Molecular Characterization of $PGF_{2\alpha}$ Synthase-like Protein in Old and New World Species of Leishmania

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or for any other award.

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This thesis is dedicated to my daughters, Christine Amali Muderwa and Shiloh Nelly.

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LIST OF ABBREVIATIONS AND ACRONYMS

A	Absorbance
AA	Arachidonic acid
AKRs	Ald-keto reductases
AP	Adaptor Protein
ATCC	American type culture collection
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cAMP	Cyclic AMP
CDD	Conserved Domain Database
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
CK	Casein kinase
COX	Cyclooxygenase
DAT	Direct agglutination test
DCL	Diffuse cutaneous leishmaniasis
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DP	Prostaglandin D_2 receptor
ECL	Enhanced chemiluminiscence
EDTA	Ethylenediaminetetraamine
ELISA	Enzyme linked immunosorbent assay
ELM	Eukaryotic linear motif
FHA	Forkhead-associated
GSK3	Glycogen synthase kinase
GST	Glutathione S-transferase
HIV	Human immunodeficiency virus
IFN-γ	Interferon gamma
IL	Interleukin

JmjC	Jumonji
kDa	kilodalton
LIG	Ligand binding sites
***Lm*PGFS**	*Leishmania major* Prostaglandin F synthase
LPG	Lipophosphoglycan
MAPK	Mitogen-activated protein kinase
MOD	Post-translational modification site
MTX	Methotrexate
MUSCLE	Multiple Sequence Comparison by Log Expectation
NCBI	National Center for Biotechnology Institute
NES	Nuclear Export Signal
N-GLC	N-glycosylation
NO	Nitric oxide
NSAIDs	Non steroidal anti-inflammatory drugs
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGFS	Prostaglandin F synthase
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PGs	Prostaglandins
PIKK	Phosphoinositide-3-OH-kinase related kinases
PKA	Protein kinase A
PKB	Protein kinase B
PKDL	Post-Kala azar dermal leishmaniasis
PLA2	Phospholipase A_2
PP1	Protein phosphatase 1
ProDKin	Proline-directed kinases
PVDF	Polyvinylidene fluoride

rgp63	Recombinant 63 kilodalton glycoprotein
RNA	Ribonucleic acid
SbV	Pentavalent antimony
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMC	Structural maintenance of chromosomes
SUMO	Small ubiquitin-related modifiers
***Tb*PGFS**	*Trypanosoma brucei* Prostaglandin F synthase
TBST	Tris buffered saline containing Tris
***Tc*OYE**	*Trypanosoma cruzi* old yellow enzyme
***Tc*PGFS**	*Trypanosoma cruzi* prostaglandin F synthase
Th 1/2	T helper 1/2
TNF-α	Tumor necrosis factor alpha
TRAF	Tumor necrosis factor receptor-associated factors
TRG	Targeting sites
TXA_2	Thromboxane A_2
USP	Ubiquitin-specific protease
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Leishmaniasis is a disease caused by protozoan parasites of the genus *Leishmania* and spread by the bite of infected sand fly species. Infection of mammals by *Leishmania* results in increased production of prostaglandins in the host. These prostaglandins which are lipid mediators are responsible for some of the symptoms observed in leishmaniasis including fever and immunosuppression. Prostaglandin $F_{2\alpha}$ is among the prostaglandins that are synthesized by *Leishmania* species. The present study was undertaken to identify and characterize the *$PGF_{2\alpha}$* synthase gene and to identify and characterize the $PGF_{2\alpha}$ synthase protein in Old and New World Species of *Leishmania* in order to provide insight into the role of these proteins in the parasite. Isolates of *L. major, L. donovani, L. tropica, L. amazonensis, L. braziliensis, L. mexicana and L. chagasi* were obtained from the cryobank of American Type Culture Collection (ATCC, Manassas USA) and cultured to obtain promastigotes. Total RNA was extracted from the cell pellets of each of the cultured *Leishmania* species. To detect gene expression at transcription level, polymerase chain reaction was carried out using *L. major PGF$_{2\alpha}$* synthase gene specific primers and complementary DNA obtained from promastigotes of each of the species mentioned above. To detect expression at translation level, total protein obtained from cultured promastigotes of the above parasites was analyzed on a Western blot using *T. brucei*-specific rabbit anti-$PGF_{2\alpha}$ synthase polyclonal antibodies. At the transcription level, $PGF_{2\alpha}$ synthase gene expression was detected in Old World species *L. major, L. donovani* and *L. tropica*, but was absent in the New World *L. amazonensis* and *L. mexicana.* It was expressed at low levels in the New World *L. chagasi.* Western blot analysis confirmed the presence of $PGF_{2\alpha}$ synthase - like proteins in Old World and not in New World species. These findings suggest that New World *Leishmania* may have evolved new ortholog genes to produce $PGF_{2\alpha}$. Alternatively, the ancestral *$PGF_{2\alpha}$* synthase gene is present in the New World species but has mutated or been lost due to speciation during evolution. Phenotypic functional studies such as enzyme assays ought to be carried out to ascertain presence and function of PGFS in promastigotes and amastigotes of Old and New World species of *Leishmania.*

CHAPTER ONE

1.0 INTRODUCTION

1.1 Leishmaniasis

Leishmaniases are vector-borne diseases caused by protozoan parasites of the genus *Leishmania*. These parasites belong to the order *Kinetoplastidae* and family *Trypanosomatidae*, which is characterized by the presence of a single flagellum and a DNA-rich kinetoplast. The *Leishmania* parasite exists in two morphological forms as an intracellular amastigote in vertebrate hosts and as a promastigote found in sandfly vectors (Singh, 2006). Leishmaniasis is prevalent throughout the tropical and sub-tropical regions of Africa, Asia, the Mediterranean, Southern Europe (Old World) and Central and South America (New World). Approximately 21 species of *Leishmania* are pathogenic to humans. These are transmitted through the bite of *Phlebotomus* sandflies in the Old World and *Lutzomyia* in the New World (Sharma & Singh, 2008)

1.2 Life cycle of *Leishmania*

In vertebrates, *Leishmania* exists as a non-motile spherical amastigote, approximately 2.5 to 5 µm in diameter, which proliferates inside the phagolysosome of host macrophages (Descoteaux & Turco, 1999). The sandfly ingests amastigote-containing macrophages and monocytes in its blood meal. These amastigotes are released into the sandfly midgut, where they differentiate into flagellated procyclic promastigotes and attach to the midgut epithelium (Descoteaux & Turco, 1999). The promastigote then goes through metacyclogenesis where the dividing, non infective procyclic form acquires virulence capabilities and is transformed into a non dividing infective metacyclic form (Bates & Rogers, 2004).

The metacyclic promastigotes detach from the midgut epithelium and migrate into the pharynx and buccal cavity of the insect. During the next blood meal, the infective metacyclic promastigotes are passed into the vertebrate host. Promastigotes enter the

macrophages through receptor-mediated phagocytosis and are incorporated into phagolysosomes where they differentiate into the amastigote form. The amastigotes proliferate, eventually rupturing the infected macrophages and are released to infect neighbouring macrophages and the cycle begins again (Figure 1.1).

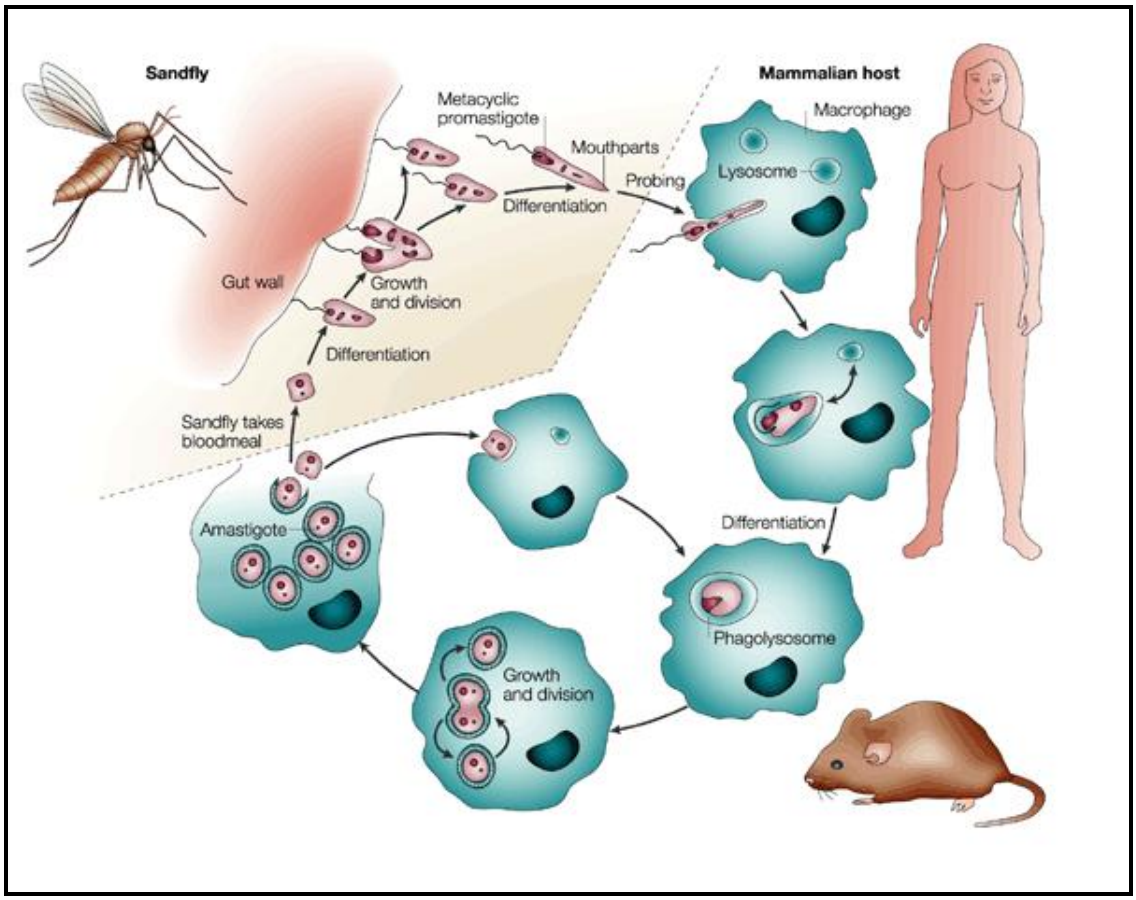

Figure 1.1: Life cycle of *Leishmania* (Sacks & Noben-Trauth, 2002)

1.3 Global distribution of Leishmaniasis

Leishmaniasis is a neglected tropical disease. It is endemic in 98 countries with more than 350 million people at risk and an estimated 1.3 million new cases and 20 000 to 30 000 deaths annually (WHO, 2014). Urbanization due to ecological, demographic and environmental changes is one of the major worldwide risk factors for

leishmaniasis and largely contributes to the persistence of the burden of the disease (Ready, 2010).

Leishmania major is found in the sparsely inhabited desert and savannah regions in the Old World including the Middle East (Al-Tawfiq & AbuKhamsin, 2004, Mohebali *et al.*, 2004), Central Asia (Strelkova *et al.*, 2001) and East Africa (el-Safi & Peters, 1991). Human infection by *L. major* is considered anthropophilic (humans are the preferred hosts), while gerbils are the sylvatic reservoir (Rassi *et al.*, 2011). *Leishmania tropica* is found in Morocco (Chaara *et al.*, 2014) Ethiopia, Kenya, Middle East (Akhoundi *et al.*, 2013), Greece (Ntais *et al.*, 2013), and India (Aara *et al.*, 2013), where it is predominantly confined to densely populated areas. *Leishmania mexicana* occurs in North and Central America, including Mexico (Gonzalez *et al.*, 2011), Texas and Oklahoma in the United States of America where it is transmitted by the *Lutzomyia* species of sandfly and is largely responsible for a cutaneous form of leishmaniasis (Clarke *et al.*, 2013). *Leishmania braziliensis* is the predominant species that causes mucocutaneous leishmaniasis in Brazil (Guerra *et al.*, 2011). *Leishmania donovani* occurs in India (Hasker *et al.*, 2013) and East Africa (Elnaiem, 2011), *L. infantum* in the Mediterranean region where dogs are the main domestic reservoir for human infection (Miro *et al.*, 2012), and *L. chagasi* is the major cause of visceral leishmaniasis in Brazil (Esch *et al.*, 2012).

1.4 Leishmaniasis in Kenya

Leishmania major, *L. donovani*, *L. aethiopica* and *L. tropica* species of *Leishmania* occur in Kenya. Visceral leishmaniasis is caused by *L. donovani* and transmitted by *Phlebotomus martini* although *Phlebotomus orientalis* has also been implicated in its transmission (Ngumbi *et al.*, 2010). Cutaneous leishmaniasis due to *L. major* is transmitted by *Phlebotomus duboscqi* and is mainly found in rodents (Schaefer *et al.*, 1994). Cutaneous leishmaniasis caused by *L. aethiopica* has been identified in Mt. Elgon. Its reservoir is the rock hyrax and it is transmitted by *Phlebotomus larroussius* (Sang *et al.*, 1993b). *Leishmania tropica* has been isolated from patients (Mebrahtu *et al.*, 1992) and rats (Massamba *et al.*, 1998) in Laikipia. Cases of

infection have also been detected in a previously uninhabited area in Utut, at the floor of the Rift Valley (Sang *et al.*, 1994). This species of *Leishmania* is transmitted by *Phlebotomus guggisbergi* (Lawyer *et al.*, 1991).

Historically, epidemics of leishmaniasis have occured in various parts of Kenya. Cases of visceral leishmaniasis were reported in Wajir and Mandera in North Eastern Kenya as early as 1935 (Peters & Killick-Kendrick, 1987). In 1940, an outbreak of visceral leishmaniasis occurred in a battalion patrolling the Northern part of Kenya during the Second World War (Cole *et al.*, 1942). A major epidemic of visceral leishmaniasis occurred in Kitui district between 1952-1953. At the peak of this epidemic 2142 cases were reported (Fendall, 1961). Visceral leishmaniasis caused by *L. donovani* is endemic in the semi-arid lowlands including West Pokot (Mutero *et al.*, 1992), Baringo (Ryan *et al.*, 2006), Kitui (Southgate & Oriedo, 1962), Machakos (Wijers & Kiilu, 1984), Meru (Wijers, 1971), Koibatek, and Kajiado (Johnson *et al.*, 1993) districts (Minter *et al.*, 1962; Ngumbi, *et al.*, 2010). Visceral leishmaniasis in these areas is transmitted by *P. martini* which lives and breeds in termite mounds (Perkins *et al.*, 1988). Several foci of cutaneous leishmaniasis have occurred in parts of Central Kenya and the Rift Valley including Nakuru, Nyandarua, Laikipia, Samburu and Isiolo (Sang *et al.*, 1993a). Baringo is the only foci where both visceral and cutaneous leishmaniasis are known to occur (Schaefer, *et al.*, 1994). Recently, climatic and demographic factors have led to emergence of leishmaniasis in previously non-endemic areas. In March 2000, cases of visceral leishmaniasis among refugees from Somalia were identified in Dadaab refugee camp, Garissa district and at Mandera hospital. There were also increasing numbers of cases of visceral leishmaniasis in Wajir hospital up to August 2001 (Boussery *et al.*, 2001). After the outbreak, there were sporadic cases of visceral leishmaniasis due to migration of infected people seeking food and security (Marlet *et al.*, 2003). Another outbreak of kala-azar was reported in Merti, Isiolo district in 2006 where a study showed that in the absence of *P. martini, P. orientalis* was a probable vector for transmission of visceral leishmaniasis (Ngumbi, *et al.*, 2010).

1.5 *Leishmania* pathogenesis

Inside the mammalian host the *Leishmania* amastigote resides within the macrophage. To sustain a chronic infection, the parasite must survive the harsh environment of phagocytic cells. *Leishmania* pathogenesis is therefore a result of the host-parasite interactions which bring about host inflammatory and immune response to the infection (Brandonisio *et al.*, 2000).

Some mice species develop *Leishmania* diseases similar to those found in humans, and as such have provided useful models for studying *Leishmania* disease pathogenesis. Using murine models, characterization of the immunological pathways that are responsible for resistance or susceptibility to *Leishmania* has given rise to the T helper 1/ T helper 2 (Th1/ Th2) paradigm of cellular/ humoral dominance of immune response in which the outcome of disease is determined by the nature and magnitude of the T-cell and cytokine responses early in infection (Sacks & Noben-Trauth, 2002). Since the *Leishmania* species differ significantly, it follows that their interaction with the mammalian host also differs. However, using murine models of *L. major*, it is generally accepted that protective immunity against *Leishmania* is mediated by an IL-12 driven CD4+ Th1- type response resulting in IFN-γ production. The immunological pathways that lead to the development of non healing disease or susceptibility are less well defined (Sacks & Noben-Trauth, 2002). However, these include an IL-4 driven Th2 response that downregulates Th1 development (Leal *et al.*, 1993) and failure to produce or respond to IL-12 (Kropf *et al.*, 1997). IL-4 downregulates production of IL-12 and IFN-γ and inhibits macrophage nitric oxide (NO) production which is critical for leishmanicidal activity (Alexander *et al.*, 1999).

The production of prostaglandins (PGs) by host macrophages increases during infection by *Leishmania* parasites (Gregory *et al.*, 2008). Prostaglandins exacerbate the outcome of infection with *Leishmania* in mice by inhibiting activation induced cell death of Th2 cells (Kaul *et al.*, 2012) suggesting a role for PGs in the

pathogenesis of these organisms. Studies indicate that PGE_2 inhibits the Th1 response and promotes the Th2 response by suppressing the natural killer cell induced IFN-γ synthesis (Walker & Rotondo, 2004). Furthermore, PGE_2 can shift the balance of CD4+ helper T cells towards a Th2 type immune response (Kuroda *et al.*, 2000) thereby playing a role in the inability of animals to resolve infections. Thus, the inability of the host to develop an effective immune response involves PGs (Reiner & Malemud, 1985).

1.6 Prostaglandins

Prostaglandins (PGs) are autocrine and paracrine lipid mediators that modulate diverse physiological and pathophysiological responses in mammals. They are not stored but are synthesized from membrane released Arachidonic acid (AA). This AA is kept esterified in glycerophospholipids until mobilized by phospholipases. Arachidonic acid then undergoes a cyclooxygenase (COX) reaction, in which it is converted to the highly unstable prostaglandin G_2 (PGG_2), followed by a peroxidase reaction, in which PGG_2 is reduced to prostaglandin H_2 (PGH_2). This intermediate then serves as the substrate for terminal prostanoid synthase enzymes to produce PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (Smith *et al.*, 2000) (Figure 1.2).

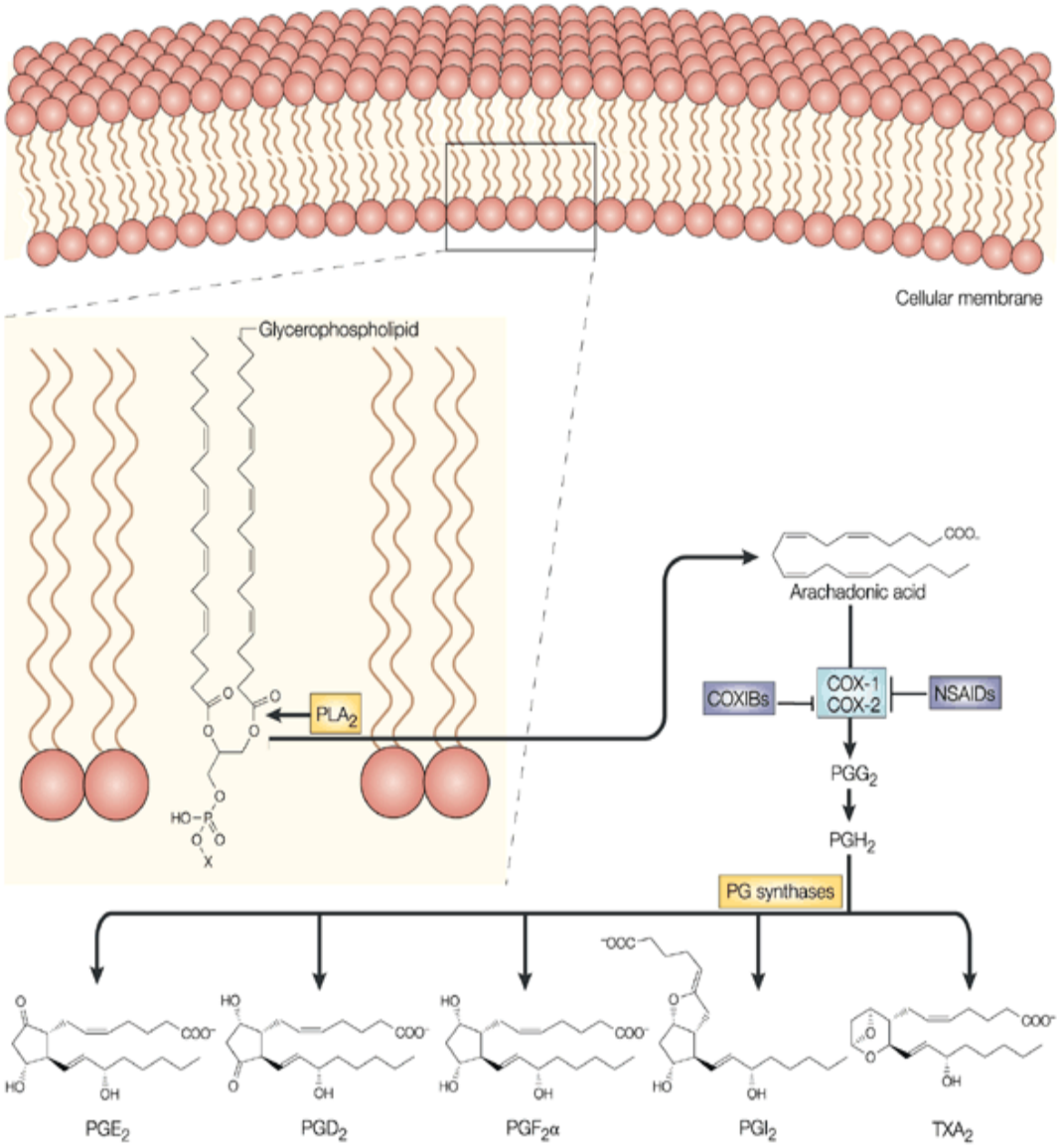

Figure 1.2: Biosynthetic pathway of prostaglandins (Gupta & Dubois, 2001)

Two isoforms of COX enzyme catalyze the formation of PGH_2 (Morita, 2002). Cyclooxygenase-1 is constitutively expressed in tissues, and its prostaglandin products are thought to mediate physiological responses such as vascular homeostasis. Cyclooxygenase-2 is often undetectable in resting cells, but is induced following extracellular stimuli by cytokines (Jones *et al.*, 1993), phorbol esters

(Kujubu *et al.*, 1993), and bacterial lipopolysaccharide (O'Sullivan *et al.*, 1992). It is expressed in various cells including monocytes (Fu *et al.*, 1990), neutrophils (Niiro *et al.*, 1997) and endothelial cells (Okahara *et al.*, 1998) at sites of inflammation (Seibert *et al.*, 1994) hence it is known as the inducible enzyme. Both forms of COX are inhibited by the classical non-steroidal anti-inflammatory drugs (NSAIDs). In humans, selective COX-2 inhibitors such as Celebrex® and Vioxx® maintain the anti-inflammatory properties of the classical nonselective NSAIDs, but without the gastrointestinal bleeding side effects (Cook, 2005).

Following their biosynthesis, prostaglandins are exported from cells across the membrane and bind to specific receptors on target cells. They can also be carried across membranes by PG transporters (Bao *et al.*, 2002). There are several forms of PG receptors known in man. Four receptor subtypes (EP_1-EP_4) bind PGE_2, two (DP_1 and DP_2) bind PGD_2 while $PGF_{2\alpha}$ and PGI_2 are bound by FP and IP receptors respectively (Tilley *et al.*, 2001). These prostanoid receptors are cell-membrane spanning G-protein coupled receptors that mediate the physiological actions of the principal prostanoid metabolites (Tilley, *et al.*, 2001).

In mammals, PGs are involved in diverse functions including bone resorption, blood vessel tone and immune responses (Funk, 2001). The PGs are recognized as the triggers of labour as elevated uterine PGs lead to contractions and labour and administration of FP antagonist delays pre term birth (Olson & Ammann, 2007). The formation of primary prostaglandins of the D, E and F series is mediated by different enzymes whose presence varies depending upon the cell type and tissue. Prostaglandin D synthases responsible for the generation of PGD_2 from arachidonate consist of at least 2 distinct enzymes. One form of the enzyme is a hematopoietic (spleen type) form belonging to the sigma GST (Glutathione S-transferase) class (Jowsey *et al.*, 2001). This enzyme is expressed in antigen presenting, langerhans and mast cells suggesting that it functions in the production of PGD_2 as an allergic and inflammatory mediator (Arima & Fukuda, 2008). A second form of PGD synthase (lipocalin type) is expressed in the brain, testes and human heart and its expression is a useful clinical marker for renal failure and atherosclerosis (Urade &

Hayaishi, 2000). In addition, it plays a pivotal role in the regulation of physiological sleep (Qu *et al.*, 2006).

Prostaglandin E synthases consist of both membrane-associated and cytosolic forms (Murakami *et al.*, 2002). Within the immune system, PGE_2 modulates the functions of cells such as T cells and macrophages which are critical to immune responses. Prostaglandin E_2 suppresses proliferation of T cells (Nataraj *et al.*, 2001) and upregulates the production of macrophage derived chemokines that recruit Th2 cells into inflammatory sites (Yamashita & Kuroda, 2002). In addition, PGE_2 influences the central nervous system in terms of temperature regulation, acting as an endogenous pyrogen (Dinarello, 2004).

Prostaglandin $F_{2\alpha}$ is synthesized by 3 pathways from PGE_2, PGD_2 or PGH_2 by the enzymes PGE 9-ketoreductase, PGD 11-ketoreductase, or PGH 9-, 11-endoperoxide reductase respectively. The enzymes which synthesize $PGF_{2\alpha}$ from PGH_2 and 9α-, 11β-$PGF_{2\alpha}$ from PGD_2 are referred to as PGF synthases (Watanabe, 2002) (Figure 1.2). Prostaglandin $F_{2\alpha}$ is produced by various tissues in mammals where it exerts a variety of biological activities such as luteolysis (Shirasuna *et al.*, 2012) and parturition (Xu *et al.*, 2013) as a constitutive part of ongoing reproductive processes of the body. Prostaglandin $F_{2\alpha}$ produced through COX-2 has been implicated in inflammation pathology as a metabolite responsible for neutrophil recruitment in the rat model of lipopolysacharide induced inflammation (de Menezes *et al.*, 2005).

Host cells are one source of PGs and mammalian host derived PGs have been studied extensively. However, accumulating evidence show that parasites can also produce PGs. It is also possible that parasite derived PGs are directly involved in the pathogenesis of parasitic diseases regulating the physiological processes in the host and enabling the parasite to persist in the body.

1.7 Problem statement

Leishmaniasis is a neglected tropical disease that affects the lowest socio-economic populations especially in developing countries (Alvar *et al.*, 2006). In addition, there is an increase in the prevalence and geographic distribution of Leishmaniasis to areas that were previously unaffected by leishmaniasis (Bashaye *et al.*, 2009, Razmjou *et al.*, 2009). These problems are compounded by the emergence of *Leishmania*/ HIV co-infection (Alvar *et al.*, 2008). Treatment by the first-line pentavalent antimonial drugs is often accompanied by side effects including cardiotoxicity, and hepatotoxicity (Esfandiarpour *et al.*, 2012; Oliveira *et al.*, 2009). In addition, the recommended dose of pentavalent antimonials for treatment of visceral leishmaniasis is 20 mg/ kg of body weight per day for 30 days by intramuscular or intravenous route (Freitas-Junior *et al.*, 2012) hence the patients have to be hospitalized for proper management. Emergence of antimonial resistant strains of *Leishmania donovani* in India (Stauch *et al.*, 2012) threatens to diminish the importance of this drug. Lipid formulations of Amphotericin B are used as the second line treatment. Due to targeted delivery to the cells and organs affected by leishmaniasis, tolerance and adverse effects are greatly reduced (Bern *et al.*, 2006). Although a preferential pricing agreement with WHO has reduced the price of liposomal amphotericin B for endemic regions to $20 (Ksh 1908) per 50 mg vial, when a total dose of 20 mg/kg is given, this is still expensive (Olliaro & Sundar, 2009). Considering these factors, Leishmaniasis presents a serious public health problem of global concern. Therefore, there is an urgent need to study the *Leishmania*/host interaction to acquire enabling knowledge for the discovery of hitherto unknown drug targets in the parasite's biochemical pathways for rational development of effective anti-*Leishmania* drugs.

1.8 Justification of the study

Prostaglandins are mediators of physiological and pathological responses, including pain, fever and immunosuppression which are observed in leishmaniasis. The kinetoplastid parasite *Trypanosoma brucei* has been shown to produce the

prostaglandins PGD_2, PGE_2 and $PGF_{2\alpha}$. Among all the PGs synthesized, $PGF_{2\alpha}$ was the major prostanoid produced by trypanosome lysates (Kubata *et al.*, 2000). The characterization of *Trypanosoma brucei* Prostaglandin F Synthase (*Tb*PGFS) and its gene provided a basis for studying related genes in other parasitic protozoa in order to get further insight into their role in parasitic protozoa, hence the analysis of $PGF_{2\alpha}$ synthase in *Leishmania* species. Furthermore, *Leishmania* parasites have been shown to produce prostaglandins (Kabututu *et al.*, 2003). However, the molecular mechanisms by which the production of prostaglandins is up regulated during these infections are poorly understood. Coupled to this is the need to identify new drug targets due to the increasing frequency of drug resistance in leishmaniasis and the enhanced risk of co-infection with HIV. Understanding the mechanisms of production of prostaglandins may lead to the identification of drug targets which may in turn prove useful in the design of novel strategies for treatment of leishmaniasis.

1.9 Objectives of the Study

1.9.1 General Objective

Molecular characterization of $PGF_{2\alpha}$ synthase-like protein from Old and New world species of *Leishmania*

1.9.2 Specific Objectives

1. To identify the *$PGF_{2\alpha}$* synthase gene in Old and New World Species of *Leishmania*
2. To characterize the *$PGF_{2\alpha}$* synthase gene in Old and New World Species of *Leishmania*
3. To identify the $PGF_{2\alpha}$ synthase protein in Old and New World Species of *Leishmania*
4. To characterize the $PGF_{2\alpha}$ synthase protein in Old and New World Species of *Leishmania*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Diagnosis of Leishmaniasis

Infections of visceral leishmaniasis are often diagnosed by visualizing the amastigote form of the parasite by microscopic examination of aspirates from lymph nodes, bone marrow or spleen (Chappuis *et al.*, 2007). To detect cutaneous leishmaniasis, the biopsy specimen or scraping smears are subjected to Giemsa staining and the amastigotes visualized using light microscopy (Goto & Lindoso, 2010). The clinical material may also be inoculated into a susceptible experimental animal to improve the yield before culturing (Singh, 2006).

A number of antibody detection methods have been developed to exploit the host-parasite interaction for the diagnosis of leishmaniasis. Enzyme linked immunosorbent assay (ELISA) is one of the most sensitive tests useful for laboratory analysis and to screen large number of samples at a rapid pace (Maalej *et al.*, 2003). The direct agglutination test (DAT) in which parasites are agglutinated by serum antibodies is popularly used as it is highly sensitive (Boelaert *et al.*, 2004). Testing with a commercially available immunochromatographic strip that uses a recombinant leishmanial antigen cloned from *L. donovani* has shown tremendous potential for the diagnosis of visceral leishmaniasis (Sivakumar *et al.*, 2006). However despite the availability of a large number of serological tests, no method is available for the detection of cutaneous and mucocutaneous leishmaniasis because of the low antibody titer (Singh, 2006). The use of PCR has been evaluated, especially on peripheral blood samples, and has been used successfully for diagnosis of visceral leishmaniasis (Fraga *et al.*, 2010).

2.2 *Leishmania* clinical features

Leishmania species are divided into the subgenera *L. viannia, L. leishmania* and *L. sauroleishmania.* Species that are recognized to cause disease in humans include *L. donovani, L. major, L. tropica* and *L. aethiopica* in the Old World, and *L. braziliensis* and *L. mexicana* in the New World (Handman, 2001). The various species cause a wide spectrum of diseases collectively known as leishmaniases that vary in their clinical manifestations and symptoms. These include: cutaneous, visceral, mucocutaneous, Post-Kala azar dermal and diffuse cutaneous leishmaniases.

Cutaneous leishmaniasis presents as sores or ulcers that develop at the site of the sandfly bite. There is a variable tendency for these lesions to self-cure and this spontaneous healing usually results in lifelong immunity from the disease which may or may not be restricted to the same *Leishmania* species (Reithinger *et al.*, 2007). Several *Leishmania* species can cause cutaneous leishmaniasis. These include *L. major* and *L. tropica* in the Old World and *L. mexicana* in the New World. In visceral leishmaniasis, the amastigotes reside within the cells of the viscera including the spleen, lymph nodes, liver and intestine. Patients infected with visceral leishmaniasis typically present with fever, cough, abdominal pain, diarrhoea, splenomegaly and hepatomegaly (Guerin *et al.*, 2002).

Mucocutaneous leishmaniasis is characterized by the ability of the parasite to mestasize to mucous tissues. The disease begins with nasal inflammation followed by ulceration of the nasal mucosa. In some cases the lips, cheeks, soft palate, pharynx or larynx are also affected. Mucosal leishmaniasis never heals spontaneously, and is very difficult to treat. Secondary bacterial infections are common and this disease is potentially fatal (David & Craft, 2009). Post-Kala azar dermal leishmaniasis (PKDL) is caused by *L. donovani* following cure of the initial visceral leishmaniasis. PKDL usually appears within 2 years of complete cure of the visceral infection, and commences with the appearance of mottling of the skin (Zijlstra *et al.*, 2003). Diffuse cutaneous leishmaniasis (DCL) is usually a

manifestation of infection with parasites which cause simple cutaneous leishmaniasis asscociated with an immunological lack of response. The lesions may be restricted, or may be widespread all over the body appearing as raised patches which are painless, but grossly disfiguring. DCL is difficult to treat and patients do not heal spontaneously (Ashford, 2000).

*Leishmania/*HIV co-infection has emerged as a major complication of leishmaniasis. Visceral leishmaniasis is the clinical form of leishmaniasis most frequently associated with HIV/AIDS. While the incidence of *Leishmania*/HIV co-infection has increased in Eastern Africa and the Indian sub-continent (Mathur *et al.*, 2006), the greatest prevalence of co-infection has been in the Mediterranean Basin with most cases notified to the WHO coming from Spain, Italy, France and Portugal (Cruz *et al.*, 2006).

2.3 Treatment of Leishmaniasis

Pentavalent antimonials (Sodium stibogluconate (Pentostam ®) and meglumine antimoniate (Aminosidine) have long been the mainstay of therapy for leishmaniasis (Murray *et al.*, 2005). However, pentavalent antimonial therapy is often accompanied by adverse effects including pain, hepatotoxicity and cardiotoxicity (Esfandiarpour, *et al.*, 2012; Oliveira, *et al.*, 2009). Combined antimony therapy and immunostimulation of macrophages has been found to be more effective than the drug or immune stimulation alone in clearing *L. donovani* amastigotes in vitro (Haidaris & Bonventre, 1983). This could serve to reduce the dosage of antimonial drug required to cure Leishmaniasis (Haidaris & Bonventre, 1983). However, there is increasing concern over the emergence of drug resistance in treatment of visceral leishmaniasis endemic areas (Stauch, *et al.*, 2012).

Amphotericin B is the second line treatment for visceral leishmaniasis. However it is acutely toxic hence the use of liposome encapsulated Amphotericin B (Ambisome®) which is highly effective against visceral leishmaniasis and better tolerated than the

conventional preparation. Unfortunately, its use in the treatment of visceral leishmaniasis in endemic regions is limited by high cost (Olliaro & Sundar, 2009). The usefulness of Pentamidine as an antileishmanial drug has been limited by its toxicity, although since its introduction it has had value as a second-line drug for visceral, cutaneous and diffuse cutaneous leishmaniasisis (Croft & Coombs, 2003).

Miltefosine, initially developed as an anticancer drug, is the first recognized oral treatment for leishmaniasis and is now being used for the full range of clinical leishmaniasis (Berman, 2008). The 8-aminoquinoline derivative sitamaquine, is an orally active drug that has undergone phase II clinical trials for the treatment of visceral leishmaniasis in Kenya (Wasunna *et al.*, 2005) and India (Jha *et al.*, 2005). This drug showed high efficacy rate at doses of 1.5 - 3 mg/kg/day for 28 days by oral route. However in populations where glucose-6-phosphate dehydrogenase deficiency which affects red blood cells is prevalent, methemoglobinemia and hemolysis occurred. For the 8-aminoquinoline to be developed against *Leishmania*, low levels of hemolytic toxicity at higher doses should be demonstrated (Richard & Werbovetz, 2010). Paromomycin, an aminoglycoside antibiotic, was originally identified as an antileishmanial in the 1960s. This drug was registered for the treatment of visceral leishmaniasis in India in 2006. Although it is given parenterally (by injection or infusion into the body), low cost and high efficacy have made paramomycin a useful weapon against visceral leishmaniasis (Sundar *et al.*, 2009).

2.4 Vaccine Development

The current control of leishmaniasis is based on therapeutic treatments which are toxic (Oliveira, *et al.*, 2009), expensive (Olliaro & Sundar, 2009) and associated with resistance (Stauch, *et al.*, 2012) therefore the development of a safe and effective vaccine against leishmaniasis remains a priority. (Tonui *et al.*, 2001) demonstrated that it is possible to limit the transmission of *L. major* by immunizing susceptible BALB/c mice with *L. major* derived antigens namely recombinant 63 kDa glycoprotein (rgp63), lipophosphoglycan (LPG), a cocktail of rgp63 and LPG or

whole parasite antigen. Parasite development in sandflies which had fed on immunized and infected mice was inhibited. Furthermore the epithelium of the midgut of sandflies which had fed on immunized bloodmeals showed degeneration and lysis (Tonui, *et al.*, 2001).

Although there have been significant advances in vaccine development against *Leishmania* parasites, to date there is no vaccine against *Leishmania* in routine use anywhere in the world. A wide range of protein formulations such as recombinant proteins, DNA vaccines and recombinant virus have been tested as vaccine candidates against several *Leishmania* species with results ranging from limited to no immunogenicity (Dumonteil, 2009). There is also a renewed interest in killed as well as live attenuated *Leishmania* vaccines which may have greater immunogenicity due to the large number of antigens they contain (Giunchetti *et al.*, 2008). The first defined *Leishmania* vaccine to enter clinical trials was the LEISH-F1 + MPL-SE comprising the *L. major* homologue of eukaryotic thiol-specific antioxidant, the *L. major* stress-inducible protein-1 and the *L. braziliensis* elongation and initiation factor (Velez *et al.*, 2009). This vaccine was found to be safe and immunogenic in human volunteers.

2.5 Vector and Reservoir control

Indoor residual spraying of houses with insecticide is the most widely used intervention for controlling sand flies that are endophillic (resting indoors) as it is a simple and cost-effective method (Davies *et al.*, 2003). Dichlorodiphenyltrichloroethane (DDT) still remains the insecticide of choice because of its low cost, high efficacy, long residual action and relative safety when used for indoor residual spraying (Sharma & Singh, 2008). Lambda-cyhalothrin, a pyrethroid insecticide has also been used in indoor residual spraying. In a trial done in Venezuela, indoor spraying led to a significant reduction in the sandfly population (Feliciangeli *et al.*, 2003).

Where sand flies are endophagic (feed indoors) and most active when people are asleep, bed nets provide considerable protection. This has been demonstrated in studies where people using untreated nets were 70% less likely to develop visceral leishmaniasis than people without nets. Treatment of the nets with pyrethroids further enhances the protection as sandflies are still very sensitive to these insecticides (Bern *et al.*, 2000).

Animal reservoir eradication as a control for visceral leishmaniasis is based on culling seropositive dogs. In a study done in Brazil, dog culling as part of the national control programme did not prevent a rise in the number of human or canine cases (Costa & Vieira, 2001) and is therefore not an effective method of control. On the other hand, experimental trials have shown that deltamethrin treated collars reduce the risk of dogs being infected by *L. infantum* as children living in the treated villages had significantly less risk of infection (Gavgani *et al.*, 2002). Permethrin, a synthetic pyrethroid used to treat dogs during sand flies season, significantly reduced the risk of *L. infantum* infection (Ferroglio *et al.*, 2008). The efficiency of *Bacillus sphaericus* against *P. martini* as a biological control has been demonstrated as treatment caused larval mortality, and reduced fecundity of the females emerged from the surviving pupae (Wahba, 2000).

Due to their immunomodulatory role, prostaglandins may be useful in development of antileishmanial interventions. However, little is known about the role of prostaglandins in the *Leishmania* parasite. Much of the information on prostaglandins has been obtained from studying mammalian prostaglandins but there are increasing reports of prostaglandins and prostaglandin-like molecules being produced by parasites (Angeli *et al.*, 2001, Ramaswamy *et al.*, 2000, Sommer *et al.*, 2003) Reviewing the production of prostaglandins by these parasites may give insight into the role of prostaglandins in *Leishmania.*

2.6 Prostaglandins and parasites

2.6.1 Production of prostaglandins by helminthes

Helminth infections are associated with elevated host prostaglandin production which modulates host responses and causes disease symptoms (Noverr *et al.*, 2003). The trematode *Schistosoma mansoni* has an active arachidonate cascade and uses the host's skin essential fatty acids to produce eicosanoids. Successful cercarial penetration of the skin and transformation of cercariae to schistosomulae are dependent on both skin essential fatty acid levels and resulting cercarial eicosanoid production (Fusco *et al.*, 1986). Among the various life cycle stages, the tissue migratory stages of the parasite appear to produce the most abundant PGE_2 which modulates cytokine production in the host, thereby favoring parasite establishment (Ramaswamy, *et al.*, 2000). Interestingly, the mammalian COX-2 inhibitors indomethacin, aspirin and nimesulide do not inhibit PGE_2 production by cercariae or schistosomula hence *S. mansoni* may possess a biochemical pathway that is different from the mammalian counterpart for the production of PGE_2 (Ramaswamy, *et al.*, 2000)

A 28-kDa glutathione-S-transferase has been identified as the enzyme responsible for production of PGD_2 by *Schistosoma mansoni* (Herve *et al.*, 2003). Skin infection of mice with *S. mansoni* activates and retains Langerhans cells in the epidermis (Herve *et al*, 2003). Parasite derived PGD_2 inhibits the TNF-α triggered migration of the epidermal Langerhans cells hence presenting a strategy for the parasite to modulate the host immune system (Angeli, *et al.*, 2001). A PGD_2 receptor (DP) deficiency or a DP antagonist restored Langerhans cells migration in infected mice (Angeli, *et al.*, 2001, Herve, *et al.*, 2003).

Microfilariae of the nematodes *Brugia malayi* and *Wuchereria bancrofti* synthesize and release PGs, primarily PGE_2 and prostacyclin from either endogenous or exogenous stores of arachidonic acid. The formation of these prostaglandins is enzymatic and can be blocked by inhibitors of mammalian COX. Therefore they possess a key eicosanoid-forming enzyme that is functionally similar to mammalian

COX (Liu *et al.*, 1990). These PGs inhibit platelet aggregation and vasoconstriction, thereby permitting microfilariae to pass through capillary vessels, enabling the parasite to sustain its life cycle in the host (Liu & Weller, 1992).

Prostaglandin E_2 has been detected in various stages of the tissue-dwelling filarial parasite *Onchocerca volvulus*. A release of PGE_2 in the tissue where *O. volvulus* reside, (the onchocercomas or the skin) leads to a possible influence on immune cells like macrophages, T cells and mast cells (Brattig *et al.*, 2006). The progression of chronic inflammation depends on angiogenesis which is in turn promoted by the increasing levels of PGE_2 and mast cells at sites of inflammation (Ghosh, 2003). Filarial PGE_2 may therefore promote the formation of onchocercoma. Additionally, *O. volvulus* possess a unique glutathione dependent PGD synthase (OvGST1) shown to metabolize AA to PGD_2 at the parasite-host interface. The enzyme participates in the modulation of immune responses by contributing to the production of parasite-derived prostanoids and restraining the host's effector responses (Sommer, *et al.*, 2003).

A soluble Glutathione-S-Transferase (GSH) of the parasitic nematode *Ascaridia galli* has been purified and characterized. This enzyme shows specific activity in the GSH-dependent isomerization of PGH to PGE. However, whether the parasite actually produces PGE_2 is yet to be determined (Meyer *et al.*, 1996). Arachidonic acid metabolism has also been demonstrated in the tissue larval stage of *Taenia taeniaformis,* a cestode parasite of rodents and cats. This parasite was found to generate Thromboxane, PGI_2 and PGE_2 from arachidonic acid which may play a role in modulation of the host immune response (Leid & McConnell, 1983).

2.6.2 Production of prostaglandins by protozoa

The protozoan parasite, *Entamoeba histolytica* produces PGE_2 through a COX-like enzyme in the presence of exogenous AA (Dey *et al.*, 2003). This enzyme is distinct from other known enzymes as multiple alignment of this protein and DNA sequence showed little homology with COX sequences from mammals. Furthermore, the

enzyme does not synthesize $PGF_{2\alpha}$ or PGD_2 from arachidonate and its activity is only inhibited by a high concentration of aspirin and not by indomethacin or nimesulide (Dey, *et al.*, 2003). Further research has identified parasite derived PGE_2 as one of the major virulence factors produced by *E. histolytica* that stimulates production of chemokines and interleukins by neutrophils thereby triggering an acute host inflammatory response. This may play a major role in the acute inflammation associated with amebiasis (Dey & Chadee, 2008).

Plasmodium falciparum produces the prostaglandins PGD_2, PGE_2 and $PGF_{2\alpha}$ in a stage-specific manner since these metabolites are more actively produced at the trophozoite and schizont stages as compared to the ring stage (Kilunga Kubata *et al.*, 1998). This may contribute to malaria disease as malaria fever coincides with the rupture of schizonts. The PGs play a role in modulating the host defence mechanisms due to the immunosuppressive properties of PGE_2 thereby allowing the survival of the parasite in the host. Investigations have also shown that PG production in these parasites is not affected by the mammalian COX inhibitors aspirin and indomethacin hence the arachidonate cascade in *P. falciparum* is distinct from the mammalian cascade (Kilunga Kubata, *et al.*, 1998).

Trypanosomes secrete prostaglandins including the somnogenic PGD_2 which can lead to sleep alterations which is the most prominent manifestation in human African trypanosomiasis (Kristensson *et al.*, 2010)**.** The kinetoplastid *Trypanosoma brucei* produces PGD_2, PGE_2 and $PGF_{2\alpha}$ from endogenous or exogenous stores of AA. Prostaglandin production in these parasites is not inhibited by aspirin and indomethacin (Kubata, *et al.*, 2000). Prostaglandin $F_{2\alpha}$ is mainly produced in the fast dividing slender bloodstream form and the procyclic insect form of the trypanosome and is scarcely secreted into the media under *in vitro* culture conditions, while PGD_2 is mainly produced by the non dividing stumpy bloodstream form and is primarily secreted (Kubata, *et al.*, 2000). Since $PGF_{2\alpha}$ was the major prostanoid synthesized by the parasite, it was purified and characterized. Investigations revealed that the *T. brucei* prostaglandin F synthase (*Tb*PGFS) gene is translated into an enzyme belonging to the aldo-keto reductase superfamily (Kubata, *et al.*, 2000).

More recent reports indicate a PGD_2 induced inhibitory effect on growing trypanosomes, through apoptosis. This has been proposed as a mechanism to control cell density in infected animals (Figarella *et al.*, 2005). As PGD_2 undergoes dehydration *in vivo* and *in vitro* to form biologically active PGs of the J series (Shibata *et al.*, 2002), investigations have shown that these downstream metabolites serve to amplify the PGD_2-induced programmed cell death in trypanosomes through production of reactive oxygen species (Figarella *et al.*, 2006).

The production of PGs has also been investigated in *Trypanosoma cruzi*. This parasite produces significant amounts of PGD_2, PGE_2 and $PGF_{2\alpha}$ using endogenous or exogenous sources of AA through a pathway which is not inhibited by mammalian COX inhibitors (Kubata *et al.,* 2002). Further work identified the prostaglandin F synthase gene in *T. cruzi* which is homologous to the *Tb*PGFS gene. This gene was cloned but failed to express any protein even though *T. cruzi* parasites synthesize $PGF_{2\alpha}$. Failure to express the protein led to the discovery that unlike *T. brucei*, *T. cruzi* utilizes *T. cruzi* old yellow enzyme (*Tc*OYE), a flavin-dependent oxidoreductase, to synthesize $PGF_{2\alpha}$ (Kubata, *et al.*, 2002).

Previous investigations have revealed that *Leishmania* possess the enzymatic machinery to synthesize PGs. *Leishmania* parasites metabolize AA to PGs (Kabututu, *et al.*, 2003). Prostaglandin production in these parasites is not inhibited by aspirin or indomethacin. These classical non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit mammalian cyclooxygenase activity thereby preventing the formation of prostanoids (Wilson *et al.*, 2004). Thus, the enzyme pathway for the production of prostaglandins in *Leishmania* is distinct from the mammalian counterpart and may be explored as a drug target for the treatment of *Leishmaniasis*.

A database search identified a *Tb*PGFS homolog in *Leishmania*, the P100/11E gene; a developmentally regulated gene which is expressed at higher abundance in promastigotes relative to amastigotes (Kidane *et al.,* 1989). The product of this gene had previously been identified as a member of the aldo-keto reductase superfamily

(Samaras & Spithill, 1989). Complementary DNA of *L. major*, *L. donovani* and *L. tropica* were cloned, sequenced and over expressed in bacteria. The 34-kDa cytosolic recombinant proteins were shown to catalyze the reduction of PGH_2 to $PGF_{2\alpha}$ and were identified as $PGF_{2\alpha}$ synthases (Kabututu*, et al.*, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 *Leishmania* parasites

Isolates of *L. major* (ATCC 50122), *L. donovani* (ATCC 50127), *L. tropica* (ATCC 30012), *L. amazonensis* (ATCC 50131), *L. braziliensis* (ATCC 50135), *L. mexicana* (ATCC 50157) and *L. chagasi* (ATCC 50133) promastigotes were obtained from the cryobank of American Type Culture Collection (Manassas, VA. USA). These were grown in Schneider's Insect Medium (Sigma-Aldrich, St. Louis, USA) containing 20% heat inactivated fetal bovine serum (Hyclone Laboratories, Logan, USA), supplemented with 100 U/ ml penicillin, 10mg/ml streptomycin sulfate and 25µg/ml amphotericin B (Sigma-Aldrich, St. Louis, USA) at 25ºC. Promastigotes were sub cultured to increase culture volume and their growth monitored daily.

The promastigotes were harvested by centrifugation at 1200 x g for 10 minutes, at 4ºC and the media discarded to obtain the cell pellet. To determine the total number of cells harvested per *Leishmania* species, an aliquot was taken from the harvest volume and placed on a glass slide. The cells were counted using a Neubauer hemocytometer under a light microscope at 40X magnification.

3.2 Extraction of total RNA

Total RNA was extracted from the cell pellets (10^6 cells) of each of the *Leishmania* species in section 3.1 using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Briefly, the cell pellets were resuspended in cold phosphate buffered saline (PBS) and then centrifuged at 300 x g for 5 minutes, at 4°C. One milliliter of Trizol reagent was added to each of the tubes and the cells lysed by repetitive pipetting. The suspensions were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. To

carry out phase separation, 0.2 ml of chloroform was added to each sample suspension and the tubes covered. The samples were vortexed for 15 seconds and then incubated at room temperature for 3 minutes. They were then centrifuged at 12 000 x g for 15 minutes at 4°C. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase, an interphase and a colourless upper aqueous phase containing the RNA. The RNA was carefully transferred by pipetting into a fresh tube.

To precipitate the RNA, 0.5 ml of isopropyl alcohol was added to each sample followed by incubation at 30°C for 10 minutes. The samples were then centrifuged at 12 000 x g for 10 minutes at 4°C to form a gel-like pellet of RNA. The supernatant was removed and the RNA pellets washed twice by adding 1 ml of 75% ethanol per sample, vortex mixing and centrifugation at 7 500 x g for 5 minutes at 4°C. All the ethanol was removed from the samples and the pellets air dried for 10 minutes. The RNA pellets were resuspended in 30µl of RNase free water followed by incubation at 60°C for 10 minutes to aid solubilization of the RNA pellet.

3.3 Quantification of RNA

The extracted RNA from each of the *Leishmania* species was quantified using the Gene Quant spectrophotometer (Amersham Biosciences, NJ, USA). In ultraviolet (UV) spectrophotometry, the amount of UV radiation absorbed by a solution of RNA is directly proportional to the amount of RNA in the sample. At 260nm, an absorbance of 1.0 corresponds to 40 µg of RNA per ml. One microliter of RNA from each of the *Leishmania* species was diluted with 39µl of RNAse free water (1:40 dilution). Appropriate volumes of these were applied to a cuvette and the absorbance of each sample read at 260nm and 280nm to determine sample concentration and purity.

3.4 cDNA synthesis and Polymerase chain reaction (PCR) amplification of *Leishmania* PGFS

First strand cDNA synthesis was performed using 1µg of RNA from each species as template, 100 units of Superscript II reverse transcriptase and 0.5 µg Oligo dT primers (Invitrogen, Carlsbad, USA) in a total reaction volume of 20µl. The reaction mixture was incubated at 42°C for 50 minutes followed by inactivation at 70°C for 15 minutes. Polymerase chain reaction was carried out using the first strand cDNA from each of the *Leishmania* species as template. The reaction mixture contained 1µl of cDNA from each *Leishmania* species as template, Ex Taq buffer (Takara Bio Inc. Otsu, Shiga, Japan), 2.5mM dNTP mixture, 0.5µM sense and antisense primers, 1.5U Ex Taq polymerase (Takara Bio Inc. Otsu, Shiga, Japan) and distilled water to give a total reaction volume of 25µl per species sample. To amplify the $PGF_{2\alpha}$ synthase gene in the seven *Leishmania* species, *L. major* Prostaglandin F Synthase (*LmPGFS*) gene specific primers (Kabututu, *et al.*, 2003) were used

Table 3.1: Prostaglandin $F_{2\alpha}$ Synthase primer sequences

Gene	Sequence (5' to 3')	Direction
Prostaglandin $F_{2\alpha}$ synthase	CGCGGATCCATGGCTGGCGTTGATAAG	Sense
Prostaglandin $F_{2\alpha}$ synthase	CGCGGATCCTTAGAACTGCGCCTCATC	Antisense

Cycling parameters included an initial denaturation at 95ºC for 1 minute, followed by 35 cycles each of denaturation at 94ºC for 1 minute, annealing at 58ºC for 30 seconds and extension at 72ºC for 1 minute. These were followed by a final extension of 72ºC for 10 minutes (Kabututu *et al*., 2003).

Ten microlitres of each PCR product was electrophoresed at 100V in 1X Tris/ Borate/ EDTA buffer (TBE: 0.89M Tris base,0.89M Boric acid and 0.02M

Na_2EDTA) on a 1% agarose gel (Takara Bio Inc. Otsu, Shiga, Japan) stained with 0.5µg/ml ethidium bromide (Sigma-Aldrich, St. Louis, USA). A 1-Kb ladder (Promega, Madison, WI, USA) was used as the molecular weight marker. The gel was visualized under UV light in an AlphaImager gel imaging system (Alpha Innotech corp, San Leandro, USA).

3.5 Purification of DNA from gel bands

Following PCR and gel electrophoresis, the gel bands from the gene of interest were cut from the gel under UV light using a scalpel and then purified using GFX PCR DNA and Gel Band purification Kit (Amersham Biosciences, NJ, USA) according to the manufacturer's instructions. Briefly, capture buffer was added to each gel slice in a 15ml centrifuge tube and vortexed vigorously. The sample was incubated at 60 ºC in a water bath until the agarose completely dissolved in the buffer.

The samples were applied to the GFX columns (Amersham Biosciences, NJ, USA) placed in collection tubes and incubated at room temperature for 1 minute then centrifuged at 16 873 x g for 30 seconds. After discarding the flow through, 500µl of wash buffer was applied to the columns which were again centrifuged at 16 873 x g for 30 seconds. The collection tubes were discarded and the columns transferred to clean 1.5ml microcentrifuge tubes.

Fifty microliters of elution buffer (10mM Tris-HCl, pH 8.0) was applied directly to the top of the matrix in each GFX column (Amersham Biosciences, NJ, USA). The samples were incubated for 1 minute at room temperature and then centrifuged at 16 873 x g for 1 minute to recover the purified DNA. A second elution was performed using 50µl of elution buffer in a fresh tube to recover the entire DNA which was then stored at -20°C until use.

3.6 Sequencing of purified DNA fragment

The nucleotide sequences were determined by using the Sanger dideoxy chain termination chemistry (BigDye Terminator) on the Applied Biosystems 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was done at the Department of Molecular and Behavioral Biology, Osaka Bioscience Institute. Osaka, Japan.

3.7 Lysis of promastigotes to obtain total proteins

Leishmania promastigote cell pellets (10^{10} cells) were ruptured as described by Kabututu *et al*., (2003) using hypotonic lysis with double distilled water containing Complete™ (Roche Applied Science, IN, USA), a cocktail of reversible and irreversible protease inhibitors. Since the surrounding fluid environment had a lower salt concentration than the cell interior, there was movement of water into the cells by osmosis through the cell membrane. This led to cytolysis since the cell membrane could not withstand the osmotic pressure of the water inside. The soluble proteins were then obtained in the supernatant after centrifugation at 3000 x g for 30 minutes at 4º C.

3.8 Determination of protein concentration

The concentration of protein in the cell lysates was determined using bicinchoninic acid (BCA) reagent (Pierce Chemical Co. IL, USA) with Bovine serum albumin (BSA) as a standard according to the manufacturer's protocol. Briefly, 50 µl of each standard and unknown sample replicate were pippeted into appropriately labelled tubes. One milliliter of the working reagent (BCA Reagent A containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide mixed with BCA Reagent B, containing 4% cupric sulfate) was

added to each tube and mixed well. The tubes were covered and incubated at 37ºC in a water bath for 30 minutes. The tubes were then left to cool to room temperature.

The Gene Quant spectrophotometer (Amersham Biosciences, NJ, USA) was set to 562nm and the instrument zeroed on a cuvette containing water only. The absorbance of all the samples was then measured. The average absorbance measurements of the blank standard replicates were subtracted from the 562nm absorbance measurement of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected 562nm measurement for each BSA standard versus it's concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

3.9 Western Blot analysis of PGFS

This was done as previously described (Kabututu, *et al.*, 2003). To determine the presence or absence of PGFS, 25µg of *Leishmania* promastigote lysates from each of the 7 species were resolved on 13% (wt/vol) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. One gel was assessed by staining with Coomassie Brilliant Blue (Daiichi Pure Chemicals, Tokyo, Japan). Broad range prestained protein marker (New England Biolabs, Hertfordshire, UK) was used as the molecular weight standard. Proteins on the second gel were transferred to an Immun-Blot polyvinylidene difluoride (PVDF) membrane (BIO-RAD Laboratories, Hercules, CA, USA) at 200mA for 1 hour before blocking with 5% non fat dried milk (Kenya Highland brand) in Tris Buffered Saline containing 0.05% (vol/vol) Tween-20 (TBST) at 4° C overnight. The membrane was then incubated at 4º C with rabbit anti-*T. brucei* $PGF_{2\alpha}$ synthase polyclonal antibody (obtained from Department of Molecular and Behavioral Biology, Osaka Bioscience Institute. Osaka, Japan) applied in 5% non fat dried milk (Kenya Highland brand) and TBST. *Trypanosoma brucei* $PGF_{2\alpha}$ synthase shares 61% amino acid identity with *Lm*PGFS. Therefore, in this study, *Tb*PGFS was used to generate rabbit polyclonal antibodies for screening for *Leishmania* PGFS.

Following this, the membrane was washed thrice in TBST, and then incubated in horseradish peroxidase conjugated donkey anti-rabbit IgG (Amersham Biosciences, NJ, USA) at a concentration of 10µg/ml for 1 hour at 4º C. The membrane was again washed thrice with TBST at 4º C, and then developed with luminol using reagents for enhanced chemiluminescence (ECL) Western blotting detection (Amersham Biosciences, NJ, USA) according to the manufacturer's instructions. Briefly, 1 ml of detection solution 1 was mixed with an equal volume of detection solution 2. After draining off the excess wash buffer from the membrane, it was placed protein side up on a clean plastic container. The mixed detection reagent was pipetted directly onto the membrane. The membrane was incubated for 1 minute at room temperature on a rocking platform and then sealed in a Ziploc ® bag while taking care to remove all the air bubbles.

In the dark room, the wrapped blot was placed in an x-ray film cassette. A sheet of chemiluminiscent film was placed on top of the blot and the cassette closed and exposed for 25 minutes. The film was then developed using Developer (hydroquinone1-5%, potassium carbonate1-5%, potassium hydroxide1-5%, sodium sulfite1-5% and water) and Fixer replenisher (Ammonium thiosulfate 1-5%, Sodium sulfite 1-5%, sodium acetate 1-5% and water) reagents from Chimifoto Ornano (Gorgonzola MI – Italy).

3.10 Bioinformatic Analyses

3.10.1 Database Similarity Searches

Following sequencing, the resulting nucleotide sequence data was used to characterize the encoded genes. Search for similar genes to the one encoded by the sequence was performed on the Genbank database at the National Center for Biotechnology Institute (NCBI) using the Basic Local Alignment Search Tool (BLAST; (Altschul *et al.*, 1990) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.10.2 Nucleotide sequence alignment

To further characterize the gene fragment, the nucleotide sequence was aligned against sequences which had shown the highest similarity (highest score bits) following the BLAST search. The sequence alignment was carried out using the Multiple Sequence Comparison by Log Expectation (MUSCLE) software version 3.6 (Edgar, 2004).

3.10.3 Search for Open reading frames

In order to locate any contiguous nucleotides in the gene fragment encoding proteins in the unknown sequence, a search for open reading frames in the gene fragment was performed using the Discovery Studio Gene™ (DS Gene; Accelrys Inc.).

3.10.4 Eukaryotic Linear Motif (ELM) analysis of the encoded protein(s)

To further characterize the gene fragments in the identified open reading frames, their nucleotide sequences were translated into a polypeptide sequences using DS Gene followed by protein-protein BLAST of the resulting sequences. The amino acid sequences with the highest similarity to the query sequence were then searched against the ELM resource (http://elm.eu.org/) for prediction of functional sites in the polypeptide fragments.

CHAPTER FOUR

4.0 RESULTS

4.1 Culture of *Leishmania* promastigotes in Complete Schneider's medium

Promastigotes of all the seven species adapted well to the Schneider's complete medium. *Leishmania major* and *L. donovani* showed the fastest growth rates while *L. braziliensis* promastigotes showed the slowest growth rates *in vitro*. Rosette formation was observed during the culture of *L. braziliensis* whereby the promastigotes were seen to be growing in clusters. This pattern was not observed in the other species (Figure 4.1).

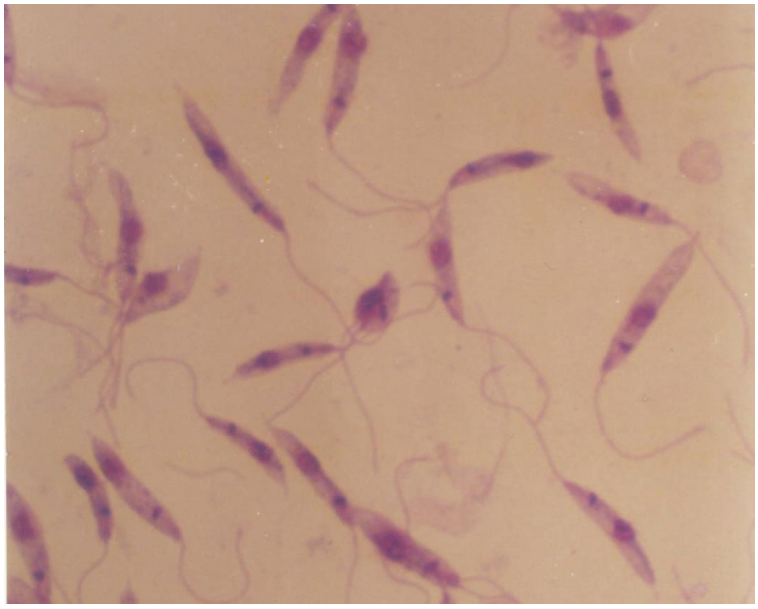

Figure 4.1: Light micrograph of *L. donovani* promastigotes.

100X under oil immersion lens

4.2 Total number of *Leishmania* cells harvested

The number of *Leishmania* promastigote cells harvested ranged between 2.55×10^6 - 5.15×10^6 cells (Table 4.1). The highest values were recorded in *L. chagasi* while the lowest concentration of cells was in *L. braziliensis.*

Table 4.1: Summary of number of cells harvested from promastigote cultures of *Leishmania* cells.

Species name	**Number of cells**
L. major	3.05×10^6
L. donovani	3.56×10^6
L. tropica	4.14×10^6
L. amazonensis	4.24×10^6
L. braziliensis	2.55×10^6
L. mexicana	2.79×10^6
L. chagasi	5.15×10^6

4.3 Concentration and purity of *Leishmania* promastigote RNA

The concentration of RNA ranged between 0.88 µg/ µl – 2.01 µg/ µl (Table 4.2). *Leishmania amazonensis* promastigotes had the highest concentration of RNA while *L. tropica* promastigotes had the lowest concentration. The purity of the specimens measured within the ideal A_{260}/A_{280} ratio for RNA of between 1.8 and 2.1 (Table 4.2) indicating absence of protein contaminants. *Leishmania major* RNA had a A_{260}/A_{280} ratio of 1.79 indicating a moderate degree of protein contamination.

Table 4.2: Concentration and purity of RNA in the *Leishmania* promastigote cells

Species name	RNA concentration (ng/µl)	A 260	A 280	260/280	dilution factor	RNA concentration (µg/µl)
L. major	135.0	6.123	3.412	1.79	10	1.35
L. donovani	160.0	7.241	3.774	1.92	10	1.60
L. tropica	87.5	6.245	3.242	1.93	10	0.88
L. amazonensis	200.5	6.805	3.684	1.85	10	2.01
L. braziliensis	92.5	5.742	2.74	2.10	10	0.93
L. mexicana	185.0	5.043	2.68	1.88	10	1.85
L. chagasi	172.5	7.142	3.552	2.01	10	1.73

4.4 Transcription of $PGF_{2\alpha}$ synthase – like gene in Old and New World species of *Leishmania*

A prominent 855-bp fragment corresponding to the $PGF_{2\alpha}$ synthase open reading frame was amplified and detected from the cDNA of the Old World species *L. major*, *L. donovani* and *L. tropica* (Figure 4.2, lanes 1, 2 & 3). However, a less prominent band of the same size was detected using the cDNA of a single New World species *L. chagasi* as the template (Figure 4.2, lane 7). There was no amplification for the other two New World species *L. amazonensis* and *L. mexicana* and (Fig. 4.2, lanes 4 and 6). Surprisingly, a prominent 2kb fragment was amplified when cDNA from *L. braziliensis* was used as the template (Figure 4.2, lane 5).

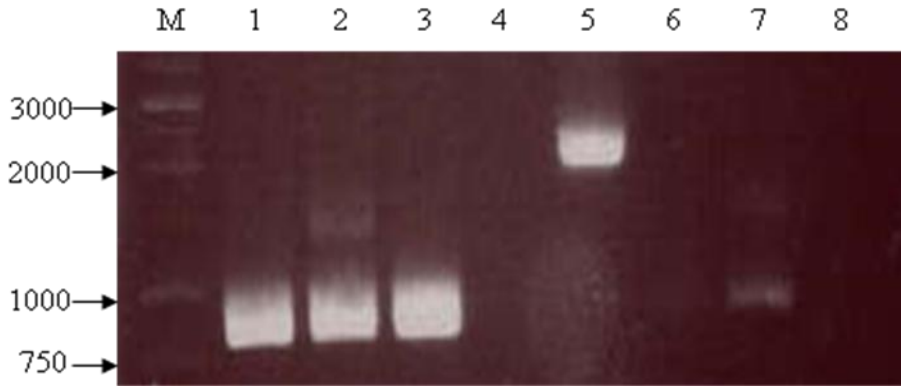

Figure 4.2: RT-PCR analysis of $PGF_{2\alpha}$ synthase gene expression from *Leishmania* species. Lane 1 = *L. major*; lane 2 = *L. donovani*; lane 3 = *L. tropica*; lane 4 = *L. amazonensis*; lane 5 = *L. braziliensis*; lane 6 = *L. mexicana*; lane 7 = *L. chagasi*; lane 8 = negative control without cDNA**.**

4.5 Large scale amplification of *Leishmania braziliensis* gene fragment

A PCR amplicon was not expected in the New World species of *L. braziliensis*, prompting the need for further characterization of the 2kb amplicon. In order to purify the *L. braziliensis* amplicon shown in Figure 4.2, a PCR reaction containing *L. braziliensis* cDNA as template in a total volume of 200µl was carried out and the entire PCR product run on agarose gel. 25µl of RT-PCR product was loaded per lane. This yielded one prominent band at 2000 bp and another weaker band at 4000 bp (Figure 4.3).

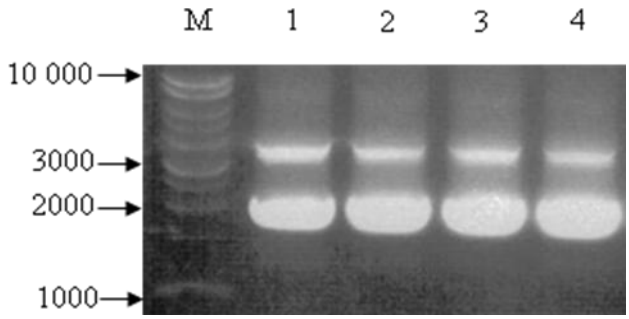

Figure 4.3: Agarose gel electrophoresis of a large-scale PCR amplicon of *L. braziliensis* $PGF_{2\alpha}$ synthase gene from cDNA. Lanes 1 to 4 are RT-PCR products of *L. braziliensis* $PGF_{2\alpha}$ synthase gene fragment run on 1% agarose gel. M = Promega 1 Kb ladder.

4.6 Sequence of the gene fragment obtained from *Leishmania braziliensis*

Nucleotide sequencing of the 4kb PCR product was not successful. However, the nucleotide sequence of the 2kb PCR product was successfully determined and was shown to contain 1983 nucleotides (Figure 4.4).

```
   1 ATGGCGGCCG CGGGAATTCG ATTCGCGGAT CCTTAGAACT GCGCCTCATC CAGTGAGGCC
  61 GAGAGCGACT TCTTGAGCTG CTGCCGCTGC AGAATCCAGG CAACGCCCTC GGCGTACTGC
 121 TCTGCCGCGA TGCGGATGGC CTCGTCACGC TCGGCGATGG TGCGAACCAC CAACTCCCAC
 181 TCGTCCAGTA TGCCACTACG GTCGTCGTGA AGCTTGCGGT ACTCGTTGGC GACACGGTCC
 241 AGCTCCCCCT GCACGTGCTG CGTGGCCAGC ACCTCCTCGT CAAGCTGCTT GCGCTGCTTC
 301 CGCACGCCGT TCTCGAGCTT CTCGATGCGC TGGGTGAGCT GCTTCACCTT TGTTTCATCC
 361 ATCTTGGAGT ACTGCGCGAT GGCGAGCTCA TCCTCCTCCT TCTGCCGGCG GGCCTCGTCC
 421 CACTGCTCCA GCTCCTCCTG GTTGTAGTCC AGTGAAGTCT TGAGCTCCTC GAGCTTCAGA
 481 TTTCCACGAA AGATGCGGTC CTGTACCGAC GTCAGCCGGT CTCGGACGTC CTCGGAGTTT
 541 TGCTCCAGCT GCGTTTGCCG CTGCTGCAGC CGCGCGCATT CGCGCTCCAT GACGCGGCAC
 601 ATGCTCTCCT CCGACTCCAC CTCGCGCTTC TTTGTCTCGG AGAGGCTCTG TGTGTTGACA
 661 ATTTCTGCTC GGACATTGCT GAGGTGCTCC TTCATAAACT GCAGTCGCCG CCGCTGGTCT
 721 TCGACGCTGC CCTGCCGCTC CTCCAGCTGC TGCTCGAAGC GCGTGAGCTG GGCGGTGAGC
 781 TCTTTGTTCG TGTTGTTGAG CAGCTCAAGG GGCAGCGCCT CGGCAGTCGC GTCCACGACA
 841 TCGAGACTCA TGTTGCACCA CTAGGTGTTG TGGTGCGTAT CGGTGTGTGC ATGTACACAG
 901 AACCAACAGC AAGACGCACG AAGTCCTTGC CAAGGCGTGA GGCTGATATG CGCCGAAGAG
 961 AGAAAGCAGT GGAGGGGGTA GTCGAAGGAG GGACTGGAGA AGAGTTGGAG GTGCGGCGGC
1021 AACAGCATGT GAAGGCACCG AGATGATGAG GGTACGTGTG GGTTCGGGGA TAGGGAAGAG
1081 AGAGAAGGAC ATCGAAGCTG CTTGCATTCG TGGTGACACG CGCTCCGCAT CCTCTTCCTC
1141 GTTGTCTTGA GGAATCGAAG AGCACAGTTC GCACAGCTGT TTCAGGAGTG AGTGGGTATC
1201 ACGAAACTGC GAAACGGCAT CAACAATCCT GAATAAAATC CGCAGAACGT GCAGAAGGAT
1261 GGAGAGGTGA GAAGCTCGGC AGTCTGAGGT GCGGCGGCCA TACAGGCGGG AGGCGTGGGG
1321 TGAATGTGAT AAGAAATGTG GCGCCCAAGA GCCGACTCGC TTTTCTGTCA AGGCCCCGCC
1381 GTCATTAGCT CGGCAACAAA GCTCGCTCGT CGTTTCAGCC TTTTTAGTGT CGTATCGTGC
1441 CGGCTTCCGC CGACGTGGGC TTGCGCTTGC TTCGAAGTCT GCCGCTAACA ACTCCCTTCC
1501 TCGTTGTCCC TGCCGAAGAA GCCCTCACCT CACCTCTCTT CGCCCCTTCC CCTTCCGAGC
1561 AGCTTTGTTT GTGTCGGCAC AGCAACCCAC GAGGGGTGTT CAGTGGCGCA GAAGTTCGGG
1621 GCAGCAATGT GGGGATAGTG ATACCTTAGC GTTGGGCCCC TAACTCGATG CGTTACCGTT
1681 AGCAGAGGAC ACCACGTGCA GCAGCCCTGC ATCCCGCAGC GTCTCAACGA GCAGCAGTTG
1741 GTTCTCCGCC ACATCCTCGG GATCGTCGAA GCCCGGGAAG GGCAGCGCAG CCACTAACAT
1801 TGTCTTTGGG TAGCTCGATA GCAGTGTGGC AATTGCTGGC GCAAATTCGG CCTCAAAGCG
1861 CAGCTCGCCG AGCTCCCCAG CGAGGCAGAC GGCCGAATTT CGTCCAATGT GATAAACCCG
1921 CGCCTCGCCA GGCACGTTCA GCAACAACTG CATGCAGCTG CGAGAGATGA GGCGCAGTTC
1981 TAA
```

Figure 4.4: Nucleotide sequence obtained upon sequencing the 2kb amplicon of the *L. braziliensis* $PGF_{2\alpha}$ synthase gene fragment.

4.7 Concentration of protein in lysates of different *Leishmania* species

The protein concentration ranged between 11.09 µg/µl - 19.67 µg/µl (Table 4.3). Lysates of *Leishmania donovani* contained the highest concentration while lysates of *L. mexicana* had the lowest concentration of protein.

Table 4.3: Concentration of protein in lysates of the different *Leishmania* species

Species name	**Protein concentration (µg/µl)**
L. major	13.13
L. donovani	19.67
L. tropica	16.58
L. amazonensis	16.93
L. braziliensis	11.60
L. mexicana	11.09
L. chagasi	12.22

4.8 SDS-PAGE analysis of protein lysates obtained from the different species of *Leishmania*

The SDS-PAGE analysis of the *Leishmania* protein lysates yielded the profiles shown in Figure 4.5 below. All the species showed a similar banding pattern and a prominent band of 63kDa.

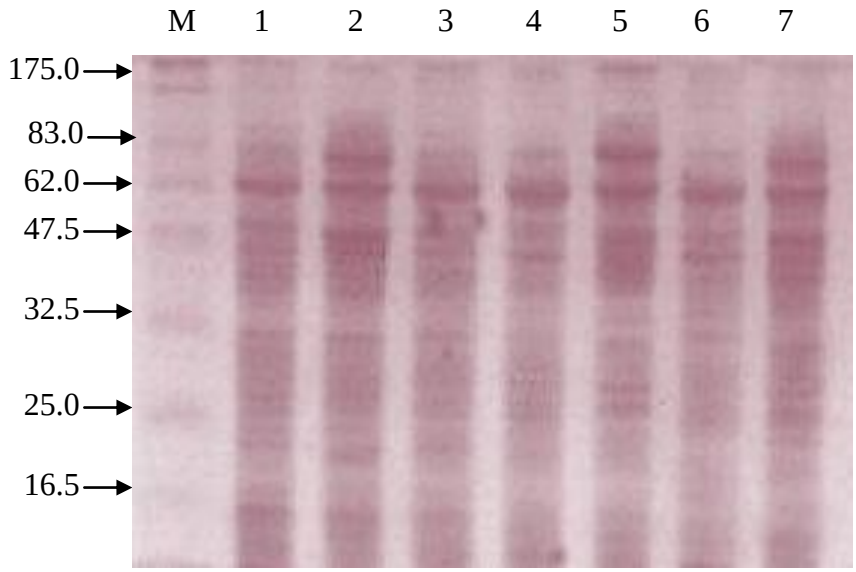

Figure 4.5: Analysis of Protein lysates from *Leishmania* species after SDS-PAGE. M = protein ladder (New England Biolabs); lane 1 = *L. major*; lane 2 = *L. donovani*; lane 3 = *L. tropica*; lane 4 = *L. amazonensis*; lane 5 = *L. braziliensis*; lane 6 = *L. mexicana*; lane 7 = *L. chagasi*.

4.9 Western Blot analysis of *Leishmania* protein lysates to detect $PGF_{2\alpha}$ synthase

Rabbit anti-*T. brucei* $PGF_{2\alpha}$ synthase polyclonal antibody detected a 34-kDa immunoreactive protein in lysates derived from to the Old World species, *L. major*, *L. donovani* and *L. tropica* (Figure 4.6; lanes 1-3). No $PGF_{2\alpha}$ synthase protein was detected in the New World species of *L. amazonensis*, *L. braziliensis L. mexicana*, *L. braziliensis* and *L. chagasi* (Figure 4.6; lanes 4-7).

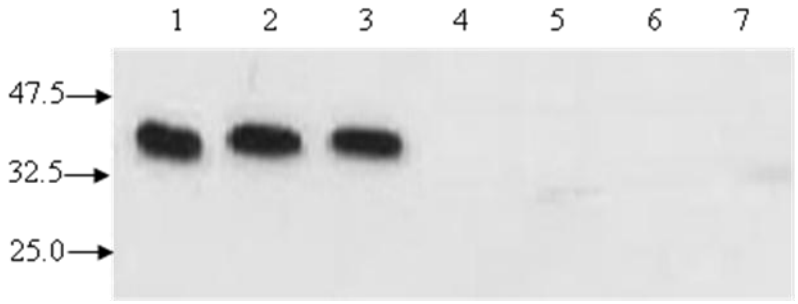

Figure 4.6: Western blot analysis of protein lysates to detect $PGF_{2\alpha}$ synthase in *Leishmania.* Lane 1 = *L. major*; lane 2 = *L. donovani*; lane 3 = *L. tropica*; lane 4 = *L. amazonensis*; lane 5 = *L. braziliensis*; lane 6 = *L. mexicana*; lane 7 = *L. chagasi*.

4.10 Sequences displaying homology to the putative *L. braziliensis* $PGF_{2\alpha}$ *synthase* gene fragment

Homology search against the NCBI's non-redundant database performed using NCBI's nucleotide BLAST tool showed that indeed the nucleotide sequence belonged to *Leishmania braziliensis* (Table 4.4). The amplified nucleotide sequence showed the highest similarity to a conserved gene of *L. braziliensis* MHOM/BR/75/M2904 coding for a partial mRNA of a hypothetical protein, (LbrM31_V2.0340) found on chromosome 31 (Table 4.4). The bit score value of the hit was 3524 with associated query coverage of 99% and an e-value of 0.0 (Table 4.4). Other high scoring hits included a partial mRNA of a conserved *L. major* hypothetical protein (LmjF31.0230) found on chromosome 31 and a partial mRNA of hypothetical protein (LinJ31.0230) from *Leishmania infantum* JPCM5 (Table 4.4).

Table 4.4: BLASTn comparison of the putative *L. braziliensis* $PGF_{2\alpha}$ synthase sequence against the non redundant DNA database at the NCBI. The top six hits belong to proteins from *Leishmania.*

Accession	Description	Max Score	Total Score	Query coverage	E value	Max ident
AM494968.1	Leishmania braziliensis chromosome 31	3524	3524	99%	0.0	99%
XM_001566985.1	Leishmania braziliensis MHOM/BR/75/M2904 hypothetical protein, conserved (LbrM31_V2.0340) partial mRNA	1485	1485	41%	0.0	99%
AM502249.1	Leishmania infantum chromosome 31	1040	1400	61%	0.0	88%
XM_001684955.1	Leishmania major hypothetical protein, conserved (LmjF31.0230) partial mRNA	1037	1037	41%	0.0	89%
XM_001467247.1	Leishmania infantum JPCM5 hypothetical protein (LinJ31.0230) partial mRNA	1009	1009	41%	0.0	89%
XM_001566986.1	Leishmania braziliensis MHOM/BR/75/M2904 hypothetical protein, conserved (LbrM31_V2.0350) partial mRNA	586	586	16%	2e-163	100%
XM_001467248.1	Leishmania infantum JPCM5 hypothetical protein (LinJ31.0240) partial mRNA	337	337	16%	2e-88	85%

4.11 Identification of regions of nucleotide similarities between the putative *L. braziliensis* $PGF_{2\alpha}$ synthase and the *LbrM31_V2.0340* genes

Pairwise nucleotide sequence alignment was carried out to compare the *L. braziliensis* gene fragment and the partial mRNA of a hypothetical protein, (LbrM31_V2.0340) found on chromosome 31 of *L. braziliensis* (Figure 4.7). This was performed to identify regions of similarity between the sequences that may indicate functional or structural relationships between the sequences.

Similarity searches showed that the putative *L. braziliensis* $PGF_{2\alpha}$ synthase nucleotide sequence lies between position 1768 and position 2580 on the *L. braziliensis* partial mRNA on chromosome 31 (Figure 4.7). The sequences were identical except for three synonymous changes at positions 2083(C→T), 2485(A→G) and 2576(T→C) (Figure 4.7).

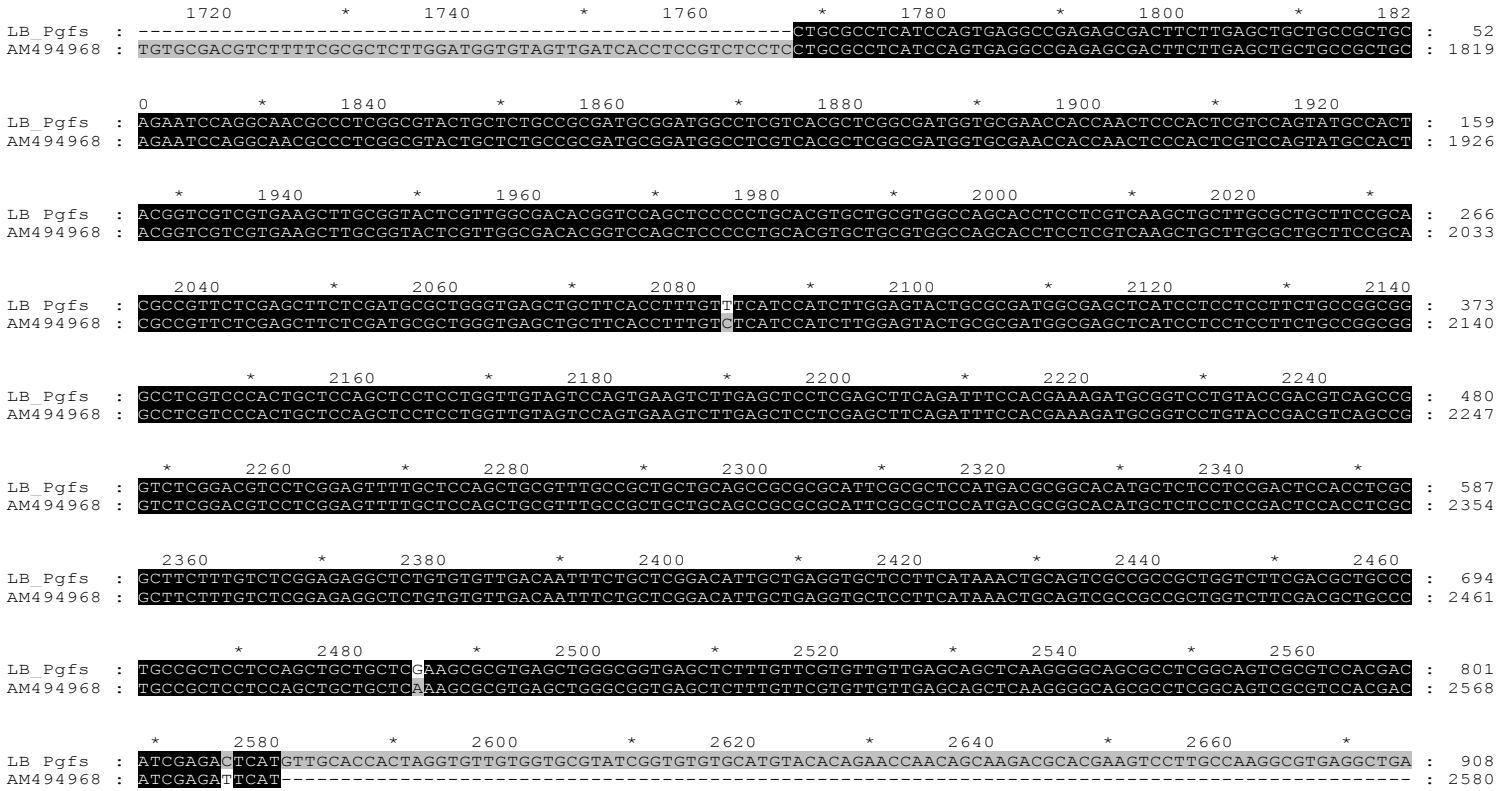

Figure 4.7: Pairwise nucleotide sequence alignment of the putative *L. braziliensis* $PGF_{2\alpha}$ synthase gene fragment (*Lb*PGFS) and the partial mRNA of a hypothetical protein found on *L. braziliensis* chromosome 31(AM 494968).

The AM 494968 nucleotide sequence had the highest bit score in the BLASTn analysis.

4.12 Identification of open reading frames (ORFs) encoded in the putative *L. braziliensis* $PGF_{2\alpha}$ synthase gene fragment

In order to ascertain that the amplified gene fragment coded for a protein(s), the nucleotide sequence was analyzed for presence of open reading frames (ORFs) in all the six possible frames. This search revealed 16 possible ORFs (Figure 4.8). Ten of these were on the complementary strand whereas six were on the main strand. Of the six ORFs in the forward direction on the main strand, four were in +2 frame, one was in +1 frame and one was in +3 frame (Figure 4.8). Among these, the largest ORF was the third one in the +2 frame covering a region of 371 nucleotides coding for 123 amino acids. The other five were small ORFs, coding for proteins of 26, 40, 51, 35 and 50 amino acids respectively. Upon translation and search of the resulting amino acid sequences against the redundant protein database at the NCBI, none of these peptides yielded homology to a known protein (result not shown). Of the 10 ORFs in the reverse direction, three were in -1 ORF, four in -2 ORF and three in -3 ORF. Upon search, eight of the frames did not give peptides with homologies to proteins in GenBank but the two largest ORFs in frame -2 and -1 gave peptides with homologies.

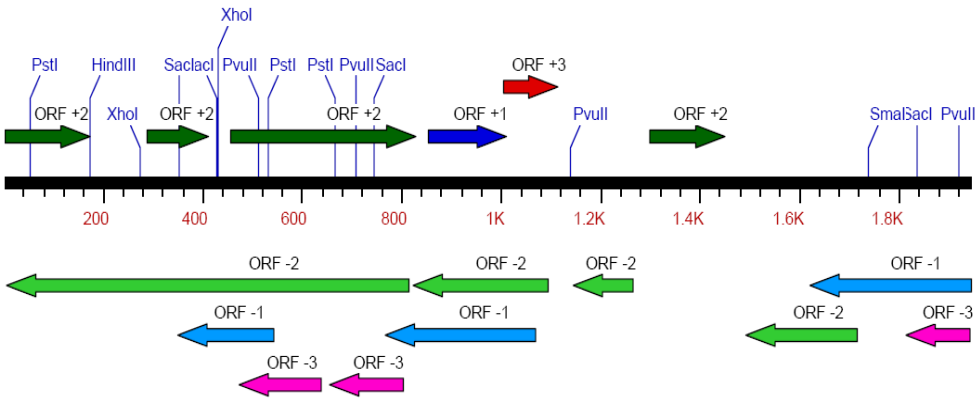

Figure 4.8: Identification of ORFs in the putative *L. braziliensis* $PGF_{2\alpha}$ synthase gene fragment.

4.12.1 Characterization of ORF-1

To characterize ORF-1 the nucleotide sequence was translated into amino acid code generating a 585 amino acid protein (Figure 4.9).

```
  1 MGISAKKRAR ICTRAVESAQ LQAPTNSTSA QGERRRGNVL GDVVDHPLLL QQHHYLLVLF
 61 VFLFFAHATA VRQNWSDRAH TPTHQYTMTK STSLKNGNGG KRASEAPSQK VVLKKAKAEV
121 AATLRKSSHG SQRPLNFFSW LLKTSRVEFF RKYFEKKPLV ASHGNCEYFA RGLPGVVPPV
181 DWSTKRMLEH IKMHPSRYGT DLNVVKFDSK LKKRVSYRTE GLVDAAELER CMKTGWSVRF
241 LRPHEFLESN SAFIGCMEQE FNCYCGVNSY WTPANSQGFA PHYDDVDVFL LQLEGEKLWR
301 LYDPPEEVDV LTRHSSEDYA PEQLPTPNHT ITLKAGDVLY MPRGTVHQGK TNLKTHSLHI
361 TFSANQMNSW ADFMIRSAQY TIETLAANKL EWRCALPRDV PQVMGAINHP VFRNTHGLAV
421 LSENQQERRA SLQKKLREMA AEVTLLLTDE SNMDVCTDVY GKETIRKMQP PSMAYSGAAS
481 SSTGLDHASH VRLISRSCMQ LLLNVPGEAR VYHIGRNSAV CLAGELGELR TLLSSYPKTM
541 LVAALPFPGF DDPEDVAENQ LLLVETLRDA GLLHVVSSAN GNASS
```

Figure 4.9: Translation of ORF-1.

When the resulting amino acid code of the translation was searched against GenBank at the NCBI using BLASTp, the top four homologous proteins were identified and are described below (Table 4.5). The highest similarity was to a hypothetical protein from *L. braziliensis* which showed 100% identity, a maximum score of 1173 and with an E-value of 0.0 (Table 4.5). This protein whose database entry accession number is XP_001567036.1 has 585 amino acids. According to the database annotation, XP_001567036.1 is a conserved protein which is uncharacterized and whose function is unknown.

Table 4.5: BLAST of translation of ORF-1

Descriptions

Legend for links to other resources: UniGene GEO Gene Structure Map Viewer PubChem BioAssay

Accession	Description	Max score	Total score	Query coverage	E value	Links
XP_001567036.1	hypothetical protein [Leishmania braziliensis MHOM/BR /75/M2904]	1173	1173	100%	0.0	G
CAM42455.2	conserved hypothetical protein [Leishmania braziliensis MHOM/BR/75/M2904]	1172	1172	100%	0.0	
CBZ29080.1	conserved hypothetical protein [Leishmania mexicana MHOM/GT/2001/U1103]	850	850	90%	0.0	
XP_001685008.1	hypothetical protein [Leishmania major strain Friedlin] >emb|CAJ08208.1| conserved hypothetical protein [Leishmania major strain Friedlin]	837	837	86%	0.0	G

The second protein identified to be homologous to the query protein was a *L. brazilensis* protein whose database entry accession number is CAM42455.2. This protein showed 100% identity to the query protein, a maximum score of 1172 and an E-value of 0.0. This protein is composed of 595 amino acids. Similar to the first hit of the database described above, this is a hypothetical protein whose function is not known. The third hit in the database was a *L. mexicana* conserved hypothetical protein which had 90% identity to the query sequence with an E-value of 0.0. This match with accession number CBZ29080 has 624 amino acids and is annotated as an uncharacterized protein and whose function is unknown. The fourth match was a protein whose accession number is XP_001685008.1 and had 86% identity to the query sequence with an E-value of 0.0. This protein is derived from *L. major* strain Friedlin and is a 624 amino acid conserved hypothetical protein whose function is unknown.

To further characterize ORF-1, the amino acid code of the protein was searched against the Conserved Domain Database (CDD) at the NCBI. This search identified a Cupin - 4 multi domain cluster in the middle (amino acid 140-400) of the protein (Figure 4.10).

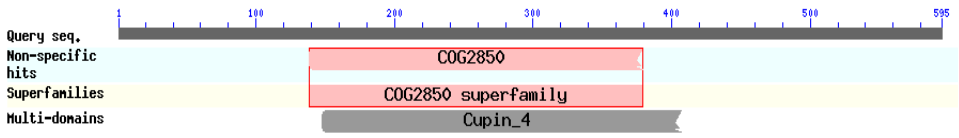

Figure 4.10: Conserved domain predicted ORF-1.

The amino acid sequence was also searched against the Eukaryote Linear Motif (ELM) resource (http://elm.eu.org/) for prediction of functional sites (Figure 4.11). The ELM search showed that this protein contains a domain of the Jumonji (JmjC) protein family which extends from amino acid residue 216-274. Furthermore, the ELM search identified several ligand binding site motifs. These include the LIG_14_3_3_3 motif observed on the C terminus of the protein and covering amino acid residues 492-497, the Clathrin box motif designated as LIG_Clathr_ClatBox_1 observed between amino acid residues 571-575 on the C terminus of the protein and the cyclin recognition site designated as LIG_CYCLIN_1 observed at the N terminus of the protein covering residues 133-137, 157-160 and 171-174.

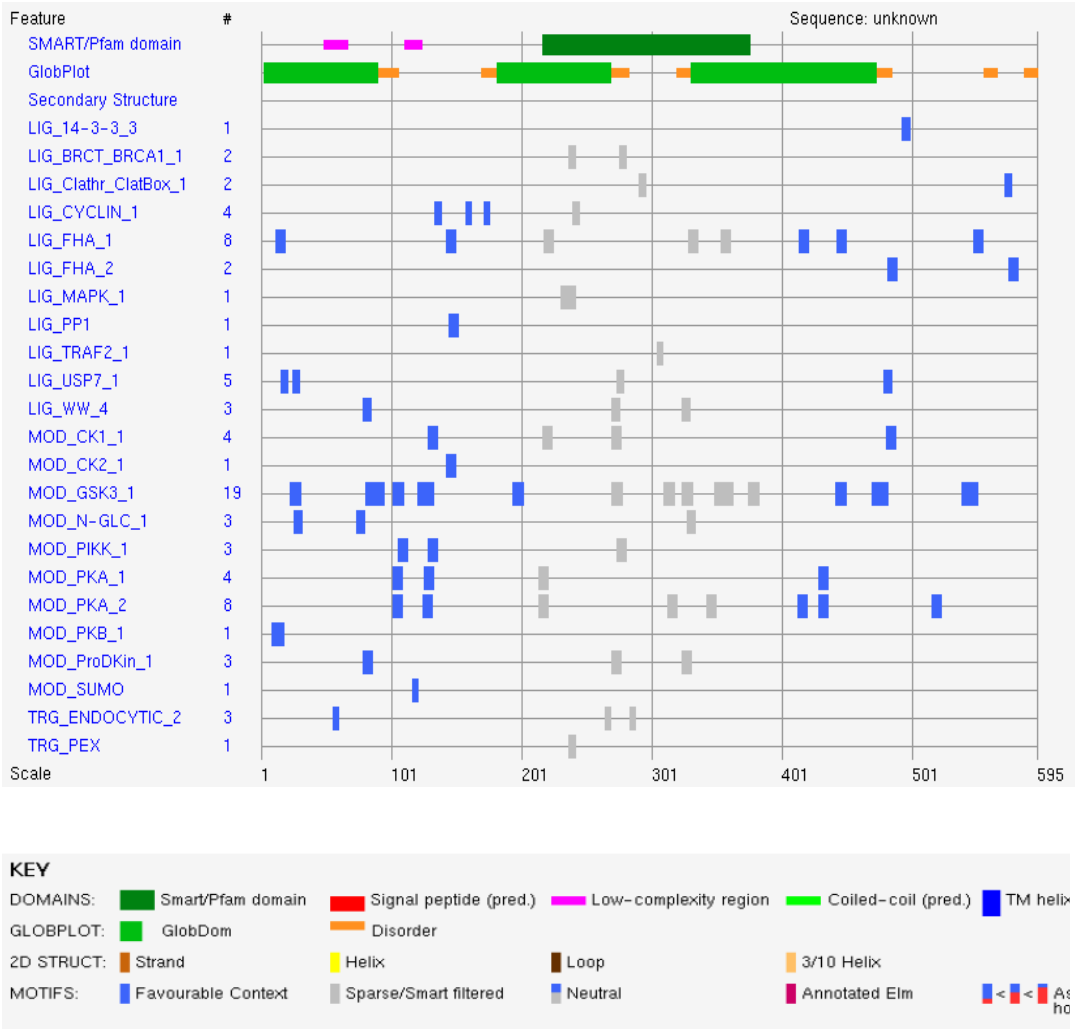

Figure 4.11: ELM analysis of full length ORF-1. The numeral next to each feature represents the number of each motif feature.

The other motifs identified are the forkhead associated domains LIG_FHA_1 and LIG_FHA_2. The LIG_FHA_1 has 2 sites on the N terminus and covers amino acid residues 11-17 and 142-148 while the 3 sites on the C terminus cover amino acid residues 413-419, 442-448 and 547-553. The LIG_FHA_2 has 2 sites towards the C terminus covering amino acid residues 481-487 and 574-580. Also observed were LIG_PP1 site covering amino acid residues 144-150, LIG_WW_4 covering amino acid residues 78-83 and LIG_USP7_1 found at amino acid residues 15-19, 24-28 and 478-482. Post-translational modification site motifs were also observed. These

include MOD_CK1_1 found at both the N and C terminus at amino acid residues 128-134 and 480-486 respectively and MOD_CK2_1 covering amino acid residues 142-148. The GSK3 phosphorylation recognition site designated as MOD_GSK3_1 motif was found at several sites at both the C and N-termini. The N terminal sites were found at amino acid residues 22-29, 86-93, 101-108, 124-131 and 193-200 while the C terminal sites were found at amino acid residues 441-448, 473-480 and 542-549. Others posttranslation modification sites observed include MOD_PIKK_1 covering amino acid residues 105-111 and 128-134, MOD_PKA_1 covering residues 101-107, 125-131 and 428-434, MOD_PKA_2 covering residues 101-107, 124-130, 412-418, 428-434 and 515-521, MOD_PKB_1 covering residues 8-16 and finally MOD_ProDKin_1 covering amino acid residues 78-84. The N-glycosylation motif designated as MOD_N-GLC_1 was observed on the N-terminus of the protein covering residues 25-30 and 73-78 while a MOD_SUMO motif which is recognized for modification by SUMO_1 was identified covering amino acid residues 116-119. The targeting site motif, TRG_ENDOCYTIC_2 motif was observed at the N terminus of the protein covering amino acid residues 55-58.

4.12.2 Characterization of ORF-2

To characterize ORF-2 the nucleotide sequence was translated into amino acid code generating an 859 amino acid protein (Figure 4.12).

```
  1 MNLDVVDATA EALPLELLNN TNKELTAQLT RFEQQLEERQ GSVEDQRRRL QFMKEHLSNV
 61 RAEIVNTQSL SETKKREVES EESMCRVMER ECARLQQRQT QLEQNSEDVR DRLTSVQDRI
121 FRGNLKLEEL KTSLDYNQEE LEQWDEARRQ KEEDELAIAQ YSKMDETKVK QLTQRIEKLE
181 NGVRKQRKQL DEEVLATQHV QGELDRVANE YRKLHDDRSG ILDEWELVVR TIAERDEAIR
241 IAAEQYAEGV AWILQRQQLK KSLSASLDEA QEETEVINYT IQEREKTSHK LQEAVPVLTQ
301 QVQSVQDEVD ALREKASRAT RDKRAVILQL EETITEIERR KRELAMTEKG RTTAAEKLKE
361 EEMTANDLQK QAQFIAQLLK DAEMASHKIT RDIEQLKTTT FRANQELAKV RAAQTTSLSE
421 ISGAQAQGKN YKAKIDQLDG ESFAQQGVLY NIEFSVQQIE KRVSRAKGER TEEERKELRR
481 KIDLLQTTLD ELEKQNRILQ NQVKRVREEM RKSSILIEKL EATKKRSLEE VLEMDLRCTH
541 SDREEKQLEK QREDLLIKVD TLELQLGRLR NLLRVKDAEL LTLEEKKRQL EADVAEREAE
601 IEVHHRLLRM EAKLAEEERK RLVTELLERQ KNLTAVKNRQ EVLVGRMDPA HARLSQVQLV
661 IAAAKEREDL QYRGDSLDAR IRRMEKEMLK LEKTIAIIRA SNEQYRHQFD KVSDKDEEVQ
721 TQKALKNKFK ELKSAISRRT LEVNDFQATT RNKQEELRAL QFEKQRVGHM QEQLLKEYEA
781 VTQDILALRE AAIRYDQAIE KAKGNVDAAV VGDIELVCTR ERLDNTIAQL LSLSREAGEE
841 VLDVVKQMLT AHQLSIEGA
```

Figure 4.12: Translation of ORF-2.

The amino acid code of the translation was searched against GenBank at the NCBI using protein BLAST and the top five homologous proteins identified. The First match was an *L. braziliensis* conserved hypothetical protein with 100% identity, a maximum score of 1690 and an E-value of 0.0 (Table 4.6). This protein whose database entry accession number is XP_001567035.1 has 859 amino acids and the database annotation indicates that its function is unknown.

Table 4.6: BLAST of translation of ORF-2.

Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Accession	Description	Max score	Total score	Query coverage	E value	Links
XP_001567035.1	hypothetical protein [Leishmania braziliensis MHOM/BR/75/M2904] >emb|CAM42454.1| conserved hypothetical protein [Leishmania braziliensis MHOM/BR/75/M2904]	1690	1690	100%	0.0	G
CBZ36424.1	unnamed protein product [Leishmania donovani BPK282A1]	1488	1488	100%	0.0	
XP_001467284.1	hypothetical protein [Leishmania infantum JPCM5] >emb|CAM70339.1| conserved hypothetical protein [Leishmania infantum JPCM5]	1487	1487	100%	0.0	G
XP_001685007.1	hypothetical protein [Leishmania major strain Friedlin] >emb|CAJ08207.1| conserved hypothetical protein [Leishmania major strain Friedlin]	1481	1481	100%	0.0	G
CBZ29079.1	conserved hypothetical protein [Leishmania mexicana MHOM/GT/2001/U1103]	1469	1469	100%	0.0	

The second match identified an *L. donovani* 859 amino acid protein with database entry accession number CBZ36424.1. This protein, whose function is unknown, had 100% identity, a maximum score of 1488 and an E-value of 0.0 (Table 4.6). Similarly, the third match identified an 859 amino acid protein from *L. infantum* JPCM5 with 100% identity and an E-value of 0.0. This protein with database entry accession number XP_001467284.1 is annotated as a hypothetical protein whose function is unknown. The fourth match identified an *L. major* 859 amino acid protein with database entry accession number XP_001685007.1. This protein has 100% identity to the query protein and an E-value of 0.0. The final match identified an *L. braziliensis* 859 amino acid hypothetical protein with 100% identity and an E-value of 0.0. This protein whose accession number is CBZ29079.1 is annotated as a hypothetical protein whose function is also unknown.

To further characterize this protein whose function is unknown, the amino acid code of the protein was searched against the Conserved Domain Database (CDD) at the NCBI as well as the ELM resource (http://elm.eu.org/) for prediction of functional sites. The CDD search identified a multi-domain cluster of Structural Maintenance of Chromosomes (SMC) proteins (Figure 4.13).

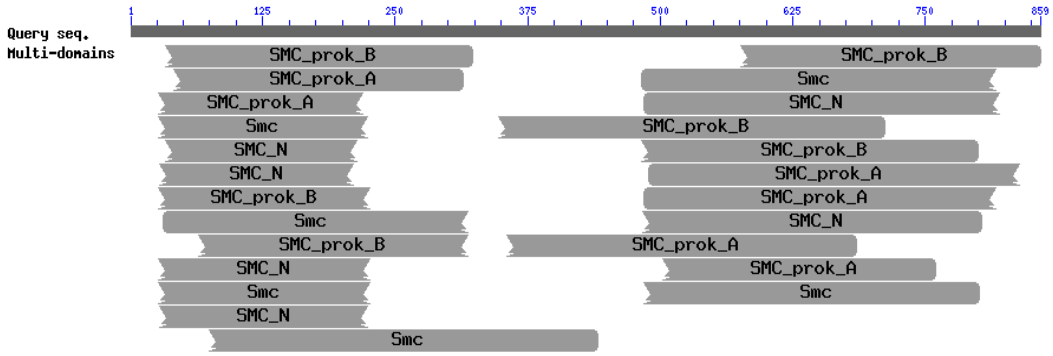

Figure 4.13: Conserved domains predicted in ORF-2.

The domains were identified by searching the CDD at NCBI using the sequence of ORF-2.

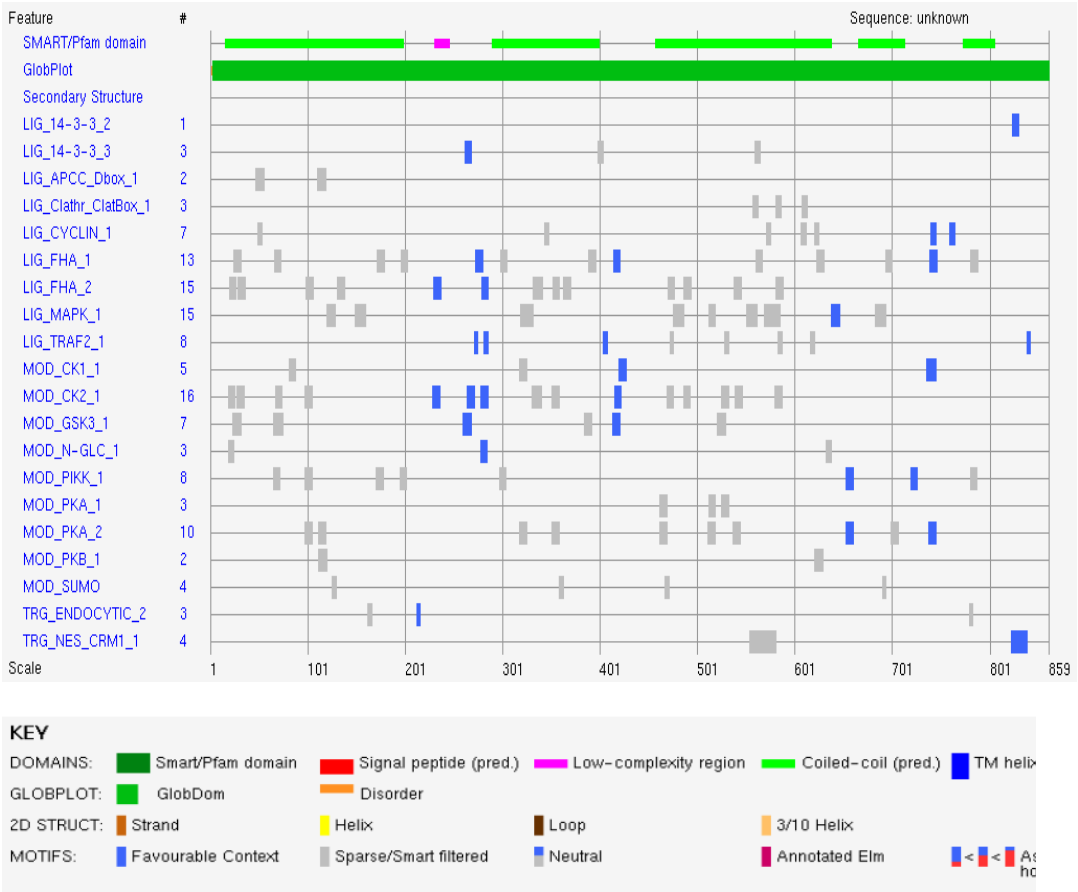

Figure 4.14: ELM analysis of full length ORF-2

A translation of the gene fragment in *L. braziliensis* was searched against the ELM resource to identify the various motif features shown above. The numeral next to each feature represents the number of each motif feature.

The ELM analysis identified several ligand binding site motifs. Thus, the LIG_14_3_3_2 motif was observed on the C terminal of the protein covering amino acid residues 822-828 while a LIG_14_3_3_3 motif was observed on the N terminal and covering amino acid residues 261-266. Two substrate recognition sites that interact with cyclin and thereby increases phosphorylation by cyclin/cdk complexes and designated as LIG_CYCLIN_1 were observed at amino acid residues 739-743 and 758-762. Fork head associated motifs identified included LIG_FHA_1 at amino acid residues 272-278, 413-419 and 738-744 while LIG_FHA_2 motif was observed on the N terminal of the protein on amino acid residues 229-235 and 278-284. A

single LIG_MAPK_1 motif was identified on the C terminal of the protein from amino acid residues 637-644 while several LIG_TRAF2_1 domains were identified at amino acid residues 270-273, 280-283, 403-406 and 837-840. Post translational modification site domains identified include Casein kinase (CK) phosphorylation sites designated as MOD_CK1_1 and MOD_CK2_1. MOD_CK1_1 motifs were observed at amino acid residues 419-425 and 734-740 while MOD_CK2_1 motifs were observed at amino acid residues 228-234, 263-269, 277-283 and 414-420. GSK3 phosphorylation recognition sites designated as MOD_GSK3_1 were observed at amino acid residues 259-266 and 412-419. MOD_PIKK_1 domains were identified at amino acid residues 652-658 and 718-724 on the C terminal of the protein while MOD_PKA_2 domains were identified at amino acid residues 652-658 and 737-743 also on the C terminal of the protein. The motif for N-glycosylation, MOD_N-GLC_1 was identified at amino acid residues 277-282 towards the N terminal of the protein. Two sub-cellular targeting sites were observed. These included TRG_ENDOCYTIC_2 identified at amino acid residue 211-214 and the TRG_NES_CRM1_1 was identified at amino acid residues 821-836.

4.13 Analysis of *L. major* Prostaglandin F synthase ORF

The *Leishmania major* genome has been fully sequenced and *L. major* PGFS identified and characterized. Therefore, in order to compare the functional sites present in *L. major* Prostaglandin F synthase ORF and the putative *L. braziliensis* Prostaglandin F synthase ORF, the *L. major* Prostaglandin F synthase ORF was obtained from GeneDB (Figure 4.15) and searched against GenBank at the NCBI using BLASTp. The search identified Aldo-keto reductase superfamily domains within the protein (Figure 4.16).

```
  1 MAVKCTHVTL SNGVQVPQLG IGTWEAKDGN EVVNIKWAVN AGYRHVDTAH YYKNEKGVGQ
 61 GISECGVPRS DIFVTTKLWN YDHGYESALA AFEQSRQALG VEYVDLYLIH WPGPNRSYIE
121 TWRAFEKLYE MKKVRAIGVS NFEPHHLDDL LANCTVPPMV NQVEMHPHFQ QKALRAYCAE
181 KNIAVTAWRP LGKGALLTEP QLVELAEKHK RSAAQVIIRW LIQLGVIAIP KSSHEERIKQ
241 NFDVFDFELS PEDMRRIESM DRNSRIGSSP ETFFPTERK
```

Figure 4.15: *L. major* PGFS ORF. The 279 amino acid protein was extracted from GenDB.

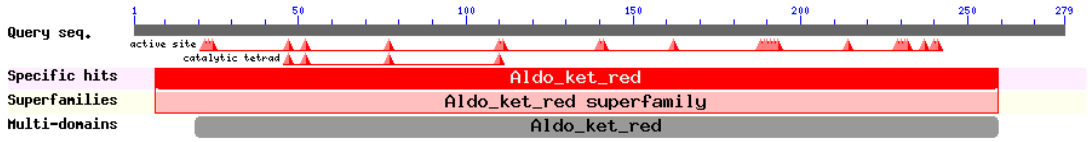

Figure 4.16: conserved domains within the *L. major* PGFS ORF.

The domains were identified by searching the CDD at NCBI using the amino acid sequence of *L. major* PGFS.

4.13.1 ELM Analysis of *L. major* Prostaglandin F synthase ORF

To further compare the *L. major* PGFS protein with the putative *L. braziliensis* PGFS, the amino acid code of the protein was searched against the ELM resource (http://elm.eu.org/) for prediction of functional sites (Figure 4.17). The ELM analysis identified an Aldo keto reductase protein family domain extending from amino acid residue 18-262.

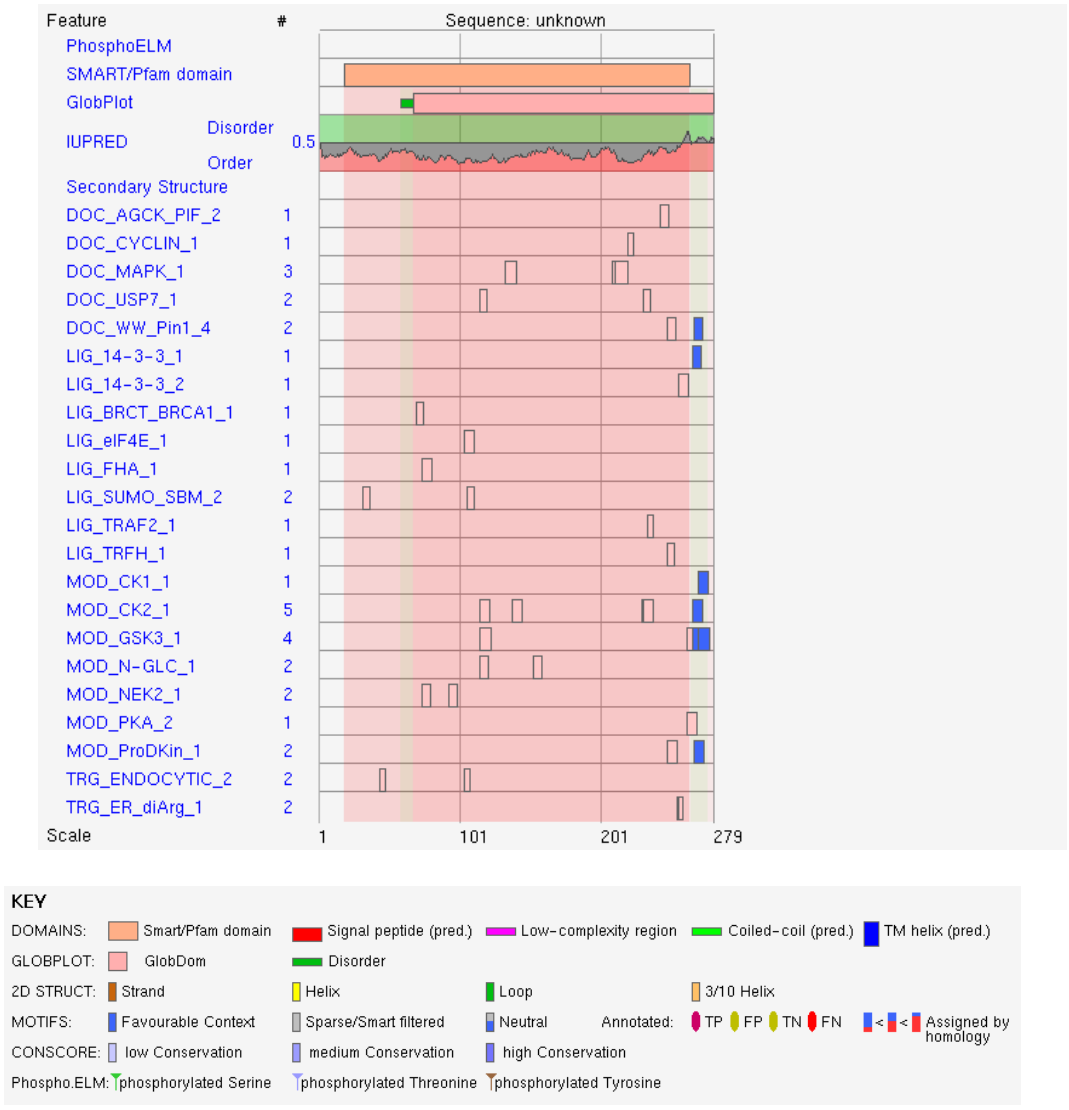

Figure 4.17: ELM analysis of *L. major* Prostaglandin F synthase.

The *L. major* Prostaglandin F synthase ORF was searched against the ELM resource to identify the various motif features shown above. The numeral next to each feature represents the number of each motif feature.

One ligand binding site motif designated as LIG_14-3-3_1 was identified on the C terminal of the protein extending from amino acid 265-270. Another ligand binding site motif designated as LIG_WW_Pin1_4 was identified on the C terminal of the protein extending from amino acid residue 266-271. Two casein kinase modification sites designated as MOD_CK1_1 and MOD_CK2_1 were identified on the C

terminal of the protein extending from amino acid residue 269-275 and 265-271 respectively. Two modification site motifs for GSK3 phosphorylation were identified on the C terminal of the protein. These were on amino acid residues 265-272 and 269-276. Finally, one motif for phosphorylation of MAP kinase designated as MOD_ProDKin_1 was identified on amino acid residues 266-272.

4.14 Identification of regions of amino acid similarities between *L. major* $PGF_{2\alpha}$ synthase, *L. braziliensis* $PGF_{2\alpha}$ synthase, ORF-1 and ORF-2

After the bulk of this work was done, the genome of *L. braziliensis* was fully sequenced and is available at GeneDB (http://www.genedb.org). Using sequence data, orthologues to *L. major* PGFS have been inferred in other kinetoplastids, including *L. braziliensis* (LbrM.31.2410: pep). Multiple sequence alignment was carried out to identify regions of similarity between *L. braziliensis* PGFS, *L. major* PGFS, ORF-1 and ORF-2 (Figure 4.18). The search showed that *L. braziliensis* PGFS and *L. major* PGFS were similar while ORF-1 and ORF-2 shared some similarity. However, both *L. braziliensis* PGFS and *L. major* PGFS differed with ORF-1 and ORF-2 to a large extent (Figure 4.18).

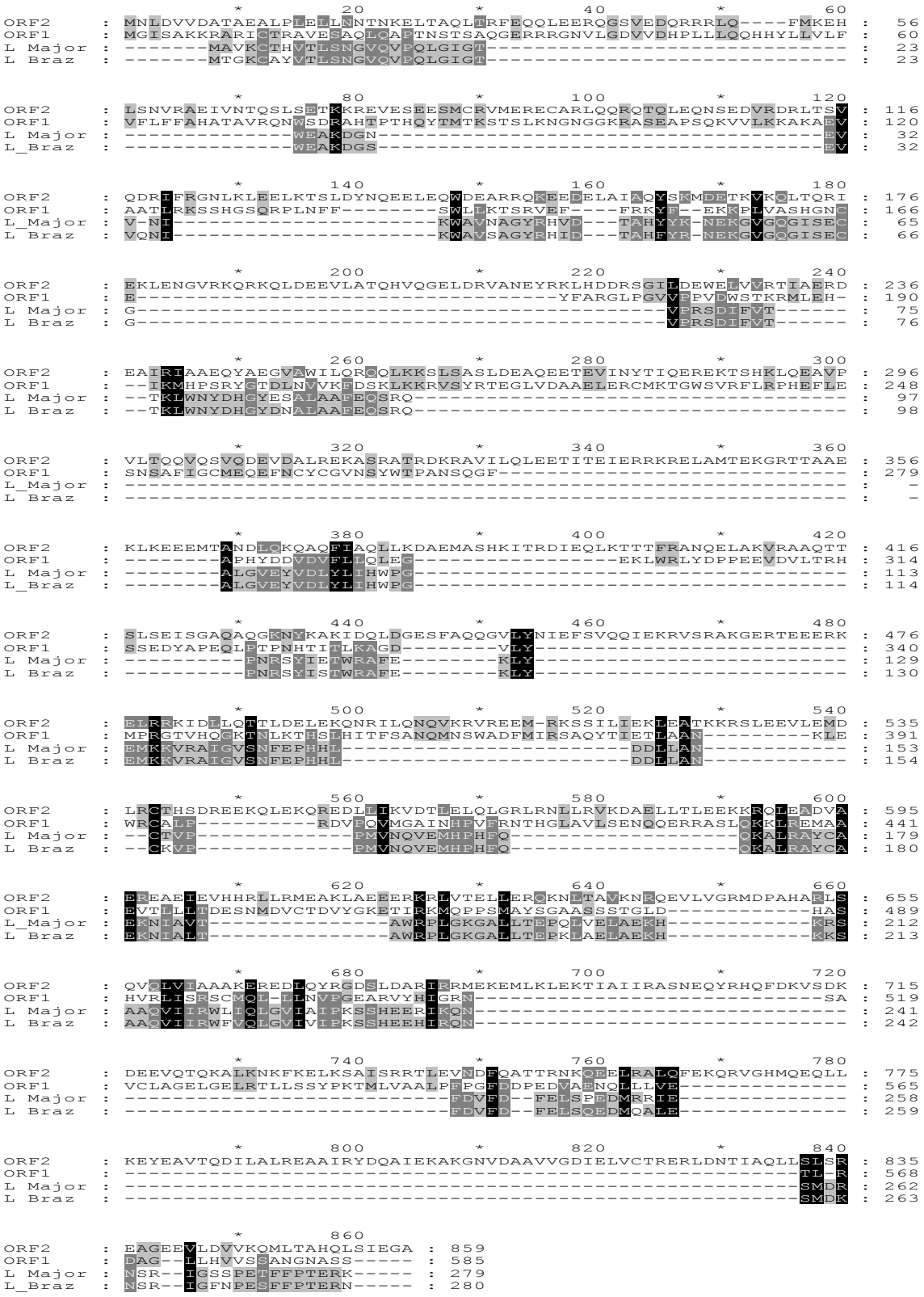

Figure 4.18: Comparison of *L. major* PGFS, *L. braziliensis* PGFS, ORF-1 and ORF-2.

4.15 Identification of regions of amino acid similarities between *L. major* $PGF_{2\alpha}$ synthase and *L. braziliensis* $PGF_{2\alpha}$ synthase

Since the Rabbit anti-*T. brucei* $PGF_{2\alpha}$ synthase polyclonal antibody detected the $PGF_{2\alpha}$ synthase protein in Old but not New World species of *Leishmania*, pairwise sequence alignment was carried out to identify amino acid similarity between, *L. major* PGFS and *L. braziliensis* PGFS (Figure 4.19). The *L. major* and *L. braziliensis* sequences were obtained from GeneDB. This was done in order to explain why the polyclonal antibody recognized the $PGF_{2\alpha}$ synthase in the Old but not the New World species of *Leishmania*. The search showed that this proteins share 92.5 % identity.

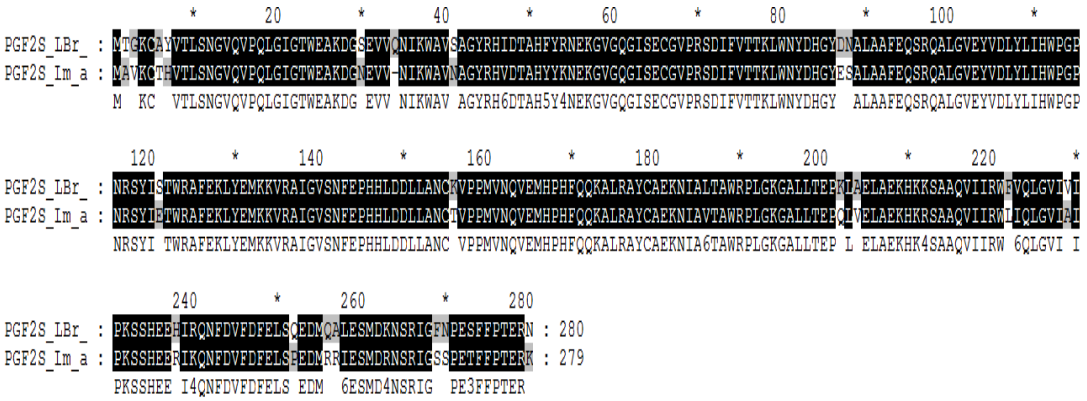

Figure 4.19: Comparison of *L. major* PGFS and, *L. braziliensis* PGFS.

4.16 Determination of annealing position of primers on *L. braziliensis* gene fragment

In order to determine the positions at which the *L. major PGFS* gene specific primers had annealed on the *L. braziliensis* sequence, the gene fragment along with the set of primers were analyzed using the Primer Show tool in The Sequence Manipulation Suite (Stothard, 2000). The resulting map showing the annealing positions of the primers indicated that the reverse primer annealed on both the 5' and 3' positions of

the *L. braziliensis* gene fragment while the forward primer did not anneal (Figure 4.20)

Results for 1983 residue sequence starting "ATGGCGGCCG".

```
   1                                        TTAGAACTGCGCCTCATC 3' Rev
   1 ATGGCGGCCGCGGGAATTCGATTCGCGGATCCTTAGAACTGCGCCTCATCCAGTGAGGC
  61 GAGAGCGACTTCTTGAGCTGCTGCCGCTGCAGAATCCAGGCAACGCCCTCGGCGTACTGC
 121 TCTGCCGCGATGCGGATGGCCTCGTCACGCTCGGCGATGGTGCGAACCACCAACTCCCA
 181 TCGTCCAGTATGCCACTACGGTCGTCGTGAAGCTTGCGGTACTCGTTGGCGACACGGTC
 241 AGCTCCCCCTGCACGTGCTGCGTGGCCAGCACCTCCTCGTCAAGCTGCTTGCGCTGCTT
 301 CGCACGCCGTTCTCGAGCTTCTCGATGCGCTGGGTGAGCTGCTTCACCTTTGTTTCATC
 361 ATCTTGGAGTACTGCGCGATGGCGAGCTCATCCTCCTCCTTCTGCCGGCGGGCCTCGTC
 421 CACTGCTCCAGCTCCTCCTGGTTGTAGTCCAGTGAAGTCTTGAGCTCCTCGAGCTTCAG
 481 TTTCCACGAAAGATGCGGTCCTGTACCGACGTCAGCCGGTCTCGGACGTCCTCGGAGTT
 541 TGCTCCAGCTGCGTTTGCCGCTGCTGCAGCCGCGCGCATTCGCGCTCCATGACGCGGCA
 601 ATGCTCTCCTCCGACTCCACCTCGCGCTTCTTTGTCTCGGAGAGGCTCTGTGTGTTGAC
 661 ATTTCTGCTCGGACATTGCTGAGGTGCTCCTTCATAAACTGCAGTCGCCGCCGCTGGTC
 721 TCGACGCTGCCCTGCCGCTCCTCCAGCTGCTGCTCGAAGCGCGTGAGCTGGGCGGTGAG
 781 TCTTTGTTCGTGTTGTTGAGCAGCTCAAGGGGCAGCGCCTCGGCAGTCGCGTCCACGAC
 841 TCGAGACTCATGTTGCACCACTAGGTGTTGTGGTGCGTATCGGTGTGTGCATGTACACA
 901 AACCAACAGCAAGACGCACGAAGTCCTTGCCAAGGCGTGAGGCTGATATGCGCCGAAGA
 961 AGAAAGCAGTGGAGGGGGTAGTCGAAGGAGGGACTGGAGAAGAGTTGGAGGTGCGGCGG
1021 AACAGCATGTGAAGGCACCGAGATGATGAGGGTACGTGTGGGTTCGGGGATAGGGAAGA
1081 AGAGAAGGACATCGAAGCTGCTTGCATTCGTGGTGACACGCGCTCCGCATCCTCTTCCT
1141 GTTGTCTTGAGGAATCGAAGAGCACAGTTCGCACAGCTGTTTCAGGAGTGAGTGGGTAT
1201 ACGAAACTGCGAAACGGCATCAACAATCCTGAATAAAATCCGCAGAACGTGCAGAAGGA
1261 GGAGAGGTGAGAAGCTCGGCAGTCTGAGGTGCGGCGGCCATACAGGCGGGAGGCGTGGG
1321 TGAATGTGATAAGAAATGTGGCGCCCAAGAGCCGACTCGCTTTTCTGTCAAGGCCCCGC
1381 GTCATTAGCTCGGCAACAAAGCTCGCTCGTCGTTTCAGCCTTTTTAGTGTCGTATCGTG
1441 GGCTTCCGCCGACGTGGGCTTGCGCTTGCTTCGAAGTCTGCCGCTAACAACTCCCTTCC
1501 TCGTTGTCCCTGCCGAAGAAGCCCTCACCTCACCTCTCTTCGCCCCTTCCCCTTCCGAG
1561 AGCTTTGTTTGTGTCGGCACAGCAACCCACGAGGGGTGTTCAGTGGCGCAGAAGTTCGG
1621 GCAGCAATGTGGGGATAGTGATACCTTAGCGTTGGGCCCCTAACTCGATGCGTTACCGT
1681 AGCAGAGGACACCACGTGCAGCAGCCCTGCATCCCGCAGCGTCTCAACGAGCAGCAGTT
1741 GTTCTCCGCCACATCCTCGGGATCGTCGAAGCCCGGGAAGGGCAGCGCAGCCACTAACA
1801 TGTCTTTGGGTAGCTCGATAGCAGTGTGGCAATTGCTGGCGCAAATTCGGCCTCAAAGC
1861 CAGCTCGCCGAGCTCCCCAGCGAGGCAGACGGCCGAATTTCGTCCAATGTGATAAACCC
1921                                                CTACTCCGCGTCAA
1921 CGCCTCGCCAGGCACGTTCAGCAACAACTGCATGCAGCTGCGAGAGATGAGGCGCAGTT
1981 ATT 5' Rev
1981 TAA
```

Figure 4.20: Sequence map showing the annealing positions of the primers on the *L. braziliensis* gene fragment.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Identification and characterization of *$PGF_{2\alpha}$* synthase gene in Old and New World Species of *Leishmania*

Using RT-PCR and western blot analyses, this study showed that *$PGF_{2\alpha}$* synthase - like gene is transcribed in Old World species *L. major*, *L. donovani* and *L. tropica*, but not in two of the New World species *L. amazonensis* and *L. mexicana*. The gene is however transcribed in *L. chagasi* but at low levels. The detection of transcription of *$PGF_{2\alpha}$* synthase - like gene in Old but not in most New World species of *Leishmania* suggests that New World *Leishmania* have evolved orthologous genes to produce $PGF_{2\alpha}$ or that ancestral *$PGF_{2\alpha}$* synthase gene is present in the New World species but has changed or been lost due to speciation during evolution. This is possible because the Old and New World parasites occupy different ecologic niches. *Leishmania* and other parasites are believed to have originated in Africa and were transported to Europe, Asia and the Americas during migration of man and animal reservoirs (Nozais, 2003). The breakup of Africa and South America has also been used to support the split between Old and New World species of *Leishmania* (Fernandes *et al.*, 1993) after which varied adaptations to changing environmental conditions led to the constitution of specific species.

It is interesting to note that *L. chagasi* which is a New World species transcribed the *$PGF_{2\alpha}$* synthase – like gene, albeit at low levels compared to the Old World species. *Leishmania chagasi* belongs to the *L. donovani* species complex which also includes *L. archibaldi*, *L. donovani*, and *L. infantum.* Quantitative comparisons of nuclear DNA fragment patterns to establish molecular evolutionary relationships within the major lineages of *Leishmania* indicate that the New World *L. chagasi* is very closely related to the Old World *L. infantum* and *L. donovani* indicating a recent separation (Beverley *et al.*, 1987) therefore this could explain the transcription of this gene in this New World species. Complete genome sequencing and comparison of the three *Leishmania* species *L. major*, *L. infantum* and *L. braziliensis* has shown that

degeneration of existing genes leading to loss of function accounts for about 80% of the species differences in *Leishmania* (Peacock *et al.*, 2007). However, it is not possible to deduce whether this gene is functional or a pseudogene.

One remarkable finding was the amplification of a 1983 nucleotide sequence in *L. braziliensis.* Preliminary analysis of this sequence revealed that this sequence lies on chromosome 31 of *L. braziliensis.* Following identification of possible open reading frames in this sequence, further analysis was done to predict the functions of these gene products. A protein BLAST of the first open reading frame (ORF-1) identified it to be a *L. braziliensis* protein whose function is unknown. Further analysis in the CDD database identified Cupin domain clusters in the protein sequence. The cupin name comes from a β barrel structural domain identified in a superfamily of prokaryotic and eukaryotic proteins. This superfamily is one of the most functionally diverse protein families known and is comprised of 20 families with members performing various functions ranging from enzymatic activities like dioxygenases, decarboxylases, hydrolases, isomerases and epimerases to non-enzymatic functions such as binding to auxin, nuclear transcription factors and seed storage (Agarwal *et al.*, 2009). Use of the ELM resource demonstrated the presence of a JmjC domain within this protein. Jumonji domains are part of the cupin metalloenzyme superfamily and this finding is consistent with the identification of cupin domains using the CDD database. Jumonji domain containing proteins are involved in regulation of chromatin and gene expression through various signaling pathways (Clissold & Ponting, 2001).

The analysis of ORF-1 translation also identified various motifs in the protein, several of them involved in cell cycle regulation, proliferation, differentiation and cell death. These include the 14_3_3 proteins. These are 28-33 kDa acidic polypeptides found in all eukaryotes. They are able to interact with a diverse array of proteins due to their phosphoserine/phosphothreonine binding activity and are therefore involved in cellular processes such as metabolism, apoptosis and cell cycle control (Morrison, 2009). Identification of the LIG_CYCLIN_1 motif indicates that the cognate protein interacts with cyclin. Cyclins are the activating partners of cyclin

dependent kinases (CDKs). These play important roles in the regulation of the eukaryotic cell cycle. Progression through the cell cycle depends upon the activation and deactivation of cyclin-dependent kinases. Each of these kinases is active for only a short period of the cell cycle during which time it phosphorylates a number of substrates required for entry into the next phase of the cell cycle (Takeda *et al.*, 2001). The FHA motif was also found in this protein translation. The FHA domain is a signal transduction module which is specific for phosphothreonine containing peptides on the ligand proteins. FHA domains are associated with proteins involved in many signaling processes but are especially prevalent in nuclear proteins that are involved in cell cycle checkpoint, DNA repair and transcriptional regulation (Durocher & Jackson, 2002).

Post translation modification site motifs MOD_CK1_1 and MOD_CK2_1 were identified in the protein translation. The MOD_CK1_1 motif is recognised by Casein Kinase 1 for serine/threonine (Ser/Thr) phosphorylation. The enzyme appears to have roles in cell division, DNA repair and glycogen metabolism (Cheong & Virshup, 2011) while MOD_CK2_1 motif is recognized by the ubiquitously expressed Casein Kinase 2 which is a messenger-independent Ser/Thr protein kinase with numerous related intracellular protein substrates. Many of these substrates are growth-associated proteins, evidence that implicates casein kinase 2 importance in cell signaling networks which are crucial to cell fate decisions (Hanif *et al.*, 2010).

Other post translation modification site motifs which were identified in the protein translation are the MOD_ProDKin_1 motif and the MOD_PIKK_1 motif. MOD_ProDKin_1 is the site at which Proline-directed kinases phosphorylate a Ser/Thr residue preceding a Pro residue. This site is recognised by a large family of MAP and CDK-like kinases. These cascades contain at least three protein kinases in a series that culminate in the activation of a multi-functional MAP kinase. MAP kinases are major components of pathways controlling embryogenesis, cell differentiation, cell proliferation, and cell death (Pearson *et al.*, 2001). The phosphoinositide-3-OH-kinase related kinases (PIKKs) are a group of ancient

eukaryotic protein kinases characterized by their large size (>200 KDa) and by the presence of a highly conserved phosphoinositide 3-kinase-like catalytic domain. PIKKs are the principal components of the DNA damage checkpoint pathway (Yang *et al.*, 2004). The LIG_PP1 motif was also identified. This Protein phosphatase 1 catalytic subunit (PP1) interacting motif binds targeting proteins that dock to the substrate for dephosphorylation. PP1 is a major Ser/Thr protein phosphatase of the eukaryotic cell. Reversible protein serine/threonine phosphorylation is an important component of the intracellular signaling machinery that controls many processes such as neurotransmission, muscle contraction, glycogen synthesis, T-cell activation, neuronal plasticity and cell proliferation (Aggen *et al.*, 2000).

The cognate protein of the ORF-1 translation may be involved in protein trafficking as evidenced by the presence of the LIG_Clathr_ClatBox_1 motif. Clathrin is the main component of the coat that decorates the cytosolic face of carrier vesicles mediating protein and lipid transport in the endocytic and late secretory pathways. Clathrin boxes are 5-residue-motifs found on cargo adaptor proteins, mostly near their C-terminus or central region. They are found in a large variety of accessory proteins involved in endocytosis and vesicular trafficking (Dell'Angelica, 2001). The presence of several LIG_USP7_1 motifs in the ORF-1 translation predicts the presence of a deubiquitinating system in the protein. Ubiquitin specific protease 7 (USP7), also known as HAUSP, is a deubiquitinating enzyme which cleaves ubiquitin moieties from its substrates. Ubiquitin specific protease 7 is a prominent member of the USP subfamily. It is involved in the regulation of stress response pathways, epigenetic silencing, cell survival pathway and the progress of infections by DNA viruses (Amerik & Hochstrasser, 2004). Related to this is the LIG_WW_4 motif which was also observed in the ORF-1 translation. WW domains are small modular domains of 38-40 residues long that mediate protein-protein interaction. WW domains recognize proline-containing ligands and fold as a stable, triple stranded β-sheet in absence of ligands or disulfide bridges. WW domain-containing proteins are involved in many cellular processes such as ubiquitin-mediated protein degradation and mitotic regulation (Macias *et al.*, 2002).

The N-glycosylation motif designated as MOD_N-GLC_1 was observed on the N-terminus of the protein translation. N-glycosylation is the most common modification of secretory and membrane-bound proteins in eukaryotic cells. N-linked glycosylation is a co-translational process involving the transfer of a oligosaccharide chain to asparagine residue in the protein. The MOD_SUMO motif was located in the protein predicting a sumoylation site. The SUMO proteins are small ubiquitin-related modifiers that are covalently conjugated onto lysine residues within target sequences. Sumoylation has an important function in nucleocytoplasmic trafficking. As well as controlling protein subcellular targeting, SUMO has a role in controlling gross subnuclear architecture. Finally, the control of transcription by sumoylation might also involve the recruitment of the modified transcription factors to particular subcellular domains (Seeler & Dejean, 2003).

The targeting site motif, TRG_ENDOCYTIC_2 which is the tyrosine-based sorting signal responsible for the interaction with mu subunit of Adaptor Protein (AP) complex motif was observed at the N terminus of the protein translation, covering amino acid residues 55-58. Intracellular traffic of many membrane-bound proteins requires sequences facing the cytoplasm. In many instances the sorting information is encoded in short peptide motifs, referred to as sorting signals. These motifs determine which vesicular traffic pathway is used to transport a particular molecule and hence determine its final destination. The YXXφ motif, where X is any amino acid and φ is a hydrophobic residue, is a sorting signal found in the cytosolic tail of various membrane proteins as an endocytic motif and also directs traffic within the endosomal and the secretory pathways. This motif interacts with mu subunit of AP complex (Bonifacino & Traub, 2003).

Other motifs involved in regulation of diverse cell functions were seen in the ORF-1 translation. These include MOD_PKA_1, MOD_PKA_2 and MOD_PKB_1 motifs. Protein kinase A (PKA) and protein kinase B (PKB) belong to a large set of related AGC kinases having a preference for phosphorylating basophilic sites. Cyclic AMP (cAMP) dependent PKA is the major target for cAMP action in eukaryotic cells. The enzyme is allosterically activated by binding of cAMP to two regulatory (R) subunits

which induces dissociation of two catalytic (C) subunits. The active kinase is then free to phosphorylate substrates on serine, or less commonly threonine residues in recognition motifs. The enzyme acts on a wide number of proteins involved in several biochemical processes including glucose metabolism, fatty acid synthesis and pyruvate oxidation. Protein kinase B (PKB/Akt/Rac-protein kinase) is a key player in the signaling pathways of numerous essential eukaryotic cellular processes including glucose metabolism, cell proliferation, apoptosis, cell migration and transcription (Brazil & Hemmings, 2001). Several MOD_GSK3_1 motifs recognised by glycogen synthase kinase (GSK3) for Ser/Thr phosphorylation were found. Glycogen synthase kinase comprises two highly related proteins (GSK3-alpha and GSK3-beta) that phosphorylate a wide variety of target proteins. Glycogen synthase kinase 3 has a key role in the regulation of many cell functions, including glycogen metabolism, signalling by insulin, growth factors and nutrients, and the specification of cell fates during embryonic development (Cohen & Frame, 2001).

Analysis of the second open reading frame (ORF-2) identified SMC protein domains. SMC proteins are a highly conserved and ubiquitous family of proteins found in eukaryotes and most prokaryotes. In eukaryotes SMC form several specialized protein complexes consisting of at least four essential and highly conserved subunits namely SMC1, SMC3, SCC1 and SCC3 whose functions include chromosome condensation, sister chromatid cohesion, DNA recombination and repair and sex chromosome gene dosage compensation in nematodes (Strunnikov & Jessberger, 1999). Homologs of separase and the components of the cohesin complex are all found in the trypanosome genome, and functional studies on SMC3, SCC1, and separase demonstrated that they are all essential for chromosome segregation (Gluenz *et al.*, 2008).

Eukaryotic liner motif analysis of ORF-2 translation identified several motifs which are involved in cell cycle regulation as seen in ORF-1. These include LIG_14_3_3_2 , LIG_14_3_3_3, LIG_CYCLIN_1, LIG_FHA, MOD_CK1_1, MOD_CK2_1 and MOD_PIKK_1. Motifs identified in the translation which are involved in regulation of diverse cell functions are the MOD_PKA_2 and MOD_GSK 3_1. Both have been

described for ORF-1. The MOD_N-GLC_1 motif involved in modification of secretory and membrane bound proteins was identified on the N-terminal of the protein while motifs implicated in protein trafficking were also identified. These are TRG_ENDOCYTIC_2 which has been described for ORF-1 and TRG_NES_CRM1_1. The Nuclear Export Signal (NES) is a linear motif involved in the regulated export of macromolecules from the nucleus via the nuclear pores. The import-export protein traffic through the nuclear envelope is mediated by soluble transport carriers called importins and exportins that bind to specific signals present within their substrates. The best-characterized export carrier is CRM1 (also known as exportin1/Xpo1), an evolutionarily well-conserved protein (Kutay & Guttinger, 2005). In summary, the motifs identified in the translations of both ORF-1 and ORF-2 belong to cell cycle regulation proteins, cell proliferation and differentiation, cell death as well as cellular trafficking. Taken together, presence of these motifs in the amplified gene segment implies that the translation product may not be a PGFS since none of these analyses are associated with PGFS.

In an effort to understand the outcome of the sequence analysis of the amplified gene segment from *L. braziliensis*, the primer sequence was analyzed. The results of this analysis indicated that that the reverse primer annealed on both the 5' and 3' positions of the *L. braziliensis* gene fragment while the forward primer did not anneal. Therefore, due to poor primer design, a non-specific product was amplified. The primer design for carrying out PCR in this study was based on the *L. major* PGFS ORF. Since the enzyme in this species has been well characterized, a comparison between this enzyme and ORF-1 and ORF-2 was made. A BLASTp search and ELM analysis identified Aldo-keto reductase superfamily domains within the *L. major* PGFS protein as expected. However, analysis of translations of ORF-1 and ORF-2 from *L. braziliensis* did not reveal any Aldo-keto reductase domains. This implies that ORF-1 and ORF-2 translations may not code for *L. braziliensis* PGFS. In addition, translations of ORF-1 and ORF-2 displayed numerous motifs, many of which are absent in *L. major* PGFS protein. This further supports the conclusion that the amplified gene, though being of *L. braziliensis* origin, is unlikely to be a *L. braziliensis* PGFS. The only motifs shared by the ORF-1 and ORF-2

translations with *L. major* PGFS protein include LIG_14_3_3_1, LIG_WW_ Pin1_4, MOD_CK1_1, MOD_CK2_1, MOD_GSK3_1 and MOD_ProDKin_1 motifs all located on the C-terminal of the protein. Finally, from these analyses, it is clear that although *L. major* PGFS protein shares some motifs with ORF-1 and ORF-2, it is from a different protein family.

Leishmania major PGFS belongs to the Ald-keto reductases (AKRs) superfamily. AKRs are a superfamily of soluble NAD (P) (H) oxidoreductases whose main function is to reduce aldehydes and ketones to primary and secondary alcohols. In a study to investigate the role of changes in RNA abundance during promastigote to amastigote differentiation in *L. donovani*, an SMC protein and prostaglandin F synthase were among the genes down regulated late in the differentiation process (Saxena *et al.*, 2007), hence both proteins seem to play significant roles in the promastigote stage as compared to the amastigote stage of the *Leishmania* parasite. Despite this common role, analysis of ORF-1 and ORF-2 from *L. braziliensis* does not show functional similarity to prostaglandin F synthase as was expected.

5.2 Identification and characterization of $PGF_{2\alpha}$ synthase protein in Old and New World Species of *Leishmania*

As a preliminary to the Western blot analysis, total protein from each of the *Leishmania* species was separated on a protein gel. The proteins showed similar banding patterns in all the species. Even though it is not possible to distinguish all the individual bands, a prominent band of 63 kDa appears in all the species. This may represent leishmanolysin or GP63, which is the most abundant surface protein of *Leishmania* promastigotes (Yao *et al.*, 2003).

*Tb*PGFS shares 61% amino acid identity with *Lm*PGFS (Kubata, *et al.*, 2000). Therefore, in this study, *Tb*PGFS was used to generate rabbit polyclonal antibodies for screening for *Leishmania* PGFS. Western blot analysis confirmed the presence of PGFS in Old World species and not in New World species. $PGF_{2\alpha}$ is transcribed at

very low levels in the New World *L. chagasi which* is closely related to *L. donovani* and *L. infantum.* However, this low level of transcription does not lead to any appreciable levels of expression. Despite the absence of PGFS on the western blot in the New World species of *Leishmania*, some of these parasites are known to produce $PGF_{2\alpha}$ (Kabututu, *et al.*, 2003). Similar observations have been described in trypanosomatids whereby the synthesis of $PGF_{2\alpha}$ is carried out by two different families of proteins. In the African trypanosome *T. brucei*, $PGF_{2\alpha}$ synthesis is catalyzed by an aldo keto reductase (Kubata, *et al.*, 2000). The aldo keto reductase superfamily consists of enzymes that catalyze redox reactions involved in biosynthesis, metabolism and detoxification (Barski *et al.*, 2008). On the other hand, $PGF_{2\alpha}$ synthesis in the American trypanosome *T. cruzi*, is catalyzed by *T. cruzi* old yellow enzyme (*Tc*OYE) which is a flavoprotein. This enzyme is also involved in the metabolism of trypanocidal compounds in the parasite with the generation of free radicals. This was discovered while searching for a $PGF_{2\alpha}$ synthase homologue in *T. cruzi*, when attempts to clone $PGF_{2\alpha}$ synthase gene and purify the protein from *T. cruzi* proved unsuccessful as the gene was a pseudogene (Kubata, *et al.*, 2002). Since genomic (RT-PCR) and serologic (western blot) results of this study demonstrates that PGFS is absent in the New world species of *Leishmania*, it is likely that a different enzyme is responsible for $PGF_{2\alpha}$ production representing a system analogous to that seen in trypanosomes.

During the course of this study, completed genomes of the four *Leishmania* species: *L. (L.) major*, *L. (L.) infantum* and *L. (V) braziliensis* and *L. mexicana* became publicly available at the GeneDB (http://www.genedb.org). Using sequence data, orthologues to *L. major* prostaglandin F synthase have been inferred in several kinetoplastids, namely *L. infantum* (LinJ.31.2210: pep), *L. braziliensis* (LbrM.31.2410: pep), *T. cruzi* (TcCLB.511287.49: pep, TcCLB.506213.50:pseudogenic_transcript: pep) and *T. brucei* (Tb11.02.2310: pep). In an effort to explain why the rabbit anti - *T. brucei* polyclonal antibody recognized the Old World and not the New World *Leishmania* species, pair wise analysis of the *L. major* PGFS and *L. braziliensis* PGFS obtained from the database was carried out. Analysis showed that these two proteins are virtually identical with an amino acid

identity of 92.5 %. This leads us to the conclusion that the PGFS protein is not expressed in the New World species. In addition, pair-wise analysis of the database *L. braziliensis* gene and that obtained and characterized in this study show no homology implying that what was amplified and characterized here is not the cognate *L. braziliensis* PGFS.

Whereas $PGF_{2\alpha}$ is associated with parturition (Xu, *et al.*, 2013) and luteolysis (Shirasuna, *et al.*, 2012) in mammals, its role in *Leishmania* parasites is not clear. However, studies have shown differential expression of this protein when promastigotes are subjected to drug pressure. In a study that carried out proteomic analysis on *L. major* promastigotes to determine the effect of Methotrexate (MTX) exposure and resistance on protein expression, $PGF_{2\alpha}$ synthase was one several proteins that was over-expressed in mutants. However, further experiments showed that $PGF_{2\alpha}$ synthase had no role in MTX resistance but was probably required for partial compensation of growth defects caused by that resistance (Drummelsmith *et al.*, 2004). In a similar study that carried out comparative proteomics of Pentavalent antimony (SbV) sensitive and resistant *L. tropica* promastigotes to identify proteins with differential expression, $PGF_{2\alpha}$ synthase was one of 4 proteins that were under expressed in resistant isolates (Hajjaran *et al.*, 2012). This suggests that $PGF_{2\alpha}$ synthase plays a role in the stress response of the parasite allowing the parasite to survive in the presence of drug pressure.

The vectors for *Leishmania* species are *Phlebotomus* sandflies for the Old World and *Lutzomyia* sandflies in the New World (Sharma & Singh, 2008). Thus, there exists a selective relationship between each parasite species and its vector based on genetic and ecological factors. For example, attachment of promastigotes to the midgut epithelium of the sand fly vectors which is an essential part of the *Leishmania* life cycle, is partly controlled by species-specific modifications of promastigote lipophosphoglycan (LPG) that selectively binds to the midgut galectin receptor (PpGalec) of the sand fly (Kamhawi *et al.*, 2004; Sacks, 2010). Since $PGF_{2\alpha}$ is more highly expressed in promastigotes than in amastigotes (Kidane, *et al.*, 1989) it is likely that selective factors in the sand fly vector could have played a role in the

divergence of $PGF_{2\alpha}$ synthase gene between Old and New World *Leishmania.* As this gene is expressed in Old World species and not in the New World species, perhaps this protein is essential in the former, but switched off in the latter for the successful metabolism and survival of the parasite in its vector.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

6.1.1 Identification of *$PGF_{2\alpha}$* synthase gene in Old and New World species of *Leishmania*

$PGF_{2\alpha}$ synthase - like gene is transcribed in promastigotes of Old World but not New World species of *Leishmania.*

6.1.2 Characterization of *$PGF_{2\alpha}$* synthase gene in Old and New World species of *Leishmania*

For the first time, the *$PGF_{2\alpha}$* synthase-like gene from *L. braziliensis* has been molecularly characterized.

6.1.3 Identification of $PGF_{2\alpha}$ synthase protein in Old and New World species of *Leishmania*

$PGF_{2\alpha}$ synthase - like protein is expressed in promastigotes of Old World but not New World species of *Leishmania*.

6.1.4 Characterization of $PGF_{2\alpha}$ synthase - like protein in Old and New World species of *Leishmania*

This study concludes that $PGF_{2\alpha}$ synthase is not expressed in promastigotes of New World species of *Leishmania*. Although *$PGF_{2\alpha}$* synthase gene has been identified in the genome of *L. braziliensis*, this study demonstrated that the protein is not expressed in the promastigote stage of this parasite.

6.2 Limitations

The design of the current work was not optimum. Whereas bioinformatic analyses of genomic data can enable prediction of protein function, it is not possible to comprehensively understand the function of a gene based on bioinformatics alone. A better design should have included aspects of phenotypic functional studies of the *L. braziliensis* protein including enzyme assays, protein-substrate binding interaction assays and proteomic approaches (immunoprecipitation assays) to ascertain presence and function of the $PGF_{2\alpha}$ synthase in *L. brazilliensis* parasites. Although, the Western blot was able to identify $PGF_{2\alpha}$ synthase – like protein in the three Old World species of *Leishmania*, enzyme kinetics should have been carried out on these proteins in order to ascertain that they are indeed the $PGF_{2\alpha}$ synthases.

6.3 Recommendations

Functional studies should be carried out to identify the active *L. braziliensis* PGFS and determine its role in the parasite with a view to explore it as a potential target for novel antileishmania therapy.

REFERENCES

Aara, N., Khandelwal, K., Bumb, R. A., Mehta, R. D., Ghiya, B. C., Jakhar, R., Dodd, C., Salotra, P. and Satoskar, A. R. (2013). Clinco-epidemiologic study of cutaneous leishmaniasis in Bikaner, Rajasthan, India. *Am J Trop Med Hyg*, **89**, 111-115.

Agarwal, G., Rajavel, M., Gopal, B. and Srinivasan, N. (2009). Structure-based phylogeny as a diagnostic for functional characterization of proteins with a cupin fold. *PLoS One*, **4**, e5736.

Aggen, J. B., Nairn, A. C. and Chamberlin, R. (2000). Regulation of protein phosphatase-1. *Chem Biol*, **7**(1), R13-23.

Akhoundi, M., Hajjaran, H., Baghaei, A. and Mohebali, M. (2013). Geographical distribution of *leishmania* species of human cutaneous leishmaniasis in Fars province, Southern Iran. *Iran J Parasitol*, **8**, 85-91.

Al-Tawfiq, J. A. and AbuKhamsin, A. (2004). Cutaneous leishmaniasis: a 46-year study of the epidemiology and clinical features in Saudi Arabia (1956-2002). *Int J Infect Dis*, **8**(4), 244-250.

Alexander, J., Satoskar, A. R. and Russell, D. G. (1999). *Leishmania* species: models of intracellular parasitism. *J Cell Sci*, **112**, 2993-3002.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol*, **215**, 403-410.

Alvar, J., Aparicio, P., Aseffa, A., Den Boer, M., Canavate, C., Dedet, J. P., Gradoni, L., Ter Horst, R., Lopez-Velez, R. and Moreno, J. (2008). The relationship between leishmaniasis and AIDS: the second 10 years. *Clin Microbiol Rev*, **21**, 334-359.

Alvar, J., Yactayo, S. and Bern, C. (2006). Leishmaniasis and poverty. *Trends Parasitol*, **22**, 552-557.

Amerik, A. Y. and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta*, **1695**(1-3), 189-207.

Angeli, V., Faveeuw, C., Delerive, P., Fontaine, J., Barriera, Y., Franchimont, N., Staels, B., Capron, M. and Trottein, F. (2001). *Schistosoma mansoni* induces

the synthesis of IL-6 in pulmonary microvascular endothelial cells: role of IL-6 in the control of lung eosinophilia during infection. *Eur J Immunol*, **31**, 2751-2761.

Arima, M. and Fukuda, T. (2008). Prostaglandin D_2 receptors DP and CRTH2 in the pathogenesis of asthma. *Curr Mol Med*, **8**, 365-375.

Ashford, R. W. (2000). The leishmaniases as emerging and reemerging zoonoses. *Int J Parasitol*, **30**, 1269-1281.

Bao, Y., Pucci, M. L., Chan, B. S., Lu, R., Ito, S. and Schuster, V. L. (2002). Prostaglandin transporter PGT is expressed in cell types that synthesize and release prostanoids. *Am J Physiol Renal Physiol*, **282**, F1103-1110.

Barski, O. A., Tipparaju, S. M. and Bhatnagar, A. (2008). The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metab Rev*, **40**(4), 553-624.

Bashaye, S., Nombela, N., Argaw, D., Mulugeta, A., Herrero, M., Nieto, J., Chicharro, C., Canavate, C., Aparicio, P., Velez, I. D., Alvar, J. and Bern, C. (2009). Risk factors for visceral leishmaniasis in a new epidemic site in Amhara Region, Ethiopia. *Am J Trop Med Hyg*, **81**, 34-39.

Bates, P. A. and Rogers, M. E. (2004). New insights into the developmental biology and transmission mechanisms of *Leishmania*. *Curr Mol Med*, **4**, 601-609.

Berman, J. J. (2008). Treatment of leishmaniasis with miltefosine: 2008 status. *Expert Opin Drug Metab Toxicol*, **4**, 1209-1216.

Bern, C., Adler-Moore, J., Berenguer, J., Boelaert, M., den Boer, M., Davidson, R. N., Figueras, C., Gradoni, L., Kafetzis, D. A., Ritmeijer, K., Rosenthal, E., Royce, C., Russo, R., Sundar, S. and Alvar, J. (2006). Liposomal amphotericin B for the treatment of visceral leishmaniasis. *Clin Infect Dis*, **43**, 917-924.

Bern, C., Joshi, A. B., Jha, S. N., Das, M. L., Hightower, A., Thakur, G. D. and Bista, M. B. (2000). Factors associated with visceral leishmaniasis in Nepal: bed-net use is strongly protective. *Am J Trop Med Hyg*, **63**, 184-188.

Beverley, S. M., Ismach, R. B. and Pratt, D. M. (1987). Evolution of the genus *Leishmania* as revealed by comparisons of nuclear DNA restriction fragment patterns. *Proc Natl Acad Sci U S A*, **84**, 484-488.

Boelaert, M., Rijal, S., Regmi, S., Singh, R., Karki, B., Jacquet, D., Chappuis, F., Campino, L., Desjeux, P., Le Ray, D., Koirala, S. and Van der Stuyft, P. (2004).

A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. *Am J Trop Med Hyg*, **70**, 72-77.

Bonifacino, J. S. and Traub, L. M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem*, **72**, 395-447.

Boussery, G., Boelaert, M., van Peteghem, J., Ejikon, P. and Henckaerts, K. (2001). Visceral leishmaniasis (kala-azar) outbreak in Somali refugees and Kenyan shepherds, Kenya. *Emerg Infect Dis*, **7**, 603-604.

Brandonisio, O., Panaro, M. A., Sisto, M., Acquafredda, A., Fumarola, L. and Leogrande, D. (2000). Interactions between *Leishmania* parasites and host cells. *Parassitologia*, **42**, 183-190.

Brattig, N. W., Schwohl, A., Rickert, R. and Buttner, D. W. (2006). The filarial parasite *Onchocerca volvulus* generates the lipid mediator prostaglandin E(2). *Microbes Infect*, **8**, 873-879.

Brazil, D. P. and Hemmings, B. A. (2001). Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem Sci*, **26**(11), 657-664.

Chaara, D., Haouas, N., Dedet, J. P., Babba, H. and Pratlong, F. (2014). Leishmaniases in Maghreb: An endemic neglected disease. *Acta Trop*, **132**C, 80-93.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J. and Boelaert, M. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol*, **5**, 873-882.

Cheong, J. K. and Virshup, D. M. (2011). Casein kinase 1: Complexity in the family. *Int J Biochem Cell Biol*, **43**(4), 465-469.

Clarke, C. F., Bradley, K. K., Wright, J. H. and Glowicz, J. (2013). Case report: Emergence of autochthonous cutaneous leishmaniasis in northeastern Texas and southeastern Oklahoma. *Am J Trop Med Hyg*, **88**, 157-161.

Clissold, P. M. and Ponting, C. P. (2001). JmjC: cupin metalloenzyme-like domains in jumonji, hairless and phospholipase A2 beta. *Trends Biochem Sci*, **26**(1), 7-9.

Cohen, P. and Frame, S. (2001). The renaissance of GSK3. *Nat Rev Mol Cell Biol*, **2**(10), 769-776.

Cole, A. C. E., Cosgrove, P. C. and Robinson, G. (1942). A preliminary report of an outbreak of kala-azar in a battalion of king's African rifles. *Trans R Soc Trop Med Hyg*, **36**, 25-34.

Cook, J. A. (2005). Eicosanoids. *Crit Care Med*, **33**, S488-491.

Costa, C. H. and Vieira, J. B. (2001). Changes in the control program of visceral leishmaniasis in Brazil. *Rev Soc Bras Med Trop*, **34**, 223-228.

Croft, S. L. and Coombs, G. H. (2003). Leishmaniasis--current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol*, **19**, 502-508**.**

Cruz, I., Chicharro, C., Nieto, J., Bailo, B., Canavate, C., Figueras, M. C. and Alvar, J. (2006). Comparison of new diagnostic tools for management of pediatric Mediterranean visceral leishmaniasis. *J Clin Microbiol*, **44**, 2343-2347.

David, C. V. and Craft, N. (2009). Cutaneous and mucocutaneous leishmaniasis. *Dermatol Ther*, **22**, 491-502.

Davies, C. R., Kaye, P., Croft, S. L. and Sundar, S. (2003). Leishmaniasis: new approaches to disease control. *BMJ*, **326**, 377-382.

de Menezes, G. B., dos Reis, W. G., Santos, J. M., Duarte, I. D. and de Francischi, J. N. (2005). Inhibition of prostaglandin $F_{2\alpha}$ by selective cyclooxygenase 2 inhibitors accounts for reduced rat leukocyte migration. *Inflammation*, **29**, 163-169.

Dell'Angelica, E. C. (2001). Clathrin-binding proteins: got a motif? Join the network! *Trends Cell Biol*, **11**(8), 315-318.

Descoteaux, A. and Turco, S. J. (1999). Glycoconjugates in *Leishmania* infectivity. *Biochim Biophys Acta*, **1455**, 341-352.

Dey, I. and Chadee, K. (2008). Prostaglandin E_2 produced by *Entamoeba histolytica* binds to EP4 receptors and stimulates interleukin-8 production in human colonic cells. *Infect Immun*, **76**, 5158-5163.

Dey, I., Keller, K., Belley, A. and Chadee, K. (2003). Identification and characterization of a cyclooxygenase-like enzyme from *Entamoeba histolytica*. *Proc Natl Acad Sci U S A*, **100**(2), 13561-13566.

Dinarello, C. A. (2004). Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. *J Endotoxin Res*, **10**, 201-222.

Drummelsmith, J., Girard, I., Trudel, N. and Ouellette, M. (2004). Differential protein expression analysis of *Leishmania major* reveals novel roles for methionine adenosyltransferase and S-adenosylmethionine in methotrexate resistance. *J Biol Chem*, **279**, 33273-33280.

Dumonteil, E. (2009). Vaccine development against *Trypanosoma cruzi* and *Leishmania* species in the post-genomic era. *Infect Genet Evol*, **9**, 1075-1082.

Durocher, D. and Jackson, S. P. (2002). The FHA domain. *FEBS Lett*, **513**(1), 58-66.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*, **32**, 1792-1797.

el-Safi, S. H. and Peters, W. (1991). Studies on the leishmaniases in the Sudan. 1. Epidemic of cutaneous leishmaniasis in Khartoum. *Trans R Soc Trop Med Hyg*, **85**(1), 44-47.

Elnaiem, D. E. (2011). Ecology and control of the sand fly vectors of *Leishmania donovani* in East Africa, with special emphasis on *Phlebotomus orientalis*. *J Vector Ecol*, **36** Suppl 1, S23-31.

Esch, K. J., Pontes, N. N., Arruda, P., O'Connor, A., Morais, L., Jeronimo, S. M. and Petersen, C. A. (2012). Preventing zoonotic canine leishmaniasis in northeastern Brazil: pet attachment and adoption of community *Leishmania* prevention. *Am J Trop Med Hyg*, **87**, 822-831.

Esfandiarpour, I., Farajzadeh, S., Rahnama, Z., Fathabadi, E. A. and Heshmatkhah, A. (2012). Adverse effects of intralesional meglumine antimoniate and its influence on clinical laboratory parameters in the treatment of cutaneous leishmaniasis. *Int J Dermatol*, **51**, 1221-1225.

Feliciangeli, M. D., Mazzarri, M. B., Blas, S. S. and Zerpa, O. (2003). Control trial of *Lutzomyia longipalpis s.l.* in the Island of Margarita, Venezuela. *Trop Med Int Health*, **8**, 1131-1136.

Fendall, N. R. (1961). The spread of kala-azar in Kenya. *East Afr Med J*, **38**, 417-419.

Fernandes, A. P., Nelson, K. and Beverley, S. M. (1993). Evolution of nuclear ribosomal RNAs in kinetoplastid protozoa: perspectives on the age and origins of parasitism. *Proc Natl Acad Sci U S A*, **90**, 11608-11612.

Ferroglio, E., Poggi, M. and Trisciuoglio, A. (2008). Evaluation of 65% permethrin spot-on and deltamethrin-impregnated collars for canine *Leishmania infantum* infection prevention. *Zoonoses Public Health*, **55**, 145-148.

Figarella, K., Rawer, M., Uzcategui, N. L., Kubata, B. K., Lauber, K., Madeo, F., Wesselborg, S. and Duszenko, M. (2005). Prostaglandin D_2 induces programmed cell death in *Trypanosoma brucei* bloodstream form. *Cell Death Differ*, **12**, 335-346.

Figarella, K., Uzcategui, N. L., Beck, A., Schoenfeld, C., Kubata, B. K., Lang, F. and Duszenko, M. (2006). Prostaglandin-induced programmed cell death in *Trypanosoma brucei* involves oxidative stress. *Cell Death Differ*, **13**, 1802-1814.

Fraga, T. L., Brustoloni, Y. M., Lima, R. B., Dorval, M. E., Oshiro, E. T., Oliveira, J., Oliveira, A. L. and Pirmez, C. (2010). Polymerase chain reaction of peripheral blood as a tool for the diagnosis of visceral leishmaniasis in children. *Mem Inst Oswaldo Cruz*, **105**, 310-313.

Freitas-Junior, L. H., Chatelain, E., Kim, H. A. and Siqueira-Neto, J. L. (2012). Visceral leishmaniasis treatment: What do we have, what do we need and how to deliver it? *Int J Parasitol Drugs Drug Resist.*, **2**, 11-19.

Fu, J. Y., Masferrer, J. L., Seibert, K., Raz, A. and Needleman, P. (1990). The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *J Biol Chem*, **265**, 16737-16740.

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*, **294**, 1871-1875.

Fusco, A. C., Salafsky, B. and Delbrook, K. (1986). *Schistosoma mansoni*: production of cercarial eicosanoids as correlates of penetration and transformation. *J Parasitol*, **72**, 397-404.

Gavgani, A. S., Hodjati, M. H., Mohite, H. and Davies, C. R. (2002). Effect of insecticide-impregnated dog collars on incidence of zoonotic visceral leishmaniasis in Iranian children: a matched-cluster randomised trial. *Lancet*, **360**, 374-379.

Ghosh, A. K. (2003). Regulation by prostaglandin E_2 and histamine of angiogenesis in inflammatory granulation tissue. *Yakugaku Zasshi*, **123**, 295-303.

Giunchetti, R. C., Correa-Oliveira, R., Martins-Filho, O. A., Teixeira-Carvalho, A., Roatt, B. M., de Oliveira Aguiar-Soares, R. D.,… and Reis, A. B. (2008). A

killed *Leishmania* vaccine with sand fly saliva extract and saponin adjuvant displays immunogenicity in dogs. *Vaccine*, **26**, 623-638.

Gluenz, E., Sharma, R., Carrington, M. and Gull, K. (2008). Functional characterization of cohesin subunit SCC1 in *Trypanosoma brucei* and dissection of mutant phenotypes in two life cycle stages. *Mol Microbiol*, **69**, 666-680.

Gonzalez, C., Rebollar-Tellez, E. A., Ibanez-Bernal, S., Becker-Fauser, I., Martinez-Meyer, E., Peterson, A. T. and Sanchez-Cordero, V. (2011). Current knowledge of *Leishmania* vectors in Mexico: how geographic distributions of species relate to transmission areas. *Am J Trop Med Hyg*, **85**, 839-846.

Goto, H. and Lindoso, J. A. (2010). Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Rev Anti Infect Ther*, **8**, 419-433.

Gregory, D. J., Sladek, R., Olivier, M. and Matlashewski, G. (2008). Comparison of the effects of *Leishmania major* or *Leishmania donovani* infection on macrophage gene expression. *Infect Immun*, **76**(3), 1186-1192.

Guerin, P. J., Olliaro, P., Sundar, S., Boelaert, M., Croft, S. L., Desjeux, P., Wasunna, M. K. and Bryceson, A. D. (2002). Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect Dis*, **2**, 494-501.

Guerra, J. A., Prestes, S. R., Silveira, H., Coelho, L. I., Gama, P., Moura, A., Amato, V., Barbosa, M. and Ferreira, L. C. (2011). Mucosal Leishmaniasis caused by *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) guyanensis* in the Brazilian Amazon. *PLoS Negl Trop Dis*, **5**, e980.

Gupta, R. A. and Dubois, R. N. (2001). Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nat Rev Cancer*, **1**, 11-21.

Haidaris, C. G. and Bonventre, P. F. (1983). Efficacy of combined immunostimulation and chemotherapy in experimental visceral Leishmaniasis. *Am J Trop Med Hyg*, **32**(2), 286-295.

Hajjaran, H., Azarian, B., Mohebali, M., Hadighi, R., Assareh, A. and Vaziri, B. (2012). Comparative proteomics study on meglumine antimoniate sensitive and resistant *Leishmania tropica* isolated from Iranian anthroponotic cutaneous leishmaniasis patients. *East Mediterr Health J*, **18**, 165-171.

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

Hanif, I. M., Hanif, I. M., Shazib, M. A., Ahmad, K. A. and Pervaiz, S. (2010). Casein Kinase II: an attractive target for anti-cancer drug design. *Int J Biochem Cell Biol*, **42**(10), 1602-1605.

Hasker, E., Kansal, S., Malaviya, P., Gidwani, K., Picado, A., Singh, R. P., Chourasia, A., Singh, A. K., Shankar, R., Menten, J., Wilson, M. E., Boelaert, M. and Sundar, S. (2013). Latent infection with *Leishmania donovani* in highly endemic villages in Bihar, India. *PLoS Negl Trop Dis*, **7**, e2053.

Herve, M., Angeli, V., Pinzar, E., Wintjens, R., Faveeuw, C., Narumiya, S., Capron, A., Urade, Y., Capron, M., Riveau, G. and Trottein, F. (2003). Pivotal roles of the parasite PGD_2 synthase and of the host D prostanoid receptor 1 in schistosome immune evasion. *Eur J Immunol*, **33**, 2764-2772.

Jha, T. K., Sundar, S., Thakur, C. P., Felton, J. M., Sabin, A. J. and Horton, J. (2005). A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. *Am J Trop Med Hyg*, **73**, 1005-1011.

Johnson, R. N., Ngumbi, P. M., Gachihi, G. S., Mwanyumba, J. P., Mbugua, J., Mosonik, N., Were, J. B. and Roberts, C. R. (1993). A new focus of kala-azar due to *Leishmania donovani sensu lato* in Kenya. *Trans R Soc Trop Med Hyg*, **87**, 142-144.

Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A. and Prescott, S. M. (1993). Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. *J Biol Chem*, **268**, 9049-9054.

Jowsey, I. R., Thomson, A. M., Flanagan, J. U., Murdock, P. R., Moore, G. B., Meyer, D. J., Murphy, G. J., Smith, S. A. and Hayes, J. D. (2001). Mammalian class Sigma glutathione S-transferases: catalytic properties and tissue-specific expression of human and rat GSH-dependent prostaglandin D_2 synthases. *Biochem J*, **359**, 507-516.

Kabututu, Z., Martin, S. K., Nozaki, T., Kawazu, S., Okada, T., Munday, C. J., Duszenko, M., Lazarus, M., Thuita, L. W., Urade, Y. and Kubata, B. K. (2003). Prostaglandin production from arachidonic acid and evidence for a 9,11-

endoperoxide prostaglandin H_2 reductase in *Leishmania*. *Int J Parasitol*, **33**, 221-228.

Kamhawi, S., Ramalho-Ortigao, M., Pham, V. M.,Kumar, S., Lawyer, P. G., Turco, S. J., Barillas-Mury, C., Sacks, D. L. and Valenzuela, J. G. (2004). A role for insect galectins in parasite survival. *Cell*, **119**(3), 329-341.

Kaul, V., Van Kaer, L., Das, G. and Das, J. (2012). Prostanoid receptor 2 signaling protects T helper 2 cells from BALB/c mice against activation-induced cell death. *J Biol Chem*, **287**(30), 25434-25439.

Kidane, G. Z., Samaras, N. and Spithill, T. W. (1989). Cloning of developmentally regulated genes from *Leishmania major* and expression following heat induction. *J Biol Chem*, **264**(7), 4244-4250.

Kilunga Kubata, B., Eguchi, N., Urade, Y., Yamashita, K., Mitamura, T., Tai, K., Hayaishi, O. and Horii, T. (1998). *Plasmodium falciparum* produces prostaglandins that are pyrogenic, somnogenic, and immunosuppressive substances in humans. *J Exp Med*, **188**, 1197-1202.

Kristensson, K., Nygard, M., Bertini, G. and Bentivoglio, M. (2010). African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. *Prog Neurobiol*, **91**, 152-171.

Kropf, P., Etges, R., Schopf, L., Chung, C., Sypek, J. and Muller, I. (1997). Characterization of T cell-mediated responses in nonhealing and healing *Leishmania major* infections in the absence of endogenous IL-4. *J Immunol*, **159**, 3434-3443.

Kubata, B. K., Duszenko, M., Kabututu, Z., Rawer, M., Szallies, A., Fujimori, K., Inui, T., Nozaki, T., Yamashita, K., Horii, T., Urade, Y. and Hayaishi, O. (2000). Identification of a novel prostaglandin $F_{2\alpha}$ synthase in *Trypanosoma brucei*. *J Exp Med*, **192**, 1327-1338.

Kubata, B. K., Kabututu, Z., Nozaki, T., Munday, C. J., Fukuzumi, S., Ohkubo, K., Lazarus, M., Maruyama, T., Martin, S. K., Duszenko, M. and Urade, Y. (2002). A key role for old yellow enzyme in the metabolism of drugs by *Trypanosoma cruzi*. *J Exp Med*, **196**(9), 1241-1251.

Kujubu, D. A., Reddy, S. T., Fletcher, B. S. and Herschman, H. R. (1993). Expression of the protein product of the prostaglandin synthase-2/TIS10 gene in mitogen-stimulated Swiss 3T3 cells. *J Biol Chem*, **268**, 5425-5430.

Kuroda, E., Sugiura, T., Zeki, K., Yoshida, Y. and Yamashita, U. (2000). Sensitivity difference to the suppressive effect of prostaglandin E_2 among mouse strains: a possible mechanism to polarize Th2 type response in BALB/c mice. *J Immunol*, **164**, 2386-2395.

Kutay, U. and Guttinger, S. (2005). Leucine-rich nuclear-export signals: born to be weak. *Trends Cell Biol*, **15**(3), 121-124.

Lawyer, P. G., Mebrahtu, Y. B., Ngumbi, P. M., Mwanyumba, P., Mbugua, J., Kiilu, G., Kipkoech, D., Nzovu, J. and Anjili, C. O. (1991). *Phlebotomus guggisberg*i (Diptera: Psychodidae), a vector of *Leishmania tropica* in Kenya. *Am J Trop Med Hyg*, **44**, 290-298.

Leal, L. M., Moss, D. W., Kuhn, R., Muller, W. and Liew, F. Y. (1993). Interleukin-4 transgenic mice of resistant background are susceptible to *Leishmania major* infection. *Eur J Immunol*, **23**, 566-569.

Leid, R. W. and McConnell, L. A. (1983). PGE_2 generation and release by the larval stage of the cestode, *taenia taeniaeformis*. *Prostaglandins Leukot Med*, **11**, 317-323.

Liu, L. X., Serhan, C. N. and Weller, P. F. (1990). Intravascular filarial parasites elaborate cyclooxygenase-derived eicosanoids. *J Exp Med*, **172**, 993-996.

Liu, L. X. and Weller, P. F. (1992). Intravascular filarial parasites inhibit platelet aggregation. Role of parasite-derived prostanoids. *J Clin Invest*, **89**, 1113-1120.

Maalej, I. A., Chenik, M., Louzir, H., Ben Salah, A., Bahloul, C., Amri, F. and Dellagi, K. (2003). Comparative evaluation of ELISAs based on ten recombinant or purified *Leishmania* antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. *Am J Trop Med Hyg*, **68**, 312-320.

Macias, M. J.,Wiesner, S. and Sudol, M. (2002). WW and SH3 domains, two different scaffolds to recognize proline-rich ligands. *FEBS Lett*, **513**(1), 30-37.

Marlet, M. V., Sang, D. K., Ritmeijer, K., Muga, R. O., Onsongo, J. and Davidson, R. N. (2003). Emergence or re-emergence of visceral leishmaniasis in areas of Somalia, north-eastern Kenya, and south-eastern Ethiopia in 2000-01. *Trans R Soc Trop Med Hyg*, **97**, 515-518.

Massamba, N. N., Mutinga, M. J. and Kamau, C. C. (1998). Characterisation of Leishmania isolates from Laikipia District, Kenya. *Acta Trop*, **71**(3), 293-303.

Mathur, P., Samantaray, J. C., Vajpayee, M. and Samanta, P. (2006). Visceral leishmaniasis/human immunodeficiency virus co-infection in India: the focus of two epidemics. *J Med Microbiol*, **55**, 919-922.

Mebrahtu, Y. B., Lawyer, P. G., Ngumbi, P. M., Kirigi, G., Mbugua, J., Gachihi, G., Wasunna, K., Pamba, H., Sherwood, J. A., Koech, D. K. and Roberts, C. R. (1992). A new rural focus of cutaneous leishmaniasis caused by *Leishmania tropica* in Kenya. *Trans R Soc Trop Med Hyg*, **86**(4), 381-387.

Meyer, D. J., Muimo, R., Thomas, M., Coates, D. and Isaac, R. E. (1996). Purification and characterization of prostaglandin-H E-isomerase, a sigma-class glutathione S-transferase, from *Ascaridia galli*. *Biochem J*, **313** (Pt 1), 223-227.

Minter, D. M., Wijers, D. J., Heisch, R. B. and Manson-Bahr, P. E. (1962). *Phlebotomus martini* a probable vector of kala-azar in Kenya. *Br Med J*, **2**, 835.

Miro, G., Checa, R., Montoya, A., Hernandez, L., Dado, D. and Galvez, R. (2012). Current situation of *Leishmania infantum* infection in shelter dogs in northern Spain. *Parasit Vectors*, **5**, 60.

Mohebali, M., Javadian, E., Yaghoobi-Ershadi, M. R., Akhavan, A. A., Hajjaran, H. and Abaei, M. R. (2004). Characterization of *Leishmania* infection in rodents from endemic areas of the Islamic Republic of Iran. *East Mediterr Health J*, **10**(4-5), 591-599.

Morita, I. (2002). Distinct functions of COX-1 and COX-2. *Prostaglandins Other Lipid Mediat*, **68-69**, 165-175.

Morrison, D. K. (2009). The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. *Trends Cell Biol*, **19**(1), 16-23.

Murakami, M., Nakatani, Y., Tanioka, T. and Kudo, I. (2002). Prostaglandin E synthase. *Prostaglandins Other Lipid Mediat*, **68-69**, 383-399.

Murray, H. W., Berman, J. D., Davies, C. R. and Saravia, N. G. (2005). Advances in leishmaniasis. *Lancet*, **366**, 1561-1577.

Mutero, C. M., Mutinga, M. J., Ngindu, A. M., Kenya, P. R. and Amimo, F. A. (1992). Visceral leishmaniasis and malaria prevalence in West Pokot District, Kenya. *East Afr Med J*, **69**, 3-8.

Nataraj, C., Thomas, D. W., Tilley, S. L., Nguyen, M. T., Mannon, R., Koller, B. H. and Coffman, T. M. (2001). Receptors for prostaglandin E_2 that regulate cellular immune responses in the mouse. *J Clin Invest*, **108**, 1229-1235.

Ngumbi, P. M., Kaburi, J. C., Anjili, C. O. and Haas, F. (2010). *Phlebotomus (Larroussius) orientalis* (Diptera: Psychodidae) as a probable secondary vector of visceral leishmaniasis in Kenya. *J Vector Borne Dis*, **47**, 58-60.

Niiro, H., Otsuka, T., Izuhara, K., Yamaoka, K., Ohshima, K., Tanabe, T., Hara, S., Nemoto, Y., Tanaka, Y., Nakashima, H. and Niho, Y. (1997). Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. *Blood*, **89**, 1621-1628.

Noverr, M. C., Erb-Downward, J. R. and Huffnagle, G. B. (2003). Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clin Microbiol Rev*, **16**, 517-533.

Nozais, J. P. (2003). The origin and dispersion of human parasitic diseases in the old world (Africa, Europe and Madagascar). *Mem Inst Oswaldo Cruz*, **98**, Suppl 1, 13-19.

Ntais, P., Sifaki-Pistola, D., Christodoulou, V., Messaritakis, I., Pratlong, F., Poupalos, G. and Antoniou, M. (2013). Leishmaniases in Greece. *Am J Trop Med Hyg*, **89**, 906-915.

O'Sullivan, M. G., Huggins, E. M., Jr., Meade, E. A., DeWitt, D. L. and McCall, C. E. (1992). Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. *Biochem Biophys Res Commun*, **187**, 1123-1127.

Okahara, K., Sun, B. and Kambayashi, J. (1998). Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells. *Arterioscler Thromb Vasc Biol*, **18**, 1922-1926.

Oliveira, A. L., Brustoloni, Y. M., Fernandes, T. D., Dorval, M. E., Cunha, R. V. and Bóia, M. N. (2009). Severe adverse reactions to meglumine antimoniate in the treatment of visceral leishmaniasis: a report of 13 cases in the southwestern region of Brazil. *Tropical doctor*, **39**, 180-182.

Olliaro, P. and Sundar, S. (2009). Anthropometrically derived dosing and drug costing calculations for treating visceral leishmaniasis in Bihar, India. *Trop Med Int Health*, **14**, 88-92.

Olson, D. M. and Ammann, C. (2007). Role of the prostaglandins in labour and prostaglandin receptor inhibitors in the prevention of preterm labour. *Front Biosci*, **12**, 1329-1343.

Peacock, C. S., Seeger, K., Harris, D., Murphy, L., Ruiz, J. C., Quail, M. A.,… and Berriman, M. (2007). Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nat Genet*, **39**, 839-847.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K. and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev*, **22**(2), 153-183.

Perkins, P. V., Githure, J. I., Mebrahtu, Y., Kiilu, G., Anjili, C., Ngumbi,…and Koech, D, K. (1988). Isolation of *Leishmania donovani* from *Phlebotomus martini* in Baringo district, Kenya. *Trans R Soc Trop Med Hyg*, **82**, 695-700.

Peters, W. and Killick-Kendrick, R.(1987). *The Leishmaniases in Biology and Medicine (* Academic Press*)*. London.

Qu, W. M., Huang, Z. L., Xu, X. H., Aritake, K., Eguchi, N., Nambu, F., Narumiya, S., Urade, Y. and Hayaishi, O. (2006). Lipocalin-type prostaglandin D synthase produces prostaglandin D_2 involved in regulation of physiological sleep. *Proc Natl Acad Sci U S A*, **103**, 17949-17954.

Ramaswamy, K., Kumar, P. and He, Y. X. (2000). A role for parasite-induced PGE_2 in IL-10-mediated host immunoregulation by skin stage schistosomula of *Schistosoma mansoni*. *J Immunol*, **165**, 4567-4574.

Rassi, Y., Oshaghi, M. A., Azani, S. M., Abaie, M. R., Rafizadeh, S., Mohebai, M., Mohtarami, F. and Zeinali, M. (2011). Molecular detection of *Leishmania* infection due to *Leishmania major* and *Leishmania turanica* in the vectors and reservoir host in Iran. *Vector Borne Zoonotic Dis*, **11**, 145-150.

Razmjou, S., Hejazy, H., Motazedian, M. H., Baghaei, M., Emamy, M. and Kalantary, M. (2009). A new focus of zoonotic cutaneous leishmaniasis in Shiraz, Iran. *Trans R Soc Trop Med Hyg*, **103**, 727-730.

Ready, P. D. (2010). Leishmaniasis emergence in Europe. *Euro Surveill*, **15**, 19505.

Reiner, N. E. and Malemud, C. J. (1985). Arachidonic acid metabolism by murine peritoneal macrophages infected with *Leishmania donovani*: *in vitro* evidence for

parasite-induced alterations in cyclooxygenase and lipoxygenase pathways. *J Immunol*, **134**, 556-563.

Reithinger, R., Dujardin, J. C., Louzir, H., Pirmez, C., Alexander, B. and Brooker, S. (2007). Cutaneous leishmaniasis. *Lancet Infect Dis*, **7**, 581-596.

Richard, J. V. and Werbovetz, K. A. (2010). New antileishmanial candidates and lead compounds. *Curr Opin Chem Biol*, **14**, 447-455.

Ryan, J. R., Mbui, J., Rashid, J. R., Wasunna, M. K., Kirigi, G., Magiri, C., Kinoti, D., Ngumbi, P. M., Martin, S. K., Odera, S. O., Hochberg, L. P., Bautista, C. T. and Chan, A. S. (2006). Spatial clustering and epidemiological aspects of visceral leishmaniasis in two endemic villages, Baringo District, Kenya. *Am J Trop Med Hyg*, **74**, 308-317.

Sacks, D. (2010). Molecular aspects of parasite–vector interactions in Leishmaniasis. In *National Institute of Allergy and Infectious Diseases, NIH*, 143-149. Springer.

Sacks, D. and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat Rev Immunol*, **2**, 845-858.

Samaras, N. and Spithill, T. W. (1989). The developmentally regulated P100/11E gene of *Leishmania major* shows homology to a superfamily of reductase genes. *J Biol Chem*, **264**, 4251-4254.

Sang, D. K.,Njeru, W. K. and Ashford, R. W. (1994). A zoonotic focus of cutaneous leishmaniasis due to *Leishmania tropica* at Utut, Rift Valley Province, Kenya. *Trans R Soc Trop Med Hyg*, **88**(1), 35-37.

Sang, D. K.,Okelo, G. B. and Chance, M. L. (1993a). Cutaneous leishmaniasis due to *Leishmania aethiopica*, on Mount Elgon, Kenya. *Ann Trop Med Parasitol*, **87**, 349-357.

Sang, D. K.,Okelo, G. B. and Chance, M. L. (1993b). Cutaneous leishmaniasis due to *Leishmania aethiopica*, on Mount Elgon, Kenya. *Ann Trop Med Parasitol*, **87**(4), 349-357.

Saxena, A., Lahav, T., Holland, N., Aggarwal, G., Anupama, A., Huang, Y., Volpin, H., Myler, P. J. and Zilberstein, D. (2007). Analysis of the *Leishmania donovani* transcriptome reveals an ordered progression of transient and permanent changes in gene expression during differentiation. *Mol Biochem Parasitol*, **152**, 53-65.

Schaefer, K. U., Kurtzhals, J. A., Sherwood, J. A., Githure, J. I., Kager, P. A. and Muller, A. S. (1994). Epidemiology and clinical manifestations of visceral and cutaneous leishmaniasis in Baringo District, Rift Valley, Kenya. A literature review. *Trop Geogr Med*, **46**, 129-133.

Seeler, J. S. and Dejean, A. (2003). Nuclear and unclear functions of SUMO. *Nat Rev Mol Cell Biol*, **4**(9), 690-699.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L. and Isakson, P. (1994). Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci U S A*, **91**, 12013-12017.

Sharma, U. and Singh, S. (2008). Insect vectors of *Leishmania*: distribution, physiology and their control. *J Vector Borne Dis*, **45**, 255-272.

Shibata, T., Kondo, M., Osawa, T., Shibata, N., Kobayashi, M. and Uchida, K. (2002). 15-deoxy-delta 12,14-prostaglandin J2. A prostaglandin D_2 metabolite generated during inflammatory processes. *J Biol Chem*, **277**, 10459-10466.

Shirasuna, K., Akabane, Y., Beindorff, N., Nagai, K., Sasaki, M., Shimizu, T., Bollwein, H., Meidan, R. and Miyamoto, A. (2012). Expression of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor and its isoforms in the bovine *corpus luteum* during the estrous cycle and $PGF_{2\alpha}$-induced luteolysis. *Domest Anim Endocrinol*, **43**(3), 227-238.

Singh, S. (2006). New developments in diagnosis of leishmaniasis. *Indian J Med Res*, **123**, 311-330.

Sivakumar, R., Sharma, P., Chang, K. P. and Singh, S. (2006). Cloning, expression, and purification of a novel recombinant antigen from *Leishmania donovani*. *Protein Expr Purif*, **46**, 156-165.

Smith, W. L., DeWitt, D. L. and Garavito, R. M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem*, **69**, 145-182.

Sommer, A., Rickert, R., Fischer, P., Steinhart, H., Walter, R. D. and Liebau, E. (2003). A dominant role for extracellular glutathione S-transferase from *Onchocerca volvulus* is the production of prostaglandin D_2. *Infect Immun*, **71**, 3603-3606.

Southgate, B. A. and Oriedo, B. V. (1962). Studies in the epidemiology of East African leishmaniasis. 1. The circumstantial epidemiology of kala-azar in the Kitui District of Kenya. *Trans R Soc Trop Med Hyg*, **56**, 30-47.

Stauch, A., Duerr, H. P., Dujardin, J. C., Vanaerschot, M., Sundar, S. and Eichner, M. (2012). Treatment of visceral leishmaniasis: model-based analyses on the spread of antimony-resistant *L. donovani* in Bihar, India. *PLoS Negl Trop Dis*, **6**, e1973.

Stothard, P. (2000). The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques*, **28**, 1102, 1104.

Strelkova, M. V., Eliseev, L. N., Ponirovsky, E. N., Dergacheva, T. I., Annacharyeva, D. K., Erokhin, P. I. and Evans, D. A. (2001). Mixed leishmanial infections in *Rhombomys opimus*: a key to the persistence of *Leishmania major* from one transmission season to the next. *Ann Trop Med Parasitol*, **95**(8), 811-819.

Strunnikov, A. V. and Jessberger, R. (1999). Structural maintenance of chromosomes (SMC) proteins: conserved molecular properties for multiple biological functions. *Eur J Biochem*, **263**, 6-13.

Sundar, S., Agrawal, N., Arora, R., Agarwal, D., Rai, M. and Chakravarty, J. (2009). Short-course paromomycin treatment of visceral leishmaniasis in India: 14-day vs 21-day treatment. *Clin Infect Dis*, **49**, 914-918.

Takeda, D. Y., Wohlschlegel, J. A. and Dutta, A. (2001). A bipartite substrate recognition motif for cyclin-dependent kinases. *J Biol Chem*, **276**(3), 1993-1997.

Tilley, S. L., Coffman, T. M. and Koller, B. H. (2001). Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest*, **108**, 15-23.

Tonui, W. K., Mbati, P. A., Anjili, C. O., Orago, A. S., Turco, S. J., Githure, J. I. and Koech, D. K. (2001). Transmission blocking vaccine studies in leishmaniasis: I. Lipophosphoglycan is a promising transmission blocking vaccine molecule against cutaneous leishmaniasis. *East Afr Med J*, **78**, 84-89.

Urade, Y. and Hayaishi, O. (2000). Biochemical, structural, genetic, physiological, and pathophysiological features of lipocalin-type prostaglandin D synthase. *Biochim Biophys Acta*, **1482**, 259-271.

Velez, I. D., Gilchrist, K., Martinez, S., Ramirez-Pineda, J. R., Ashman, J. A., Alves, F. P.,… and Piazza, F. M. (2009). Safety and immunogenicity of a defined vaccine for the prevention of cutaneous leishmaniasis. *Vaccine*, **28**, 329-337.

Wahba, M. M. (2000). The influence of *Bacillus sphaericus* on the biology and histology of *Phlebotomus papatasi*. *J Egypt Soc Parasitol*, **30**, 315-323.

Walker, W. and Rotondo, D. (2004). Prostaglandin E_2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-gamma synthesis. *Immunology*, **111**(3), 298-305.

Wasunna, M. K., Rashid, J. R., Mbui, J., Kirigi, G., Kinoti, D., Lodenyo, H., Felton, J. M., Sabin, A. J., Albert, M. J. and Horton, J. (2005). A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya. *Am J Trop Med Hyg*, **73**, 871-876.

Watanabe, K. (2002). Prostaglandin F synthase. *Prostaglandins Other Lipid Mediat*, **68-69**, 401-407.

WHO. 2014. *Leishmaniasis Fact sheet*. Retrieved from http://www.who.int/mediacentre/factsheets/fs375/en.

Wijers, D. J. (1971). A ten years' study of kala-azar in Tharaka (Meru district, Kenya). I. Incidence studies from the records at Marimanti. *East Afr Med J*, **48**, 533-550.

Wijers, D. J. and Kiilu, G. (1984). Studies on the vector of kala-azar in Kenya, VIII. The outbreak in Machakos District; epidemiological features and a possible way of control. *Ann Trop Med Parasitol*, **78**, 597-604.

Wilson, J. E., Chandrasekharan, N. V., Westover, K. D., Eager, K. B. and Simmons, D. L. (2004). Determination of expression of cyclooxygenase-1 and -2 isozymes in canine tissues and their differential sensitivity to nonsteroidal anti-inflammatory drugs. *Am J Vet Res*, **65**(6), 810-818.

Xu, C., Long, A., Fang, X., Wood, S. L., Slater, D. M., Ni, X. and Olson, D. M. (2013). Effects of $PGF_{2\alpha}$ on the expression of uterine activation proteins in pregnant human myometrial cells from upper and lower segment. *J Clin Endocrinol Metab*, **98**(7), 2975-2983.

Yamashita, U. and Kuroda, E. (2002). Regulation of macrophage-derived chemokine (MDC, CCL22) production. *Crit Rev Immunol*, **22**, 105-114.

Yang, L., Dan, H. C., Sun, M., Liu, Q., Sun, X. M., Feldman, R. I.,…and Cheng, J. Q. (2004). Akt/protein kinase B signaling inhibitor-2, a selective small molecule

inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. *Cancer Res*, **64**(13), 4394-4399.

Yao, C., Donelson, J. E. and Wilson, M. E. (2003). The major surface protease (MSP or GP63) of *Leishmania sp*. Biosynthesis, regulation of expression, and function. *Mol Biochem Parasitol*, **132**, 1-16.

Zijlstra, E. E., Musa, A. M., Khalil, E. A., el-Hassan, I. M. and el-Hassan, A. M. (2003). Post-kala-azar dermal leishmaniasis. *Lancet Infect Dis*, **3**, 87-98.

APPENDICES

Appendix 1: KEMRI SSC APPROVAL

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ESACIPAC/SSC/100162 **8th March, 2012**

Janet Majanja

Thro'**Director, CBRD**
NAIROBI

REF: SSC No. 2228 (New) – Analysis of $PGF_{2\alpha}$ Synthetase in Old and New world species of *Leishmania*

Reference is made to the above mentioned protocol. The KEMRI SSC during its 188th meeting held on Tuesday March 6, 2012 made the following observations:-

1) The work proposed in the protocol had already been concluded. Subsequently, your application was considered as a request for retrospective approval.
2) The study was conducted as a research project for MSc course undertaken at ITROMID programme.
3) According to a support letter from your supervisor, the ITROMID programme did not require SSC and/or ERC approval of student proposals during the period which the protocol was developed.
4) Currently a letter showing Ethical approval is required by the ITROMID program as a pre requisite to submission of a thesis.
5) That you encountered significant challenges during the course of preparing your protocol and in the conduct of the study and had changes of supervisors in the course of the studies which resulted in the delay in completing your studies.
6) The study is of minimal risk as you did not work with human participants, but restricted to laboratory experimentation.

In view of the foregoing, the KEMRI SSC has agreed to give retrospective approval of the scientific work done in the study. However, it is important to note that this is not standard practice and any future research protocols should be reviewed and approval obtained before implementation of the study.
Kindly submit four (4) copies of the revised protocol for onward transmission to the ERC for consideration of ethical approval.

Sammy Njenga, PhD
SECRETARY, SSC

Appendix 2: KEMRI ERC APPROVAL

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **April 27, 2012**

TO: **Ms. JANET MAJANJA (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. KIMANI GACHUHI**
THE DIRECTOR, CBRD,
NAIROBI

7/5/12

Dear Madam,

RE: **SSC PROTOCOL No. 2208- REVISED (*INITIAL SUBMISSION*): ANALYSIS OF PGF2α SYNTHASE IN OLD AND NEW WORLD SPECIES OF LEISHMANIA**

This is to inform you that at the 200th meeting of the KEMRI Ethics Review Committee held on 17th April 2012, the above referenced proposal was discussed.

The Committee noted that:

1. The laboratory-based study was implemented in 2004 and that at the time, the requirement for ITROMID students to obtain scientific and ethics review and approval prior to implementation of a research project was not stipulated. Additionally, that you faced certain challenges in research project supervision.

2. The proposal was presented to the ITROMID faculty in February 2004.

3. The following publications have arisen from the study:
   (a) Molecular Basis for Prostaglandin Production in Hosts and Parasites.Kubata BK, Duszenko M, Martin KS, Urade Y. Trends Parasitol. 2007 Jul;23(7):325-31. Epub 2007 May 24.

(b) Analysis of PGF2α synthase in Old and New World Species of Leishmania. Majanja, J.; Kubata, B.K. International Journal of Infectious Diseases. v. 12(Suppl 1) 2008 p. e384

The Committee regrets that even though the study was laboratory based, you were not provided with timely information and guidance regarding research conduct and responsibilities that would foster responsible conduct of research. This is most unfortunate because the ERC cannot grant retrospective approval to a study which has either commenced or has been completed without prior ethical approval. It can however express an opinion on the ethical propriety or otherwise of such study. In the above case, the Committee is of the view that had the proposal been presented to it for review in 2004, when it should have been so presented, a favourable opinion would have been given and it would have been granted ethical approval.

Please seek further counsel from the ITROMID programme regarding the submission of your MSc thesis to the university.

Yours truly,

AMBROSE RACHIER,
CHAIR,
KEMRI ETHICS REVIEW COMMITTEE