glucose dissolved in 1000ml distilled water and autoclaved at 121°C for 30 minutes. 100U/ml penicillin (GIBCO) and 100U/ml streptomycin were added to it and one milliliter of the solution was then added as overlay to the NNN solid phase medium before use (Evans, 1989).

3.4. Parasite Culture

Samples taken from patients were inoculated into the liquid phase of the medium and incubated at 26 ^{O}C in an incubator. The growth of the parasite was monitored by

microscopy. Using a sterile plastic pipette, one to two drops of the overlay was taken and screened for promastigotes under a compound light microscope. The presence of motile promastigotes was recorded as culture positive. The microscopic observation was done every four days for a maximum of one month with serial subculturing within one-week interval. Absence of motile promastigotes within one month was taken as negative and thus the culture medium was discarded.

The positive cultures were subcultured into new NNN media and into RPMI 1640 containing 10% heat inactivated fetal calf serum supplemented with 2ml of 100U/ml penicillin (GIBCO) and 100U/ml streptomycin combined (PS).

The promastigotes were harvested when they grew sufficiently. The overlay/FCS was spinned at 721xg in Beckman centrifuge (California, USA) to pellet down the parasites. The pellet was then divided into two. One half was used for genetic study and the other half was preserved for other studies following standard protocols. Briefly, the pellet was resuspended in a 2ml freezing medium (90ml FCS mixed with 10ml dimethylsulfoxide). One milliliter of the mixture was then aliquoted into 2ml cryotubes (NUNC, Denmark) and cryopreserved. Cryopreservation was done following the modified protocol of Evans (1989): the tubes that contain promastigotes in a freezing medium were kept in a freezing can that had been kept in a refrigerator for more than one hour. Then, the freezing can with the cryotubes was placed at –80 freezer for one day. The next day, the tubes were transferred from –80 freezer to liquid nitrogen.

3.5. Reference Strains Used

Reference strains of different *Leishmania* species were used as controls for strain typing of the experimental isolates. These were *L. aethiopica* L-100, *L. infantum* LEM-75, *L. infantum* MON-183, *L. major* 5ASKH, and *L. tropica* K-27. These reference strains were those recommended by the Expert Committee on Leishmaniasis (WHO 1989). All were kindly provided by the National Center for Tropical Medicine, Madrid, Spain.

3.6. Species Typing

Species typing of the isolates was done using two methods. These are isoenzyme electrophoresis and PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism). These experiments were done at the leishmaniasis research section of the National Center for Tropical Medicine, Madrid, Spain.

3.6.1. Isoenzyme Electrophoresis

Isoenzyme electrophoresis was done on one of the isolates (1093/02) using glucose-phosphate-isomerase (GPI EC 5.3.1.9) following the procedure of Rioux *et al* (1990). It involved three major steps: crude protein extraction, electrophresis and staining.

The crude protein was extracted from the promastigotes using lysis reagent (0.5ml Triton X-100 (Sigma), 0.825g sucrose in 10ml sterile distilled water). One hundred microliter of the solution was added onto a promastigote pellet. The mixture was then homogenized using a plastic rod until a uniform suspension was obtained.

Thick starch gel was prepared one day before electrophoresis. Twenty- two gram of potato starch (Fluka Biochemica, Switzerland) was dissolved in 200ml of gel buffer (4.5ml of 10x electrode buffer, 1%NADP dissolved in 400ml sterile distilled water). The electrode buffer was made by dissolving 121g Tris, 116g malic acid, 33.6g EDTA and 20g $MgCl_2 \cdot 6H_2O$ in 1liter sterile distilled water. After mixing the starch with the gel buffer, the suspension was heated over the flame of a Bunsen burner for complete dissolution. The gel was then poured onto a gel cast, covered by a glass cover taking care not to produce air bubble. Pressure was applied on the gel with a flask containing about 500ml tap water. The gel was left to solidify overnight. On the next day, wells were made by inserting a comb into the gel. The whole-cell extract was loaded into the gel using filter paper strips of about 1cm length that were kept in it for about two minutes. The gel was then subjected to electrophoresis at 100V and 65mA for four hours in the cold room.

After electrophoresis, the paper strips were discarded and the gel was cut into three slices using a stretched string. The slices were put in staining dishes. Meanwhile, specific substrate and staining solutions were prepared as follows. Ten milligram of fructose-6-phosphate (Sigma) was added into a tube containing 10ml 0.2M Tris-HCl P^H 8.0, 1ml 0.5M $MgCl_2 \cdot 6H_2O$, 0.5ml 1%NADP, 1ml 1% NAD and 1ml 1% NBT. The mixture was incubated at 37 ^OC for 30 minutes and sixteen units of glucose-6-phosphate dehydrogenase (Sigma), 0.5ml 1% phenazine methosulfate (PMS) (Sigma) and 5ml 2% agarose were added to it. The suspension was immediately poured onto the gel slices and incubated at room temperature for about 1h. The distance traveled by the enzyme was then measured and compared with that of the reference strain.

3.6.2. PCR-RFLP

PCR-RFLP was done on one of the isolates (1093/02) following the modified protocol of Minodier *et al* (1997). The procedure involved PCR-amplification of a repetitive genomic DNA followed by restriction digestion of the product.

Total genomic DNA was extracted from promastigotes using EZ-DNA isolation kit (Kibbutz Beit Haemek, Israel). Briefly, the promastigotes were treated with 600μl EZ-DNA solution and incubated at 90 ^{o}C for 5min with regular vortexing. Eight hundred microliters of 100% ethanol was mixed to it by inverting the tube 10 times and left for 3min at room temperature. It was then spinned at 13000 rpm for 6 min. The supernatant was discarded and the pellet washed with 95% ethanol and air-dried. The air-dried DNA was finally dissolved in 100μl sterile distilled water ready for PCR.

Polymerase chain reaction was done using T2 and B4 primers. The nucleotide sequences of the primers were 5'-CGG CTT CGC ACC ATG CGG TG-3' and 5'-ACA TCC CTG CCC ACA TAC GC-3' respectively. Each PCR mixture contained 5μl of 10x DNA polymerase buffer, 1μl of dNTP mix (100mM final concentration), 15 picomol of each primer, 1.4μl of 1U/μl *Tth* DNA polymerase, 10μl of genomic DNA and 30.6μl of sterile distilled water in 50μl total reaction volume. The PCR was started with prior heating at 80 ^{o}C for 2min, and at 94 ^{o}C for 5min followed by 30 cycles of amplification (with one cycle consisting of 30s at 94 ^{o}C, 30s at 65 ^{O}C and 30s at 72 ^{O}C) and final extension at 72 ^{o}C for 5min in automatic thermocycler (Eppendorf, Switzerland).

Hae-III (Pharmacia) was used for the restriction enzyme analysis. This enzyme can identify the sequence 5'-GG↓ CC-3'. Following the recommendation of the supplier, 17μl of the PCR product was added to 2μl of the *Hae-III* enzyme buffer and 1μl (10U) of the enzyme. This mixture was incubated at 37 ^{O}C for 1h and the product was analyzed by running electrophoresis on 1.5 % agarose gel at 100V and 50mA for 1h.

3.7. CHARACTERIZATION OF SUPEROXIDE DISMUTASE GENE

3.7.1. DNA Extraction

Genomic DNA was extracted from promastigotes using modified protocol of Paramchuk *et al* (1997). Five hundred microliter of lysis buffer (10mM Tris-HCl P^{H} 8.3, 50mM EDTA pH 8.0 and 1%SDS) was added to the promastigote pellet and boiled for 15min. Twenty-five microliter of 2mg/ml RNAase A (Pharmacia) was added to the suspension and incubated at 37 ^{o}C for 1h. This was followed by addition of 5μl of 10mg/ml proteinase K (GIBCO) and incubation at 42 ^{o}C overnight. The next day, phenol-chloroform-isoamyl alcohol (PCIA) extraction was done. Briefly, 300μl of PCIA was added, vortexed for 5s and spinned at 12000rpm for 5min. The upper-phase was collected in a separate microcentrifuge tube. After adding 350μl of isopropanol, the mixture was incubated at –20 ^{o}C for 30min. It was spinned at 12000rpm for 15min and the supernatant removed. Ethanol precipitation was performed following the method described in section 3.6.2 above. The DNA pellet was dissolved in 1x TE (Tris-EDTA) buffer and stored in a refrigerator for further work. The presence and purity of the genomic DNA were confirmed by electrophoresis on 1% agarose gel.

3.7.2. Polymerase Chain Reaction

PCR was done to amplify open reading frames (ORFs) of FeSODB1 and FeSODB2 genes following the modified protocol of Paramchuk *et al* (1997). The primers used were those previously designed to amplify FeSOD genes from *L. chagasi*. *BamH-I* restriction site was incorporated in each primer to effect cloning in expression vectors like P^{GEX-2T}. The nucleotide sequence of the primers were 1) FeSODB1 ATG/*BamH-I* 5'-TTT CCC GGG GGG ATC CAT GCC GTT CGC TGT TCA GCC-3', 2) FeSODB1TAA/*BamH-I* 5'-TCG CAG GGA TCC TTA AAG CTG GCT AGT GGC-3', and 3) FeSODB2TAA/*BamH*-I 5'-TCC TCC CGG GGG ATC CTT ACA GAT CAC TGT TG-3'. FeSODB1 was amplified using primers 1 and 2. Whereas, FeSODB2 gene was amplified using 1 and 3 as forward and reverse primers respectively.

Promastigote genomic DNA was subjected to PCR in a total reaction volume of 25μl PCR mix with Ready-to go PCR beads (Pharmacia P-L Biochemicals, Upsala, Sweden). Each bead contains *Taq* DNA polymerase, dNTPs and PCR buffer. To these beads were added 1μl of 50 pmol/μl of each primer, 3μl of about 0.1μg/μl of promastigote DNA and sterile distilled water. Amplification reaction involved denaturation at 94 ^{o}C for 1min, primer annealing at 50°C for 30s and extension at 72 ^{o}C for 1min for a total of 30 cycles in a thremocycler (Hybaid). In the reaction, both positive and negative (blank) controls were included. The positive control was *L. donovani* genomic DNA (Gift of Professor Lashitew Gedamu, University of Calgary)

previously used to amplify FeSOD genes. In the blank reaction tube, all the ingredients of PCR were added except the genomic DNA.

A second PCR was also done to increase the amount and concentration of the amplified genes. The types of primers as well as the reaction conditions were the same as for the first PCR. However, in these reactions, the first PCR products rather than the genomic DNA were used as template DNA.

3.7.3. Gel Electrophoresis

The PCR products were visualized by performing agarose gel electrophoresis. 1.5% (w/v) agarose (Sigma) was used for this purpose. The gel was prepared as follows. 0.75g agarose was dissolved in 50ml of 1x TBE buffer (10.8g Tris base, 5.5g boric acid, 4ml 0.5M EDTA P^H 8.0 in 1liter distilled water) in a micro-wave oven. When the agarose melted completely, it was allowed to cool to about 50 ^oC. 2.5μl of 10mg/ml ethidium bromide (Sigma) was added and the gel mixture was poured onto a gel cast tray. The agarose was left for about one hour to solidify. The gel was put on an electrophoresis tank (Pharmacia) containing 250μl of 1x TBE and 12.5μl of ethidium bromide.

Five microliter of the PCR product was taken from each reaction and mixed with 4μl of gel loading buffer (0.5% bromophenol blue, 50%glycerol w/v, 50mM Tris HCl P^H 7.5 and 5Mm EDTA) in a microcentrifuge tube. Each mix was then loaded into a separate well in the gel and subjected to electrophoresis at 80V and 40mA for one hour. In the first well, 3μl of 0.1μg/μl 1kb DNA ladder (Invitrogen) was loaded as a

reference to compare the molecular size of the amplified fragment. The gel was visualized by UVP Transilluminator and the picture was taken and printed (Sambrook *et al*, 1989).

3.7.4. DNA Purification

The PCR products were purified using Microspin™ Column purification kit (Pharmacia). S-400 spin column was chosen for this purpose. The resin in the column was resuspended by vortexing for a few seconds. The cap was loosen one-fourth turn and the bottom closure was snapped off. The column was placed in a 1.5ml microcentrifuge tube for support and was pre-spinned at 3000rpm for 1min. The column was placed in a new 1.5ml tube and the cap removed. The sample was applied slowly to the top center of the resin and spinned at 3000rpm for 2min. The purified sample was collected in the bottom of the support tube ready for further analysis.

3.7.5. Restriction Digestion of the PCR Product

In order to confirm that the PCR products are FeSODB1 and FeSODB2 genes, the amplification products were subjected to restriction digestion using *EcoR-I* (New England Biolabs). The enzyme has a recognition sequence of G ↓AATTC. Five microliter of each of the second PCR products were subjected to restriction digestion by 1μl of 20000U/ml enzyme following the instruction of the manufacturer. The digestion mixtures were incubated at 37 °C for 1h. After digestion, the products were incubated at 60 °C for 20min to stop the reaction. The negative controls had all the constituents of the reaction except the enzyme, *EcoR-I*.

The digestion products were analyzed by agarose gel electrophoresis. Ten microliter of the digest was subjected to electrophoresis on 1.5% agarose gel following the method described in section 3.7.3. The gel picture was taken and the molecular sizes of the restriction fragments recorded.

3.7.6. Sequencing

The open reading frames of *L. aethiopica* FeSODB1 and FeSODB2 genes from two isolates (1093/02 and 1184/02) were sequenced at University of Calgary, Canada. The second PCR products were subjected to purification using QIAquick PCR purification kit (Qiagen). The purified DNAs were then sent to the DNA sequencing facility, University of Calgary, Canada.

4. RESULT

4.1. Study Population

As it is shown in Table-1 below, the patients came from different places in the country including Addis Ababa (25%). The age ranged from 7 to 65 years with a mean value of 25.6 years. Males constitute 65% of the study population. The average duration of the lesion was 16.25 months with a range of 2 months to 7 years. The uncovered part of the body (face) is the most frequently affected region with cheek accounting 25% followed by nose (20%) and lip (15%).

Table 1. Clinico-Epidemiological Data of the Study Subjects

No	Code	Age	Sex	Address	Duration lesion(Mo)	Site of lesion	Culture
1	1067/02	28	M	Gojjam	5	Arm, leg	N
2	1068/02	26	F	Bedele	4	Cheek	N
3	1093/02	26	M	Muhger	2	Cheek	P
4	1105/02	18	F	AA	3	Eyelid	N
5	1117/02	13	F	Adigrat	24	Nose	N
6	1118/02	65	M	Guraghe	2	Neck, eyelid	P
7	1120/02	26	M	Gojjam	84	Lip	N
8	1131/02	19	M	AA	72	Nose, ear	N
9	1180/02	53	M	Bulga	6	Hand	N
10	1181/02	7	M	Butajira	8	Cheek	P
11	1183/02	26	M	D/Ziet	6	Cheek	N
12	1184/02	15	F	Gayent	12	Cheek	P
13	1185/02	7	F	Bulga	12	Arm, cheek	P
14	1200/02	20	M	Arsi	6	Nose	N
15	1207/02	17	M	Gojjam	36	Lip	N
16	1208/02	42	M	Wollo	2	Nose	N
17	1209/02	21	M	AA	6	Forehead	N
18	1210/02	45	M	AA	24	Nose, ear	N
19	1211/02	20	F	Sendaffa	8	Nose	N
20	1213/02	18	F	AA	3	Lip	N

F = Female, M = Male, Mo = Month, N = Negative, P = Positive

4.2. Specimen Collection

Of the total of 20 skin slit specimens collected and cultured, only 5 (25%) were found to be positive for motile promastigotes. Isolates from patients 1093/02 and 1185/02

grew well in the primary culture. Unlike 1093/02 and 1185/02, the other three (1118/02, 1181/02 and 1184/02) showed scanty growth in the primary culture. In the secondary culture (those transferred from the primary culture), the isolates showed better growth than their respective primary cultures.

4.3. Species Typing

4.3.1. Isoenzyme Electrophoresis

Isoenzyme electrophoresis of glucose-phosphate-isomerase (GPI) from the isolate 1093/02 gave a distinct band equal in size with *L. aethiopica* L-100. *L. major*, *L. tropica* and *L. infantum* showed a different band pattern from *L. aethiopica* reference strain and the isolate, 1093/02.

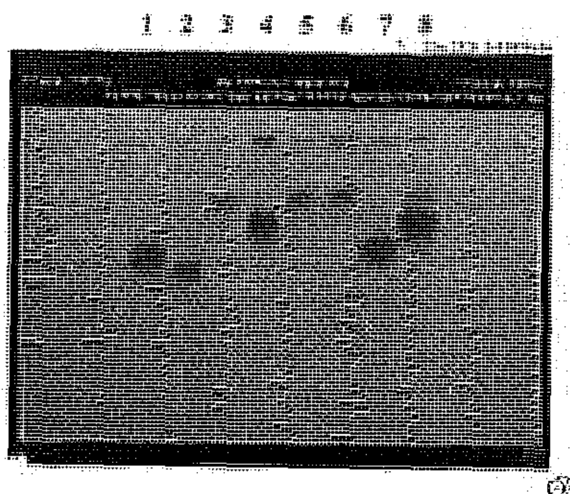


Figure 5. Isoenzyme Electrophoresis of GPI.

(Lane-1= *L. infantum* LEM-75, Lane-2= *L. infantum* MON-183, Lane-3= Isolate 1093/02, Lane-4= *L. tropica* K-27, Lane-5= Isolate 1093/02, Lane-6= *L. aethiopica* L-100, Lane-7= *L. infantum* LEM-75, and Lane-8= *L. major* 5ASKH)

As it is shown in the Figure-5, *L. major* 5ASKH (lane-8) and *L. tropica* K-27 (lane-4) gave bands that migrated farther in the gel than both bands of *L. aethiopica* reference

strain (lane-6) and isolate 1093/02 (lane 3 and 5). In addition, *L. major* gave two separate bands (one intense and one faint) in one lane (lane-8). All the bands of the three *L. infantum* strains migrated farther from the cutaneous leishmaniasis causing strains. Bands from 1093/02 (lanes 3 and 5) aligned in the same line as *L. aethiopica* reference strain (L-100).

The zymogram was calculated for each strain as the distance traveled by the protein of a given strain divided by the distance traveled by *L. infantum* (LEM-75) multiplied by hundred. The result is summarized in Table-2.

Table 2. Zymogram of the Different *Leishmania* Strains.

Name of the strain	Lane No	Absolute distance (cm)	Zymogram
L. infantum (LEM-75)	1	2.0	100
L. infantum (MON-183)	2	2.4	120
1093/02 (Isolate)	3	1.0	50
L. tropica (K-27)	4	1.7	85
1093/02 (Isolate)	5	1.0	50
L. aethiopica (L-100)	6	1.0	50
L. infantum (LEM-75)	7	2.0	100
L. major (5ASKH)	8	1.6	80

4.3.2. PCR-RFLP

T2 and B4 primers amplified repetitive genomic DNA of 250bp size from the isolates (1093/02 and 1184/02) as well as from the reference strains (*L. aethiopica* L-100 and *L. infantum* LEM-75).

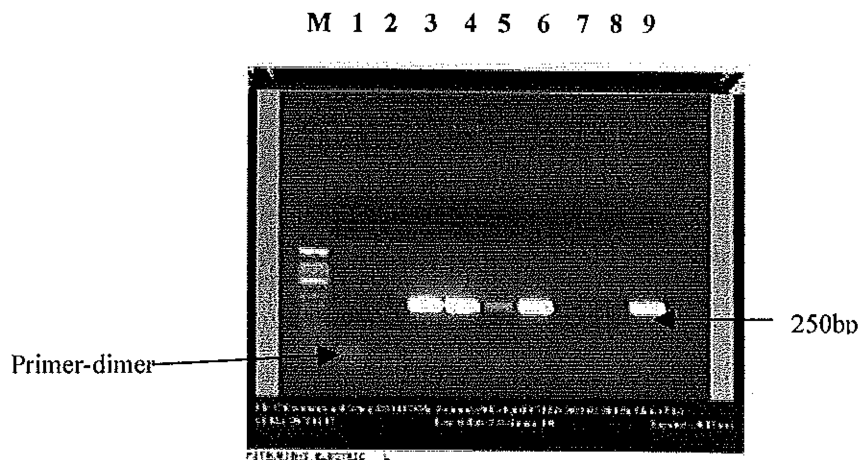


Figure 6. PCR Product of Amplification of the Repetitive Genomic DNA.

(M= O/X174 molecular size marker, Lane-1 and Lane-2= negative controls for genomic DNA extraction and PCR respectively, Lane-3 and Lane-4= 1093/02, Lane-5= 1184/02, Lane-6= *L. aethiopica* L-100, Lane-7 and Lane-8= Negative controls, and Lane-9= *L. infantum* LEM-75)

Both the isolate and the reference strains showed amplification of the 250bp gene. Whereas, the negative controls didn't show any band (Lanes 1, 2, 7 and 8, Figure-6).

Restriction digestion of the above PCR products with *Hae-III* revealed similarity of our isolate (1093/02) with the *L. aethiopica* reference strain. As it is shown in Figure-7, both the reference strain (*L. aethiopica* L-100) (lane-4) and the isolate (1093/02) (lanes-1 and –2) resulted in two bands of about 215bp and 35bp in size. Whereas, *L. infantum* produced only one band of about 250bp in size (lane-5). The isolate 1184/02 didn't produce visible bands (lane-3).

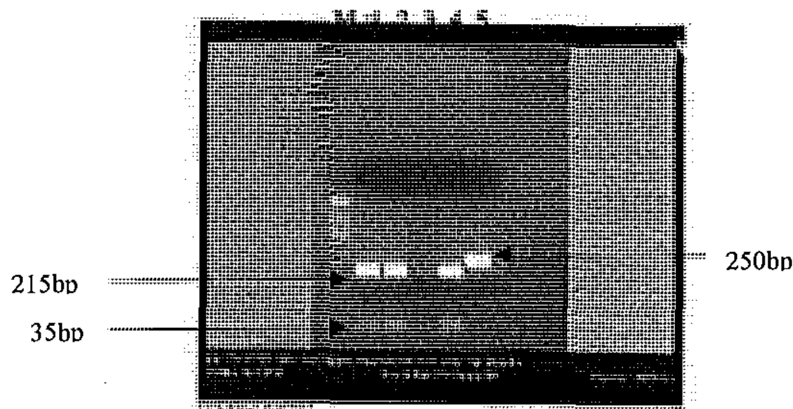


Figure 7. Restriction Digestion Pattern of the PCR Product.

(M= molecular size marker, Lane-1 and Lane-2= isolate 1093/02, Lane-3= 1184/02, Lane-4= *L. aethiopica* L-100, Lane-5= *L. infantum* LEM-75)

4.4. CHARACTERIZATION OF SOD GENES

4.4.1. Polymerase Chain Reaction

Total genomic DNA was extracted and open-reading frames (ORFs) of SODB1 and SODB2 were amplified from both the isolates and the positive control (*L. donovani*).

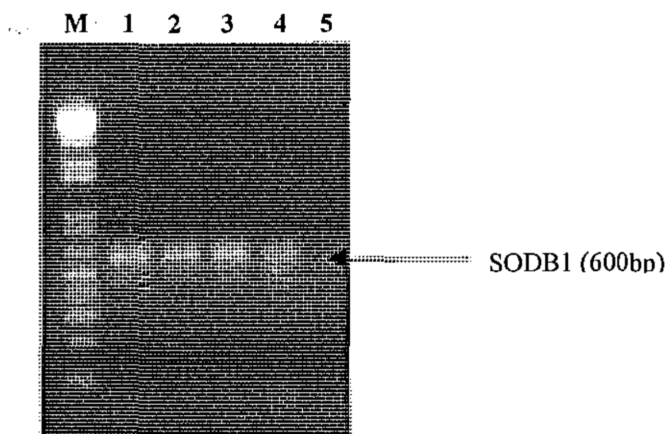


Figure 8. First PCR Products of SODB1

(M= 1kb molecular size marker, Lane-1= 1093/02, Lane-2= 1184/02, Lane-3= 1185/02, Lane-4= *L. donovani*, and Lane-5= negative control (PCR blank)).

As it is shown in the Figure-8 above, SODB1 ORF was amplified from the isolates 1093/02, 1184/02 and 1185/02 (lanes1, 2, and 3 respectively) as well as from the

positive control (lane-4). This gene has a molecular size of about 600bp. The fifth lane was the negative control and it didn't give any band. On the other hand, PCR amplification of the SODB2 gene resulted in a fragment of about 650bp in size. Like SODB1 amplification, lane-5 didn't give any band (Figure-9).

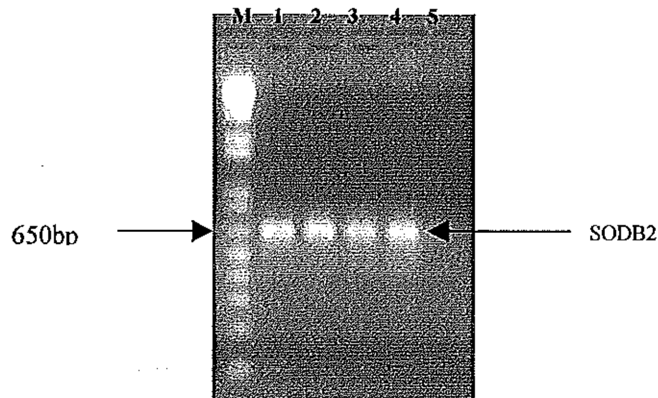


Figure 9. First PCR Products of SODB2

(M= 1kb molecular size marker, Lane-1= 1093/02, Lane-2= 1184/02, Lane-3= 1185/02, Lane-4= *L. donovani*, and Lane-5= negative control (PCR blank)).

Second PCR was done for each of SODB1 and SODB2 ORFs using the first PCR products as a template. These reactions resulted in bands with similar molecular size as the first PCR products. However, the bands were more intense than the first PCR products showing that the concentrations increased in the second reaction. Furthermore, the amount of the product was increased by making quadriplates of each of the first PCR products. The products of each isolate were pooled for sequencing.

4.4.2. Restriction Digestion of SOD Genes

Restriction digestion of SODB1 and SODB2 with *EcoR-I* resulted in two different band patterns. As shown in Figure-10, digestion of SODB1 gave two bands of about

250bp and 340bp (lane-1). Whereas, digestion of the SODB2 resulted in one big band of about 350bp in size (lane-3). In both cases the controls gave one band each. That is, SODB1 gave one band of size about 600bp (lane-2) and SODB2 gave a band with a size of about 670bp (lane-4).

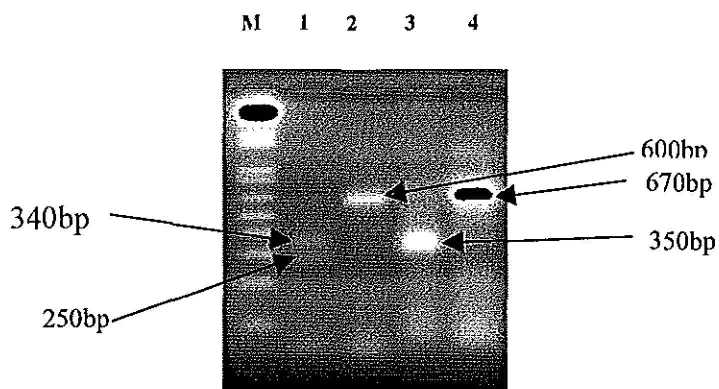


Figure 10. Restriction Digestion of SODB1 and SODB2

(M= 1kb molecular size marker, Lane-1= digested SODB1, Lane-2= undigested SODB1 (negative control), Lane-3= digested SODB2, and Lane-4= undigested SODB2 (negative control)).

4.4.3. Sequencing

Nucleic acid sequencing showed that LaethSODB1 and LaethSODB2 ORFs are 588bp and 627bp long respectively. These sequences were translated to predicted amino acid sequences using vector NTI software. Nucleic acid alignments of *L. aethiopica*, *L. chagasi* and *L. major* SODB1 and SODB2 as well as the phylogentic trees are presented in Appendix-2.

Nucleic acid sequence of LaethSODB1 (1093/02) demonstrated higher identity to LcFeSODB1 (94.9%) than LmFeSODB1 (73%). As it is indicated in Table-3 below, unlike the differences in nucleic acid sequence, the amino acid sequence showed that LaethSODB1 is not very different from LmFeSODB1 (90.8% identity).

Comparison of the predicted amino acid sequence of SODB1 from *L. aethiopica* with that of LcFeSODB1 (Paramchuk *et al*, 1997) and manganese SOD of the bacteria, *Thermus thermophilus* (Ludwig *et al*, 1991) indicated that *L. aethiopica* SODB1 fall within the category of iron SOD. Amino acid sequence of LaethFeSODB1 from 1093/02 showed 89.8% identity to *Lc*FeSODB1 and 40.6% to manganese SOD of *Thermus thermophilus* (Ludwig *et al*, 1991).

```
                                       1              R-1            * 30
Translation of L aeth SODB1-1093   (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHE
Translation of L aeth SODB1-1184   (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHE
     Translation of LcFeSODB1      (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHE
     Translation of LmFeSODB1      (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHE
                    Consensus      (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHE
                                       31 R-2                          60
Translation of L aeth SODB1-1093  (31) KHHKGYAMKLNAAAESNSALASKSLVEIIK
Translation of L aeth SODB1-1184  (31) KHHKGYAMKLNAAAESNSALASKSLVEIIK
     Translation of LcFeSODB1     (31) KHHKGYAVKLNAAAESNSGLASKSLVDIIK
     Translation of LmFeSODB1     (31) KHHKGYAVKLTAAAESNSALASKSLVDIIK
                    Consensus     (31) KHHKGYAMKLNAAAESNSALASKSLVDIIK
                                       61               *            90
Translation of L aeth SODB1-1093  (61) SEKGPAFNCAAQIFNHDFFWRCLSPQGGGE
Translation of L aeth SODB1-1184  (61) SEKGPAFNCAAQIFNHDFFWRCLSPQGGGE
     Translation of LcFeSODB1     (61) SEKGPAFNCAAQIFNHDFFWRCLSREAGGE
     Translation of LmFeSODB1     (61) SEKGPAFNCAAQIYNHDFFWRCLSPCGGGE
                    Consensus     (61) SEKGPAFNCAAQIFNHDFFWRCLSPQGGGE
                                       91                           120
Translation of L aeth SODB1-1093  (91) PSGNLASAINASFGSFASFKEEFTAAANGH
Translation of L aeth SODB1-1184  (91) PSANLASAINASFGSFASFKEEFTAAANGH
     Translation of LcFeSODB1     (91) PSGPLASAIVDSFGTFASFKKEFTDAPNGH
     Translation of LmFeSODB1     (91) PSGNLASAIIDSFGSFSNFKEEFTAAANGH
                    Consensus     (91) PSGNLASAINDSFGSFASFKEEFTAAANGH
                                        R-3                         150
Translation of L aeth SODB1-1093 (121) FGSGWAWLVKDKSNDKLKVLQTHDAGCPLT
Translation of L aeth SODB1-1184 (121) FGSGWAWLVKDKSNGKLKVLQTHDAGCPLT
     Translation of LcFeSODB1    (121) FGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
     Translation of LmFeSODB1    (121) FGSGWAWLVKDKSSGKLKVFQTHDAGCPLT
                    Consensus    (121) FGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
                                       151         *  *R-4          180
Translation of L aeth SODB1-1093 (151) EPNLVPILTCDVWEHAYYIDYRNDRAAYVN
Translation of L aeth SODB1-1184 (151) EPNLVPILTCDVWEHAYYIDYRNDRAAYVN
     Translation of LcFeSODB1    (151) EPNLVPMLTCDIWEHAYYIDYRNDRASYVN
     Translation of LmFeSODB1    (151) EPDLVPILTCDVWEHAYYIDYKNDRASYVS
                    Consensus    (151) EPNLVPILTCDVWEHAYYIDYRNDRAAYVN
                                          R-5       196
Translation of L aeth SODB1-1093 (181) AFWNMVNWDFATSQL-
Translation of L aeth SODB1-1184 (181) AFWNMVNWDFATSQL-
     Translation of LcFeSODB1    (181) AFWNMVDWDFASSQL-
     Translation of LmFeSODB1    (181) AFWNMVDWDFASSQL-
                    Consensus    (181) AFWNMVNWDFASSQL
```

Figure 11. Multiple Amino Acid Sequence Alignment of *Leishmania* Iron SODs

(Open boxes represent conserved amino acid sequences in several Fe and Mn SODs, and Asterisk represents the invariant amino acids involved in coordinating the metal ion)

The five boxes in the multiple amino acid sequence alignment above indicate the conserved regions of iron and manganese SODs as defined by Heinzen *et al* (1992).

Table 3. Comparison of Similarity of LaethFeSODs with SODs of other organisms

	LaethFeSODB1		LaethFeSODB2	
Organism	Nucleic acid(%)	Amino acid (%)	Nucleic acid (%)	Amino acid (%)
L. chagasi	94.9	89.8	95.7	92.8
L. major	73	90.8	72.1	90.4
Thermus thermophilus	55.5	40.6	54	38.5

Percent identities were tabulated from pairwise alignments of *L. aethiopica* FeSODB1 sequences with iron-containing SODs from *L. chagasi* and *L. major* as well as manganese-containing SOD from *Thermus thermophilus* using vector NTI software. *L. aethiopica* SODs displayed appreciably higher amino acid identity with the FeSODs than with the MnSODs.

The invariant amino acid residues involved in Fe and Mn metal binding, three histidines and one aspartate residue (Parker and Blake, 1988), are conserved in LaethFeSODB1 and LaethFeSODB2. The histidine residues are found at amino acid positions 28,76, and 165 and the aspartate residue is located at position 161.

The two *L. aethiopica* SODs (SODB1 and SODB2) showed less identity (86.6%) than each enzyme separately with other organisms. Furthermore, *L aethiopica* FeSODB2 contains a 13 amino acid extension at the amino terminus that is absent in LaethFeSODB1.

4. DISCUSSION

The objectives of this study were to identify SOD genes in *L. aethiopica*, to see their similarities and differences to those of other *Leishmania* species as well as to show the role of the enzyme SOD in protecting the parasite from the host immune system. In order to address these objectives, isolation of the parasites from cutaneous leishmaniasis patients and species typing of the isolates were considered as the first step of the study. Thus, the study included characterization of both the parasite and their SOD genes.

In order to characterize SOD genes of *L. aethiopica*, we preferred working on clinical isolates than on reference strains. This is because actual clinical strains were considered more wild type and therefore more appropriate.

We needed to characterize SOD on 5 to 10 different strains to confirm that the gene in *L. aethiopica* was not extremely variable. Testing on one isolate may not represent the true nature among a species because of the possibility that the single isolate may be a changed variant. Thus, a total of twenty suspected cutaneous leishmaniasis patients were enrolled in the study.

According to the clinico-epidemiological data, the clinical isolates obtained from cutaneous leishmaniasis patients were representative of circulating strains. The age, sex, address, duration of lesion as well as site of lesion fit with known pattern in the literature. Younger segment of the population is affected more by the disease. Sixteen (80%) of 20 suspected patients enrolled in this study were below 30 years of age.

Furthermore, the most frequently affected part of the body was the face (Mengistu *et al*, 1992). The latest is attributed to the higher susceptibility of this part of the body to the bite of sandflies.

In our study population, *Leishmania* culture postivity was low (25%). When diagnosis was made using *Leishmania*-specific PCR from the biopsy specimens of these patients, about 75% were found to be *Leishmania* positive (data not shown). The low isolation rate in the study population might be due to technical difficulties in specimen collection and culturing the parasite.

As it is shown in Table-1, in all culture positive cases the duration of lesion was less or equal to 12 months. It seems logical that as the lesion becomes chronic, the possibility of secondary infection increases which in turn impairs the *in vitro* growth of the parasite.

Species identification of the clinical isolates was done using two types of identification techniques: isoenzyme electrophoresis (IE) and PCR-RFLP. Isoenzyme electrophoresis has been considered as a gold-standard for typing *Leishmania* world wide (Rioux *et al*, 1990). Whereas, although it has not been established for all species, PCR-RFLP is a relatively rapid and easy method (Minodier *et al*, 1997).

In order to type a strain and give it an international code, IE is expected to be done for at least 13 enzymes (Le Blancq *et al*, 1986 and Rioux *et al*, 1990). However, in this study only glucose phosphate isomerase was analyzed. This was because the parasite

number at the time of typing was not enough to perform IE for 13 or more enzymes. GPI was selected for two reasons: it can differentiate *L. aethiopica* from other Old World CL causing species and the bands produced in IE of GPI are known to be clear and easier to interpret (Le Blancq *et al*, 1986 and Rioux *et al*, 1990). Moreover, according to Le Blancq and his colleagues (1986), out of 13 enzymes analyzed, *L. aethiopica* had only one enzyme pattern (esterase) in common with *L. tropica* and none with *L. major*. These justify that IE of only GPI can be used for species identification of local *Leishmania* isolates.

As it is shown in Figure-5 and Table-2, GPI from the isolate 1093/02 gave a band at similar position to that of GPI from *L. aethiopica* L-100 but at a different position from other *Leishmania* reference strains tested. In the work of Rioux *et al* (1990), only *L. aethiopica* strains showed a zymogram of 53 for GPI. No other species gave less than 55. In our work, IE for GPI resulted in a zymogram of 50 (for *L. aethiopica* L-100 and 1093/02), 80 (for *L. major* 5ASKH), 85 (for *L. tropica* K-27) and 120 (for *L. infantum* MON-183).

PCR-RFLP was done to supplement the result of IE. The technique has been used in typing of species in epidemiological studies (Minodier *et al*, 1997). It is advantageous over other techniques in that it is rapid and relatively easy to perform. Its disadvantage is that it is not a well-established technique for all *Leishmania* species. However, it is known that it can differentiate *L. aethiopica* from *L. major*. Thus, in our study, we employed PCR-RFLP to rule out *L. major* and to further confirm the findings by IE.

PCR-RFLP involves PCR amplification of a 250bp repetitive genomic DNA followed by restriction digestion with *Hae-III.* This results in only one band of 250bp for *L. infantum*, four bands of 215, 155, 95 and 35bp for *L. major* and two bands of 215 and 35bp for *L. tropica* and *L. aethiopica* strains (Minodier *et al* 1997). In our experiment, two bands of 215 and 35bp were obtained from the isolate 1093/02 and *L. aethiopica* L-100 thus confirming that the isolate is not *L. major*.

In our study, the clinical isolate (1093/02) was confirmed as being *L. aethiopica* using IE and PCR-RFLP. It is this isolate that was used for the genetic study.

The genes that encode superoxide dismutase were characterized by sequence analysis of the PCR product. The genomic DNA from promastigotes of *L. aethiopica* isolates was extracted and PCR amplification of the ORFs of LaethFeSODs.

Amplification of LaethFeSODB genes required optimization of the type of primer and the PCR conditions. PCR was first done using degenerate primers previously designed to amplify *L. chagasi* FeSODs (GFP+ labeled LcFeSODB1ATG and LcFeSODB1TAA). In the first few attempts, no amplification was obtained. Upon modification of the nucleotide sequence of primers, amplification of the desired gene was obtained. However, the products were not pure. That is, multiple non-specific bands appeared on the gel together with the desired gene. Finally, the PCR program was changed in such a way that the annealing time was reduced from one minute to 30 seconds. This modified PCR cycling condition in combination with the new primers resulted in correct amplification of FeSODB1 and FeSODB2 from *L. aethiopica*

genomic DNA. The bands of LaethFeSODB1 lay in between 500bp and 650bp molecular size marker and that of LaethFeSODB2 lay around 650bp molecular size marker. Upon sequencing of these PCR products, the size was found to be 588bp for LaethFeSODB1 and 627bp for LaethFeSODB2.

Restriction digestion of the PCR products with *EcoR-I* was used as a presumptive confirmation of SOD genes. Paramchuk *et al* (1997) confirmed that *L. chagasi* SODs have an *EcoR-I* site 334 bases downstream of the start codon. Digestion of LaethFeSODB1 resulted in two bands with approximate size of 340bp and 250bp on 1.5% agarose gel (Figure-10 lane-1). Whereas, digestion of LaethFeSODB2 generated a single band of about 350bp on the gel (lane-3). These results were in agreement with the published data and that of the sequence information. That is, digestion of SODB1 produces two bands of size 334bp and 254bp and SODB2 two bands of size 334 and 293bp (Paramchuk *et al*, 1997). The appearance of only one intense band instead of two in LaethFeSODB2 digestion (lane-3) might be because of the incapability of the gel to separate these two almost equal sized bands. On the other hand, the appearance of a single band at around 600bp for SODB1 (lane-2 of Figure-10) and 650bp for SODB2 (lane-4) (positive controls) clearly confirms that the digestion protocol worked well.

The primary objective of this work was to compare the similarity of *L. aethiopica* with other *Leishmania* species with respect to superoxide dismutase gene. Studies on *L. chagasi*, *L. major* and *L. tropica* have indicated that SOD and the genes encoding SOD are conserved across different *Leishmania* species (Paramchuk *et al*, 1997;

Ghosh *et al*, 2003). In agreement with these works, *L. aethiopica* SOD amino acid sequences showed high degree of identity (similarity) with iron-containing SODs of *L. chagasi* (89.8%) and *L. major* (90.8%). However, small variations were found in the predicted amino acid sequences of *L. aethiopica* SODs. For example, as shown in Figure 11, valine is substituted for methionine at position 38, and aspartate for glutamate at position 57. The variation is also seen even in a region (region-5, Figure-11) which has been found to be conserved in several Fe and MnSODs (Heinzen *et al*, 1992). That is, aspartate is substituted for asparagine at position 187. This substitution changes the charge of the amino acid and thus might affect the properties of the protein.

It was also found out that the invariant amino acids that are thought to be involved in coordinating the metal ion at the active site of SODs are conserved in LaethFeSODB. These are three histidine residues and one asparate residue at positions 28, 76, 165 and 161 respectively.

The relatively higher degree of variation in the nucleic acid sequences of LaethFeSODB1 and LmFeSODB1 (73%) can be utilized to develop a molecular diagnostic tool. Upon identification of the variable region one can develop specific primers that selectively amplify species-specific segment of the SOD gene. This, in effect, could help us distinguish these species in places like Ethiopia where both *L. aethiopica* and *L major* infections occur.

CONCLUSION

In this study the genes that code for superoxide dismutase in *L. aethiopica* were characterized using molecular biological techniques. The SODB1 and SODB2 genes were PCR-amplified from the genomic DNA of promastigotes and sequenced. The sequence information showed that *L. aethiopica* SODB genes are under the family of iron-containing SODs. Predicted amino acid comparison indicated that *L. aethiopica* SODB has high degree of similarity with SODBs of other *Leishmania* species. This similarity is an important finding in order to use new antileishmanial drugs targeting SODs of other *Leishmania* species (if any) to treat CL caused *by L. aethiopica.* However, small variations were seen in certain regions including those that have been found to be conserved in Fe- and Mn-SODs. The impact of these variations in the structure and function of the enzyme needs further investigation. On the other hand, the discrepancy in the nucleotide sequence of LaethFeSODB and other *Leishmania* species could be utilized to develop PCR based diagnostic techniques that differentiate *L. aethiopica* from other species. The attempt made to clone the genes and express in *Escherichia coli* should be strengthened so as to further study the properties of the enzyme and keep on trying to utilize it as a possible drug target or vaccine candidate against CL in Ethiopia.

REFERENCES

Akuffo, H., Kaplan, G., Kiessling, R., Teklemariam, S., Dietz, M., McElrath, J., and Cohn, Z. (1990) Administration of recombinant interleukin-2 reduces the local parasite load of patients with disseminated cutaneous leishmaniasis. *J. Infect. Dis.* **161**: 775-80.

Ashford, R. W, Bray, M. A., Hutchinson, M. P. and Bray, R. S. (1973) The epidemiology of cutaneous leishmaniasis in Ethiopia. *Trans. Roy.Soc.Trop.Med. and Hyg.* 67: 568-601.

Beaman, L. and Beaman, B. L. (1990) Monoclonal antibodies demonstrate that superoxide dismutase contributes to protection of N*ocardia asteroids* within the intact host. *Infect. Immun.* **58**: 3122-28.

Benov, L. and Fridovich, I. (1998) Growth in iron-enriched medium partially compensates E*schrichia coli* from the lack of manganese and iron superoxide dismutases. *J.Biol.Chem.* **273**: 10313-16.

Blank, C., Bogdan, C., Bauer, C., Erb, K. and Moll, H. (1996) Murine epidermal Langerhan cells do not express inducible nitric oxide synthase. *Eur.J.Immunol.* **26**: 792-96.

Bogdan, C., Gessner, A., Solbach, W., and Rollinghoff, M. (1996) Invasion, control and persistence of *Leishmania* parasite. *Curr.Opin.Immunol.* **3**:517-25.

Bogdan, C., Rollinghoff, M., and Solbach, W. (1990) Evasion strategies of *Leishmania* parasites. *Parasitol. Today.* **6**: 183-86.

Buettner, G. R. (1998) Antioxidant enzymes and functions. http://www. medicine.uiowa.edu /frrb/SRFRSsunrisefreraschool198buettner.pdf pp. 1-20.

Crawford, R. M., Leiby, A. D., Green, S. J., Nacy, C. A., Frontier, A. H., and Meltzer, M. S. (1994) Macrophage activation: a riddle of immunological resistance. *In*: Zwilling,B.S. and Eisentein, T. K. (eds) *Macrophage-pathogen interaction.* pp. 29-46. Marcel Dekker,Inc. New York, Basel and Hong Kong.

Desjeux, P. (1996) Leishmaniasis: public health aspects and control. *Clinics in Dermatology* **14**: 417 - 23.

El Hassan, A. M. (2001) Old World cutaneous leishmaniasis. *Africa Health* **11**: 9 - 11.

Evans, D. (1989) Handbook on isolation, characterization and cryopreservation of *Leishmania*. UNDP/World Bank/ WHO.

Fang, F. C. (1997) Mechanisms of nitric oxide-related antimicrobial activity. *J.Clin.Invest.* **99**: 2818-25.

Franzon, V. L., Arondel, J., and Sansonetti, P.J. (1990) Contribution of superoxide dismutase and catalase activities to *Shigella flexneri* pathogenesis. *Infect. Immun.* **58**: 529-35.

Fridovich, I. (1989) Superoxide dismutase: an adaptation to a paramagnetic gas. *J.Biol.Chem.* **264**: 7761-4.

Fridovich, I. (1997) Superoxide anion radical ($O_2^{\cdot-}$), superoxide dismutases, and related matters. *J.Biol.Chem.* **272**: 18515-7.

Ghosh, S., Goswami, S., and Adhya, S. (2003) Role of superoxide dismutase in survival of *Leishmania* within the macrophage. *Biochem. J.* **369**: 447-452.

Green, S. J., Meltezer, M. S., Hibbs, J. B., and Nacy, C. A. (1990) Activated macrophages destroy intracellular *L. major* amastigotes by an L-arginine-dependent killing mechanism. *J.Immunol.* **144**: 278-83.

Hailu, A. and Frommel, D. (1993) Leishmaniasis In: Kloos, H. and Ahmed Zein, Z. (eds) *The ecology of health and disease in Ethiopia*. Pp. 375-88. Westview press. Boulder, San Francisco, Oxford.

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

Heinzen, R. A, Frazier, M. E., and Mallavia, L. P. (1992) C*oxiella burnetii* superoxide dismutase gene: cloning, sequencing and expression in *Escherichia coli*. *Infect. Immune.* **60**: 3814-23.

http:/metallo.scripps.edu/PROMISE/FESOD.html

Ismail, S. O., Paramchuk, W., Skeiky, Y. A., Reed, S. G., Bhatia, A., and Gedamu, L. (1997) Molecular cloning and characterization of two iron superoxide dismutase cDNAs from *Trypanosoma cruzi*. *Mole. and Biochem. Parasitol.* **86**: 187-97.

Katakura, K. and Kobayashi, A. (1988) Acid phosphatase activity of virulent and avirulent clones of *L. donovani* promastigotes. *Infect. Immun.* **56**: 2856-60.

Kelleher, M., Moody, S. F., Mirabile, P., Osborn, A. B., and Handman, E. (1995) Lipophosphoglycan blocks attachment of *L. major* amastigotes to macrophages. *Infect. Imuun.* **63**:43-50

Le Blanq, S. M, Belehu, A, and Peters, W. (1986) *Leishmania* in the Old world: 3 the distribution of *L. aethiopica* zymodemes. *Trans. Roy. Soc. Trop. Med. and Hyg.* **80**: 360-66

Leiw, F. Y., Millott, S., Parkinson, C., Palmer, M. J., and Moncada, S. (1990) Macrophage killing of *Leishmania* parasite *in vivo* is mediated by nitric oxide from L-arginine. *J.Immunol.* **144**: 4794-97.

Ludwig, M. L., Metzeger, A. L., Pattridge, K. A. and Stallings, W. C. (1991) Manganese superoxide dismutase from *Thermus thermophilus*. *J. Mol. Biol.* **219**: 335-58.

Mengistu, G., Laskay, T., Gemetchu, T., Humber, D., Ersamo, M., Evans, D., Teferedegne, H., Phelouzat, M. A., and Frommel, D. (1992) Cutaneous leishmaniasis in southwestern Ethiopia: Ocholo revisited. *Trans. Roy. Soc. Trop. Med. and Hyg.* **86**: 149-53.

Minodier, P., Piarroux, R., Gambarelli, F., Joblet, C and Dumon, H. (1997) Rapid identification of causitive species in patients with Old world leishmaniasis. *J.Clin. Microbiol.* **35**: 2551-55.

Moncayo, A., Kuzue, F. and Modabber, F. (1994) Trypanosomiasis and leishmaniasis. In: Lankinen, K. S., Bergstrom, S., Makela, P. H., and Peltomaa, M. (eds.) *Health and Disease in Developing Countries.* Pp. 211 - 20. MacMillan.

Mosser, D. M. and Brittingham, A. (1997) *Leishmania*, macrophage and complement: a tale of subversion and exploitation. *Parasitology* **115**: S9 - S23.

Paramchuk, W. J., Ismail, S. O., Bhatia, A., and Gedamu, L (1997) Cloning, characterization and overexpression of two iron superoxide dismutase cDNAs

from *Leishmania chagasi*: role in pathogensis. *Mole.and Biochem.Parasitol.* **90**:203-21.

Parker, M. W. and Blake, C. C (1988) Iron- and manganese-containing superoxide dismutases can be distinguished by their primary structure. *FEBS Lett.* **229**: 377-82

Pearson, R. D., and Steingbiel, R. T. (1980) Mechanism of lethal effect of human serum upon *L. donovani*. *J.Immunol.* **125**: 2195-2201.

Pesci, E. C., Cottel, D. L., and Pickett, C. L. (1994) Genetic, enzymatic and pathogenic studies on the iron superoxide dismutase of *Campylobacter jejuni*. *Infect. Immun.* **62**: 2687-94.

Richard, G., Cynthia, M., Anuraj, H., and Laurie, R. (1994) Interaction between *L. major* and macrophages. In: Zwilling, B. S. and Eisentein, T. K. (eds) *Macrophage-pathogen interaction*. Pp437-60. Marcel Dekker,Inc. New York, Basel and Hong Kong.

Rioux, J. A., Lanotte, G., Serres, E., Pratlong, F., Bastien, P. and Perieres, J. (1990) Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. *Ann. Parasitol.Hum.Comp.* **65**:111-125

Robinson, J. M., and Badway, J. A. (1994) Production of active oxygen species by phagocytic leukocytes. In: Zwilling, B. S. and Eisentein, T. K. (eds) *Macrophage-pathogen interaction*. Pp159-78. Marcel Dekker,Inc. New York, Basel and Hong Kong.

Russel, D. G., and Talamas-Rohana, P. (1989) *Leishmania* and macrophage: a marriage of inconveniece. *Immunol. Today* **10**: 328-33.

Sambrook, J., Fritch, E. F., and Maniatis, T.(1989) Molecular cloning: a laboratory manual. 2nd edition.Vol.-I. Pp. 6.3-6.60 Cold Spring Harbor Laboratory Press.

Sarojini, P. A., Humber, D. P., Yemane-Berhan, T., Fekede, E., Belehu, A., Mock, B., and Warndorff, J. A. (1984) Cutaneous leishmaniasis cases seen in two years at the All African Leprosy Rehabilitation and Training Center Hospital. *Ethiop. Med. J.* **22**: 7-11.

Talamas-Rohana, P., Wright, S. D., Lennartz, M. R., and Russell, D. G. (1990) Lipopolysaccharide from *L. mexicana* promastigotes binds to members of the CR3, $p^{150,95}$ and LFA-1 family of leukocyte integrins. *J.Immunol.* **144**: 4817-24.

Tannich, E., Bruchhaus, I., Walter, R. D. and Horstmann, R. D. (1991) Pathogenic and nonpathogenic *Entamoeba histolytica*; identification and molecular cloning of an iron-containing superoxide dismutase. *Mole. and Biochem. Parasitol.* 49: 61-72.

WHO (2000) The leishmaniases and *Leishmania*/HIV co-infections. *Fact Sheet* N<u>o</u> **116.**

Woods, M. L., Mayer, J., Evans, T. G., Hibbs, J. B. (1994) Antiparasitic effects of nitric oxide in an *in vitro* model of *Chlamydia trachomatis* infection and in an *in vivo* murine model of *L. major* infection. *In*: Zwilling, B.S. and Eisentein, T.K. (eds) *Macrophage-pathogen interaction.* pp. 179-95. Marcel Dekker,Inc. New York, Basel and Hong Kong.

APPENDIX

Appendix 1. Data Acquisition Format

1. Name..Patient Code.......................
2. Age.......................... Sex a. Male...... b. Female.........
3. Patient's address Region........... Zone.................Woreda......... Kebele........... House Number..........
4. Duration of lesion...................................
5. Type of lesion.......................................
6. Site of lesion a. Nose....... b. Ear.......c. Lip i. upper.......ii. lower.........d. Forehead.......e. others, Specify..........
7. Change in the nasal and mucus membrane a. yes....... b. No......
8. Cutaneous scars a. present......... b. absent.........
9. Clinical form.............
10. Specimens submitted for lab. examination:

    a. Mucosal smear.......b. Biopsy........c. Lymph node aspirate........d. Blood..............
11. Type and Duration of Treatment (if any)

....

....

....

12. Response to Treament.................

Complications

Pulmonary infections......................

Anaemia.....................

Super-infection...............................

```
                                1                                                                         75
L aeth SODB1-1093       (1)  MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAMKLNAAAESNSALASKSLVEIIKSEKGPAFNCAAQIFN
  Laeth SODB1-1184      (1)  MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAMKLNAAAESNSALASKSLVEIIKSEKGPAFNCAAQIFN
         LcFeSODB1      (1)  MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLNAAAESNSGLASKSLVDIIKSEKGPAFNCAAQIFN
         LdFeSODB1      (1)  MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLNAAAESNSGLASKSLVDIIKSEKGPAFNCAAQIFN
         LmFeSODB1      (1)  MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLTAAAESNSALASKSLVDIIKSEKGPAFNCAAQIYN
         Consensus      (1)  MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLNAAAESNSALASKSLVDIIKSEKGPAFNCAAQIFN
                             76                                                                       150
L aeth SODB1-1093      (76)  HDFFWRCLSPQGGGEPSGNLASAINASFGSFASFKEEFTAAANGHFGSGWAWLVKDKSNDKLKVLQTHDAGCPLT
  Laeth SODB1-1184     (76)  HDFFWRCLSPQGGGEPSANLASAINASFGSFASFKEEFTAAANGHFGSGWAWLVKDKSNGKLKVLQTHDAGCPLT
         LcFeSODB1     (76)  HDFFWRCLSREAGGEPSGPLASAIVDSFGTFASFKKEFTDAENGHFGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
         LdFeSODB1     (76)  HDFFWRCLSPRGGGEPSGPLASAIVDSFGSFASFKKEFTDAANGHFGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
         LmFeSODB1     (76)  HDFFWRCLSPCGGGEPSGNLASAIIDSFGSFSNFKEEFTAAANGHFGSGWAWLVKDKSSGKLKVEQTHDAGCPLT
         Consensus     (76)  HDFFWRCLSP GGGEPSGNLASAIVDSFGSFASFKEEFTAAANGHFGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
                             151                                          196
L aeth SODB1-1093     (151)  EPNLVPILTCDVWEHAYYIDYRNDRAAYVNAFWNMVNWDFATSQL
  Laeth SODB1-1184    (151)  EPNLVPILTCDVWEHAYYIDYRNDRAAYVNAFWNMVNWDFATSQL
         LcFeSODB1    (151)  EPNLVPMLTCDIWEHAYYIDYRNDRASYVNAFWNMVDWDFASSQL
         LdFeSODB1    (151)  EPNLVPILTCDIWEHAYYIDYRNDRASYVNAFWNMVDWDFASSQL
         LmFeSODB1    (151)  EPDLVPILTCDVWEHAYYIDYKNDRASYVSAFWNMVDWDFASSQL
         Consensus    (151)  EPNLVPILTCDVWEHAYYIDYRNDRASYVNAFWNMVDWDFASSQL
```

Leishmania SODB1 Protein Alignment

March 20, 2003

```
                                1                                                                         75
L aeth SODB2-1093     (1)  ATGCCGTTCGCTGTTCAGCCGCTGCCGTACCCCCACGATGCGCTCGCGTCGAAGGGCATGTCGAAGGAGCAGGTC
L aeth SODB2-1184     (1)  ATGCCGTTCGCTGTTCAGCCGCTGCCGTACCCCCACGATGCGCTCGCGTCGAAGGGCATGTCGAAGGAGCAGGTC
        LcFeSODB2     (1)  ATGCCGTTCGCTGTTCAGCCGCTGCCGTACCCCCACGATGCGCTCGCGTCGAAGGGCATGTCGAAGGAGCAGGTC
        LdFeSODB2     (1)  ATGCCGTTTGCGGTGCAGCCGCTGCCGTATCCGCATGATGCGCTGGCGAGCAAAGGCATGAGCAAAGAACAGGTG
        LmFeSODB2     (1)  ATGCCGTTTGCGGTGCAGCCGCTGCCGTATCCGCATGATGCGCTGGCGAGCAAAGGCATGAGCAAAGAACAGGTG
        Consensus     (1)  ATGCCGTTCGCTGTTCAGCCGCTGCCGTACCCCCACGATGCGCTCGCGTCGAAGGGCATGTCGAAGGAGCAGGTC
                           76                                                                       150
L aeth SODB2-1093    (76)  AGCTTCCACCACGAGAAGCACCACAAGGGCTACGCCATGAAGCTGAACGCTGCCGCGGAGTCAAACTCGGCTCCT
L aeth SODB2-1184    (76)  AGCTTCCACCACGAGAAGCACCACAAGGGCTACGCCATGAAGCTGAACGCTGCCGCGGAGTCAAACTCGGCTCCT
        LcFeSODB2    (76)  AGCTTCCACCACGAGAAGCACCACAAGGGCTACGCCGTGAAGCTGAACGCTGCCGCGGAGTCAAACTCGGGTCTT
        LdFeSODB2    (76)  AGCTTTCATCATGAAAAACATCATAAAGGCTATGCCGTGAAACTGAACGCGGCGGCGGAAAGCAACAGCGGCCTG
        LmFeSODB2    (76)  AGCTTTCATCATGAAAAACATCATAAAGGCTATGCCGTGAAACTGAGCGCGGCGGCGGAAAGCAACAGCGGCCTG
        Consensus    (76)  ACCTTCCACCACGAGAAGCACCACAAGGGGTACGCCGTGAAGCTGAACGCTGCCGCGGAGTCAAACTCGGCTCTT
                           151                                                                      225
L aeth SODB2-1093   (151)  GCGTCGAAGTCGCTGGTGGAGATCATCAAGTCTGAGAAGGGCCCCGCCTTCAACTGTGCGGCGCAGATTTTCAAC
L aeth SODB2-1184   (151)  GCGTCGAAGTCGCTGGTGGAGATCATCAAGTCTGAGAAGGGCCCCGCCTTCAACTGTGCGGCGCAGATTTTCAAC
        LcFeSODB2   (151)  GCGTCGAAGTCGCTGGTGGAGATCATCAAGTCTGAGAAGGGCCCCGCCTTCAACTGTGCGGCGCAGATTTTCAAC
        LdFeSODB2   (151)  GCGAGCAAAAGCCTGGTGGATATTATTAAAAGCGAAAAAGGCCCGGCGTTTAACTGCGCGGCGCAGATTTTTAAC
        LmFeSODB2   (151)  GCGAGCAAAAGCCTGGTGGATATTATTAAAAGCGAAAAAGGCCCGGCGTTTAACTGCGCGGCGCAGATTTATAAC
        Consensus   (151)  GCGTCGAAGTCGCTGGTGGA ATCATCAAGTCTGAGAAGGGCCCCGCCTTCAACTGTGCGGCGCAGATTTTCAAC
                           226                                                                      300
L aeth SODB2-1093   (226)  CACGACTTCTTCTGGCGCTGCCTGTCGCCACGCGGCGGGTCGAAGCCGCACGGAAAGATCGCGAGTGCGATCAAC
L aeth SODB2-1184   (226)  CACGACTTCTTCTGGCGCTGCCTGTCGCCACGCGGCGGGTCGAAGCCGCACGGAAAGATCGCGAGTGCGATCAAC
        LcFeSODB2   (226)  CACGACTTCTTCTGGCGCTGCCTGTCGCCGAGAGGCGGGTCGAAGCCGCACGGAGAGATCGCGAGTGCGATCGTC
        LdFeSODB2   (226)  CATGATTTTTTTTGGCGTTGCCTGAGCCCGCGTGGCGGCAGCAAACCGCATGGCGAAATTGCGAGCGCGATTGTG
        LmFeSODB2   (226)  CATGATTTTTTTTGGCGTTGCCTGAGCCCGCGTGGCGGCAGCAAACCGCATGGCGAAATTGCGAGCGCGATTATT
        Consensus   (226)  CACGACTTCTTCTGGCGCTGCCTGTCGCCGCG GGCGGGTCGAAGCCGCACGGAGAGATCGCGAGTGCGATCATC
                           301                                                                      375
L aeth SODB2-1093   (301)  GCCAGTTTTGGCAGCTTCGCGAGTTTCAAGGAGGAATTCACGGCTGCGGCCAACGGTCACTTTGGCTCCGGCTGG
L aeth SODB2-1184   (301)  GCCAGTTTTGGCAGCTTCGCGAGTTTCAAGGAGGAATTCACGGCTGCGGCCAACGGTCACTTTGGCTCCGGCTGG
        LcFeSODB2   (301)  GACAGTTTTGGCAGCTTCGCGAGCTTCAAGAAGGAATTCACGGATGCGGCCAACGGCCACTTTGGGTCCGGCTGG
        LdFeSODB2   (301)  GATAGCTTTGGCAGCTTTGCGAGCTTTAAAAAAGAATTTACCGATGCGGCGAACGGCCATTTTGGCAGCGGCTGG
        LmFeSODB2   (301)  GATAGCTTTGGCAGCTTTAGCAACTTTAAAAAAGAATTTACCGATGCGGCGAACGGCCATTTTGGCAGCGGCTGG
        Consensus   (301)  GACAGTTTTGGCAGCTTCGCGAGCTTCAAGAAGGAATTCACGGATGCGGCCAACGGCCACTTTGGCTCCGGCTGG
                           376                                                                      450
L aeth SODB2-1093   (376)  GCGTGGCTCGTGAAGGACAAGTCGAATGGCAAGCTGAAGGTGCTCCAAACGCACGACGCGGGCTGCCCGCTCACG
L aeth SODB2-1184   (376)  GCGTGGCTCGTGAAGGACAAGTCGAATGGCAAGCTGAAGGTGCTCCAAACGCACGACGCGGGCTGCCCGCTCACG
        LcFeSODB2   (376)  GCGTGGCTCGTGAAGGACAAGTCGAGTGGCAAGCTGAAGGTGCTCCAAACGCACGACGCGGGCTGCCCGCTCACG
        LdFeSODB2   (376)  GCGTGGCTGGTGAAAGATAAAAGCAGCGGCAAACTGAAAGTGCTGCAGACCCATGATGCGGGCTGCCCGCTGACC
        LmFeSODB2   (376)  GCGTGGCTGGTGAAAGATAAAAGCAGCGGCAAACTGAAAGTGTTTCAGACCCATGATGCGGGCTGCCCGCTGACC
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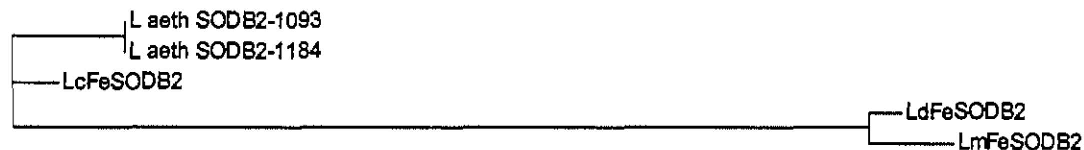
        Consensus  (376) GCGTGGCTCGTGAAGGACAAGTCGAGTGGCAAGCTGAAGGTGCTCCAAACGCACGACGCGGGCTGCCCGCTCACG
                         451                                                                       525
 L aeth SODB2-1093  (451) GAGCCCAACCTCGTGCCGATCTTGACGTGCGATGTATGGGAGCACGCGTACTACATCGACTACAGGAACGACCGC
 L aeth SODB2-1184  (451) GAGCCCAACCTCGTGCCGATCTTGACGTGCGATGTATGGGAGCACGCGTACTACATCGACTACAGGAACGACCGC
         LcFeSODB2  (451) GAGCCCAACCTCGTGCCGATCTTGACGTGCGATATATGGGAGCACGCGTACTATATCGACTACAGGAACGACCGC
         LdFeSODB2  (451) GAACCGAACCTCGTGCCGATTCTGACCTGCGATATTTGGGAACATGCGTATTATATTGATTATCGTAACGATCGT
         LmFeSODB2  (451) GAACCGGATCTCGTGCCGATTCTGGCCTGCGATGTGTGGGAACATGCGTATTATATTGATTATCGTAACGATCGT
         Consensus  (451) GAGCCCAACCTCGTGCCGATCTTGACGTGCGATGTATGGGAGCACGCGTACTATATCGACTACAGGAACGACCGC
                         526                                                                       600
 L aeth SODB2-1093  (526) GCGGCCTACGTAAACGCATTTTGGAACATGGTCAATTGGTCGCACGCCAACCACTGCTACAGGGACGCTGGCGGC
 L aeth SODB2-1184  (526) GCGGCCTACGTAAACGCATTTTGGAACATGGTCAATTGGTCGCACGCCAACCACTGCTACAGGGACGCTGGCGGC
         LcFeSODB2  (526) GCGTCCTACGTGAACGCATTTTGGAACATGGTAAATTGGTCGCACGCCAACCGCTGCTACAGGGCTGCTGGTGGC
         LdFeSODB2  (526) GCGAGCTATGTGAACGCGTTTTGGAACATGGTCAACTGGAGCCATGCCAACCGTTGCTATCGTGCGGCGGGCGGC
         LmFeSODB2  (526) GCGAGCTATGTGAGCGCGTTTTGGAACATGGTCAACTGGAGCCATGCCAACCATTGCTATCGTGCGGCGGGCGGC
         Consensus  (526) GCG CCTACGTGAACGCATTTTGGAACATGGTGAATTGGTCGCACGCCAACCACTGCTACAGGGC GCTGGCGGC
                         601                        627
 L aeth SODB2-1093  (601) TCCCACTACGTCAACAGTGATCTGTAA
 L aeth SODB2-1184  (601) TCCCACTACGTCAACAGTGATCTGTAA
         LcFeSODB2  (601) TCCCACTACGTCAACAGTGATCTGTAA
         LdFeSODB2  (601) AGCCATTATGTGAACAGCGATCTGTAA
         LmFeSODB2  (601) AGCCATTATGTGAACAGCGATCTGTAA
         Consensus  (601) TCCCACTACGTCAACAGTGATCTGTAA
```



Leishmania Nucleic Acid Alignment

March 20, 2003

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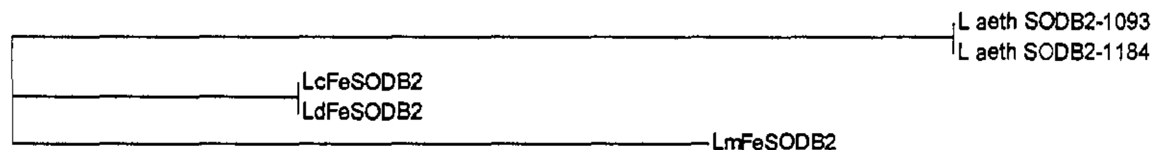
                        1                                                                         75
L aeth SODB2-1093   (1) MPFAVQPLPYPHDALASKGMSKEQVSFHHEKHHKGYAMKLNAAAESNSAPASKSLVEIIKSEKGPAFNCAAQIFN
L aeth SODB2-1184   (1) MPFAVQPLPYPHDALASKGMSKEQVSFHHEKHHKGYAMKLNAAAESNSAPASKSLVEIIKSEKGPAFNCAAQIFN
        LcFeSODB2   (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLNAAAESNSGLASKSLVDIIKSEKGPAFNCAAQIFN
        LdFeSODB2   (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLNAAAESNSGLASKSLVDIIKSEKGPAFNCAAQIFN
        LmFeSODB2   (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLTAAAESNSALASKSLVDIIKSEKGPAFNCAAQIYN
        Consensus   (1) MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLNAAAESNSALASKSLVDIIKSEKGPAFNCAAQIFN
                        76                                                                       150
L aeth SODB2-1093  (76) HDFFWRCLSPRGGSKPHGKIASAINASFGSFASFKEEFTAAANGHFGSGWAWLVKDKSNGKLKVLQTHDAGCPLT
L aeth SODB2-1184  (76) HDFFWRCLSPRGGSKPHGKIASAINASFGSFASFKEEFTAAANGHFGSGWAWLVKDKSNGKLKVLQTHDAGCPLT
        LcFeSODB2  (76) HDFFWRCLSPRGGSKPHGEIASAIVDSFGSFASFKKEFTDAANGHFGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
        LdFeSODB2  (76) HDFFWRCLSPRGGSKPHGEIASAIVDSFGSFASFKKEFTDAANGHFGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
        LmFeSODB2  (76) HDFFWRCLSPRGGSKPHGEIASAIIDSFGSFSNFKKEFTDAANGHFGSGWAWLVKDKSSGKLKVFQTHDAGCPLT
        Consensus  (76) HDFFWRCLSPRGGSKPHGEIASAIVDSFGSFASFKKEFTDAANGHFGSGWAWLVKDKSSGKLKVLQTHDAGCPLT
                        151                                                      209
L aeth SODB2-1093 (151) EPNLVPILTCDVWEHAYYIDYRNDRAAYVNAFWNMVNWSHANHCYRDAGGSHYVNSDL[illegible]
L aeth SODB2-1184 (151) EPNLVPILTCDVWEHAYYIDYRNDRAAYVNAFWNMVNWSHANHCYRDAGGSHYVNSDL[illegible]
        LcFeSODB2 (151) EPNLVPILTCDIWEHAYYIDYRNDRASYVNAFWNMVNWSHANRCYRAAGGSHYVNSDL[illegible]
        LdFeSODB2 (151) EPNLVPILTCDIWEHAYYIDYRNDRASYVNAFWNMVNWSHANRCYRAAGGSHYVNSDL[illegible]
        LmFeSODB2 (151) EPDLVPILACDVWEHAYYIDYRNDRASYVSAFWNMVNWSHANHCYRAAGGSHYVNSDL[illegible]
        Consensus (151) EPNLVPILTCDVWEHAYYIDYRNDRASYVNAFWNMVNWSHANHCYRAAGGSHYVNSDL
```



Leishmania SODB2 Protein Alignment

March 20, 2003