MOLECUAR ANALYSIS OF PEROXIDOXIN GENES FROM *LEISHMANIA AETHIOPICA*

BY

DAGIM JIRATA BIRRI

A thesis submitted to research and graduate programs office of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Biology (Applied microbiology)

June 2004

Acknowledgements

I am very much thankful to Professor Lashitew Gedamu, my external advisor, for identifying the research topic, giving lectures and laboratory courses in molecular biology, providing expensive reagents free of charge, arranging for the sequencing the genes, and giving me encouragement and constructive comments. Professor Lashitew also deserves thanks due to his excellent personality. He has an understanding character. He is also easily approachable and has a fatherly interaction with and love for his students.

My heartfelt thanks also go to Dr. Abraham Aseffa and Ato Asrat Hailu, my advisors, for their genuine guidance, critical evaluation of the draft of my thesis, and immediate feedbacks and valuable comments. I appreciate them for giving consideration to my requests even when they were busy. Their humble and easily approachable personality, which is an excellent example for everybody, warmed my heart and always motivated me to work comfortably under their supervision. In addition, Dr. Abraham is worthy to receive special gratitude as a Deputy Director of AHRI for his admirably immediate responses to my requests and his concerns for students.

I am indebted to AHRI for covering the cost of research project, allowing me to work in their well-equipped and high standard laboratory and providing transport, computer and printer facilities. I thank Dr. Howard Engers, Director of AHRI, for his significant comments and constructive criticisms, which emanate from his all-roundedness and years of experience and lead one close to perfection. I am also grateful to him for providing up-to-date information on science and his follow up of my research progress.

I am thankful to Dilla College of Teacher Education and Health Sciences, Debub University, for paying my salary during my study period, and the Department of Biology and Office of Research and Graduate Programs, Addis Ababa University, for funding the project and providing computers and other facilities.

I thank Dr. Stephen Barr for teaching me techniques in molecular biology and for sequencing the genes. I also thank Ato Abebe Genetu for teaching me laboratory techniques and for isoenzyeme typing the isolate I worked on. I am thankful to W/t Azeb Tadese for teaching me THP1 cell culturing and infection. I thank Sister Genet Amare for taking samples from patients and all the patients for their willingness to give samples for this study.

I thank Ato Teklu Kuru and W/t Liya Wassie for their valuable assistance. They are two of those colleagues I enjoyed working with. I am also thankful to Ato Girma Birhanu and Ato Endalamaw Gadissa for preparing media and maintaining *Leishmania* culture. I am thankful to all AHRI staff and my friends for every contribution they have made to my success.

I am thankful to W/o Tsedekech G/ Meskel and Ato Zelalem Wodaj for their unforgettable support during my Master's education. I am grateful to Ato Taddese Jaleta, Kefyalew Gomoro, Mesfin Tafese, Bokena Dadi, Matewos Teferi, Sileshi Desta and Mosisa Adi for their moral supports. I am also thankful to my father Jirata Birri, my mother Abebech Wolde and my sisters and brothers for their material, spiritual and moral support. Above all, I am so much thankful to God, who is the ultimate reason for my education and success.

Table of Contents

Page

List of Figures

Page

List of Tables

Page

List of Abbreviations and Symbols

AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency Syndrome
ALERT	All African Leprosy Rehabilitation and Training Center
BLAST	Basic Local Alignment Search Tool
CL	Cutaneous leishmaniasis
CuOOH	Cumene hydroperoxide
DCL	Diffuse cutaneous leishmaniasis
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
GP63	Glycoprotein 63
HIV	Human Immunodeficiency Virus
IFA	Immunoflouresent Assay
IL-12	Interleukin-12
INF-γ	Interferon gamma
LCL	Localized cutaneous leishmaniasis
LPG	Lipophosphoglycan
MEGA	Molecular Evolutionary Genetic Analysis
MCL	Mucocutaneous leishmaniasis
MHC	Major Histocompatibility Complex
NNN	Novy-MacNeal-Nicolle
mRNA	Messenger ribonucleic acid
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
Prxn	Peroxiredoxin

Pxn	Peroxidoxin
Pxn1	Peroxidoxin 1
Pxn2	Peroxidoxin 2
RACE	Rapid Amplification of cDNA Ends
RFLP	Restriction Fragment Length Polymorphism
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROOH	Alkyl hydroperoxide
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SSC	Sodium chloride/sodium citrate
Th1	T- helper 1
THP1	Human acute monocytic leukemia cell line
t-BOOH	t-butyl hydroperoxide
TryP	Tryparedoxin peroxidase
TSA	Thiol-specific antioxidant
UTRs	Untranslated regions
VL	Visceral leishmaniasis
WHO	World Health Organization

List of Appendices

Abstract

Cutaneous leishmaniasis in Ethiopia is primarily caused by *Leishmania aethiopica* and rarely by *L. tropica* and *L. major*. There is no rapid differential diagnostic method for these species. The intracellular survival mechanism of *Leishmania* parasites is not well understood although one of the mechanisms may be production of antioxidant enzymes such as peroxidoxins. Peroxidoxin genes have been isolated from various *Leishmania* species but not from *L. aethiopica*. Our objective was to identify and characterize peroxidoxin genes from *L. aethiopica*.

In this study, we identified two peroxidoxin genes (Pxn1 and Pxn2) from *L. aethiopica* by PCR. Sequence analysis of the two genes showed that there is a high nucleotide sequence homology between *L. aethiopica* and other *Leishmania* species. Southern blot hybridization analysis showed peroxidoxins of *L. aethiopica* genes exist as multigene family. RT-PCR demonstrated that pxn1 is predominantly expressed in amastigotes and stationary phase promastigotes, suggesting its importance for infectivity and intracellular survival. Pxn2 is constitutively expressed in the different stages of the parasite. Northern blot analysis and RT-PCR showed that the overall expression of all peroxidoxin genes in *L. aethiopica* is higher in the amastigotes than in the promastigotes, suggesting that peroxidoxins are important for intracellular survival. Peroxidoxin genes appear to be important virulence factors and thus may be potential targets for drug development against leishmaniasis.The genes can be explored further as potential candidate vaccines and molecular diagnostic tools. We suggest that further research be conducted on peroxidoxins to evaluate their potential as diagnostics, vaccine candidates and drug targets.

Key words: Intracellular survival, *L. aethiopica*, peroxidoxin genes

1. Introduction

1.1 Leishmaniasis

Leishmaniasis is a complex disease caused by protozoan parasites of the genus *Leishmania* of the order kinetoplastida, family trypanosomatidae. Human infection is caused by about 21 of 30 species that infect mammals. These include the subgenus *Leishmania* (*L. donovani*, *L. infantum*, *L. chagasi*, *L. mexicana*, *L. amazonensis*, *L. venezuelensis*, *L. tropica*, *L. major*, *L. aethiopica*) and the subgenus *Viannia* (*L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) panamensis*, and *L. (V.) peruviana*).

The *Leishmania* parasite occurs in two developmental forms: the amastigote and the promastigote. The amastigote is the intracellular form that lives in macrophage phagolysosome of the vertebrate host. Amastigotes are spherical, aflagellated and nonmotile. The promastigote is the extracellular form that occurs in the sandfly vector and *in vitro* culture. Promastigotes are elongated, flagellated and motile. They assume two forms: procyclic logarithmic (noninfective and dividing) and metacyclic stationary (infective and nondividing) promastigotes (Sacks and Perkins, 1984).

1.1.1 Epidemiology and geographic distribution

The disease is prevalent in tropical and subtropical regions. According to WHO, leishmaniasis is prevalent in 88 countries (16 developed, 72 developing countries) in five continents (Figure 1) (www.who.int/health_tropics leishmaniasis.htm), being highly prevalent to North Africa, the Middle East, parts of Europe and parts of Central and South America

(Roberts *et al.*, 2000). It is absent from Australia, South East Asia and the South Pacific. It has infected 12 million people. Two million new cases and 57, 000 deaths are estimated to occur each year.

The number of cases of leishmaniasis is increasing, mainly due to 1) environmental changes that increase the chance of human exposure to sandflies 2) the movement of susceptible populations into endemic areas and 3) HIV co-infection, especially in urban areas. HIV-leishmaniasis co-infection has been reported to occur in 33 countries worldwide (www.who.int/health_tropics leishmaniasis.htm). AIDS results in the reactivation of asymptomatic or previously healed *Leishmania* infections.

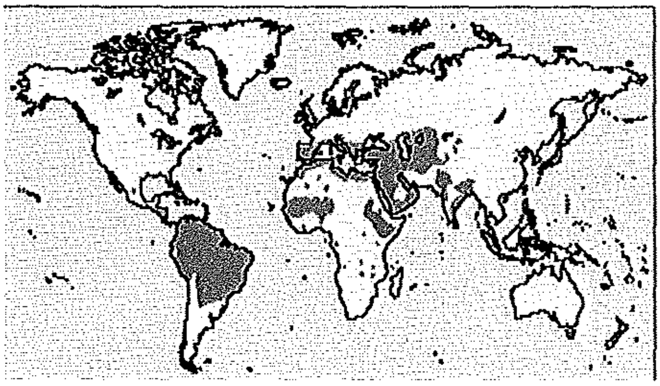

Figure 1. A map showing the worldwide distribution of leishmaniasis. **Source:** Davies *et al*, 2003.

1.1.2 Clinical manifestations

Clinically, there are two major types of leishmaniasis: visceral and cutaneous leishmaniasis. The type of leishmaniasis is determined by the *Leishmania* species, the geographic location and immune responses of the host.

Visceral leishmaniasis (VL), also called kala-azar, is most commonly caused by *Leishmania donovani* in India and Africa, *L. infantum* in the Mediterranean region, *L. chagasi* in Central and South America and occasionally by other *Leishmania* species (Berman, 1997). Ninety percent of all VL cases occur in Bangladesh, Brazil, India, Nepal and Sudan. VL is the most severe form of leishmaniasis. Death occurs within 2-3 years after infection if left untreated in almost all cases. The disease attacks reticuloendothelial cells of the visceral organs such as liver, spleen, lymph nodes and bone marrow. It is characterized by fever, weight loss, swelling of spleen and liver, and anemia.

Cutaneous leishmaniasis (CL), which is the most common form, represents 50-75% of all new cases. CL is primarily caused by *L. mexicana* complex and *L. braziliensis* complex (Grimaldi *et al.*, 1989) in the New World, and by *L. major, L. tropica* and *L. aethiopica* in the Old World (Berman, 1997). The disease attacks the mononuclear phagocytes of the skin. It presents itself as one or more skin lesions at the site of the sandfly bite on the exposed parts of the body. CL generally heals spontaneously within 3 to 6 months, leaving a scar on the skin. CL is manifested in three forms: localized cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL). In LCL, the lesions are localized to certain parts of the body whereas in DCL lesions spread throughout the body. DCL is caused by *L. mexicana, L. amazonensis* and *L. aethiopica*. DCL never heals spontaneously and relapses after treatment. Ninety percent of the global CL cases occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria.

Mucocutaneous leishmaniasis (MCL) causes disfiguring of nasal cavity and throat. It may appear even years after the initial skin ulcer has healed. Like DCL, MCL never heals spontaneously. It is common in Central and South America, where it is caused by *L. braziliensis* (Pearson *et al.*, 2000). Ninety percent of MCL cases occur in Bolivia, Brazil and Peru.

1.1.3 Hosts

The invertebrate hosts are the sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Pearson *et al.*, 2000). About 30 species of sandflies are known to be vectors of leishmaniasis (www.who.int/inf-fs/en/fact116.html). Most leishmaniases are zoonotic diseases, being mainly sylvatic. The vertebrate hosts are primarily mammals (Roberts and Janovy, 1996), including canids and rodents, which serve as natural reservoirs (Ashford and Bettini, 1987). Humans are infected only accidentally when exposed to the natural transmission cycle. Humans are the only reservoir hosts in anthroponotic forms.

1.1.4 Life cycle and Transmission

Leishmaniasis is transmitted by the bite of female sandflies, which take blood meal containing amastigotes from an infected vertebrate host. The amastigotes are transformed into the noninfective procyclic promastigotes that multiply in the midgut and migrate to the pharynx and the buccal cavity of the sandfly. The pharynx is heavily infected between the 6th and 9th day of ingestion of an infected blood meal. At this stage, the promastigotes are infective (metacyclic). During the bite of the infected sandfly vector the metacyclic promastigotes are injected into the skin of vertebrates, taken up into local macrophages by receptor-mediated phagocytosis. They end up in phagolysosomes, where they are transformed into amastigotes that survive and multiply in the hostile acidic phagolysosomes (PH 4.5-5). The macrophages eventually burst and release the amastigotes, ready to infect new macrophages. They can then be taken up by sandfly with a blood meal during a second bite, completing the cycle (Figure 2).

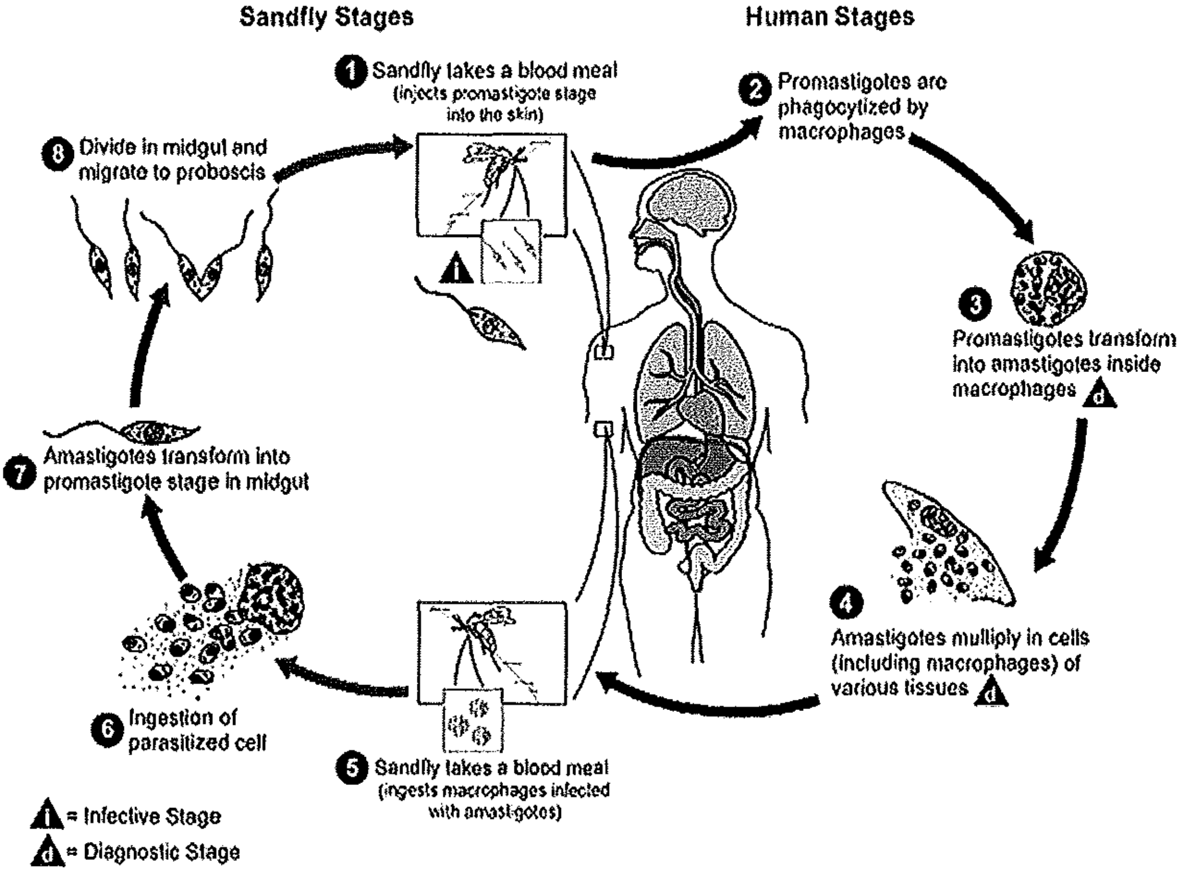

Figure 2. The life cycle of *Leishmania* parasite.

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

1.1.5 Diagnosis

Laboratory diagnosis is accomplished by parasitological, immunologic and molecular diagnostic methods. Parasitological diagnosis employs microscopy for the demonstration of amastigotes in Giemsa-stained smears of relevant tissues. The parasite (promastigotes) can also be isolated in culture in Novy-MacNeal-Nicolle (NNN) medium. Immunodiagnostic methods include serological tests to detect antibody or antigen. ELISA or IFA methods for antibody detection are of less value in CL diagnosis. Molecular diagnostic methods that include the gold standard isoenzyme gel electrophoresis and PCR, and monoclonal antibodies are differential diagnostic methods for species differentiation (Vega-Lopez, 2003). Histopathology is another diagnostic method that demonstrates amastigotes in hematoxylin-eosin stained biopsies. It is less sensitive than other methods. In Ethiopia, there is a need to develop a PCR-based diagnosis that differentiates among *Leishmania* species that cause Ethiopian CL.

1.1.6 Treatment

The drugs currently available for use against leishmaniasis include the pentavalent antimonials such as sodium stibogluconate (pentostam) and N-methylglucamine antimoniate (glucantime), pentamidine, amphotericin B and its lipid formulation AmBisome (Berman, 1997). The antimonials have been used as first line drugs for about 50 years; however, their disadvantages include toxicity, variable efficacy against VL and CL, long course of therapy and emergence of resistance strains, particularly in India (Croft and Coombs, 2003; Sundar, 2001). In Ethiopia, relapses were reported after treatment with antimonials (Berhe *et al.*, 1994). Amphotericin B is a second line drug that has been used when the antimonials fail. But, its use is limited by toxicity, high cost and difficulty to administer. The development of lipid-based formulations of amphotericin B has reduced toxicity and increased tolerance and therapeutic efficacy (Veerareddy and Vobalaboina, 2004). Three lipid formulations are now available: liposomal amphotericin B (AmBisome), amphotericin B lipid complex and amphotericin B colloidal dispersion. Pentamidine is also a second line drug that is less frequently used due to its toxicity. The aminoglycoside paromomycin (aminosidine) and other drugs for VL are in clinical trials (Croft and Coombs, 2003). Thus, less toxic drugs that are effective and can be administered orally are needed. Recently, a promising new drug, oral miltefosine, was registered in India in 2002 for use against VL (Davies *et al.*, 2003).

1.1.7 Prevention and control

Leishmaniasis can be controlled by drugs, vaccines and vector control. The drugs available and their limitations are discussed above. The most effective ones are generally the most toxic. There is no effective vaccine ready for use against leishmaniasis (Handman, 2001); Experimental vaccines are still under development. Vaccination with promastigotes is promising, but not yet ready. Thus, there is a need for the development of new drugs and vaccines, which necessitates identification of targets for candidate vaccines and drugs. Vector control is possible through the use insecticides and bednets (Davies *et al.*, 2003).

1.2 Leishmaniais in Ethiopia

All forms of leishmaniasis occur in Ethiopia. Visceral leishmaniasis due to *L. donovani* occurs in lowlands and semi-desert areas of the country such as the Segen valley around Konso (Lindtjorn and Olafsson, 1983; Ayele and Ali, 1984; Hailu, 1990), Gambella (Hailu *et al.*, 1996), Humera and Metema (Mengesha and Abuhoy, 1978), and low lands of Algena, Nakfa, Afabet, Teseney and Genale river Basin and West of Moyale (Ayele and Ali, 1984). The main sandfly vectors of VL in Ethiopia are *P. martini*, *P. orientalis* and *P. celiae* (Gebre-

Michael and Lane, 1996; Balkew *et al.*, 2002). Although the overall prevalence is not known in the country, a study conducted in 10 villages near the Segen valley, South Ethiopia, 10 years ago showed that VL prevalence and incidence was 3.1% and 1.9%, respectively (Ali and Ashford, 1994).

Cutaneous leishmaniasis commonly occurs in the highlands of Ethiopia. Ashford *et al* (1973) found LCL in three separated highland areas (Kutaber, Ocholo and Aleku), where hyraxes are the most important reservoir hosts. The disease is also found in other highland areas such as Debresina, Sebeta, Tulukuche, Goba and Adigrat (Ayele, 1982) and the Sidamo highlands (Lindtjorn, 1981). DCL is common in areas where LCL occurs (Bryceson and Leithead, 1966). Ethiopian CL is generally caused by the endemic species, *L. aethiopica* (Bryceson and Thomas, 1966; Bray *et al.*, 1973; Sarojini *et al.*, 1984) and rarely by *L. tropica* and *L. major* (Desjeux, 1991), whose vector is *P. dubscqi* (Gebre-Michel *et al.*, 1993). Like that of VL, the overall prevalence of CL in Ethiopia is not well studied, but in Ocholo, an endemic village near Arba Minch, the prevalence of CL with active lesion and scars was 10.7% and 34.4%, respectively in 1973 (Ashford *et al.*, 1973). A study conducted in the same village 19 years later, in 1992, confirmed endemicity with prevalence of 3.6% active lesions and 34.3% scars (Mengistu *et al.*, 1992). In this study, they showed that the prevalence of active lesion was higher (8.5%) in the age group 0-10. Mucocutaneous leishmaniasis is also found in Ethiopia in areas where other types of CL occur (Bryceson and Thomas, 1966; Barnetson *et al.*, 1978).

1.2.1 *Leishmania aethiopica*

Leishmania aethiopica causes a spectrum of disease ranging from the self-healing LCL to the non-self healing DCL. It is found in the highlands of Ethiopia and Kenya, Saudi Arabia and Yemen (Ashford and Bettini, 1987; Pearson and Sousa, 1996; Morsy *et al.*, 1997). The known natural vertebrate hosts of *L. aethiopica* include three species of hyraxes (*Procavia capensis, Heterohyrax brucei* and *Dendrohyrax arboreus*), the giant rat (*Cricetomys gambianus*), and man (Ashford and Bettini, 1987). The parasite is transmitted by two closely related sandfly species, *Phlebotomous longipes* and *P. pedifer,* both of which occur at high altitudes, between 1700 m and 2700 m (Ashford and Bettini, 1987). Very recently, a new sandfly vector (*P. sergenti*) for *L. aethiopica* has been discovered in the upper Awash valley (Gebre-Michael *et al.*, 2004)

Molecular genetics of *L. aethiopica* and its mechanism of intracellular survival and drug resistance are not known. In addition, there is no rapid differential diagnostic method that distinguishes among *L. aethiopica*, *L. tropica* and *L. major*, all of which occur in Ethiopia to variable degree. Ongoing global efforts to develop diagnostic, drug and vaccine targets for leishmaniasis are difficult for using *L. aethiopica* because there is no animal model for experimental infection. Attempts have been initiated in Ethiopia to identify and characterize genes from *L. aethiopica* and compare these with those in other *Leishmania* species. Therefore, the identification and characterization of genes would help to understand mechanism of intracellular survival and pathogenesis, and to identify targets for diagnostics, candidate vaccines and drugs against leishmaniasis. Moreover, the understanding of molecular genetics (gene expression and regulation) and molecular basis of drug resistance in *L. aethiopica* and other *Leishmania* species is a crucial step towards identifying target molecules for diagnostics, drugs and vaccines.

1.3 Molecular genetics of *Leishmania*

1.3.1 The genome of *Leishmania*

Hybridization studies have shown that the haploid genome of *Leishmania* is about 35 Mb in size and is distributed over 36 chromosomes which range in size from 0.35 to about 3 Mb (Wincker *et al.*, 1996; The *Leishmania* Genome Network, 1998). This study also showed that the 36 chromosomes are conserved among the Old World species of *Leishmania* (*L. major*, *L. infantum*, *L. tropica* and *L. aethiopica*). The New World *Leishmania* species *L. mexicana* complex and *L. braziliensis* complex have 34 and 35 chromosomes, respectively (Britto *et al.*, 1998). The *Leishmania* chromosomes exhibit size polymorphism such that each strain of a given species has a distinctive molecular karyotype (Lighthall and Gianni, 1992). The chromosome size variation ranges from 10- 20% between different strains and/or species (Ivens and Blackwell, 1999). Even homologous chromosomes vary in size both among species and within strains of the same species (Swindle and Tait, 1996; Wincker *et al.*, 1996; Britto *et al.*, 1998).

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

1.3.2 *Leishmania* gene organization

The *Leishmania* contains about 8600 genes, which are organized into large polycistronic units of adjacent genes on the same DNA strand (Myler *et al.*, 1999; Myler *et al.*, 2000; Myler and Stuart, 2000) and are synthenic (Wincker *et al.*, 1996; Britto *et al.*, 1998). This information is based on the *L. major* genome. The genes occur as a single pair or in multiple copies in tandem arrays (Swindle and Tait, 1996). No introns have been identified within any *Leishmania* protein coding genes (Myler *et al.*, 2000). The *Leishmania* genome has 58-60% GC content (Stiles, 1999).

1.3.3 Gene expression in *Leishmania*

The mechanism of gene expression in *Leishmania* and other trypanosomatids is different from that of higher eukaryotes. In higher eukaryotes each gene is under the control of its own promoter and is transcribed as monocistronic messenger RNA, but in *Leishmania* and other trypanosomatids, genes are usually found grouped together in tandem arrays (polycistronic transcription units) which are under the control of a single promoter and are co-transcribed to produce polycistronic precursor RNAs (Graham, 1995). Another difference is that trypanosomatid genes are devoid of introns and they do not therefore require cis-splicing to produce mature messenger RNAs. Individual mature mRNAs (monocistronic transcripts) are produced from polycistronic transcripts by a process called trans-splicing and polyadenylation (Teixeira, 1998; Monnerat *et al.*, 2004) (Figure 3).

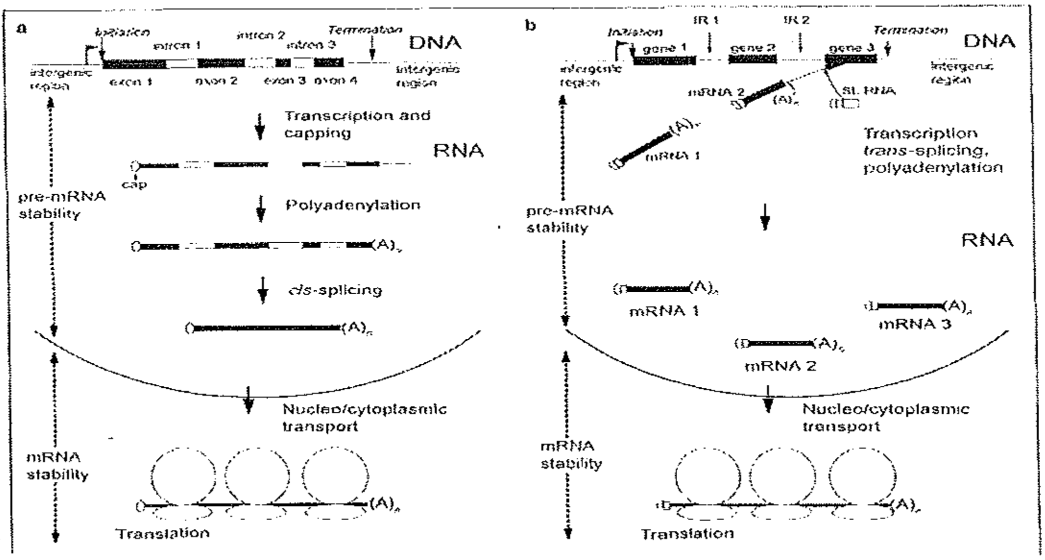

Figure 3. Transcription of gene a) higher eukaryotes b) trypanosomatids. **Source:** Graham, 1995.

1.3.4 Regulation of gene expression in *Leishmania*

In higher eukaryotes, where each gene is under the control of its own promoter, transcriptional regulation is the most common mechanism of regulation of gene expression. By contrast, in trypanosomatids, because of polycistronic organization of genes, transcriptional regulation of gene expression is not possible as this would lead to expression at the same level of all genes in the polycistronic transcriptional unit by their common promoter (Graham, 1995). But experimental evidences have shown that these genes are not expressed at the same level, and their expression is primarily post-transcriptionally regulated, possibly during RNA maturation processes, trans-splicing, and polyadenylation (Swindle and Tait, 1996; Teixeira, 1998; Monnerat *et al.*, 2004). Many genes in *Leishmania* e.g. peroxidoxin genes, histone genes, cysteine protease and SOD genes are differentially regulated in the different stages of the parasite by stage-specific modulation of mRNA abundance (Omara-Opyene and Gedamu, 1997; Barr and Gedamu, 2001; Papageorgiou and Soteriadou, 2002; Plewes *et al.*, 2003), which is mediated by sequences located in the 3' untranslated region and in intercistronic regions (Brooks *et al.*, 2001; Boucher *et al.*, 2002).

1.4 Molecular aspects of *Leishmania*-macrophage interactions

1.4.1 Macrophage defense mechanisms

Leishmania parasites live within phagolysosomes of macrophages in the vertebrate host as amastigotes. The interaction between macrophages and amastigotes determines the outcome of infection with *Leishmania*. The host uses various defense mechanisms to eliminate amastigotes. These include oxidative burst, production of reactive nitrogen species, acidification of phagolysosomes and digestion by hydrolytic enzymes.

When *Leishmania* parasites are engulfed by macrophages, the NAD(P)H oxidase in the plasma membrane of the macrophage is activated. This enzyme transfers reducing equivalents from NAD(P)H to molecular oxygen, resulting in a large increase in oxygen consumption (= oxidative or respiratory burst), which produces increased amount of reactive oxygen species (ROS). The primary ROS include superoxide anions ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). They can interact with cellular targets to produce secondary and tertiary ROS, such as hydroperoxides (ROOH) and lipid peroxides. The accumulation of ROS results in oxidative stress and consequently intracellular killing of the parasites (Murray, 1981; Murray *et al*, 1983). This is called oxidative killing (Hughes, 1988). Superoxide is formed according to the following reaction:

$$NADPH - 2e^- + 2O_2 \longrightarrow NADP^+ + H^+ + 2{}^{\cdot}O_2^-$$

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

$$H_2O_2 + O_2 \longrightarrow 2\,{}^{\cdot}O_2^- + 2\,H^+$$

Hydroxyl radical is the most biologically reactive species and can be produced according to the following reactions:

$${}^{\cdot}O_2^- + H_2O_2 \longrightarrow {}^{\cdot}OH + O_2 + OH^-$$

$${}^{\cdot}O_2^- + HOCl \longrightarrow {}^{\cdot}OH + O_2 + Cl^-$$

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$$

$$2ONOO^- + 2H^+ \longrightarrow 2\,{}^{\cdot}OH + NO_2 + NO_2^+$$

Hydrogen peroxide is produced as follows:

$$2\,{}^{\cdot}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Macrophages, upon activation, also produce reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), which are capable of killing intracellular parasites (Green *et al.*, 1989; Liew *et al.*, 1990; Barr and Gedamu, 2003). This is a nonoxidative killing and may be called nitrosative killing. Nitric oxide is produced from a terminal guanidine nitrogen atom of L-arginine (Marletta *et al.*, 1988). NO causes loss of iron from critical target enzymes of parasites (James and Hibbs, 1990). Nitric oxide and superoxide radical react to produce peroxynitrite (Bekman and Coppenol, 1996), which is more reactive than nitric oxide.

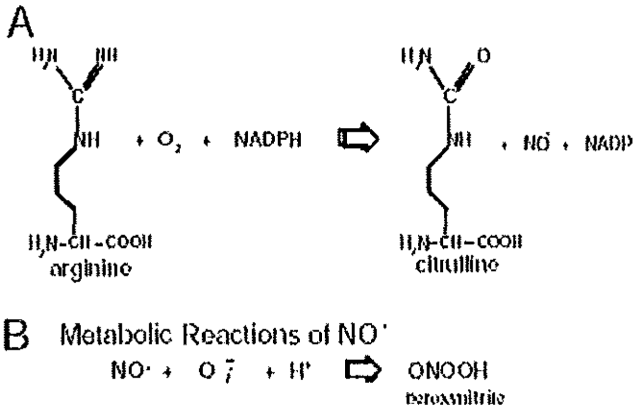

Figure 4. Formation of reactive nitrogen species. **A)** Nitric oxide formation from L-arginine. The reaction catalyzed by nitric oxide synthase. **B)** Formation of peroxynitrite

The reactive oxygen species and reactive nitrogen species have been implicated in inducing oxidation of proteins, chain breaks in polysaccharides, peroxidation of lipids and strand breaks and base modifications in nucleic acids (Chae *et al.*, 1994b). This would lead to intracellular killing of the parasites.

1.4.2 Mechanisms of survival of *Leishmania* within macrophages

The *Leishmania* parasite, on its side, has evolved various mechanisms to evade the macrophage defense responses and continues to survive and multiply in the hostile phagolysosomes (Bogdan *et al.*, 1990; Bogdan and Rollinghoff, 1998, 1999; Alexander *et al.*, 1999; Brandonisio *et al.*, 2000; Cunningham, 2002; Handman and Bullen, 2002; Zambarno-Villa *et al.*, 2002). These include inhibition of phagolysosome fusion, inhibition of hydrolytic enzymes, inhibition of cytokine production, prevention of apoptosis, inhibition of oxidative burst, inhibition of nitric oxide production, scavenging and detoxification of reactive oxygen species and reactive nitrogen species.

It has been shown that *L. donovani* amastigotes can inhibit phagosome-endosome fusion by using their lipophosphoglycan (LPG), a cell surface glycoprotein (Roberts and Janovy, 1996; Desjardins and Descoteaux, 1997; Descoteaux and Turco, 1999).

In order to survive in phagolysosomes, *Leishmania* amastigotes have to either withstand or inhibit host hydrolytic enzymes (Descoteaux and Turco, 1999). LPG may enable *Leishmania* to resist degradation by the hydrolytic enzymes (Descoteaux and Turco, 1999). In addition, the glycoprotein 63 protease, which shows optimum activity under the acidic condition of phagolysosomes, has been shown to degrade lysosomal enzymes (Cunningham, 2002).

L. donovani has been shown to prevent apoptosis of infected macrophages (Moore and Matlashewski, 1994), which results in their extended survival. Thus, by preventing apoptosis the parasites enhance their survival within macrophages.

Amastigotes of *L. amazonensis* have been found to degrade MHC class II molecules of their host (Souza-Leao *et al.*, 1995). This impairs antigen presentation by MHC molecules and thus promotes intramacrophagal survival of the parasites.

Th1 cells are responsible for protection against *Leishmania* parasite infection. These parasites have been found to inhibit production of IL-12 (Carrera *et al.*, 1996; Weinheber *et al.*, 1998), which drives Th1 (CD4+) responses and induces INF-γ production from both natural killer cells and T cells (Alexander *et al.*, 1999). INF-γ activates macrophages to produce NO. *Leishmania* is highly susceptible to killing by INF-γ activated macrophages. Therefore, this ability to suppress production of IL-12 may provide intracellular survival advantage to the parasite (Zambrano-Villa *et al.*, 2002).

Leishmania parasites have been shown to inhibit oxidative burst of mouse macrophages (Buchmuller-Rouiller and Mauel, 1987). *Leishmania* promastigotes and amastigotes contain an acid phosphatase on their surface that has been shown to inhibit oxidative burst of the macrophages. LPG and Gp63 have been associated with inhibition/suppression of oxidative burst (Descoteaux and Turco, 1999).

Leishmania possess enzymatic defense mechanisms against macrophage oxidants. The enzymatic defense is due to antioxidant enzymes such as superoxide dismutase and peroxidoxins, which bring about reduction and detoxification of reactive oxygen species and reactive nitrogen species, preventing or limiting cellular damages caused by the oxidants. Superoxide dismutase (SOD) is a very important antioxidant enzyme in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme). It catalyzes the dismutation of superoxide anion according to the following reaction:

$$2\,{}^{\cdot}O_2^{-} + 2H^{+} \xrightarrow{\underline{SOD}} O_2 + H_2O_2$$

SOD gene has been isolated from various *Leishmania* species, including *Leishmania aethiopica* (Paramchuk *et al.*, 1997; Genetu, 2003) and was found to be the first line antioxidant against superoxide (Paramchuk *et al.*, 1997). It has been shown that SOD is important for survival of *L. chagasi* and *L. tropica* in macrophages (Ghosh *et al.*, 2003; Plewes *et al.*, 2003)

1.5 Peroxidoxins

Peroxidoxins are a recently discovered family of antioxidant enzymes (peroxidases) (Hofmann *et al.*, 2002). They were first isolated from the yeast *Saccharomyces cerevisiae* (Kim *et al.,* 1988). At that time they were designated as thiol-specific antioxidants (TSA) because they were shown to protect yeast cells from oxidative damage, caused by a system generating reactive oxygen species, in the presence of a thiol compound dithithretiol (DTT), but not in the presence of ascorbate, a non-thiol molecule. Later, their specific physiological roles were shown to be reduction of peroxides and they were renamed peroxidoxins or peroxiredoxins. Since then, peroxidoxins have been identified from organisms of all kingdoms, including bacteria, protozoa, helminths, plants and mammals.

Unlike the well-known antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase, peroxidoxins do not contain tightly bound metal ions or prosthetic groups such as haeme or flavin (Kim *et al.*, 1988); nor do they show activities of these antioxidant enzymes.

1.5.1 Types of peroxidoxins

Based on the number of their cysteine residues, peroxidoxins are classified into two sub-groups: 1-cys peroxidoxins and 2-cys peroxidoxins. The 1-cys peroxidoxins have only one conserved cysteine residue that occurs in the N-terminus. The cysteine residue is surrounded by the amino acid sequence PVCT that is unique to this sub-group. The 2-cys peroxidoxins are characterized by having two conserved cysteine residues in the N-terminus (generally near residue 50) and C-terminus (generally near residue 170). Most peroxidoxins identified to date belong to this group. The N-terminus conserved cysteine is surrounded by the unique amino acid sequence FVCP (Chae *et al.*, 1994b). In addition to these two types, peroxidoxins with three cysteine residues have been recently discovered in bacteria (Hillas *et al.*, 2000; Reynolds *et al.*, 2002).

1.5.2 Function of peroxidoxins

The major function of peroxidoxins is their antioxidant (peroxidase) activity, which is dependent on thiol compounds (Kim *et al.*, 1988). Their peroxidase activity is due to their ability to reduce and detoxify reactive oxygen species such as hydrogen peroxides (Chae *et al.*, 1994a; Netto *et al.*, 1996; McGonigle *et al.*, 1998; Seo *et al.*, 2000; Wilkinson *et al.*, 2000; Barr and Gedamu, 2001, 2003; Castro *et al.*, 2002), hydroxyl radicals (Barr and Gedamu, 2001), hydroperoxides (e.g. CuOOH, t-BOOH) (Bryk *et al.*, 2000; Hillas *et al.*, 2000; Flohe *et al.*, 2002; Barr and Gedamu, 2003) and reactive nitrogen species such as peroxynitrites (Bryk *et al.*, 2000; Master *et al.*, 2002; Barr and Gedamu, 2003), protecting cells from damages induced by these species.

1.5.3 Peroxidoxins of *Leishmania*

Peroxidoxins have been identified and characterized from several species of *Leishmania*, including *Leishmania major* (Levick *et al.*, 1998; Webb *et al.*, 1998), *Leishmania chagasi* (Barr and Gedamu, 2001), *Leishmania donovani* (Barr and Gedamu, 2001; Flohe *et al.*, 2002) and *Leshmania infantum* (Castro *et al.*, 2002). They all are 2-cys peroxidoxins (cys 52 and cys 173) and have antioxidant activity that is dependent on a thiol compound called tryparedoxin. The peroxidoxin isolated from *L. major* is encoded by a multiple copy gene tandemly arranged on chromosome 15. The enzyme protects the parasite from H_2O_2, but it cannot reduce organic hydroperoxide. In this regard, it is similar to peroxidoxin 2 from *L. chagasi* (Barr and Gedamu, 2003) and *Plasmodium falciparum* (Kawazu *et al.*, 2000; Krnajksi *et al.*, 2001).

In *L. chagasi* and *L. donovani*, three differentially expressed 2-cys peroxidoxins (Pxn1, Pxn2, Pxn3) have been identified and characterized by Barr and Gedamu (2001). They showed that pxn2 and pxn3 are predominantly expressed in the promastigote stage of the parasite whereas pxn1 is predominantly expressed in the amastigote stage. Pxn1 is capable of detoxifying, alkyl hydroperoxides, hydroxyl radicals, peroxynitrites and nitric oxide whereas pxn2 detoxifies only H_2O_2 (Barr and Gedamu, 2003).

The peroxidoxin (tryparedoxin peroxidase) of *L. donovani* shows high similarity to pxn2 of *L. major* (Flohe *et al.*, 2002). This enzyme efficiently reduces H_2O_2 and has broad substrate specificity for hydroperoxides. It reacts and moderately reduces t-BOOH, CuOOH, and is marginally active with linoleic acid hydroperoxide and phophatidyl choline hydroperoxide. In this respect, it differs from peroxidoxin of *L. major* that acts poorly on t-BOOH and not at all on CuOOH.

In *L. infantum,* two peroxidoxins have been identified, with identity of only 50% (Castro *et al.*, 2002). One is localized to the cytoplasm and is encoded by a multiple copy gene. Its similarity to *L. donovani* tryparedoxin peroxidase and *L. major* pxn2 is 99% and 94%, respectively. It is 99.5% similar to pxn1 gene of *L. chagasi*. The other is found in mitochondria and is encoded by a single copy gene and it is 96.5% similar to *L. major* pxn2. Both have been found to be constitutively expressed at protein level. Although the two enzymes are found in different organelles, they have complementary antioxidant activities in protecting the parasites against oxidative stress.

1.5.4 Peroxidoxins as vaccine candidates and drug targets

Several studies in murine models have clearly demonstrated that peroxidoxin of *Leishmania* has immunogenic properties and thus can be exploited for its potential as candidate vaccine against *Leishmania*. In one of the studies, *L. major* peroxidoxin 2 was evaluated for its potential as a subunit protein vaccine (Webb *et al.*, 1998). Briefly, immunization of susceptible BALB/c mice with recombinant protein plus interleukin-12 conferred partial protection against cutaneous leishmaniasis. In addition to this, peripheral blood mononuclear cells (PBMC) from mucosal leishmaniasis patients showed strong peroxidoxin-specific proliferative responses (Webb *et al.*, 1998). PBMC from visceral leishmaniasis patients also responded to this protein although the frequency and the level of proliferation were lower (Webb *et al.*, 1998).

In another study, the immunogenicity of *L. major* peroxidoxin 2 as a DNA vaccine was studied in mouse experiments (Campos-Neto *et al.*, 2002), in which the vaccine provided solid protection against infection with virulent *L. major*. The vaccine was capable of inducing strong cellular (CD8+, CD4+ T cells) and antibody-mediated (IgG2a) immune responses.

Very recently, the vaccine potential of *L. major* peroxidoxin 2, in fusion with other two *L. major* proteins, has been evaluated in BALB/c mice that are susceptible to *L. major* infection (Coler *et al.*, 2002). This study showed that the fusion protein vaccine designated leish 111F, provided long-lasting protection against infection with *L. major* and *L. amazonensis*. The polyprotein vaccine induced gamma interferon production and IgG2a response.

Peroxidoxins may also be potential drug targets. Many researchers of peroxidoxins have suggested that enzymes of thiol metabolism such as peroxidoxins may be potential drug targets against pathogenic bacteria and parasitic protozoa, for which effective chemotherapies are not available (Hillas *et al.*, 2000; Barr and Gedamu, 2001; Son *et al.*, 2001; Kawazu *et al.*, 2001; Castro *et al.*, 2002; Flohe *et al.*, 2002; Wilkinson *et al.*, 2000). In particular, the potential of peroxidoxins has to be exploited in the development of effective drugs against trypanosomatids.

To date, no work has been done to determine the presence and role of peroxidoxins in *Leishmania aethiopica*. Our work aimed at identifying and characterizing these genes from *L. aethiopica*. We hypothesize that *L. aethiopica*, like other *Leishmania* species, possesses peroxidoxin genes that have antioxidant activity and play an important role in the intracellular survival of the parasite. We also hypothesized that peroxidoxins of *L. aethiopica* show high sequence homology to those of other *Leishmania* species.

2. Objectives of the study

2.1 General objectives

- To identify and characterize peroxidoxin genes from *Leishmania aethiopica*

2.2 Specific objectives

1. To identify peroxidoxin gene(s) from *Leishmania aethiopica*
2. To sequence of the coding region and compare the degree of homology with peroxidoxin genes of other *Leishmania* species
3. To determine peroxidoxin gene organization
4. To determine the expression pattern of the genes in the promastigote and amastigote stages of the parasite

3. Materials and methods

3.1 Parasite culture

Skin scrapings and a 4 mm biopsy were taken from the edges of lesion of two localized cutaneous leishmaniasis patients visiting ALERT hospital in Addis Ababa. Samples were taken from patients upon their informed consent. In order to culture promastigotes, the skin scrapings were inoculated onto Novy-MacNeal-Nicolle (NNN) medium and incubated at 26 °C. NNN medium is a biphasic blood agar base medium prepared from sheep blood and supplemented with Locke's overlay solution (WHO, 1990). The isolates were designated as 1093/02 and 1185/02. Five days later, promastigotes of the isolate were subcultured in a liquid medium containing RPMI 1640 medium (Sigma), 10% heat-inactivated fetal calf serum (Sigma), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (GIBCO BRL, Scotland) and were grown to logarithmic phase or stationary phase, as required for subsequent experiments. The promastigotes of the isolated 1093/02 were split into three parts: one part was used for isoenzyme typing, the second part was for nucleic acids isolation and/or infection of THP-1 cells and the third part was preserved in liquid nitrogen for further work. It was shown by isoenzyme typing that the isolate 1093/02 belongs to *L. aethiopica* (Genetu, 2003). The isolate 1185/02 was not typed.

3.2 DNA isolation

Genomic DNA was isolated from the promastigotes by the phenol-chloroform extraction and ethanol precipitation method (Sambrook and Russel, 2000). Briefly, 10^7/ml promastigotes were pelleted by centrifugation (Beckman Coulter, Allgera 6R centrifuge, USA) at 3000 rpm and the pellet was washed twice in PBS at 2000 rpm and transferred to 1.5 ml Eppendorf tube. Parasites were lysed in lysis buffer (10 mM Tris-Cl, PH 8.3, 50 mM EDTA, 1% SDS). The lysate was then incubated with RNase A (to a final concentration of 100 μg/ml) at 37 °C for 1 hour to destroy contaminating RNA and further incubated overnight with proteinase K (to a final concentration of 100 μg/ml) at 42 °C to remove proteins that would interfere with further analysis of the DNA. The DNA was extracted by adding equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and spinning in a microcentrifuge at 12, 000 g for 5 minutes. The aqueous (upper) phase containing DNA was carefully removed and transferred to a new Eppendorf tube to which 0.7 volume of isopropanol was added and incubated at –20 °C for 30 minutes. This was spun in a microcentrifuge at 12, 000 g for 15 minutes to pellet the DNA. The supernatant was discarded and the pellet was washed in 1 ml 70% ethanol, after which it was spun at 12, 000 g for 5 minutes. The precipitate was then dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), quantified in spectrophotometer at 260 nm and stored at –20 °C for further analysis. The DNA was used as a template for PCR and Southern blot analysis.

3.3 Polymerase Chain Reaction (PCR)

The coding regions of two peroxidoxin genes (pxn1 and pxn2) of *L. aethiopica* were amplified by PCR from genomic DNA isolated from the promastigotes of the isolate 1093/02. The following primers were used to amplify the coding region of pxn1: sense primer (primer 1): 5'-ACCAGGGATCC**ATG**TCCTGCGGTGACGCC-3" and antisense primer (primer 2): 5'-ACATCGGATCC**TTA**CTTATTGTGATCGACCTTCAGGCC-3'.

The pxn2 was amplified using the above sense primer and a different antisense primer (primer 3): 5'-CCGGGATCCGAATTCAGATCT**TTA**CTGTTTGCTGAAGTACC-3'. The primers have a BamH I site (underlined) and were previously used to amplify the coding regions of *L. chagasi* or *L. donovani* peroxidoxin genes (Barr and Gedamu, 2001). These primers were selected hoping that the peroxidoxin gene from *L. aethiopica* would exhibit high sequence homology that of *L. chagasi* or *L. donovani* that they could anneal to the gene.

The PCR reaction mixture contained 0.5 μg genomic DNA, 5 pmoles of forward and reverse primers, puReTaq™ Ready-To-Go PCR Bead (Amersham Biosciences, USA), which contains 2.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μm dNTPs and stabilizers including bovine serum albumin. The contents of the bead gave the respective concentrations when sterile distilled water was added to the mixture to a final volume of 25 μl. The reaction was carried out in a master cycler (Eppendorf), with the cycling conditions: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 40 °C for 2 min, 72 °C for 2 min; 72 °C for 10 min. The PCR products was verified by using internal primers: sense primer (primer 4) sequence: 5'-TATCGGATCCTCTCGACTTCACGTTTGTGCCC-3' and primer 5: 5'-TCCAGGATCCAGTTAGCGGGGCACACCTCACCGTG-3'). This is an antisense primer designed based on the highly conserved region of peroxidoxin genes of *L. chagasi* and *L. donovani* species (Barr and Gedamu, 2001). The components of this PCR were as listed above, with the following cycling conditions: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min; 72 °C for 10 minutes. The PCR product was further confirmed DNA by sequencing. The PCR product was used as a probe in Northern blot hybridization to determine the expression pattern of peroxidoxin genes in the two stages of the parasite.

3.4 Sequencing the coding regions of peroxidoxin genes

The PCR products were sent to and sequenced at the University of Calgary, The DNA Sequencing Facility, Alberta, Canada. The sequence data was analyzed in Ethiopia. The base sequence of the coding region of *L. aethiopica* peroxidoxin was compared to that of *L. major, L. tropica, L. chagasi, L. donovani* and *L. infantum* to determine the degree of sequence homology. This was done using Vector NTI software (version 6). The coding regions of peroxidoxins of *L. aethiopica* were translated into protein, and amino acid sequences were compared to peroxidoxin proteins of other *Leishmania* species using the same software. Dendrograms were constructed using MEGA 3 software by Neighbor Joining (NJ) method (Saitou and Nei, 1987).

3.5 Southern blot hybridization

Labeling of probe, prehybridization, hybridization and immunological detection were carried out using DIG High Prime DNA Labeling and Detection Starter kit I (Roche, Germany) according to manufacturer's instructions.

3.5.1 Probe preparation

DIG-labeled probes were prepared with DIG-High Prime solution (provided in the kit) by random primed labeling method. The solution contained random primers, digoxigenin-dUTP, dNTPs, Klenow DNA polymerase and 5 x reaction buffers. The PCR product was isolated from low-melting point agarose following standard procedures (Sambrook and Russel, 2000) and quantified in spectrophotometer at 260 nm. One microgram of the purified PCR product was mixed with autoclaved double distilled water to a final volume of 16 μl and denatured in a boiling water bath for 10 minutes. Then, 4 μl DIG-High Prime solution was added to the denatured DNA and the mixture was incubated at 37 °C for 20 hours, during which synthesis and labeling was accomplished.

Labeling efficiency was determined by making a series of dilutions of the labeled DNA and DIG-labeled control DNA starting from 1 ng/μl and applying to a positively charged nylon membrane, which was subjected to immunological detection. According to the manufacturer of the kit, the visibility of the 0.1 pg/μl dilution is indicative of high labeling efficiency and the probe was used at a concentration of 25 ng/ml in hybridization solution.

3.5.2 Genomic DNA blotting and hybridization

Genomic DNA (4 μg) of promastigotes was digested with the restriction enzyme EcoR I and BamH III following standard procedures (Sambrook and Russel, 2000). The digested DNA was run on 0. 8% agarose gel at 100 volts. After electrophoresis, the photograph of the gel was taken and the DNA on the gel was allowed to transfer to a positively charged nylon membrane (Roche, Germany) overnight by upward alkali (0.4 N NAOH) transfer method following standard protocols (Sambrook and Russel, 2000). The membrane was rinsed in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl, 0.001 M EDTA). The DNA was then fixed permanently to the nylon membrane by exposing to UV light (254 nm) for 5minutes. Following DNA fixation, the membrane was prehybridized with a hybridization solution, provided in the above-mentioned kit, in a shaking water bath at 68 °C for 30 minutes. The membrane was hybridized with a DIG-labeled DNA probe (25 ng/ml in 3 ml of hybridization solution) in hybridization incubator in a roller bottle overnight at 42 °C, after which stringency washes were done with decreasing salt concentration and increasing temperature. The first was done twice with 2x SSC (sodium chloride/sodium citrate) at room temperature for 5 minutes and then twice with 0.5x SSC at 68 °C for 15 minutes. Following stringency washes, immunological detection of hybrid DNA was carried out at room temperature as follows: the membrane was incubated for 30 minutes in 1x blocking solution (provided in the kit), incubated for 30 minutes in antibody solution (anti-digoxigenin antibody conjugated to alkaline phosphatase), washed twice for 15 minutes in washing buffer (0.1 M Tris-HCl, 0.15 M NaCl, PH 7.5), equilibrated for 5 minutes in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, PH 9.5) and finally incubated overnight in freshly prepared colour substrate solution containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), which react with alkaline phosphatase and bring about a color change. After color development, the reaction was stopped by washing the membrane with sterile Tris-EDTA buffer (0.1 M Tris-HCl, 1mM EDTA, PH 8) for 5 minutes. Finally, the result was photographed.

3.6 Infection of THP1 cells

The macrophage cell lines THP1 cells (American Cell Culture Collection) were used as an *in vitro* model for the infection of *L. aethiopica* (Ogukolade *et al.*, 1990; Mohamed *et al.*, 1992) and *L. donovani* (Dasgupta *et al.*, 2003). These cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum (Sigma, Germany), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (all three are from Gibco BRL, Scotland) at 37 °C in 5% CO_2 incubator. The cells were pelleted by centrifugation (Beckman Coulter, Allgera 6R centrifuge, USA) at 1500 rpm for 10 minutes at room temperature. The pellet was then washed twice with RPMI 1640 at 1500 rpm for 10 minutes at 4 °C. The pellet was suspended in RPMI 1640 and cell concentration was adjusted to 2 x 10^5 cells/ml.

The THP-1 cells were checked for viability by trypan blue exclusion and differentiated to macrophages by treatment with retinoic acid at a final concentration of 10^{-6} M. The cells were incubated with this concentration of retinoic acid at 37 °C in 5% CO_2 incubator for 5 days. The differentiated cells were washed three times with RPMI 1640 and checked for viability by trypan blue exclusion, after which they were mixed with stationary phase promastigotes at 1: 20 cell to parasite ratio and incubated in fresh medium at 37 °C in 5% CO_2 incubator for 24 hours. Verification of infection was done as follows: the cells were washed three times with RPMI 1640 at 600 rpm to remove unphagotisized promastigotes, cytocentrifgued, fixed with absolute methanol and stained with Wright stain.

3.7 RNA isolation

In order to analyze the expression pattern of *L. aethiopica* peroxidoxin genes, total RNA was isolated from promastigotes and amastigotes using TRIzol^{TM} reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, the parasites were pelleted at 3000 rpm in Beckman Coulter centrifuge and washed in 1x PBS (phosphate-buffered saline) at 2000 rpm. The pellet was resuspended in 1 ml TRIzol™ reagent and transferred to 1.5 ml Eppendorf tube and incubated at room temperature for 5 minutes. Chloroform (200 µl) was added to the suspension, shaken vigorously and left at room temperature for 3 minutes, after which it was microcentrifuged at 12,000 g at 4 °C for 15 minutes. Aqueous phase was transferred to a new tube, 500 µl isopropanol was added, mixed and incubated at room temperature for 10 minutes, after which it was microcentrifuged at 12, 000 g for 10 minutes at 4 °C. The pellet was washed in 1 ml 70% ethanol by spinning at 7, 500 g for 5 minutes. The pellet was then air-dried, resuspended in RNase free water and stored at –80 °C. The integrity of the RNA was checked by running 4 µg of the sample on 1.2% denaturing formaldehyde agarose gel before storage. In order to get amastigotes and isolate RNA from them, the macrophage cell lines THP-1 cells were infected with stationary phase promastigotes. Stationary phase promastigotes were obtained by inoculating a liquid medium with 10^6/ml parasites and harvesting them on day 6 or day 7. At this stage the parasites were nondividing and can be distinguished form the dividing logarithmic phase promastigotes (Zarley *et al.*, 1991). To determine the expression pattern of peroxidoxin genes, RNA was then analyzed by Northern blot hybridization (Sambrook and Russel, 2000) and RT-PCR. Thus the RNA was used as a template in Northern botting and in cDNA synthesis.

3.8 Complementary DNA (cDNA) synthesis

cDNA was synthesized using Omniscript Reverse Transcriptase (RT) kit (Qiagen, Germany) following manufacturer's procedures. Briefly, a reaction mixture (20 µl) of 1x RT buffer, dNTP mix (0.5 mM each dNTP), 10 units RNase inhibitor, 1 μM oligo-dT primer, 4 units Omniscript Reverse Transcriptase, 2 μg RNA and RNase free water was incubated at 37 °C for 1 hour, after which the reaction mixture was heated at 93 °C for 5 minutes to inactivate Omniscript reverse transcriptase that would interfere with further analysis of the cDNA (subsequent PCR). (All reaction reagents were components of the kit except RNase inhibitor and oligo-dT primer which were purchased from Promega, USA). This was followed by rapid chilling on ice. The cDNA was then quantified using a spectrophotometer at 260 nm and stored at –20 ° C for further use as a template for PCR.

3.9 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Three different PCR experiments were conducted as described in section 3.3 to determine expression pattern of peroxidoxin 1, peroxidoxin2 and all peroxidoxins in the different stages of *L. aethiopica*. But the template used was cDNA of amastigotes or promastigotes, not genomic DNA, according to the individual experiments. The first was carried out using internal primers (primer 4 and primer 5) to determine the overall expression level of all peroxidoxin genes present in *L. aethiopica*. The second was performed using primer 1 and primer 2 to determine expression pattern of peroxidoxin 1 and the third was conducted using primer 1 and primer 3 to determine expression pattern of peroxidoxin 2. The house-keeping α-tubulin gene of *Leishmania* was used as a loading control. The PCR product was run on 1.5% agarose gel and the intensity of bands corresponding to amastigotes and promastigotes were compared.

3.10 Northern blot hybridization

RNA samples from the promastigote, infected macrophages and uninfected THP1 cells were run on 1.2% denaturing formaldehyde agarose gel and transferred to a positively charged nylon membrane by upward capillary transfer method using 20x SSC as a transfer buffer. All solutions and water used here were made RNAse free by treating them with diethylpyrocarbonate to a final concentration of 0.1%. Hybridization and immunological detection were carried out as described for Southern blot hybridization.

4. Results

4.1 Identification of two peroxidoxin genes from *L. aethiopica*

In this study, we identified the coding regions of two peroxidoxin genes (pxn1 and pxn2) from genomic DNA of *L. aethiopica* (isolate 1093/02) promastigotes by PCR. Pxn1 was amplified using primer 1 and primer 2 whereas pxn2 was amplified using primer 1 and primer 3 (Figure 5). Sequence analysis of the PCR products showed that the coding region of the pxn1 is 570 base pairs (Figure 6a; Figure 7a) while that of pxn2 is 600 base pairs (Figure 6b; Figure 7b). The PCR products were verified by 1) comparing the PCR products of peroxidoxin genes from other *Leishmania* species on agarose gel, 2) PCR using internal primers (primer 4 and primer 5) (Figure 5) which amplified from the internal region (Figure 6c) and 3) sequencing their respective products. We have also analyzed the PCR products of the coding regions of peroxidoxin genes from two clinical isolates (designated 1185/02 and 1400/02) (Figure 6a, lane 3 and 4; Figure 6b, lane 3) taken from Ethiopian LCL patients and from *L. tropica* (Figure 6a, lane 5; Figure 6b, lane 4). These genes were sequenced and compared to those of *L. aethiopica* (see appendix C and E). This is the first report of peroxidoxin genes sequence from *L. aethiopica*, but also from *L. tropica*.

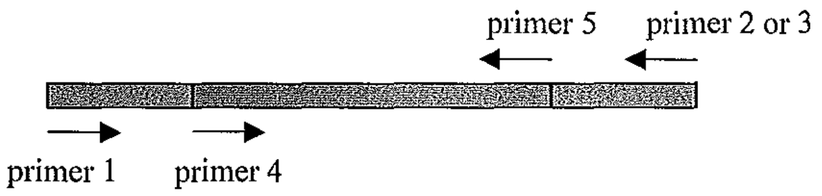

Figure 5. The coding region of peroxidoxin gene and primers used in PCR. The middle region represents the internal region of the coding region.

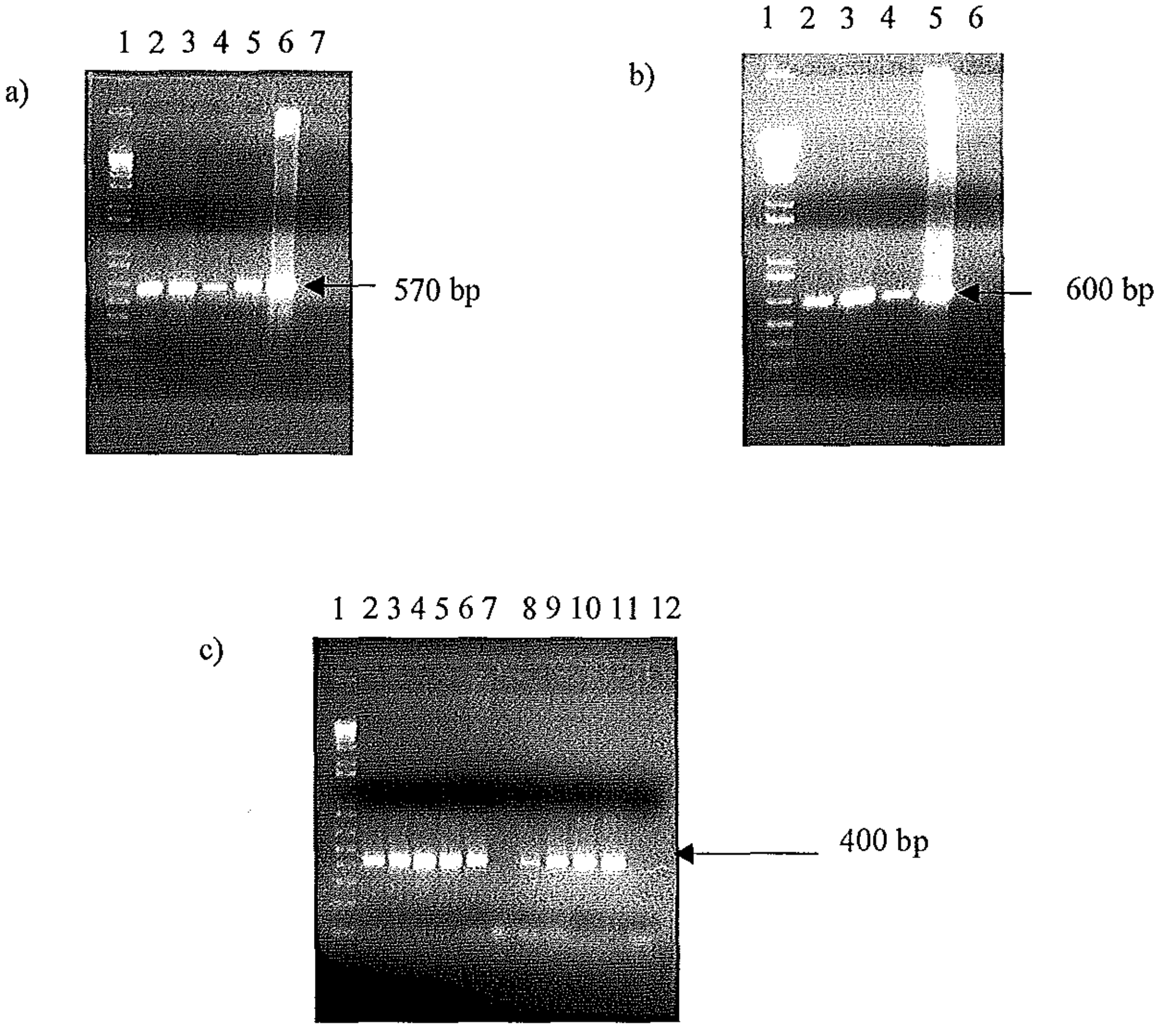

Figure 6. PCR product **a)** Pxn1 from genomic DNAs. 1 kb plus DNA ladder (lane 1), 1093/02 (*L. aethiopica*) (lane 2), 1185/02 (lane 3), 1400/02 (lane 4), *L. tropica* (lane 5), *L. chagasi* (lane 6) **b)** Pxn2 from genomic DNAs. 1 kb plus DNA ladder (lane 1), 1093/02 (*L. aethiopica*) (lane 2), 1185/02 (lane 3), *L. tropica* (lane 4), *L. chagasi* (lane 5) **c)** Internal region of Pxn1 (lane 2-7) and pxn2 (lane 8-12) from PCR products of pxn1 and pxn2, respectively. 1 kb plus DNA ladder (lane1), 1093/02 (*L. aethiopica*) (lane 2), 1185/02 (lane 3), 1400/02 (lane 4), *L. tropica* (lane 5), *L. chagasi* (lane 6), Negative control (lane 7), 1093/02 (lane 8), 1185/02 (lane 9), *L. tropica* (lane 10), *L. chagasi* (lane 11), Negative control (lane 12).

4.2 Sequence analysis of coding regions of peroxidoxin genes

The PCR products containing the coding regions of the two peroxidoxin genes (pxn1 and pxn2) were sequenced at University of Calgary, DNA sequencing facility, Alberta, Canada. The nucleotide sequences of pxn1 and pxn2, together with the amino acids they encode, are given in Figure 7. Like other *Leishmania* genes, the coding region DNA sequence is rich in G/C, which is 64.5% and 62.8% for pxn1 and pxn2, respectively.

a)

```
        M   S   R   G   D   A   K   I   N   S   P   A   P   P
  1    ATG TCC CGC GGT GAC GCC AAG ATC AAC TCT CCC GCG CCG CCC
        F   E   E   V   A   L   M   P   N   G   S   F   K   K
 43    TTC GAG GAG GTG GCG CTC ATG CCC AAC GGC AGC TTC AAG AAG
        I   S   L   S   A   Y   K   G   K   W   V   V   L   F
 85    ATC AGC CTC TCC GCC TAC AAG GGC AAG TGG GTC GTG CTC TTC
        F   Y   P   L   D   F   S   F   V   C   P   T   E   I
127    TTC TAC CCG CTC GAC TTC AGC TTC GTG TGC CCG ACA GAG ATC
        I   Q   F   S   D   S   V   S   R   F   N   E   L   N
169    ATC CAG TTC TCC GAC AGC GTG AGT CGC TTC AAC GAG CTC AAC
        C   E   V   L   A   C   S   M   D   S   E   Y   A   H
211    TGC GAG GTC CTC GCG TGC TCG ATG GAC AGC GAG TAC GCG CAC
        L   Q   W   T   L   Q   D   R   K   K   G   G   L   G
253    CTG CAG TGG ACG CTG CAG GAC CGC AAG AAG GGC GGC CTC GGG
        A   M   A   I   P   M   L   A   D   K   T   K   C   I
295    GCC ATG GCG ATC CCA ATG CTG GCC GAC AAG ACC AAG TGC ATC
        A   R   S   Y   G   V   L   E   E   S   Q   G   V   A
337    GCT CGT TCC TAC GGC GTG CTG GAG GAG AGC CAG GGC GTG GCC
        Y   R   G   L   F   I   I   D   P   H   G   M   V   R
379    TAC CGC GGT CTC TTC ATC ATC GAC CCC CAT GGC ATG GTG CGT
        Q   I   T   V   N   D   M   P   V   G   R   N   V   E
421    CAG ATC ACC GTC AAC GAC ATG CCG GTG GGC CGC AAC GTG GAG
        E   V   L   R   L   L   E   A   F   Q   F   V   E   K
463    GAG GTT CTG CGC CTG CTG GAG GCT TTT CAG TTC GTG GAG AAG
        H   G   E   V   C   P   A   N   W   K   K   G   D   P
505    CAC GGC GAG GTG TGC CCC GCG AAC TGG AAA AAG GGC GAC CCT
        G   L   K   V   D   H   N   K   *
547    GGC CTG AAG GTC GAT CAC AAT AAG TAA
```

b)

```
        M   S   C   G   D   A   K   I   N   S   P   A   P   P
  1    ATG TCC TGC GGT GAC GCC AAG ATC AAC TCT CCC GCG CCG CCC
        F   E   E   V   A   L   M   P   N   G   S   F   K   K
 43    TTC GAG GAG GTG GCG CTC ATG CCC AAC GGC AGC TTC AAG AAG
        I   S   L   S   A   Y   K   G   K   W   V   V   L   F
 85    ATC AGC CTC TCC GCC TAC AAG GGC AAG TGG GTC GTG CTC TTC
        F   Y   P   L   D   F   T   F   V   C   P   T   E   I
127    TTC TAC CCG CTC GAC TTC ACC TTC GTG TGC CCG ACA GAG ATC
        I   A   F   S   D   S   V   S   R   F   N   E   L   N
169    ATC GCG TTC TCC GAC AGC GTG AGT CGC TTC AAC GAG CTC AAC
        C   E   V   L   A   C   S   M   D   S   E   Y   A   H
211    TGC GAG GTC CTC GCG TGC TCG ATG GAC AGC GAG TAC GCG CAC
        L   Q   W   T   L   Q   D   R   Q   K   G   G   L   G
253    CTG CAG TGG ACG CTG CAG GAC CGC CAG AAG GGC GGC CTC GGG
        A   M   A   I   P   M   L   A   D   K   T   K   C   I
295    GCC ATG GCG ATC CCA ATG CTG GCC GAC AAG ACC AAG TGC ATC
        A   R   S   Y   G   V   L   E   E   S   Q   G   V   A
337    GCT CGT TCC TAC GGC GTG CTG GAG GAG AGC CAG GGC GTG GCC
        Y   R   G   L   F   I   I   D   P   H   G   M   V   R
379    TAC CGC GGT CTC TTC ATC ATC GAC CCC CAT GGC ATG GTG CGT
        Q   I   T   V   N   D   M   P   V   G   R   S   V   E
421    CAG ATC ACC GTC AAC GAC ATG CCG GTG GGC CGC AGC GTG GAG
        E   V   X   R   L   L   E   A   F   Q   F   V   E   K
463    GAG GTT NTG CGC CTG CTG GAG GCT TTT CAG TTC GTG GAG AAG
        H   G   E   V   C   P   A   N   W   K   K   G   A   P
505    CAC GGC GAG GTG TGC CCC GCG AAC TGG AAG AAG GGC GCC CCC
        T   M   K   P   E   P   K   A   S   V   E   G   Y   F
547    ACG ATG AAG CCG GAA CCG AAG GCG TCT GTC GAG GGG TAC TTC
        S   K   Q   *
589    AGC AAA CAG TAA
```

Figure 7. The nucleotide and amino acid sequences of pxn1 (a) and pxn2 (b). The two conserved cysteine residues at positions 52 and 173 are in rectangular boxes. Symbols of amino acids are given in Appendix F.

The sequences representing the two genes were compared to each other, to peroxidoxins of the clinical isolate 1185/02 and to those of other *Leishmania* species, including *L. tropica*, *L. major*, *L. chagasi*, *L. donovani* and *L. infantum*, using Vector NTI software. The similarity between pxn1 and pxn2 of *L. aethiopica* (isolate 1093/02) is 91.3%. Pxn2 contains 27 more nucleotides at its 3' end, which are absent from pxn1. Pxn1 showed the highest identity (99.5%) to the clinical isolate 1185/02, suggesting that *L. aethiopica* (1093/02) is nearly identical to the clinical isolate 1185/02 (Figure 8; Appendix C). The dendrogram in Figure 9 also shows very close relationship between the isolates 1093/02 and 1185/02. Thus, it is very likely that these two isolates belong to the same species (i.e. *L. aethiopica*).

We also found a very high degree of nucleotide sequence similarity between peroxidoxins of *L. aethiopica* and those of *Leishmania* species (Figure 8). Excluding the clinical isolate 1185/02, pxn1 showed the highest identity to *L. tropica* pxn1 (98.8%), *L. chagasi* pxn1 (96.9%) and *L. donovani* pxn1 (96.9%). Nucleotide sequence similarity between *L. aethiopica* pxn1 and those of other organisms is given in Table 1.

Table 1 Identity between *L. aethiopica* pxn1 gene and peroxidoxin genes of other organisms

Organism	**Gene**	**Accession number**	**Identity**
L. tropica	Peroxidoxin 1	Not submitted *	98.8%
L. chagasi	Peroxidoxin 1	AF205887	96.9%
L. donovani	Peroxidoxin 1	AF134161	96.9%
L. major	Peroxidoxin 2	AF069386	89.7%
L. chagasi	Peroxidoxin 2	AF312397	89.5%
L. chagasi	Peroxidoxin 3	AF312398	89.5%
L. infantum	Cytosolic peroxiredoxin	AY058210.1	89.3%
Crithidia fasiculata	Tryparedoxin peroxidase	AF055914	79.8%
Trypanosoma cruzi	Tryparedoxin peroxidase	AF106856	70.8%

* We have sequenced pxn1 and pxn2 from *L. tropica* . But, we have not yet submitted the sequences to the gene bank.

Like pxn1, pxn2 also exhibited the highest identity (99%) to clinical isolate 1185/02, followed by *L. tropica* pxn2 (98.5%), *L. major* pxn2 (97.3%) and *L. chagasi* pxn2 (97%). The identity between pxn2 of *L. aethiopica* and peroxidoxins of other organisms is given in Table 2.

Table 2 Identity between *L. aethiopica* pxn2 gene and peroxidoxin genes of other organisms

Organism	Gene	Accession number	Identity
L. tropica	Peroxidoxin 2	Not submitted	98.5%
L. major	Peroxidoxin 2	AF069386	97.3%
L. chagasi	Peroxidoxin 2	AF312397	97%
L. chagasi	Peroxidoxin 3	AF312398	96.5%
L. infantum	Cytosolic peroxiredoxin	AY058210.1	96.5%
L. chagasi	Peroxidoxin 1	AF205887	89%
L. donovani	Peroxidoxin 1	AF134161	89%
Trypanosoma cruzi	Tryparedoxin peroxidase	AF106856	76.3%
Crithidia fasiculata	Tryparedoxin peroxidase	AF055914	73.6%

Nucleotide sequence alignment of peroxidoxin genes showed that little divergence exists between peroxidoxin genes of *L. aethiopica* and those of other *Leishmania* species (Figure 8). Nucleotide sequence alignment of peroxidoxins of different species is given in Appendix A.

```
                                  1                                                                   70
         1093 pxn1       (1)     ATGTCCCGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
         1093 pxn2       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
         1185 pxn1       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
         1185 pxn2       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
    L. chagasi pxn1       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
    L. chagasi pxn2       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
    L. chagasi pxn3       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
   L. donovani pxn1       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
   L. infantum Prxn       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
      L. major pxn2       (1)     ATGTCCTGCGGTAACGCCAAGATCAACTCTCCCGCGCCGTCCTTCGAGGAGGTGGCGCTCATGCCCAACG
    L. tropica pxn1       (1)     ATGACCTGCGGGGAAGCCAAGATCCACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
    L. tropica pxn2       (1)     ATGTCCTGCGGTGAAACCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGATGGCGCTCATGCCCAACG
         Consensus       (1)     ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
                                  71                                                                 140
         1093 pxn1      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
         1093 pxn2      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
         1185 pxn1      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
         1185 pxn2      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
    L. chagasi pxn1      (71)     GCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
    L. chagasi pxn2      (71)     GCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
    L. chagasi pxn3      (71)     GCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
   L. donovani pxn1      (71)     GCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
   L. infantum Prxn      (71)     GCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
      L. major pxn2      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCTCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
    L. tropica pxn1      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
    L. tropica pxn2      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
         Consensus      (71)     GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
                                  141                                                                210
         1093 pxn1     (141)     CTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
         1093 pxn2     (141)     CTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
         1185 pxn1     (141)     CTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
         1185 pxn2     (141)     CTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
    L. chagasi pxn1     (141)     TTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGAAAACATAAGTCGCTTCAACGAGCTCAAC
    L. chagasi pxn2     (141)     CTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGAAAACGTGAGTCGCTTCAACGAGCTCAAC
    L. chagasi pxn3     (141)     CTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGAAAACGTGAGTCGCTTCAACGAGCTCAAC
   L. donovani pxn1     (141)     TTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCTGAAAACGTGAGTCGCTTCAACGAGCTCAAC
   L. infantum Prxn     (141)     CTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGAAAACGTGAGTCGCTTCAACGAGCTCAAC
      L. major pxn2     (141)     CTTCACCTTCGTGTGCCCGACAGAGGTCATCGCGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
    L. tropica pxn1     (141)     CTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
    L. tropica pxn2     (141)     CTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAACGTGAGTCGCTTCAACGAGCTCAAC
         Consensus     (141)     CTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
                                  211                                                                280
         1093 pxn1     (211)     TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
         1093 pxn2     (211)     TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCCAGA
         1185 pxn1     (211)     TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
         1185 pxn2     (211)     TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
    L. chagasi pxn1     (211)     TGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
    L. chagasi pxn2     (211)     TGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
    L. chagasi pxn3     (211)     TGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
   L. donovani pxn1     (211)     TGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
   L. infantum Prxn     (211)     TGCGAGGTCCTTGCGTGCTCCATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
      L. major pxn2     (211)     TGCGAGGTCCTCGCGTGCTCGATAGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
    L. tropica pxn1     (211)     TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
    L. tropica pxn2     (211)     TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
         Consensus     (211)     TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
                                  281                                                                350
         1093 pxn1     (281)     AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
         1093 pxn2     (281)     AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
         1185 pxn1     (281)     AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
         1185 pxn2     (281)     AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
    L. chagasi pxn1     (281)     AGGGCGGCCTCGGCGCCATGGCGATTCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTGCCTACGG
    L. chagasi pxn2     (281)     AGGGCGGCCTCGGCGCCATGGCGATTCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTGCCTACGG
    L. chagasi pxn3     (281)     AGGGCGGCCTCGGCGCCATGGCGATTCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTGCCTACGG
   L. donovani pxn1     (281)     AGGGCGGCCTCGGCACCATGGCGATTCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTGCCTACGG
   L. infantum Prxn     (281)     AGGGCGGCCTCGGCGCCATGGCGATTCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTGCCTACGG
      L. major pxn2     (281)     AGGGCGGCCTCGGGACCATGGCGATCCCAATGCTAGCCGACAAGACCAAGAGCATCGCTCGTTCCTACGG
    L. tropica pxn1     (281)     AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
    L. tropica pxn2     (281)     AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTTCCTACGG
         Consensus     (281)     AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTTCCTACGG
```

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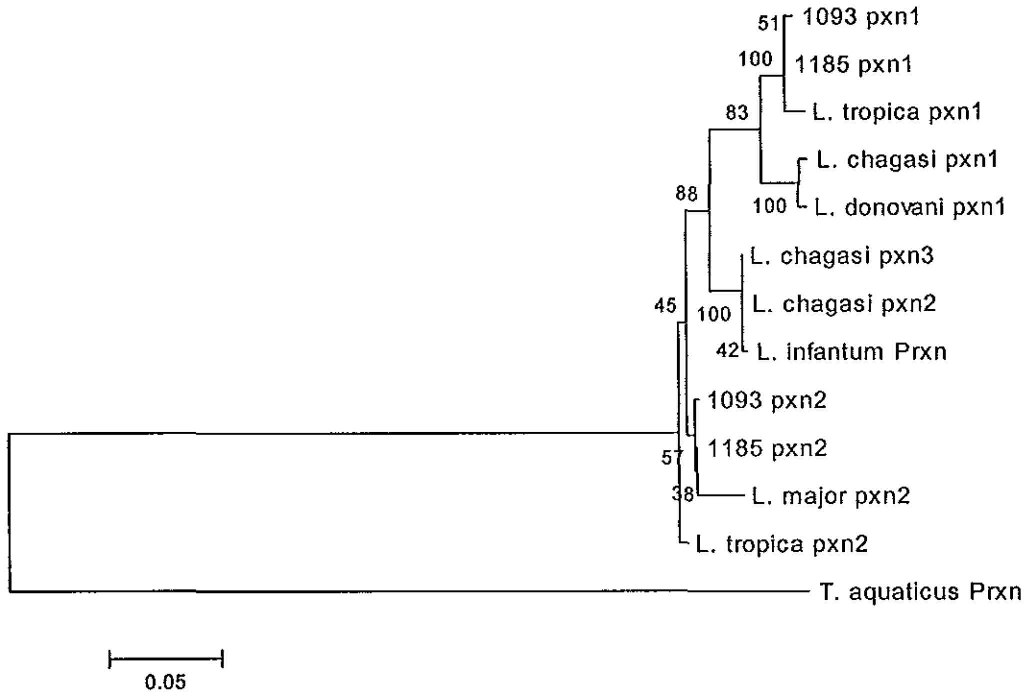
                        351                                                                420
       1093 pxn1  (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
       1093 pxn2  (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
       1185 pxn1  (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
       1185 pxn2  (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
 L. chagasi pxn1  (351) CGTGCTGGAGGAGAAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGC
 L. chagasi pxn2  (351) CGTGCTGGCGGAGAAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGC
 L. chagasi pxn3  (351) CGTGCTGGCGGAGAAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGC
L. donovani pxn1  (351) CGTGCTGGAGGAGAAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGC
L. infantum Prxn  (351) CGTGCTGGCGGAGAAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGC
   L. major pxn2  (351) CGTGCTGGAGGAGAGCCGGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGCTGCGT
 L. tropica pxn1  (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
 L. tropica pxn2  (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
       Consensus  (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
                        421                                                                490
       1093 pxn1  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
       1093 pxn2  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAGCGTGGAGGAGGTTNTGCGCCTGCTGGAGGCTTTTC
       1185 pxn1  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
       1185 pxn2  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAGCGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
 L. chagasi pxn1  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
 L. chagasi pxn2  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
 L. chagasi pxn3  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
L. donovani pxn1  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
L. infantum Prxn  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
   L. major pxn2  (421) CAGATCACCGTCAATGACATGCCGGTGGGCCGCAGCGTGGAGGAGGTTCTACGCCTGCTGGAGGCTTTTC
 L. tropica pxn1  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
 L. tropica pxn2  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTGC
       Consensus  (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
                        491                                                                560
       1093 pxn1  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAAAAGGGCGACCCTGGCCTGAAGGTCGA
       1093 pxn2  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
       1185 pxn1  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCCTGGCCTGAAGGTCGA
       1185 pxn2  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
 L. chagasi pxn1  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCCTGGCCTGAAGGTCGA
 L. chagasi pxn2  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
 L. chagasi pxn3  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
L. donovani pxn1  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCCTGGCCTGAAGGTCGA
L. infantum Prxn  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
   L. major pxn2  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
 L. tropica pxn1  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCCTGGCCTGAAGGTCGA
 L. tropica pxn2  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
       Consensus  (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCCCACGATGAAGCCGGA
                        561                                  600
       1093 pxn1  (561) TCACAATAAGTAA---------------------------
       1093 pxn2  (561) ACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
       1185 pxn1  (561) TCACAATAAGTAA---------------------------
       1185 pxn2  (561) ACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
 L. chagasi pxn1  (561) TCACAATAAGTAA---------------------------
 L. chagasi pxn2  (561) GCCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
 L. chagasi pxn3  (561) GCCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAGCTGTGA
L. donovani pxn1  (561) TCACAATAAGTAA---------------------------
L. infantum Prxn  (561) GCCGAAGGCGTCTGTCGAGGGGTACTTCAGTAAGCAGTAA
   L. major pxn2  (561) ACCGAAGGCGTCTGTCGAGGGATACTTCAGCAAGCAGTAA
 L. tropica pxn1  (561) TCTCAATAAGTAA---------------------------
 L. tropica pxn2  (561) ACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
       Consensus  (561)  CCGAAGGCGTCTGTCGAGGGGTACTTCAGCAA CAGTAA
```

Figure 8. Nucleotide sequence alignment of peroxidoxins of *Leishmania* species.

a)



51
1093 pxn1
100
1185 pxn1
83
L. tropica pxn1
L. chagasi pxn1
88
100
L. donovani pxn1
L. chagasi pxn3
45
100
L. chagasi pxn2
42
L. infantum Prxn
1093 pxn2
57
1185 pxn2
38
L. major pxn2
L. tropica pxn2
T. aquaticus Prxn
0.05

b)

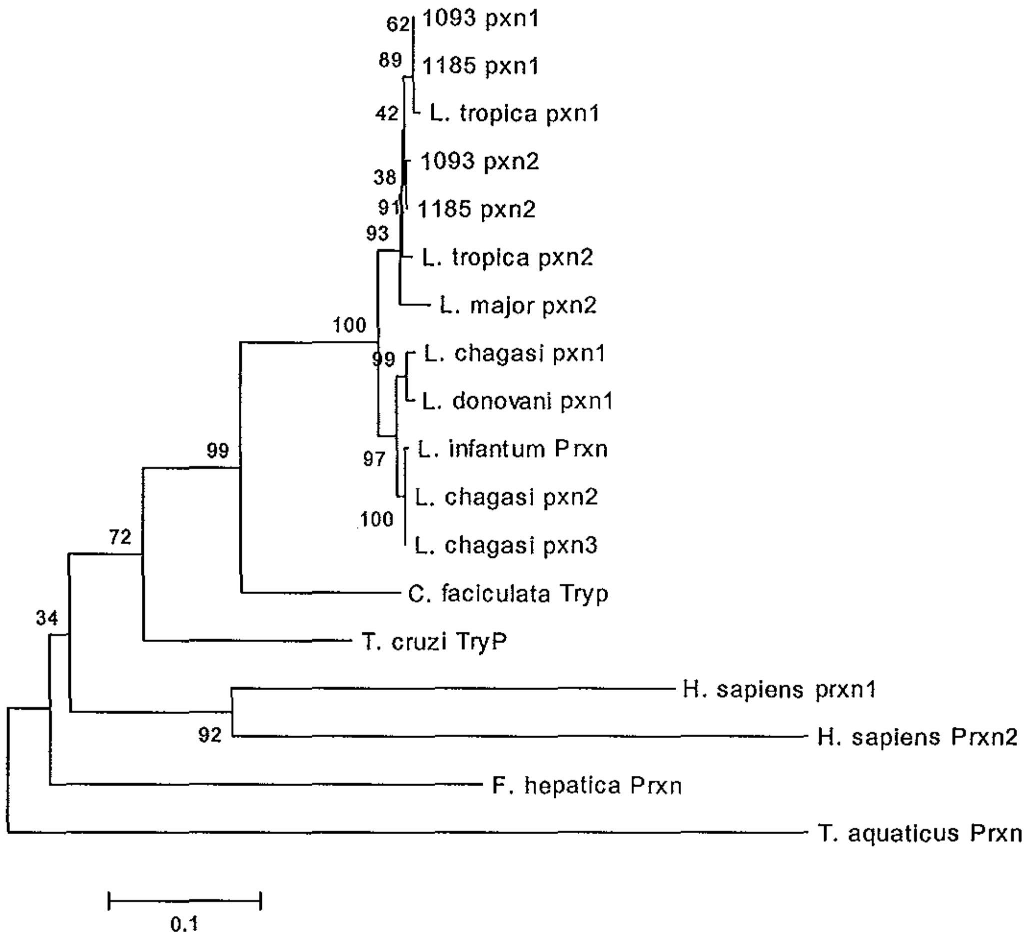


Figure 9. Dendrogram of peroxidoxins constructed based on nucleotide sequence alignment **(a)** *Leishmania* species **(b)** *Leishmania* and other organisms. Note that 1093 is *L. aethiopica.*

4.3 Peroxidoxin protein sequence analysis

In order to predict the proteins encoded by the open reading frames of pxn1 and pxn2 genes, the nucleotide sequences of the corresponding genes were translated into amino acid sequences by using Vector NTI software (Figure 7). We found that each one of the open reading frames encode for a single polypeptide. Pxn1 and pxn2 encode for a protein containing 190 and 199 amino acids, respectively. The molecular weight and isoelectric point of pxn1 protein is 21.29 kD and 6.49, respectively whereas those of pxn2 are 22.137 kD and 5.98, respectively as predicted using the same software. The two predicted proteins differ mainly in their primary structure, especially in the carboxyl terminus region. Pxn2 contains 9 more amino acids at carboxyl terminus, which are absent in pxn1. Amino acid sequence alignment using the same software revealed that 88.4% similarity exists between pxn1 and pxn2 proteins.

Predicting the amino acid sequence also helped us perform protein database search for homologous proteins to peroxidoxin proteins of *L. aethiopica* from other organisms. BLAST search for homologous proteins in protein database revealed that pxn1 and pxn2 proteins belong to the peroxidoxin protein family, which are found in organisms of all kingdoms, including humans (Chae *et al*, 1994b). The BLAST search also revealed that very high similarity exists between peroxidoxin proteins of *L. aethiopica* and other *Leishmania* species.

The degree of pxn1 protein sequence similarity to those of *Leishmania* species shows a similar pattern of homology as obtained using the nucleotide sequence. It is clear form Table 3 that pxn1 protein shows the highest identity to *L. tropica* pxn1 (97.4%), followed by *L. chagasi* pxn1 (94.7%), and *L. donovani* pxn1 (94.7%). It also exhibited high similarity to those of other organisms (Table 3). Pxn2 protein exhibits the highest identity to *L. tropica* pxn2 (95.5%) and peroxidoxin protein of *L. major* (94.5%), followed by peroxidoxin 2 and peroxidoxin 3 of *L. chagasi*, to both of which it shows 93% identity. Pxn2 similarity to peroxidoxin proteins of other organisms is given in Table 4.

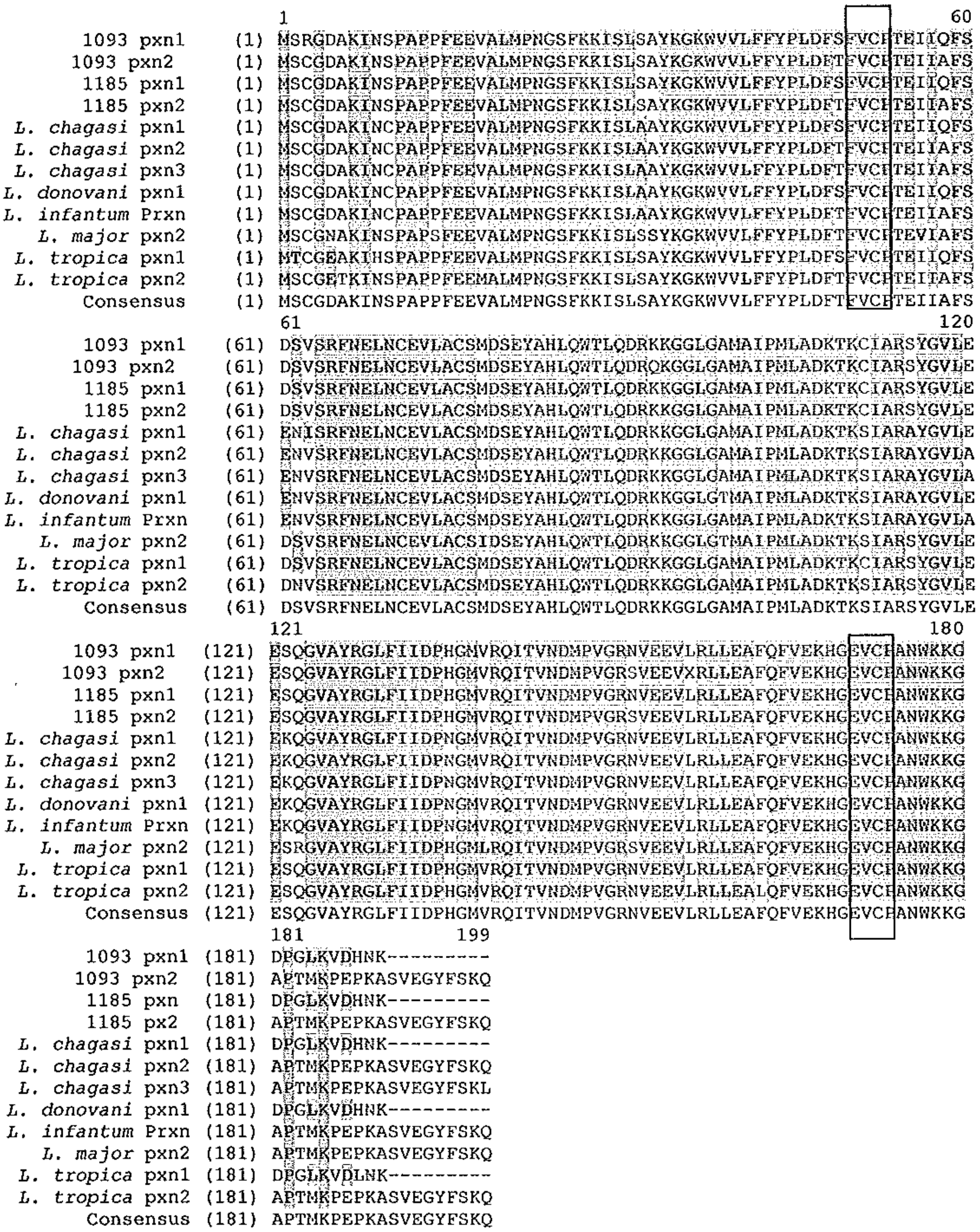
Table 3. Identity between *L. aethiopica* pxn1 protein and protein homologues of other organisms

Organism	**Protein**	**Accession number**	**Identity**
L. tropica	Peroxidoxin 1	Not submitted	97.4%
L. chagasi	Peroxidoxin 1	AAG40074.1	94.7%
L. donovani	Peroxidoxin 1	AAK82654.1	94.7%
L. major	Peroxidoxin 2	AAC79432.1	84.9%
L. chagasi	Peroxidoxin 2	AAK69586.1	85.4%
L. chagasi	Peroxidoxin 3	AAK69587.1	85.4%
L. infantum	TSA protein	AAK58478.1	85.4%
Crithidia fasiculata	Tryparedoxin peroxidase	AAC72300.1	73.7%
Trypanosoma brucei	Tryparedoxin peroxidase	AAG45225.1	68.3%
Shistosoma mansoni	Thioredoxin peroxidase	AAD17299.1	60%
Ratus norvegicus	Peroxiredoxin 1	NP 476455.1	56.8%
Homo sapiens	Peroxiredoxin 1	XP 001393.2	56.8%
Diroflaria immitis	Thioredoxin peroxidase	AAC38831.1	55.5%
Onchocerca volvulus	Peroxidoxin 2	AAC32810.1	55%

Table 4. Identity between *L. aethiopica* pxn2 protein and protein homologues of other organisms

Organism	**Protein**	**Accession number**	**Identity**
L. tropica	Peroxidoxin 2	Not submitted	95.5%
L. major	Peroxidoxin 2	AAC79432.1	94.5%
L. chagasi	Peroxidoxin 2	AAK69586.1	94%
L. infantum	TSA protein	AAK58478.1	94%
L. chagasi	Peroxidoxin 3	AAK69587.1	93.5%
L. chagasi	Peroxidoxin 1	AAG40074.1	84.4%
L. donovani	Peroxidoxin 1	AAK82654.1	84.4%
Crithidia fasiculata	Tryparedoxin peroxidase	AAC72300.1	69.8%
Trypanosoma brucei	Tryparedoxin peroxidase	AAG45225.1	68.8%
Shistosoma mansoni	Thioredoxin peroxidase	AAD17299.1	64%
Onchocerca volvulus	Thioredoxin peroxidase	AAC48312.1	61.3%
Homo sapiens	Peroxiredoxin 1	XP 001393.2	60.5%
Ratus norvegicus	Peroxiredoxin 1	NP 476455.1	60%
Onchocerca volvulus	Peroxidoxin 2	AAC32810.1	59.7%
Diroflaria immitis	Thioredoxin peroxidase	AAC38831.1	58.2%
Homo sapiens	Peroxidoxin 2	AAH03022.1	56.3%

Protein sequence alignment (Figure 10) shows pxn1 and pxn2 contain two conserved motifs, which are characteristic features of all 2-cys peroxidoxins (McGonigle *et al.*, 1998). The two motifs comprise the active site of peroxidoxin enzymes (Wood *et al.*, 2003). The first, which contains the amino acid sequence EVCP, is located in the amino terminus whereas the second, which contains FVCP is found in the carboxyl terminus of the polypeptide. Each of the two motifs contains a highly conserved cysteine residue, making up a total of two highly conserved cysteine residues in peroxidoxin protein. The first conserved cysteine residue is located in the first motif at position 52 and the second conserved cysteine residue is located in the second motif at position 173, like those of other *Leishmania* species. The presence of the two conserved motifs containing two conserved cystiene residues clearly shows that peroxidoxins of *L. aethiopica* belong to 2-cyc peroxidoxin. Peroxidoxin protein sequence alignment of different species is given in Appendix D.



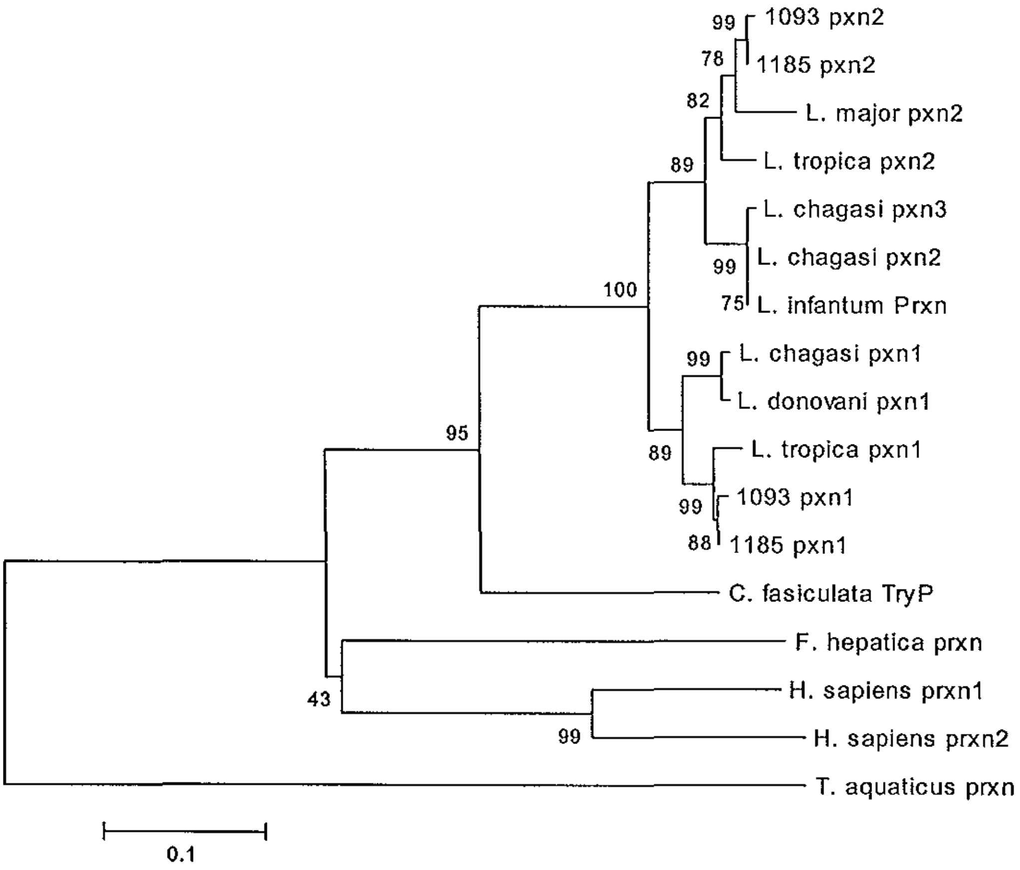
```
                                 1                                                         60
          1093 pxn1     (1) MSRGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCHTEIIQFS
          1093 pxn2     (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCHTEIIAFS
          1185 pxn1     (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCHTEIIQFS
          1185 pxn2     (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCHTEIIAFS
    L. chagasi pxn1     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFSFVCHTEIIQFS
    L. chagasi pxn2     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFTFVCHTEIIAFS
    L. chagasi pxn3     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFTFVCHTEIIAFS
   L. donovani pxn1     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFSFVCHTEIIQFS
   L. infantum Prxn     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFTFVCHTEIIAFS
      L. major pxn2     (1) MSCGNAKINSPAPSFEEVALMPNGSFKKISLSSYKGKWVVLFFYPLDFTFVCHTEVIAFS
    L. tropica pxn1     (1) MTCGEAKIHSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCHTEIIQFS
    L. tropica pxn2     (1) MSCGETKINSPAPPFEEMALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCHTEIIAFS
          Consensus     (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCHTEIIAFS
                            61                                                        120
          1093 pxn1    (61) DSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLE
          1093 pxn2    (61) DSVSRFNELNCEVLACSMDSEYAHLQWTLQDRQKGGLGAMAIPMLADKTKCIARSYGVLE
          1185 pxn1    (61) DSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLE
          1185 pxn2    (61) DSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLE
    L. chagasi pxn1    (61) ENISRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARAYGVLE
    L. chagasi pxn2    (61) ENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARAYGVLA
    L. chagasi pxn3    (61) ENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARAYGVLA
   L. donovani pxn1    (61) ENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGTMAIPMLADKTKSIARAYGVLE
   L. infantum Prxn    (61) ENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARAYGVLA
      L. major pxn2    (61) DSVSRFNELNCEVLACSIDSEYAHLQWTLQDRKKGGLGTMAIPMLADKTKSIARSYGVLE
    L. tropica pxn1    (61) DSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLE
    L. tropica pxn2    (61) DNVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARSYGVLE
          Consensus    (61) DSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARSYGVLE
                            121                                                       180
          1093 pxn1   (121) ESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
          1093 pxn2   (121) ESQGVAYRGLFIIDPHGMVRQITVNDMPVGRSVEEVXRLLEAFQFVEKHGEVCHANWKKG
          1185 pxn1   (121) ESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
          1185 pxn2   (121) ESQGVAYRGLFIIDPHGMVRQITVNDMPVGRSVEEVLRLLEAFQFVEKHGEVCHANWKKG
    L. chagasi pxn1   (121) EKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
    L. chagasi pxn2   (121) EKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
    L. chagasi pxn3   (121) EKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
   L. donovani pxn1   (121) EKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
   L. infantum Prxn   (121) EKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
      L. major pxn2   (121) ESRGVAYRGLFIIDPHGMLRQITVNDMPVGRSVEEVLRLLEAFQFVEKHGEVCHANWKKG
    L. tropica pxn1   (121) ESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
    L. tropica pxn2   (121) ESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEALQFVEKHGEVCHANWKKG
          Consensus   (121) ESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCHANWKKG
                            181                199
          1093 pxn1   (181) DPGLKVDHNK---------
          1093 pxn2   (181) APTMKPEPKASVEGYFSKQ
           1185 pxn   (181) DPGLKVDHNK---------
           1185 px2   (181) APTMKPEPKASVEGYFSKQ
    L. chagasi pxn1   (181) DPGLKVDHNK---------
    L. chagasi pxn2   (181) APTMKPEPKASVEGYFSKQ
    L. chagasi pxn3   (181) APTMKPEPKASVEGYFSKL
   L. donovani pxn1   (181) DPGLKVDHNK---------
   L. infantum Prxn   (181) APTMKPEPKASVEGYFSKQ
      L. major pxn2   (181) APTMKPEPKASVEGYFSKQ
    L. tropica pxn1   (181) DPGLKVDLNK---------
    L. tropica pxn2   (181) APTMKPEPKASVEGYFSKQ
          Consensus   (181) APTMKPEPKASVEGYFSKQ
```

Figure 10. Sequence alignment of peroxidoxin protein *Leishmania* species. The two conserved motifs are in boxes.

Evolutionary relationships among peroxidoxins of *Leishmania* species as well as between *Leishmania* species and other organisms was determined by drawing a dendrogram using

MEGA 3 software by Neighbor Joining (NJ) method (Saitou and Nei, 1987). The construction of the dendrogram was based on both nucleotide sequence (Figure 9) and amino acid sequence (Figure 11) similarity. However, both of them gave similar evolutionary relationships. These two figures show that *L. aethiopica* pxn1 is nearly identical to pxn1 of the isolate 1185/02. The same is also true for pxn2. This strongly suggests that both belong to the same species. When we come to comparison among different species, *L. aethiopica* pxn1 and *L. tropica* are very closely related. In addition, *L. aethiopica* pxn2, *L. major* pxn2 and *L. tropica* pxn2 are very closely related.

a)



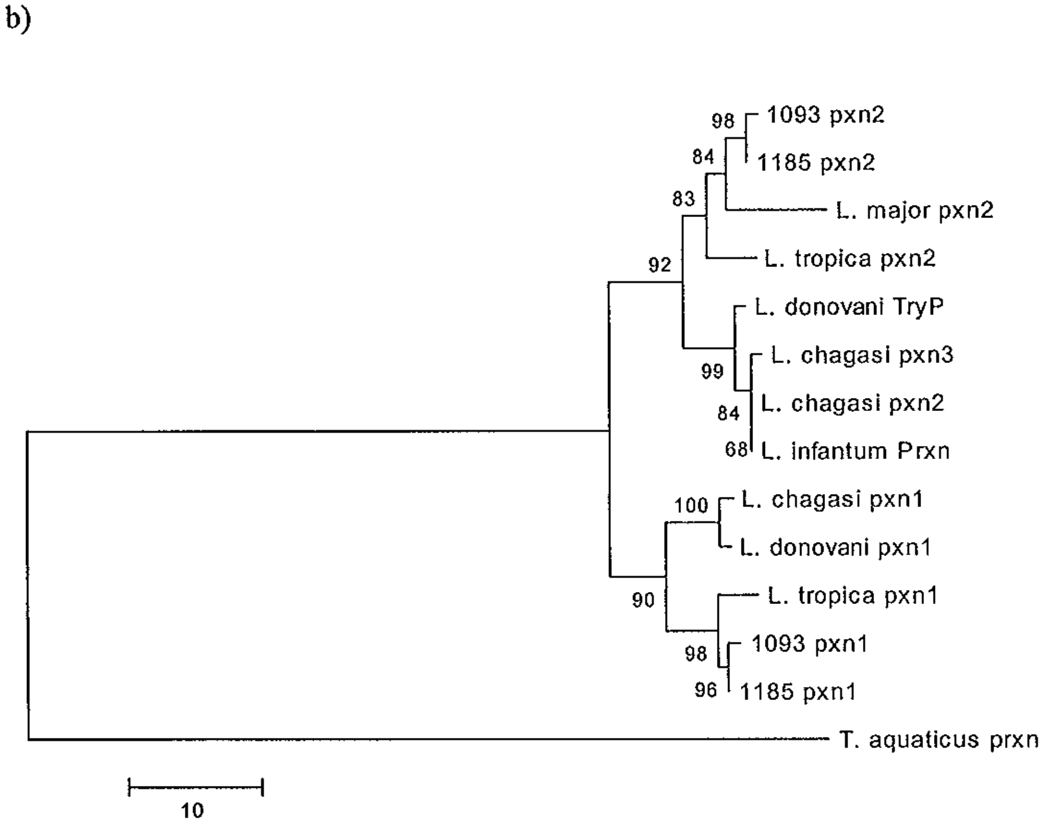


Figure 11. Dendrograms of peroxidoxins constructed based on amino acid sequence. (a) *Leishmania* species (b) *Leishmania* and other organisms. Note that 1093 is *L. aethiopica.*

4.4 Peroxidoxin gene organization

In order to determine organization of peroxidoxin genes existing in *L. aethiopica*, Southern blot analysis was carried out on genomic DNA of *L. aethiopica* promastigotes using restriction enzymes that were selected from information obtained from the restriction mapping of the nucleotide sequence of both pxn1 and pxn2 of *L. aethiopica*. The genomic DNA was digested singly with EcoR I and Hind III, both of which have no internal restriction site in the coding regions of pxn1 and pxn2. These enzymes were selected to avoid complex restriction patterns that would arise when enzymes that cut within the gene are used.

Southern blot hybridization analysis showed that EcoR I digest of genomic DNA blotted on nylon membrane with a non-radioactive digoxigenin-labeled probe gave multiple hybridizing bands (Figure 12, lane 2). This result indicates that peroxidoxins of *L. aethiopica* occur as multigene family. Digestion of genomic DNA with Hind III produced one hybridizing band, which contains all the peroxidoxin genes (Figure 12, lane 3), suggesting that all peroxidoxins of *L. aethiopica* are clustered in a single band.

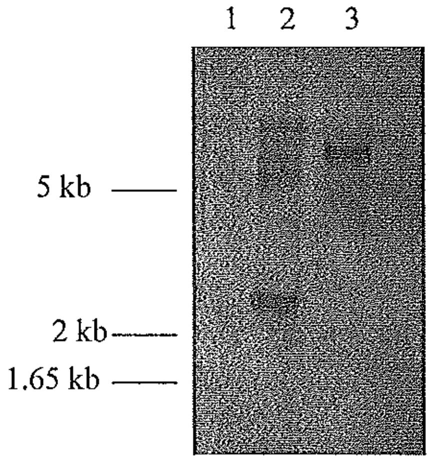


Figure 12. Southern blot hybridization. 1kb plus DNA ladder (lane 1), 4 μg of *L. aethiopica* genomic DNA digested with EcoR I (lane 2) and Hind III (lane 3).

4·5 Expression pattern of peroxidoxin genes

Using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), we determined the level of expression of peroxidoxin genes in the different developmental stages (logarithmic and stationary phase promastigotes and amastigote) of the parasite. Although we have identified only two peroxidoxin genes by PCR, more than two peroxidoxin genes exist in *L. aethiopica* as revealed by Southern blot hybridization analysis. Here, we not only studied the expression pattern of the two genes identified, but also the overall expression of all the available peroxidoxin genes in *L. aethiopica*. For this purpose, three different RT-PCR experiments were conducted. In order to determine the overall expression level of all peroxidoxin genes, the first RT-PCR experiment was performed using internal primers with equal concentrations of cDNAs from the relevant stages of the parasite. These primers were selected for this particular study because they could anneal to all peroxidoxin genes of *L. aethiopica* as they were designed based on very highly conserved regions of peroxidoxins of other trypanosomatids (Barr and Gedamu, 2001). The RT-PCR products were run on agarose gel and showed different intensity of bands corresponding to the different developmental stages of the parasite (Figure 13). Interestingly, we found that the band of the RT-PCR product amplified from cDNA of the amastigote stage (Figure 13a, lane 4) is more intense than those amplified from the cDNA of the logarithmic and stationary phase promastigotes (Figure 13a, lane 2 and 3). The band derived from cDNA of the stationary promastigotes (Figure 13a, lane 3) is more intense than that of logarithmic phase promastigote (Figure 13a, lane 2). In short, the intensity of bands increases as one goes form the logarithmic through stationary phases of promastigotes to the intracellular amastigotes. No band was observed for cDNA from uninfected THP1 cells, which was used as a negative control for cDNA infected THP1 cells.

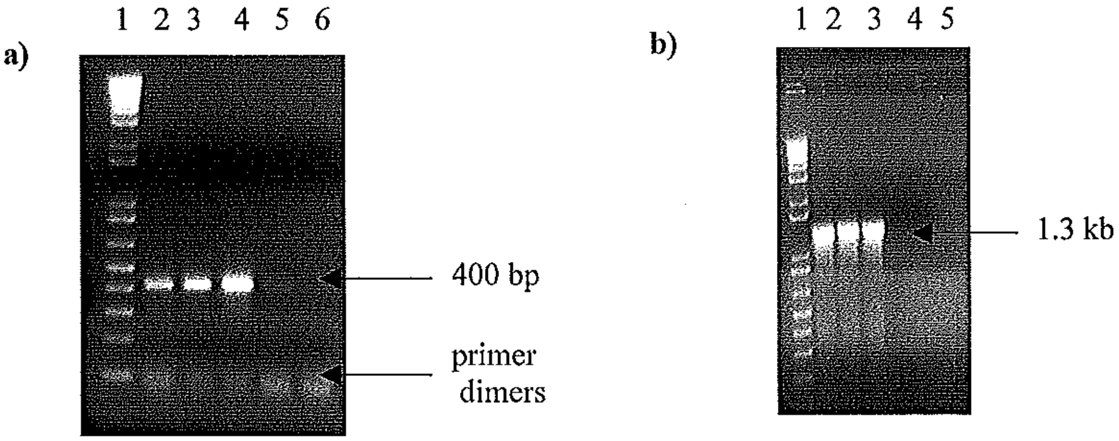


Figure 13. RT-PCR product. **a)** Peroxidoxin internal region. 1 kb plus DNA ladder (lane 1), logarithmic phase promastigote (lane 2), stationary phase promastigote (lane 3), amastigote from infected THP1 cells (lane 4), uninfected THP1 cells (lane 5), negative control (lane 6) **b)** α-tubulin gene. 1 kb plus DNA ladder (lane 1), logarithmic phase promastigote (lane 2), stationary phase promastigote (lane 3), amastigote from infected THP1 cells (lane 4), uninfected THP1 cells (lane 5), negative control (lane 6)

The house-keeping *Leishmania* α-tubulin gene was used as a loading control. The α-tubulin gene was amplified from cDNA using α-tubulin gene primers and the PCR product was run on agarose gel (Figure 13b). Gels containing RT-PCR products were photographed by UV-Sense Transilluminator Camera (UVP Laboratory Products, London) and the bands were analysed using LabWorks Image Acquisition and Analysis Software (UVP Laboratory Products, London). The maximum OD values of the bands were measured. The OD values for peroxidoxin gene RT-PCR product were divided by those of α-tubulin gene to normalize loading error and the ratios were compared between different stages of the parasite (Table 5). The ratios are directly proportional to expression level. The table shows that the highest ratio corresponds to the most intense band (amastigote) for peroxidoxin PCR product whereas the lowest ratio corresponds to the least intense band (logarithmic phase promastigote), suggesting that the expression level of peroxidoxin changes accordingly. In other words, this result indicates that the overall level of expression of peroxidoxin genes of *L. aethiopica* increases as the parasite transforms from logarithmic phase promastigote to stationary phase promastigote and reaches its highest level in the amastigote stage.

Table 5. Comparisons of OD values of internal region of peroxidoxin gene and α-tubulin gene. The ratios (column 4) are directly proportional to level of expression.

Parasite stage	**Pxn band OD**	**α-tubulin band OD**	**OD ratios** (Pxn to α-tubulin)
Logarithmic phase	1789.5	3247.9	0.55
Stationary phase	2439.9	3246.3	0.75
Amastigote	2593	3300.5	0.79

To assess the expression of each of the two amplified genes (pxn1 and pxn2), we performed RT-PCR using the pair of primers used to amplify these genes from genomic DNA of *L. aethiopica* (Figure 5). The second RT-PCR experiment was thus performed to determine expression level of pxn1 and it employed primer 1 and primer 2 (Figure 5). We found an RT-PCR product, which gave a more intense band with cDNA from the amastigote (Figure 14a, lane 4) than with cDNAs from the logarithmic and stationary phase promastigotes were used (Figure 14a, lane 1 and 2).

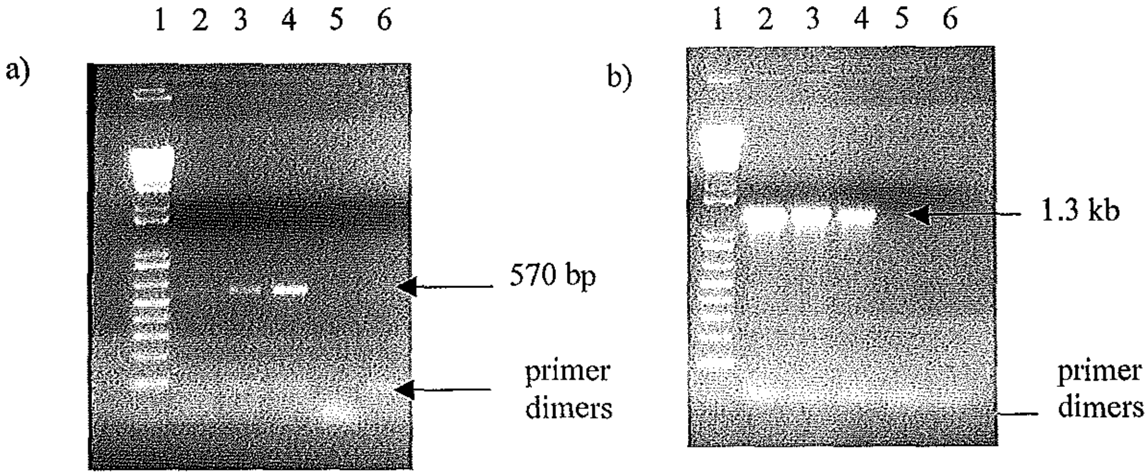


Figure 14. RT-PCR product. **a)** Peroxidoxin 1. 1 kb plus DNA ladder (lane 1), logarithmic phase promastigote (lane 2), stationary phase promastigote (lane 3), amastigote from infected THP1 cells (lane 4), uninfected THP1 cells (lane 5), negative control (lane 6) **b)** α-tubulin gene. 1 kb plus DNA ladder (lane 1), logarithmic phase promastigote (lane 2), stationary phase promastigote (lane 3), amastigote from infected THP1 cells (lane 4), uninfected THP1 cells (lane 5), negative control (lane 6)

The stationary phase cDNA produced a more intense band than the logarithmic phase promastigote cDNA. In short, the intensity of bands increases as one goes from the logarithmic phase promastigote through the stationary phase promastigote to the intracellular amastigote. α-tubulin gene was used as a loading control as described above. Ratios of the OD values of pxn1 to that of α-tubulin are given in Table 6. The ratios are directly proportional to the level of expression. This result indicates that pxn1 is predominantly expressed in the amastigote stage, followed by stationary phase promastigote. Pxn1 level of expression in amastigotes is about eight times higher than its expression in the logarithmic phase promastigote, and three times higher than its expression in the stationary phase promastigote. The expression of pxn1 in the stationary phase promastigote is about three times higher than its expression in the logarithmic phase promastigote.

Table 6. Comparisons of OD values of pxn1 gene and a-tubulin gene. The ratios (column 4) are directly proportional to level of expression.

Parasite stage	**Pxn1 band OD**	**α-tubulin band OD**	**OD ratios** (Pxn1 to α-tubulin)
Logarithmic phase	230.1	2530.1	0.09
Stationary phase	662.9	2552	0.26
Amastigote	1952.3	2649.9	0.74

A third RT-PCR was carried out to determine expression pattern of pxn2 gene and it employed primer 1 and primer 3 (Figure 5). A slightly more intense band was observed for cDNA from the logarithmic phase (Figure 15a, lane 2) than the one form the stationary phase promastigote (Figure 15a, lane 3).

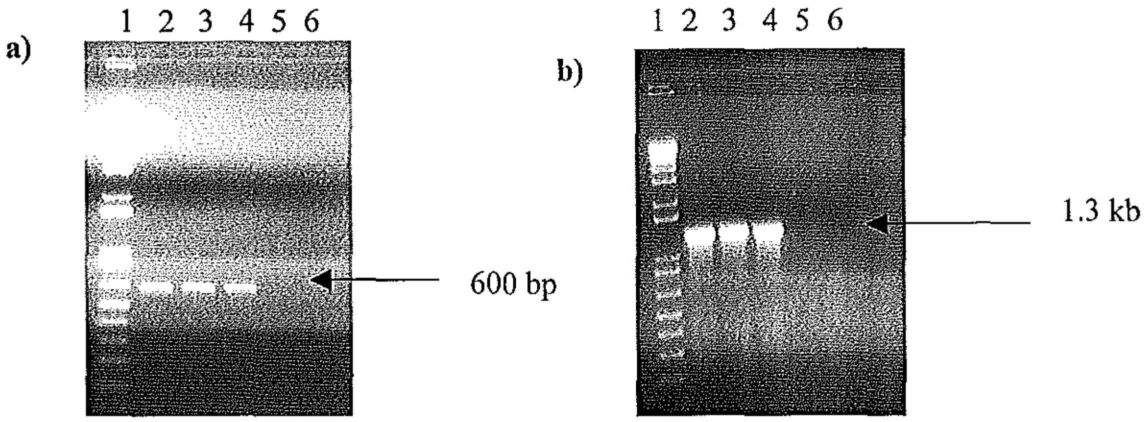


Figure 15. RT-PCR product. a) Peroxidoxin 2. 1 kb plus DNA ladder (lane 1), logarithmic phase promastigote (lane 2), stationary phase promastigote (lane 3), amastigote from infected THP1 cells (lane 4), uninfected THP1 cells (lane 5), negative control (lane 6) **b)** α-tubulin gene. 1 kb plus DNA ladder (lane 1), logarithmic phase promastigote (lane 2), stationary phase promastigote (lane 3), amastigote from infected THP1 cells (lane 4), uninfected THP1 cells (lane 5), negative control (lane 6)

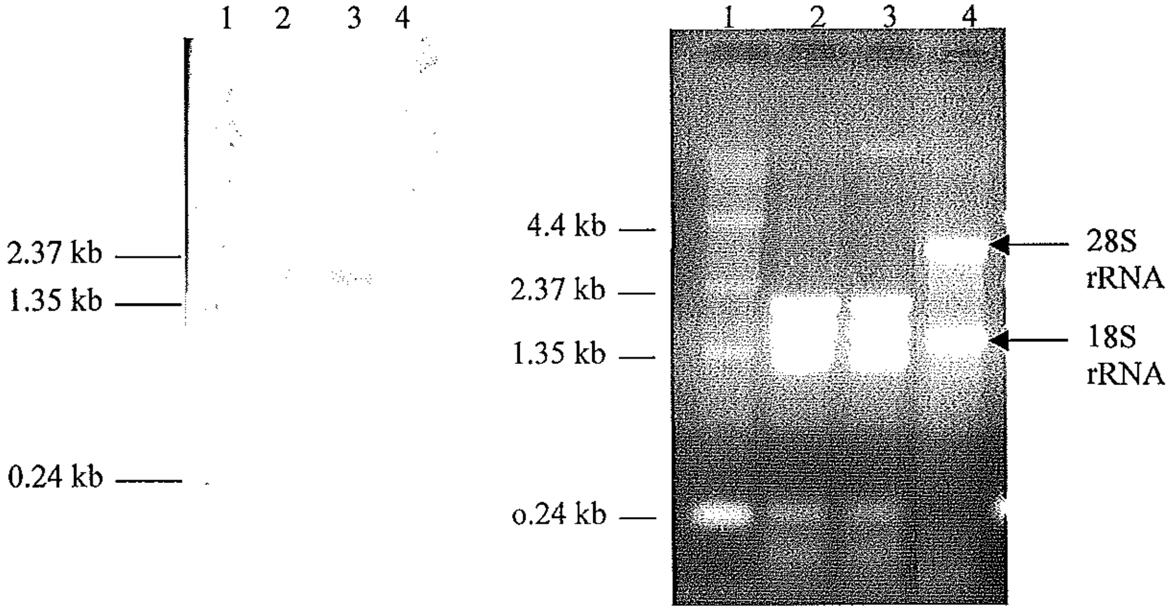
Compared to the amastigote phase the stationary phase promastigote produced a slightly more intense band. In short, band intensity shows some decrease when the logarithmic phase promastigote and the stationary phase promastigote are compared to the intracellular amastigote (Figure 15). Because this is a very small difference, it may be considered that the intensity of the bands is more or less equal. The house-keeping α-tubulin gene was used as a loading control as described above. Ratios of the OD values of pxn2 to that of α-tubulin are given in Table 7. This table shows that ratios obtained are nearly equal, indicating that pxn2 expression is more or less the same in the different stages of the parasite. There is no sufficient evidence to conclude that pxn2 is expressed differentially. Thus, the gene appears to be constitutively expressed throughout the life of the parasite.

Table 7. Comparisons of OD values of pxn2 gene and α-tubulin gene. The ratios (column 4) are directly proportional to level of expression.

Parasite stage	**Pxn2 band OD**	**α-tubulin band OD**	**OD ratios** (Pxn2 to α-tubulin)
Logarithmic phase	2176.8	3247.9	0.67
Stationary phase	2053.6	3246.3	0.63
Amastigote	2017.3	3300.5	0.61

4.6. Northern blot hybridization analysis

The first RT-PCR experiment was verified by carrying out Northern blot hybridization analysis using digoxigenin-labeled probes derived form the internal region of peroxidoxin of *L. aethiopica*. Northern blot analysis of total RNA run on 1.2% formaldehyde agarose gel (Figure 16b) produced a single hybridizing band (transcript) in both promastigote and amastigote stages of the parasite. The band corresponding to the amastigote is more intense than that of the promastigote (Figure 16a).



a)

b)

Figure 16. a) Northern blot hybridization. 0.24-9.5 kb RNA ladder (lane 1), promastigote (lane 2), infected THP1cells (lane 3) and uninfected THP1 cells (lane 4) b) Denaturing formaldehyde agarose gel electrophoresis total RNA used for Northern blotting. 0.24-9.5 kb RNA ladder (lane 1), promastigote (lane 2), infected THP1 cells (lane 3), Uninfected THP1 cells (lane 4)

5. Discussion

Peroxidoxin genes have been isolated from various *Leishmania* species (Levick *et al.*, 1998; Webb *et al.*, 1998; Barr and Gedamu, 2001; Castro *et al.*, 2002; Flohe *et al.*, 2002) and have been shown to play an important role for intracellular survival, through detoxification of host oxidants generated by macrophages in an attempt to eliminate the parasite (Barr and Gedamu 2001, 2003). In this study, consistent with our hypothesis, we demonstrate the existence of peroxidoxin genes in *L. aethiopica,* suggesting that *L. aethiopica* uses peroxidoxins as antioxidant enzymes that may protect the parasite from oxidative damage by oxidants of macrophages. That the genes are similar to those of *L. chagasi* is probably indicative of similarity in function. In *L. chagasi* peroxidoxins have been shown to detoxify ROS (H_2O_2, ROOH, OH^-) and RNS (NO, $ONOO^-$) (Barr and Gedamu, 2003). Based on this finding and the very high similarity that exists between peroxidoxin genes of the two species, we can speculate that *L. aethiopica* may detoxify these reactive species. Our finding that, like other *Leishmania* species, *L. aethiopica* and *L. tropica* possess peroxidoxin genes further confirms that these genes are conserved in all *Leishmania* species (Levick *et al.*, 1998; Webb *et al.*, 1998; Barr and Gedamu, 2001; Castro *et al.*, 2002; Flohe *et al.*, 2002).

Previous studies have shown that high nucleotide sequence similarity exists between known peroxidoxin genes of *Leishmania* species (Castro *et al.*, 2002; Flohe *et al.*, 2002). Our finding that very high similarity exists between peroxidoxins of *L. aethiopica* and other *Leishmania* species is in agreement with these studies. The high similarity suggests that these genes have a common ancestral gene in the trypanosomatids. It can also be speculated that high sequence similarity may predict high similarity in structure, function, and mechanism of action of the enzymes in the different species. The existing slight difference may provide some degree of difference in the specific function of peroxidoxins in different *Leishmania* species. In other words, peroxidoxins of different *Leishmania* species may have both overlapping and nonoverlapping functions. For example, while peroxidoxins of both *L. chagasi* and *L. major* detoxify H_2O_2, indicating overlapping function, only that of *L. chagasi*, not *L. major*, is capable of detoxifying alkyl hydroperoxides (Levick *et al.*, 1998; Barr and Gedamu 2001, 2003). Furthermore, the high similarity of peroxidoxin genes is also indicative of the existence of conserved epitopes in all *Leishmania* species. Thus, peroxidoxins appear to be important sources of conserved epitopes, which might be potential immunogens in the development of vaccine against leishmaniasis.

As mentioned earlier, in Ethiopia where CL is caused by three species of *Leishmania* (*L. aethiopica*, *L. major* and *L. tropica*), there is no rapid diagnostic tool that differentiates between these species. Based on nucleotide sequence divergence, primers can be designed and/or restriction sites could be analyzed to develop species-specific PCR/PCR-RFLP diagnostic methods, respectively. However, the divergence in peroxidoxin nucleotide sequences of these three species is very low that it may be difficult to use the coding region as a target for diagnostics. Nevertheless, the 3' untranslated regions (UTRs) of pxn1 and pxn2 showed greater divergence in peroxidoxin genes of other *Leishmania* species (Barr and Gedamu, 2001), suggesting that this may be the case for those species causing CL in Ethiopia. Thus, analysis of the 3' UTRs might help to design species-specific primers to develop a PCR based diagnostic test that may differentiate between *L. aethiopica*, *L. major* and *L. tropica* isolates from cutaneous leishmaniasis patients in Ethiopia, avoiding the laborious techniques of isoenzyme typing. Properly validated, such PCR can directly confirm

L. aethiopica infection in clinical samples from lesions, providing a rapid diagnostic alternative to culture and histology.

Our finding that peroxidoxin genes of *L. aethiopica* occur in multigene family is in agreement with previous studies on gene organization of peroxidoxin genes of other *Leishmania* species (Levick *et al.*, 1998; Webb *et al.*, 1998; Barr and Gedamu, 2001; Castro *et al.*, 2002). This suggests that the gene products of *L. aethiopica* and other *Leishmania* species could be expressed at different stages of the parasite development to play a role in its survival and potentially as a virulence factor.

Expression of pxn1 and pxn2 at mRNA level was analyzed in the logarithmic and stationary phase promastigotes as well as in the amastigote stage of the parasite. Barr and Gedamu (2001) reported that peroxidoxin genes are differentially expressed in different stages of *L. chagasi*. They demonstrated that pxn1 mRNA is predominantly expressed in the amastigote stage whereas pxn2 is predominantly expressed in the promastigote stage of the parasite. In agreement with this study and our hypothesis, we found differential expression of peroxidoxin 1 but in contrary constitutive expression of peroxidoxin 2 in the different developmental stages of *L. aethiopica*.

The higher level of pxn1 mRNA expression in the stationary phase promastigotes than in the logarithmic promastigotes suggests that pxn1 is more important for the stationary phase. In terms of virulence, one difference between logarithmic phase promastigote and stationary phase promastigote is that the latter is infective while the former is not. Since pxn1 is expressed abundantly in the infective stage, it may be involved in infectivity of *L. aethiopica*.

During transformation from logarithmic to stationary phase promastigotes, the parasite undergoes physiological and biochemical changes that prepares it for infectivity. These changes occur during this process because many genes should be turned off or their expression level reduced while at the same time other genes are turned on or their expression level increased. Our study and other studies (Barr and Gedamu, 2001) suggest that the expression of pxn1 increases during this transformation process.

The higher level of pxn1 mRNA expression in the intracellular amastigote stage suggests that pxn1 might be important for intracellular survival of the most likely involving a variety of other genes as well. In the host macrophage, the parasite encounters host oxidants (ROS and RNS) and the genes may provide a survival advantage by detoxifying reactive oxygen species and reactive nitrogen species, as indicated in previous studies (Barr and Gedamu, 2003). In short, pxn1 may enhance intracellular survival of the parasites by protecting them from oxidative stress. In addition, pxn1 may have some role in the infectivity of amastigotes. Amastigotes replicate in macrophages until macrophages burst and release them to infect more healthy macrophages. Amastigotes are generally more infective than stationary phase promastigotes (Chang and Chaudhuri, 1990). The highest level of pxn1 expression in amastigotes as compared to stationary phase promastigotes also suggests its probable importance for amastigote infectivity. Furthermore, differential expression of pxn1 may explain the previous finding that stationary phase promastigotes are more resistant to hydrogen peroxide, t-BOOH and $ONOO^-$ than logarithmic phase promastigotes (Barr and Gedamu, 2003). It may be speculated that the higher level of pxn1 in the stationary phase promastigotes makes them more resistant to the above oxidants than logarithmic phase promastigotes.

In short, expression analysis of pxn1 mRNA indicates that pxn1 is probably a virulence factor. Such proteins are potential targets for the development of antileishmanial chemotherapeutic agents. Specific inhibitors of pxn1 synthesis or activity may render the parasite 1) noninfective, preventing infection of macrophages and/or 2) susceptible to attack by host reactive oxygen species and reactive nitrogen species, inhibiting its intramacrophageal survival. There are differences in the mechanism of action between human and trypanosomatids peroxidoxins, which may be targets for specific inhibitors (Rhee *et al.*, 2001)

Our finding that pxn2 is expressed constitutively in the different stages of *L. aethiopica* is consistent with previous reports on the expression pattern of *L. major* (Levick *et al.*, 1998), to which pxn2 protein of *L. aethiopica* showed the highest similarity. But it is in contrast to pxn2 of *L. chagasi* and *L. donovani*, which is differentially expressed, with predominant expression in the promastigote stage of the parasites. Our result suggests that pxn2 is almost equally important for all stages of the parasite.

We indicated that peroxidoxins of *L. aethiopica* exist as a multigene family, among which two are pxn1 and pxn2 and others are yet to be identified. Our result that the overall level of expression of peroxidoxin genes is higher in the amastigotes suggests that they may be important for intracellular survival. This higher level of overall expression in the amastigote stage may be due to abundant expression in this stage of pxn1 as our result showed that pxn2 does not make a significant difference in abundance in the different stages of the parasite.

6. Conclusion

We conclude that peroxidoxin genes are present in *L. aethiopica* in a multigene family and we identified and characterized two peroxidoxin genes, pxn1 and pxn2. Peroxidoxin genes may play similar functions (i.e. provide antioxidant activity against host oxidants) in all *Leishmania* species. High nucleotide and amino acid sequence homology exists between *L. aethiopica* and other *Leishmania* species. Although divergence in the nucleotide sequence of the coding region of peroxidoxin genes among the different *Leishmania* species that cause cutaneous leishmaniasis in Ethiopia is very low, alternatively, the 3' UTRs of these genes can be exploited for developing differential molecular diagnostic tools. Peroxidoxin expression is either differential or constitutive. Pxn1 genes are differentially expressed, with higher level in amastigote stage followed by the stationary phase promastigotes, suggesting that peroxidoxin genes may be involved in intracellular survival and infectivity and of *L. aethiopica*. For this reason, they appear to be important targets for drug development against leishmaniasis, for which effective, less toxic and short course drugs are needed urgently. Drugs that target peroxidoxins can be used against all *Leishmania* species since the amino acid sequence is highly conserved among *Leishmania* species. Pxn2 is constitutively expressed in the different stages of the parasite, suggesting that it may be equally important for all stages of the parasite. Peroxidoxins may also be exploited as potential vaccine candidates as they are very similar among *Leishmania* species.

7. Recommendations

As we need a rapid differential diagnostic tool that differentiates among *L. aethiopica*, *L. tropica* and *L. major* for clinical management and epidemiological survey and control of cutaneous leishmaniasis in Ethiopia, the potential of peroxidoxin nucleotide sequence, specially the 3' UTRs, as a diagnostic tool has to be tested using PCR or PCR-RFLP or both. For this purpose, the 3' UTRs can be identified by RACE and be sequenced. Furthermore, a virulent protein can only be used as a drug target if its mechanism of action or synthesis is well understood. Thus, we recommend that research on peroxidoxins has to continue to provide a better knowledge on the mechanism of action or synthesis for their future use as drug targets. We also recommed that immunogenicity of peroxidoxin proteins be tested to establish future vaccine development. For this purpose, it is possible to use the already available purified *L. chagasi* peroxidoxin proteins (pxn1 and pxn2), as their amino acid sequence of peroxidoxins is highly conserved among *Leishmania* species. The absolute importance of peroxidoxin genes for infectivity and intracellular survival of *Leishmania* has to be confirmed by conducting further experiments involving gene knock out and re-introduction studies.

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9. Appendices

Appendix A. Nucleotide sequence alignment of peroxidoxins of *Leishmania* and other organisms

```
                               1                                                            62
           1093 pxn1     (1)   ATGTCCCGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
           1093 pxn2     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
           1185 pxn1     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
           1185 pxn2     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
C. faciculata Tryp       (1)   ATGTCCTGCGGTGCCGCCAAGTTGAACCACCCCGCGCCTGAGTTCGACGACATGGCGCTCAT
F. hepatica  Prxn        (1)   ------------ATGTTGCAGCCTAATATGCCCGCCCCGAATTTTTCTGGACAGGCGGTA--
   H. sapiens prxn1      (1)   ATGTCTTCAGGAAATGCTAAAATTGGGCACCCTGCCCCCAACTTCAAAGCCACAGCTGTTAT
   H. sapiens Prxn2      (1)   ATGGCCTCCGGTAACGCGCGCATCGGAAAGCCAGCCCCTGACTTCAAGGCCACAGCGGTG--
     L. chagasi pxn1     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
     L. chagasi pxn2     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
     L. chagasi pxn3     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
    L. donovani pxn1     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
    L. infantum Prxn     (1)   ATGTCCTGCGGTGACGCCAAGATCAACTGTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
       L. major pxn2     (1)   ATGTCCTGCGGTAACGCCAAGATCAACTCTCCCGCGCCGTCCTTCGAGGAGGTGGCGCTCAT
     L. tropica pxn1     (1)   ATGACCTGCGGGGAAGCCAAGATCCACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
     L. tropica pxn2     (1)   ATGTCCTGCGGTGAAACCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGATGGCGCTCAT
   T. aquaticus Prxn     (1)   ------------ATGTCTTTGGTTGGGAAAAAAGTACAACCATTCCGTGCCCAAGCGTAT--
        T. cruzi TryP     (1)   ATGTCCTGCGGAGACGCAAAGCTCAACCACCCGGCGCCCGACTTCAATGAGACGGCGCTGAT
           Consensus     (1)   ATGTCCTGCGGTGACGCCAAGATCAACT TCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCAT
                               63                                                          124
           1093 pxn1    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
           1093 pxn2    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
           1185 pxn1    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
           1185 pxn2    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
C. faciculata Tryp      (63)   GCCCAACGGCACATTCAAGAAGGTAAGCCTGTCGTCCTACAAGGGCAAGTACGTCGTGCTCT
F. hepatica  Prxn       (49)   -GTGGGCAAGGAGTTCGAAACCATCAGTTTATCAGACTACAAGGGCAAATGGGTGATTCTCG
   H. sapiens prxn1     (63)   GCCAGATGGTCAGTTTAAAGATATCAGCCTGTCTGACTACAAAGGAAAATATGTTGTGTTCT
   H. sapiens Prxn2     (61)   -GTTGATGGCGCCTTCAAAGAGGTGAAGCTGTCGGACTACAAAGGGAAGTACGTGGTCCTCT
     L. chagasi pxn1    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
     L. chagasi pxn2    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
     L. chagasi pxn3    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
    L. donovani pxn1    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
    L. infantum Prxn    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCGCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
       L. major pxn2    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCGTCCTACAAGGGCAAGTGGGTCGTGCTCT
     L. tropica pxn1    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
     L. tropica pxn2    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
   T. aquaticus Prxn    (49)   -CACAACGGTGAATTCATCGAAGTGACAGAGCAAGACTTCATGGGGAAATGGAGCATCGTTT
        T. cruzi TryP    (63)   GCCCAACGGCACCTTCAAGAAGGTGGCTCTCACCTCCTACAAGGGCAAGTGGCTGGTGCTCT
           Consensus    (63)   GCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCT
                               125                                                         186
           1093 pxn1   (125)   TCTTCTACCCGCTCGACTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGC
           1093 pxn2   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAGC
           1185 pxn1   (125)   TCTTCTACCCGCTCGACTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGC
           1185 pxn2   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAGC
C. faciculata Tryp     (125)   TCTTCTACCCCATGGACTTCACCTTCGTGTGCCCCACCGAGATCATCCAGTTCTCCGACGAC
F. hepatica  Prxn      (110)   CCTTCTATCGACTTGATTTCACGTTCGTGTGTCCAACGGAAATAATCGCGATCAGTGATCAG
   H. sapiens prxn1    (125)   TCTTTTACCCTCTTGACTTCACCTTTGTGTGCCCCACGGAGATCATTGCTTTCAGTGATAGG
   H. sapiens Prxn2    (122)   TTTTCTACCCTCTGGACTTCACTTTTGTGTGCCCCACCGAGATCATCGCGTTCAGCAACCGT
     L. chagasi pxn1   (125)   TCTTCTACCCGCTCGATTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGAAAAC
     L. chagasi pxn2   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGAAAAC
     L. chagasi pxn3   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGAAAAC
    L. donovani pxn1   (125)   TCTTCTACCCGCTCGATTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCTGAAAAC
    L. infantum Prxn   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGAAAAC
       L. major pxn2   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGGTCATCGCGTTCTCCGACAGC
     L. tropica pxn1   (125)   TCTTCTACCCGCTCGACTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGC
     L. tropica pxn2   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAAC
   T. aquaticus Prxn   (110)   GCTTCTATCCGGCGGACTTTACGTTCGTCTGCCCGACGGAGCTCGAAGATTTGCAAGATCAC
        T. cruzi TryP   (125)   TCTTCTACCCGATGGACTTCACCTTCGTCTGCCCCACAGAGATCTGCCAATTCTCGGACCGT
           Consensus   (125)   TCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCCGACAGC
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                          187                                                        248
         1093 pxn1  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGC
         1093 pxn2  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGC
         1185 pxn1  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGC
         1185 pxn2  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGC
C. faciculata Tryp  (187) GCCAAGCGCTTCGCCGAGATCAACACCGAGGTGATCTCCTGCTCCTGCGACAGCGAGTACAG
F. hepatica  Prxn   (172) ATGGAGCAGTTCGCACAACGTAACTGCGCCGTCATCTTCTGCTCTACTGACTCGGTTTATTC
  H. sapiens prxn1  (187) GCAGAAGAATTTAAGAAACTCAACTGCCAAGTGATTGGTGCTTCTGTGGATTCTCACTTCTG
  H. sapiens Prxn2  (184) GCAGAGGACTTCCGCAAGCTGGGCTGTGAAGTGCTGGGCGTCTCGGTGGACTCTCAGTTCAC
   L. chagasi pxn1  (187) ATAAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGC
   L. chagasi pxn2  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGC
   L. chagasi pxn3  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGC
  L. donovani pxn1  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCCATGGACAGCGAGTACGC
  L. infantum Prxn  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTTGCGTGCTCCATGGACAGCGAGTACGC
     L. major pxn2  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATAGACAGCGAGTACGC
   L. tropica pxn1  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGC
   L. tropica pxn2  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGC
 T. aquaticus Prxn  (172) TACGCGACGTTTAAAGAACTTGGCGTGGAAGTGTACTCGGTTTCGACCGATACACATTATAC
     T. cruzi TryP   (187) GTGAAGGAGTTCTCTGACATTGGCTGCGAGGTGCTTGCCTGCTCCATGGACAGCGAGTACTC
         Consensus  (187) GTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGC
                          249                                                        310
         1093 pxn1  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATGGCGATCCCAA
         1093 pxn2  (249) GCACCTGCAGTGGACGCTGCAGGACCGCCAGAAGGGCGGCCTCGGGGCCATGGCGATCCCAA
         1185 pxn1  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATGGCGATCCCAA
         1185 pxn2  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATGGCGATCCCAA
C. faciculata Tryp  (249) CCACCTGCAGTGGACGTCCGTGGACCGCAAGAAGGGCGGCCTCGGCCCCATGGCCATCCCCA
F. hepatica  Prxn   (234) GCATCTGCAATGGACCAAAATGGATCGTAAGGTTGGCGGTATAGGCCAGCTGAACTTCCCGC
  H. sapiens prxn1  (249) TCATCTAGCATGGGTCAATACACCTAAGAAACAAGGAGGACTGGGACCCATGAACATTCCTT
  H. sapiens Prxn2  (246) CCACCTGGCTTGGTATGAGCAGGGGCCAAAGAG-GGAGGTTGCAGCTAAGCTCACACCCTCA
   L. chagasi pxn1  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGCGCCATGGCGATTCCAA
   L. chagasi pxn2  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGCGCCATGGCGATTCCAA
   L. chagasi pxn3  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGCGCCATGGCGATTCCAA
  L. donovani pxn1  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGCACCATGGCGATTCCAA
  L. infantum Prxn  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGCGCCATGGCGATTCCAA
     L. major pxn2  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGACCATGGCGATCCCAA
   L. tropica pxn1  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATGGCGATCCCAA
   L. tropica pxn2  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATGGCGATCCCAA
 T. aquaticus Prxn  (234) TCATAAAGCATGG--CACGATACATCGCCAGCC-------ATCAGCAAAATCGAATACGTGA
     T. cruzi TryP   (249) CCATCTGGCCTGGACAAGCATTGAGCGCAAGCGTGGCGGACTTGGCCAGATGAACATCCCCA
         Consensus  (249) GCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGCGCCATGGCGATCCCAA
                          311                                                        372
         1093 pxn1  (311) --TGCTGGCCGA-------CAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAGGAG
         1093 pxn2  (311) --TGCTGGCCGA-------CAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAGGAG
         1185 pxn1  (311) --TGCTGGCCGA-------CAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAGGAG
         1185 pxn2  (311) --TGCTGGCCGA-------CAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAGGAG
C. faciculata Tryp  (311) --TGCTGGCCGA-------CAAGACCAAGGGCATCGCGCGCGCCTACGGCGTGCTGGACGAG
F. hepatica  Prxn   (296) --TGCTGGCAGA-------CAAGAATATGTCTGTCTCTCGCGCCTTTGGTGTTCTGGATGAG
  H. sapiens prxn1  (311) --TGGTATCAGA-------CCCGAAGCGCACCATTGCTCAGGATTATGGGGTCTTAAAGGCT
  H. sapiens Prxn2  (307) GGTCCTAGCAGTGTGGCTTCGTGGCCATTGCTCAACCTCTGGAACCTGCGTTTCCCCATCGT
   L. chagasi pxn1  (311) --TGCTGGCCGA-------CAAGACCAAGAGCATCGCTCGTGCCTACGGCGTGCTGGAGGAG
   L. chagasi pxn2  (311) --TGCTGGCCGA-------CAAGACCAAGAGCATCGCTCGTGCCTACGGCGTGCTGGCGGAG
   L. chagasi pxn3  (311) --TGCTGGCCGA-------CAAGACCAAGAGCATCGCTCGTGCCTACGGCGTGCTGGCGGAG
  L. donovani pxn1  (311) --TGCTGGCCGA-------CAAGACCAAGAGCATCGCTCGTGCCTACGGCGTGCTGGAGGAG
  L. infantum Prxn  (311) --TGCTGGCCGA-------CAAGACCAAGAGCATCGCTCGTGCCTACGGCGTGCTGGCGGAG
     L. major pxn2  (311) --TGCTAGCCGA-------CAAGACCAAGAGCATCGCTCGTTCCTACGGCGTGCTGGAGGAG
   L. tropica pxn1  (311) --TGCTGGCCGA-------CAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAGGAG
   L. tropica pxn2  (311) --TGCTGGCCGA-------CAAGACCAAGAGCATCGCTCGTTCCTACGGCGTGCTGGAGGAG
 T. aquaticus Prxn  (287) --TGATCGGCGAT------CCTTCGCATCAAT-TGTCGCGCATGTTTGATGTGCTTGACGAA
     T. cruzi TryP   (311) --TTCTTGCCGA-------CAAGACCAAGTGCATCATGAAGTCGTATGGTGTGCTGAAGGAG
         Consensus  (311)   TGCTGGCCGA       CAAGACCAAGAGCATCGCTCGTGCCTACGGCGTGCTGGAGGAG
                          373                                                        434
         1093 pxn1  (364) AGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGTCAGAT
         1093 pxn2  (364) AGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGTCAGAT
         1185 pxn1  (364) AGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGTCAGAT
         1185 pxn2  (364) AGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGTCAGAT
C. faciculata Tryp  (364) GACAGCGGGGTCGCCTACCGCGGCGTCTTCATCATCGACCCCAACGGCAAGCTGCGCCAGAT
F. hepatica  Prxn   (349) GAACAGGGTAATACCTACCGTGGCAATTTCCTCATCGATCCCAAGGGGGTCCTGCGCCAGAT
  H. sapiens prxn1  (364) GATGAAGGCATCTCGTTCAGGGGCCTTTTTATCATTGATGATAAGGGTATTCTTCGGCAGAT
  H. sapiens Prxn2  (369) GAAAATAATGGAAACATTGCCGCCCAAGTCTTTAAGGATGATGACAGTAATTAGCATTTGA-
   L. chagasi pxn1  (364) AAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGCCAGAT
   L. chagasi pxn2  (364) AAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGCCAGAT
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   L. chagasi pxn3  (364) AAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGCCAGAT
  L. donovani pxn1  (364) AAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGCCAGAT
  L. infantum Prxn  (364) AAACAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCGCCAGAT
     L. major pxn2  (364) AGCCGGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGCTGCGTCAGAT
   L. tropica pxn1  (364) AGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGTCAGAT
   L. tropica pxn2  (364) AGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGTCAGAT
 T. aquaticus Prxn  (340) GAACAAGGGTTGGCGCAGCGCGGCACGTTCATCATCGATCCGGACGGCGTCATCCAAGCGGT
     T. cruzi TryP  (364) GAGGATGGCGTGGCGTACCGTGGCCTTTTCATCATCGACCCGAAGCAGAACCTGCGGCAGAT
         Consensus  (373) AA CAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCAATGGCATGGTGCG CAGAT
                          435                                                         496
         1093 pxn1  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
         1093 pxn2  (426) CACCGTCAACGACATGCCGGTGGGCCGCAGCGTG-GAGGAGGTTNTGCGCCTGCTGGAGGCT
         1185 pxn1  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
         1185 pxn2  (426) CACCGTCAACGACATGCCGGTGGGCCGCAGCGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
C. faciculata Tryp  (426) CATCATCAACGACATGCCCATCGGCCGCAACGTC-GAGGAGGTGATCCGCCTGGTCGAGGCG
  F. hepatica  Prxn (411) CACGGTGAATGACGACCCGGTGGGCCGTTCCGTT-GAAGAAGCCTTGCGTCTGCTCGATGCA
  H. sapiens prxn1  (426) CACTGTAAATGACCTCCCTGTTGGCCGCTCTGTG-GATGAGACTTTGAGACTAGTTCAGGCC
  H. sapiens Prxn2  (430) --------------------------------------------------------------
   L. chagasi pxn1  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
   L. chagasi pxn2  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
   L. chagasi pxn3  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
  L. donovani pxn1  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
  L. infantum Prxn  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
     L. major pxn2  (426) CACCGTCAATGACATGCCGGTGGGCCGCAGCGTG-GAGGAGGTTCTACGCCTGCTGGAGGCT
   L. tropica pxn1  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
   L. tropica pxn2  (426) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG-GAGGAGGTTCTGCGCCTGCTGGAGGCT
 T. aquaticus Prxn  (402) CGAAATCAACGCGGACGGCATCGGCCGCAACGCCAGCACACTGATTGA-TAAAATTAAAGCG
     T. cruzi TryP  (426) CACCGTCAACGACCTCCCCGTTGGCCGCGACGTG-GACGAGGCCCTTCGCCTTGTGAAGGCG
         Consensus  (435) CACCGTCAACGACATGCCGGTGGGCCGCAACGTG GAGGAGGTTCTGCGCCTGCTGGAGGCT
                          497                                                         558
         1093 pxn1  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAAAAGGGCGACCC
         1093 pxn2  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCC
         1185 pxn1  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCC
         1185 pxn2  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCC
C. faciculata Tryp  (487) CTGCAGTTCGT---GGAGGAGCACGGCGAGGTGTGCCCGGCCAACTGGAAGAAGGGCGACGC
  F. hepatica  Prxn (472) TTCATATTC---CACGAGGAGCATGGAGAGGTCTGCCCGGCGAACTGGAAGCCTAAAAGCAA
  H. sapiens prxn1  (487) TTCCAGTTCAC---TGACAAACATGGGGAAGTGTGCCCAGCTGGCTGGAAACCTGGCAGTGA
  H. sapiens Prxn2  (430) --------------------------------------------------------------
   L. chagasi pxn1  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCC
   L. chagasi pxn2  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCC
   L. chagasi pxn3  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCC
  L. donovani pxn1  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCC
  L. infantum Prxn  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCC
     L. major pxn2  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCC
   L. tropica pxn1  (487) TTTCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCC
   L. tropica pxn2  (487) TTGCAGTTCGT---GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGCCCC
 T. aquaticus Prxn  (463) GCCCAATACGTGCGCAACCATCCGGGTGAAGTCTGCCCGGCGAAATGGAA----AGAAG---
     T. cruzi TryP  (487) TTCCAGTTCGT---GGAGAAGCATGGCGAGGTGTGCCCCGCCAACTGGAAGCCCGGTGACAA
         Consensus  (497) TTTCAGTTCGT   GGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCG CCC
                          559                                                    615
         1093 pxn1  (546) TGGCCTGAAGGTCGATCACAATAAGTAA-----------------------------
         1093 pxn2  (546) CACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA--
         1185 pxn1  (546) TGGCCTGAAGGTCGATCACAATAAGTAA-----------------------------
         1185 pxn2  (546) CACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA--
C. faciculata Tryp  (546) CAAGAAGAAGGAGGGCCACTAG-----------------------------------
  F. hepatica  Prxn (531) GACCATCGTGCCTACTCCGGATGGATCCAAAGCATATTTCTCCTCAGCCAACTAG--
  H. sapiens prxn1  (546) TACCATCAAGCCTGATGTCCAAAAGAGCAAAGAATATTTCTCCAAGCAGAAGTGA--
  H. sapiens Prxn2  (430) ---------------------------------------------------------
   L. chagasi pxn1  (546) TGGCCTGAAGGTCGATCACAATAAGTAA-----------------------------
   L. chagasi pxn2  (546) CACGATGAAGCCGGAGCCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA--
   L. chagasi pxn3  (546) CACGATGAAGCCGGAGCCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAGCTGTGA--
  L. donovani pxn1  (546) TGGCCTGAAGGTCGATCACAATAAGTAA-----------------------------
  L. infantum Prxn  (546) CACGATGAAGCCGGAGCCGAAGGCGTCTGTCGAGGGGTACTTCAGTAAGCAGTAA--
     L. major pxn2  (546) CACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGATACTTCAGCAAGCAGTAA--
   L. tropica pxn1  (546) TGGCCTGAAGGTCGATCTCAATAAGTAA-----------------------------
   L. tropica pxn2  (546) CACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA--
 T. aquaticus Prxn  (518) G-------GGCGGAAAG--GCTGAAGCCAA-GCCTTGACCTTGTCGGCAAAATTTAA
     T. cruzi TryP  (546) GACGATGAAGCCGGATCCCGAAAAGTCCAAGGAGTACTTTGGTGCTGTCGCGTAG--
         Consensus  (559)  ACGATGAAGCCGGA CC AA G GTC    GA    T CT C       AGT
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Appendix B. Amino acid sequence alignment of peroxidoxin of *Leishmania* and other organisms

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                                1                                                     55
         1093 pxn1     (1) MSRGDAKINSPAPPEEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCPTE
         1093 pxn2     (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTE
         1185 pxn1     (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCPTE
         1185 pxn2     (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTE
C. fasiculata TryP     (1) MSCGAAKLNHPAPEFDDMALMPNGTFKKVSLSSYKGKYVVLFFYPMDFTFVCPTE
  F. hepatica prxn     (1) ----MLQPNMPAPNFSGQAVV-GKEFETISLSDYKGKWVILAFYPLDFTFVCPTE
 H. sapiens prxn1     (1) MSSGNAKIGHPAPNFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTFVCPTE
 H. sapiens prxn2     (1) MASGNARIGKPAPDFKATAVV-DGAFKEVKLSDYKGKYVVLFFYPLDFTFVCPTE
   L. chagasi pxn1     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFSFVCPTE
   L. chagasi pxn2     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFTFVCPTE
   L. chagasi pxn3     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFTFVCPTE
  L. donovani pxn1     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFSFVCPTE
  L. infantum Prxn     (1) MSCGDAKINCPAPPFEEVALMPNGSFKKISLAAYKGKWVVLFFYPLDFTFVCPTE
     L. major pxn2     (1) MSCGNAKINSPAPSFEEVALMPNGSFKKISLSSYKGKWVVLFFYPLDFTFVCPTE
   L. tropica pxn1     (1) MTCGEAKIHSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCPTE
   L. tropica pxn2     (1) MSCGETKINSPAPPFEEMALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTE
     T. cruzi TryP     (1) MSCGDAKLNHPAPDFNETALMPNGTFKKVALTSYKGKWLVLFFYPMDFTFVCPTE
         Consensus     (1) MSCGDAKIN PAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTE
                           56                                                   110
         1093 pxn1    (56) IIQFSDSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
         1093 pxn2    (56) IIAFSDSVSRFNELNCEVLACSMDSEYAHLQWTLQDRQKGGLGAMAIPMLADKTK
         1185 pxn1    (56) IIQFSDSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
         1185 pxn2    (56) IIAFSDSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
C. fasiculata TryP    (56) IIQFSDDAKRFAEINTEVISCSCDSEYSHLQWTSVDRKKGGLGPMAIPMLADKTK
  F. hepatica prxn    (51) IIAISDQMEQFAQRNCAVIFCSTDSVYSHLQWTKMDRKVGGIGQLNFPLLADKNM
 H. sapiens prxn1    (56) IIAFSDRAEEFKKLNCQVIGASVDSHFCHLAWVNTPKKQGGLGPMNIPLVSDPKR
 H. sapiens prxn2    (55) IIAFSNRAEDFRKLGCEVLGVSVDSQFTHLAWINTPRKEGGLGPLNIPLLADVTR
   L. chagasi pxn1    (56) IIQFSENISRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
   L. chagasi pxn2    (56) IIAFSENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
   L. chagasi pxn3    (56) IIAFSENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
  L. donovani pxn1    (56) IIQFSENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGTMAIPMLADKTK
  L. infantum Prxn    (56) IIAFSENVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
     L. major pxn2    (56) VIAFSDSVSRFNELNCEVLACSIDSEYAHLQWTLQDRKKGGLGTMAIPMLADKTK
   L. tropica pxn1    (56) IIQFSDSVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
   L. tropica pxn2    (56) IIAFSDNVSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
     T. cruzi TryP    (56) ICQFSDRVKEFSDIGCEVLACSMDSEYSHLAWTSIERKRGGLGQMNIPILADKTK
         Consensus    (56) IIAFSD VSRFNELNCEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTK
                           111                                                  165
         1093 pxn1   (111) CIARSYGVLEESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEAFQF
         1093 pxn2   (111) CIARSYGVLEESQGVAYRGLFIIDPHGMVRQITVNDMPVGRSVEEVXRLLEAFQF
         1185 pxn1   (111) CIARSYGVLEESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEAFQF
         1185 pxn2   (111) CIARSYGVLEESQGVAYRGLFIIDPHGMVRQITVNDMPVGRSVEEVLRLLEAFQF
C. fasiculata TryP   (111) GIARAYGVLDEDSGVAYRGVFIIDPNGKLRQIIINDMPIGRNVEEVIRLVEALQF
  F. hepatica prxn   (106) SVSRAFGVLDEEQGNTYRGNFLIDPKGVLRQITVNDDPVGRSVEEALRLLDAFIF
 H. sapiens prxn1   (111) TIAQDYGVLKADEGISFRGLFIIDDKGILRQITVNDLPVGRSVDETLRLVQAFQF
 H. sapiens prxn2   (110) RLSEDYGVLKTDEGIAYRGLFIIDGKGVLRQITVNDLPVGRSVDEALRLVQAFQY
   L. chagasi pxn1   (111) SIARAYGVLEEKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQF
   L. chagasi pxn2   (111) SIARAYGVLAEKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQF
   L. chagasi pxn3   (111) SIARAYGVLAEKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQF
  L. donovani pxn1   (111) SIARAYGVLEEKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQF
  L. infantum Prxn   (111) SIARAYGVLAEKQGVAYRGLFIIDPNGMVRQITVNDMPVGRNVEEVLRLLEAFQF
     L. major pxn2   (111) SIARSYGVLEESRGVAYRGLFIIDPHGMLRQITVNDMPVGRSVEEVLRLLEAFQF
   L. tropica pxn1   (111) CIARSYGVLEESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEAFQF
   L. tropica pxn2   (111) SIARSYGVLEESQGVAYRGLFIIDPHGMVRQITVNDMPVGRNVEEVLRLLEALQF
     T. cruzi TryP   (111) CIMKSYGVLKEEDGVAYRGLFIIDPKQNLRQITVNDLPVGRDVDEALRLVKAFQF
         Consensus   (111) SIARSYGVLEE QGVAYRGLFIIDP GMVRQITVNDMPVGRNVEEVLRLLEAFQF
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                                166                              199
         1093 pxn1      (166)   VEKHGEVCPANWKKGDPGLKVDHNK---------
        1093 pxn2       (166)   VEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
         1185 pxn1      (166)   VEKHGEVCPANWKKGDPGLKVDHNK---------
         1185 pxn2      (166)   VEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
C. fasiculata TryP      (166)   VEEHGEVCPANWKKGDAKKKEGH-----------
  F. hepatica prxn      (161)   HEEHGEVCPANWKPKSKTIVPTPDGSKAYFSSAN
 H. sapiens prxn1       (166)   TDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK
 H. sapiens prxn2       (165)   TDEHGEVCPAGWKPGSDTIKPNVDDSKEYFSKHN
   L. chagasi pxn1      (166)   VEKHGEVCPANWKKGDPGLKVDHNK---------
   L. chagasi pxn2      (166)   VEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
   L. chagasi pxn3      (166)   VEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKL
  L. donovani pxn1      (166)   VEKHGEVCPANWKKGDPGLKVDHNK---------
  L. infantum Prxn      (166)   VEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
     L. major pxn2      (166)   VEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
   L. tropica pxn1      (166)   VEKHGEVCPANWKKGDPGLKVDLNK---------
   L. tropica pxn2      (166)   VEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
      T. cruzi TryP     (166)   VEKHGEVCPANWKPGDKTMKPDPEKSKEYFGAVA
         Consensus      (166)   VEKHGEVCPANWKKGAPTMKPDP  S E Y S
```

Appendix C. Nucleotide sequence alignment of peroxidoxin genes of *Leishmania* species that cause cutaneous leishmaniasis in Ethiopia

a) pxn1

```
                          1                                                                    70
      1093 pxn1      (1) ATGTCCCGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
      1185 pxn1      (1) ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
L. tropica pxn1      (1) ATGACCTGCGGGGAAGCCAAGATCCACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
      Consensus      (1) ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTCATGCCCAACG
                          71                                                                  140
      1093 pxn1     (71) GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
      1185 pxn1     (71) GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
L. tropica pxn1     (71) GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
      Consensus     (71) GCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTGCTCTTCTTCTACCCGCTCGA
                          141                                                                 210
      1093 pxn1    (141) CTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
      1185 pxn1    (141) CTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
L. tropica pxn1    (141) CTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
      Consensus    (141) CTTCAGCTTCGTGTGCCCGACAGAGATCATCCAGTTCTCCGACAGCGTGAGTCGCTTCAACGAGCTCAAC
                          211                                                                 280
      1093 pxn1    (211) TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
      1185 pxn1    (211) TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
L. tropica pxn1    (211) TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
      Consensus    (211) TGCGAGGTCCTCGCGTGCTCGATGGACAGCGAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGA
                          281                                                                 350
      1093 pxn1    (281) AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
      1185 pxn1    (281) AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
L. tropica pxn1    (281) AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
      Consensus    (281) AGGGCGGCCTCGGGGCCATGGCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGG
                          351                                                                 420
      1093 pxn1    (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
      1185 pxn1    (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
L. tropica pxn1    (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
      Consensus    (351) CGTGCTGGAGGAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
                          421                                                                 490
      1093 pxn1    (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
      1185 pxn1    (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
L. tropica pxn1    (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
      Consensus    (421) CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTGGAGGCTTTTC
                          491                                                                 560
      1093 pxn1    (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAAAAGGGCGACCCTGGCCTGAAGGTCGA
      1185 pxn1    (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCCTGGCCTGAAGGTCGA
L. tropica pxn1    (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCCTGGCCTGAAGGTCGA
      Consensus    (491) AGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGCGACCCTGGCCTGAAGGTCGA
                          561       573
      1093 pxn1    (561) TCACAATAAGTAA
      1185 pxn1    (561) TCACAATAAGTAA
L. tropica pxn1    (561) TCTCAATAAGTAA
      Consensus    (561) TCACAATAAGTAA
```

b) pxn2

```
                             1                                                         60
         1093 pxn2    (1)   ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTC
         1185 pxn2    (1)   ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTC
      L. major pxn2    (1)   ATGTCCTGCGGTAACGCCAAGATCAACTCTCCCGCGCCGTCCTTCGAGGAGGTGGCGCTC
    L. tropica pxn2    (1)   ATGTCCTGCGGTGAAACCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGATGGCGCTC
          Consensus    (1)   ATGTCCTGCGGTGACGCCAAGATCAACTCTCCCGCGCCGCCCTTCGAGGAGGTGGCGCTC
                             61                                                       120
         1093 pxn2   (61)   ATGCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTG
         1185 pxn2   (61)   ATGCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTG
      L. major pxn2   (61)   ATGCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCTCCTACAAGGGCAAGTGGGTCGTG
    L. tropica pxn2   (61)   ATGCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTG
          Consensus   (61)   ATGCCCAACGGCAGCTTCAAGAAGATCAGCCTCTCCGCCTACAAGGGCAAGTGGGTCGTG
                             121                                                      180
         1093 pxn2  (121)   CTCTTCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCC
         1185 pxn2  (121)   CTCTTCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCC
      L. major pxn2  (121)   CTCTTCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGGTCATCGCGTTCTCC
    L. tropica pxn2  (121)   CTCTTCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCC
          Consensus  (121)   CTCTTCTTCTACCCGCTCGACTTCACCTTCGTGTGCCCGACAGAGATCATCGCGTTCTCC
                             181                                                      240
         1093 pxn2  (181)   GACAGCGTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGC
         1185 pxn2  (181)   GACAGCGTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGC
      L. major pxn2  (181)   GACAGCGTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATAGACAGC
    L. tropica pxn2  (181)   GACAACGTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGC
          Consensus  (181)   GACAGCGTGAGTCGCTTCAACGAGCTCAACTGCGAGGTCCTCGCGTGCTCGATGGACAGC
                             241                                                      300
         1093 pxn2  (241)   GAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCCAGAAGGGCGGCCTCGGGGCCATG
         1185 pxn2  (241)   GAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATG
      L. major pxn2  (241)   GAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGACCATG
    L. tropica pxn2  (241)   GAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATG
          Consensus  (241)   GAGTACGCGCACCTGCAGTGGACGCTGCAGGACCGCAAGAAGGGCGGCCTCGGGGCCATG
                             301                                                      360
         1093 pxn2  (301)   GCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAG
         1185 pxn2  (301)   GCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAG
      L. major pxn2  (301)   GCGATCCCAATGCTAGCCGACAAGACCAAGAGCATCGCTCGTTCCTACGGCGTGCTGGAG
    L. tropica pxn2  (301)   GCGATCCCAATGCTGGCCGACAAGACCAAGAGCATCGCTCGTTCCTACGGCGTGCTGGAG
          Consensus  (301)   GCGATCCCAATGCTGGCCGACAAGACCAAGTGCATCGCTCGTTCCTACGGCGTGCTGGAG
                             361                                                      420
         1093 pxn2  (361)   GAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
         1185 pxn2  (361)   GAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
      L. major pxn2  (361)   GAGAGCCGGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGCTGCGT
    L. tropica pxn2  (361)   GAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
          Consensus  (361)   GAGAGCCAGGGCGTGGCCTACCGCGGTCTCTTCATCATCGACCCCCATGGCATGGTGCGT
                             421                                                      480
         1093 pxn2  (421)   CAGATCACCGTCAACGACATGCCGGTGGGCCGCAGCGTGGAGGAGGTTNTGCGCCTGCTG
         1185 pxn2  (421)   CAGATCACCGTCAACGACATGCCGGTGGGCCGCAGCGTGGAGGAGGTTCTGCGCCTGCTG
      L. major pxn2  (421)   CAGATCACCGTCAATGACATGCCGGTGGGCCGCAGCGTGGAGGAGGTTCTACGCCTGCTG
    L. tropica pxn2  (421)   CAGATCACCGTCAACGACATGCCGGTGGGCCGCAACGTGGAGGAGGTTCTGCGCCTGCTG
          Consensus  (421)   CAGATCACCGTCAACGACATGCCGGTGGGCCGCAGCGTGGAGGAGGTTCTGCGCCTGCTG
                             481                                                      540
         1093 pxn2  (481)   GAGGCTTTTCAGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGC
         1185 pxn2  (481)   GAGGCTTTTCAGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGC
      L. major pxn2  (481)   GAGGCTTTTCAGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGC
    L. tropica pxn2  (481)   GAGGCTTTGCAGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGC
          Consensus  (481)   GAGGCTTTTCAGTTCGTGGAGAAGCACGGCGAGGTGTGCCCCGCGAACTGGAAGAAGGGC
                             541                                                      600
         1093 pxn2  (541)   GCCCCCACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
         1185 pxn2  (541)   GCCCCCACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
      L. major pxn2  (541)   GCCCCCACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGATACTTCAGCAAGCAGTAA
    L. tropica pxn2  (541)   GCCCCCACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
          Consensus  (541)   GCCCCCACGATGAAGCCGGAACCGAAGGCGTCTGTCGAGGGGTACTTCAGCAAACAGTAA
```

Appendix D. Amino acid sequence alignment of peroxidoxin proteins of *Lieshmaia* species that cause cutaneous leishmaniasis

a) pxn1

```
                      1                                                                   70
L. tropica pxn1   (1) MTCGEAKIHSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCPTEIIQFSDSVSRFNELN
      1185 pxn1   (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCPTEIIQFSDSVSRFNELN
      1093 pxn1   (1) MSRGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCPTEIIQFSDSVSRFNELN
      Consensus   (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFSFVCPTEIIQFSDSVSRFNELN
                      71                                                                 140
L. tropica pxn1  (71) CEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLEESQGVAYRGLFIIDPHGMVR
      1185 pxn1  (71) CEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLEESQGVAYRGLFIIDPHGMVR
      1093 pxn1  (71) CEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLEESQGVAYRGLFIIDPHGMVR
      Consensus  (71) CEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLEESQGVAYRGLFIIDPHGMVR
                      141                                            190
L. tropica pxn1 (141) QITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCPANWKKGDPGLKVDLNK
      1185 pxn1 (141) QITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCPANWKKGDPGLKVDHNK
      1093 pxn1 (141) QITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCPANWKKGDPGLKVDHNK
      Consensus (141) QITVNDMPVGRNVEEVLRLLEAFQFVEKHGEVCPANWKKGDPGLKVDHNK
```

b) pxn2

```
                      1                                                                   70
L. tropica pxn2   (1) MSCGETKINSPAPPFEEMALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTEIIAFSDNVSRFNELN
  L. major pxn2   (1) MSCGNAKINSPAPSFEEVALMPNGSFKKISLSSYKGKWVVLFFYPLDFTFVCPTEVIAFSDSVSRFNELN
      1185 pxn2   (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTEIIAFSDSVSRFNELN
      1093 pxn2   (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTEIIAFSDSVSRFNELN
      Consensus   (1) MSCGDAKINSPAPPFEEVALMPNGSFKKISLSAYKGKWVVLFFYPLDFTFVCPTEIIAFSDSVSRFNELN
                      71                                                                 140
L. tropica pxn2  (71) CEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARSYGVLEESQGVAYRGLFIIDPHGMVR
  L. major pxn2  (71) CEVLACSIDSEYAHLQWTLQDRKKGGLGTMAIPMLADKTKSIARSYGVLEESRGVAYRGLFIIDPHGMLR
      1185 pxn2  (71) CEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKCIARSYGVLEESQGVAYRGLFIIDPHGMVR
      1093 pxn2  (71) CEVLACSMDSEYAHLQWTLQDRQKGGLGAMAIPMLADKTKCIARSYGVLEESQGVAYRGLFIIDPHGMVR
      Consensus  (71) CEVLACSMDSEYAHLQWTLQDRKKGGLGAMAIPMLADKTKSIARSYGVLEESQGVAYRGLFIIDPHGMVR
                      141                                                     199
L. tropica pxn2 (141) QITVNDMPVGRNVEEVLRLLEALQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
  L. major pxn2 (141) QITVNDMPVGRSVEEVLRLLEAFQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
      1185 pxn2 (141) QITVNDMPVGRSVEEVLRLLEAFQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
      1093 pxn2 (141) QITVNDMPVGRSVEEVXRLLEAFQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
      Consensus (141) QITVNDMPVGRSVEEVLRLLEAFQFVEKHGEVCPANWKKGAPTMKPEPKASVEGYFSKQ
```

Appendix E. Nucleotide sequences pxn1 and pxn2 of *L. tropica* and the amino acids they encode

b) *L. tropica* pxn1

```
          M   T   C   G   E   A   K   I   H   S   P   A   P   P
    1    ATG ACC TGC GGG GAA GCC AAG ATC CAC TCT CCC GCG CCG CCC
          F   E   E   V   A   L   M   P   N   G   S   F   K   K
   43    TTC GAG GAG GTG GCG CTC ATG CCC AAC GGC AGC TTC AAG AAG
          I   S   L   S   A   Y   K   G   K   W   V   V   L   F
   85    ATC AGC CTC TCC GCC TAC AAG GGC AAG TGG GTC GTG CTC TTC
          F   Y   P   L   D   F   S   F   V   C   P   T   E   I
  127    TTC TAC CCG CTC GAC TTC AGC TTC GTG TGC CCG ACA GAG ATC
          I   Q   F   S   D   S   V   S   R   F   N   E   L   N
  169    ATC CAG TTC TCC GAC AGC GTG AGT CGC TTC AAC GAG CTC AAC
          C   E   V   L   A   C   S   M   D   S   E   Y   A   H
  211    TGC GAG GTC CTC GCG TGC TCG ATG GAC AGC GAG TAC GCG CAC
          L   Q   W   T   L   Q   D   R   K   K   G   G   L   G
  253    CTG CAG TGG ACG CTG CAG GAC CGC AAG AAG GGC GGC CTC GGG
          A   M   A   I   P   M   L   A   D   K   T   K   C   I
  295    GCC ATG GCG ATC CCA ATG CTG GCC GAC AAG ACC AAG TGC ATC
          A   R   S   Y   G   V   L   E   E   S   Q   G   V   A
  337    GCT CGT TCC TAC GGC GTG CTG GAG GAG AGC CAG GGC GTG GCC
          Y   R   G   L   F   I   I   D   P   H   G   M   V   R
  379    TAC CGC GGT CTC TTC ATC ATC GAC CCC CAT GGC ATG GTG CGT
          Q   I   T   V   N   D   M   P   V   G   R   N   V   E
  421    CAG ATC ACC GTC AAC GAC ATG CCG GTG GGC CGC AAC GTG GAG
          E   V   L   R   L   L   E   A   F   Q   F   V   E   K
  463    GAG GTT CTG CGC CTG CTG GAG GCT TTT CAG TTC GTG GAG AAG
          H   G   E   V   C   P   A   N   W   K   K   G   D   P
  505    CAC GGC GAG GTG TGC CCC GCG AAC TGG AAG AAG GGC GAC CCT
          G   L   K   V   D   L   N   K   *
  547    GGC CTG AAG GTC GAT CTC AAT AAG TAA
```

b) *L. tropica* pxn2

```
      M   S   C   G   E   T   K   I   N   S   P   A   P   P
  1  ATG TCC TGC GGT GAA ACC AAG ATC AAC TCT CCC GCG CCG CCC
      F   E   E   M   A   L   M   P   N   G   S   F   K   K
 43  TTC GAG GAG ATG GCG CTC ATG CCC AAC GGC AGC TTC AAG AAG
      I   S   L   S   A   Y   K   G   K   W   V   V   L   F
 85  ATC AGC CTC TCC GCC TAC AAG GGC AAG TGG GTC GTG CTC TTC
      F   Y   P   L   D   F   T   F   V   C   P   T   E   I
127  TTC TAC CCG CTC GAC TTC ACC TTC GTG TGC CCG ACA GAG ATC
      I   A   F   S   D   N   V   S   R   F   N   E   L   N
169  ATC GCG TTC TCC GAC AAC GTG AGT CGC TTC AAC GAG CTC AAC
      C   E   V   L   A   C   S   M   D   S   E   Y   A   H
211  TGC GAG GTC CTC GCG TGC TCG ATG GAC AGC GAG TAC GCG CAC
      L   Q   W   T   L   Q   D   R   K   K   G   G   L   G
253  CTG CAG TGG ACG CTG CAG GAC CGC AAG AAG GGC GGC CTC GGG
      A   M   A   I   P   M   L   A   D   K   T   K   S   I
295  GCC ATG GCG ATC CCA ATG CTG GCC GAC AAG ACC AAG AGC ATC
      A   R   S   Y   G   V   L   E   E   S   Q   G   V   A
337  GCT CGT TCC TAC GGC GTG CTG GAG GAG AGC CAG GGC GTG GCC
      Y   R   G   L   F   I   I   D   P   H   G   M   V   R
379  TAC CGC GGT CTC TTC ATC ATC GAC CCC CAT GGC ATG GTG CGT
      Q   I   T   V   N   D   M   P   V   G   R   N   V   E
421  CAG ATC ACC GTC AAC GAC ATG CCG GTG GGC CGC AAC GTG GAG
      E   V   L   R   L   L   E   A   L   Q   F   V   E   K
463  GAG GTT CTG CGC CTG CTG GAG GCT TTG CAG TTC GTG GAG AAG
      H   G   E   V   C   P   A   N   W   K   K   G   A   P
505  CAC GGC GAG GTG TGC CCC GCG AAC TGG AAG AAG GGC GCC CCC
      T   M   K   P   E   P   K   A   S   V   E   G   Y   F
547  ACG ATG AAG CCG GAA CCG AAG GCG TCT GTC GAG GGG TAC TTC
      S   K   Q   *
589  AGC AAA CAG TAA
```

Appendix F. One letter symbol for amino acids

A	Alanine
C	cysteine
D	Aspartic acid
E	glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

Appendix G . Standard Genetic Code

	T	**C**	**A**	**G**
T	TTT Phe (F) TTC " TTA Leu (L) TTG "	TCT Ser (S) TCC " TCA " TCG "	TAT Tyr (Y) TAC TAA **Ter** TAG **Ter**	TGT Cys (C) TGC TGA **Ter** TGG Trp (W)
C	CTT Leu (L) CTC " CTA " CTG "	CCT Pro (P) CCC " CCA " CCG "	CAT His (H) CAC " CAA Gln (Q) CAG "	CGT Arg (R) CGC " CGA " CGG "
A	ATT Ile (I) ATC " ATA " **ATG** Met (M)	ACT Thr (T) ACC " ACA " ACG "	AAT Asn (N) AAC " AAA Lys (K) AAG "	AGT Ser (S) AGC " AGA Arg (R) AGG "
G	GTT Val (V) GTC " GTA " GTG "	GCT Ala (A) GCC " GCA " GCG "	GAT Asp (D) GAC " GAA Glu (E) GAG "	GGT Gly (G) GGC " GGA " GGG "