Identification of *Leishmania tropica* and *Leishmania aethiopica* by DNA Sequencing and the Detection of *Leishmania* RNA Virus

Megan Thomas

A thesis submitted to the University of Lancaster
For the degree of
Master of Science (by research)
In Biomedical Science

Lancaster University
Faculty of Health and Medicine
Division of Biomedical and Life Sciences

January 2018

Declaration

This thesis is submitted to Lancaster University in accordance with the requirements of the degree Master of Science in the division of Biomedical and Life Science. I declare that the work in this thesis is original and carried out by the author. I confirm that this work has not been submitted to any institution wholly or partially for the award of any degree.

Acknowledgements

First, I would like to thank my supervisor, Professor Paul Bates for his help and support with my thesis. I am also grateful to him for giving me the opportunity to work on this project in the field of parasitology, an area I have been interested in for many years. I would also like to thank Michelle Bates for her help and guidance through my time in the lab. Finally, I would like to my family for their support and encouragement throughout this experience.

Table of Contents

Chapter Two - Literature Review

Chapter Three - Materials and Methods

List of Tables

List of Figures

Appendix List

List of Abbreviations

PCR	Polymerase chain reaction
PKDL	Post kala-azar leishmaniasis
DALY	Disability-adjusted life years lost
HAART	Highly active antiretroviral therapy
ATP	Adenosine triphosphate
WHO	World Health Organisation
AIDS	Acquired immune deficiency syndrome
HIV	Human immunodeficiency virus
PIs	Peptidase inhibitors
LRV	*Leishmania* Ribonucleic Acid Virus
ds	Double stranded
MLEE	Electrophoretic motilities of enzymes
MLMT	Multilocus microsatellite typing
RFLP	Restriction fragment length polymorphism
TLR3	Toll like Receptor 3
RNA	Ribonucleic Acid
RdRp	Dependent RNA polymerase
RNAi	RNA interference
siRNA	Small interfering RNA
mRNA	Messenger RNA
FBS	Foetal Bovine Serum
PBS	Phosphate-buffered saline
FRET	Fluorescence resonance energy transfer
IPCC	Intergovernmental Panel on Climate Change

Abstract

Leishmaniasis is a neglected tropical disease and a major cause of morbidity and mortality in impoverished countries. Leishmaniasis is a global disease and is clinically and epidemiologically diverse, with many different species of *Leishmania* affecting humans. The aim of this study was to investigate the classification of *L. tropica* and *L. aethiopica* as different species, and to check for the presence of LRV in their isolates. *L. tropica* and *L. aethiopica* are closely related species of *Leishmania* that infect humans; and there is some debate over whether they are indeed different species. The Leishmania RNA Virus is a double stranded RNA virus which infects *Leishmania* and is thought to increase the pathology of the disease and exacerbating mucosal developments. The methods used in this study were polymerase chain reaction and sequencing for the ITS1 gene and a dot blot to detect the presence of LRV. The results of the PCR were used for identification, the formation of phylogenetic trees, and a distance pairwise matrix. The PCR results supported the classification of *L. tropica* and *L. aethiopica* as different species, supporting previous results also used for the analysis. The results of the dot blot found LRV presence in the *L. aethiopica* isolates but no evidence of the virus in *L. tropica.* The identification of an infecting species of *Leishmania* and the clinical outcomes it can cause will help provide appropriate and affective treatment. The findings of this study also demonstrate the importance of species identification and the methods used.

Chapter One: General Introduction

1.1. *Leishmania* and Leishmaniasis

Leishmaniasis is "a neglected vector-borne tropical infection that is considered to be a disease of poverty" (Pace, 2014). An infectious disease is considered to be neglected when there is the absence of effective, affordable or accessible drug treatments. The demographic population for such diseases tend to be poor, living in developing countries, and so are often ignored by the pharmaceutical industry (Yamey, 2002). However, the increase in international travel has also led to an increase in the number of leishmaniasis cases in non-endemic countries (Field et al. 2010). This makes the parasitic infection even more important to address.

Leishmaniasis is a protozoal infection. Although well recognised, the disease has been shrouded in various mysterious names for a long time; such as Dumdum fever and kala-azar, meaning “black fever” in Hindi. The disease was first described in 1903 by Leishman and Donovan (Murray, 2002). Leishmaniasis is clinically and epidemiologically diverse, and has some of the highest morbidity and mortality rates amongst parasitic diseases. The disease can lead to various clinical manifestations, from localised skin ulcers to a lethal systemic disease. Cutaneous leishmaniasis is a vector-borne parasitic disease that is transmitted by the bite of sand flies. This disease results in one or more sores or nodules on the skin that frequently heal without treatment after a few months, but often resulting in scar formation. Cutaneous leishmaniasis is the most widely distributed manifestation of leishmaniasis (Kebede et al. 2013). Cutaneous leishmaniasis has recently been found to potentially be viscerotropic (Jacobson, 2013).

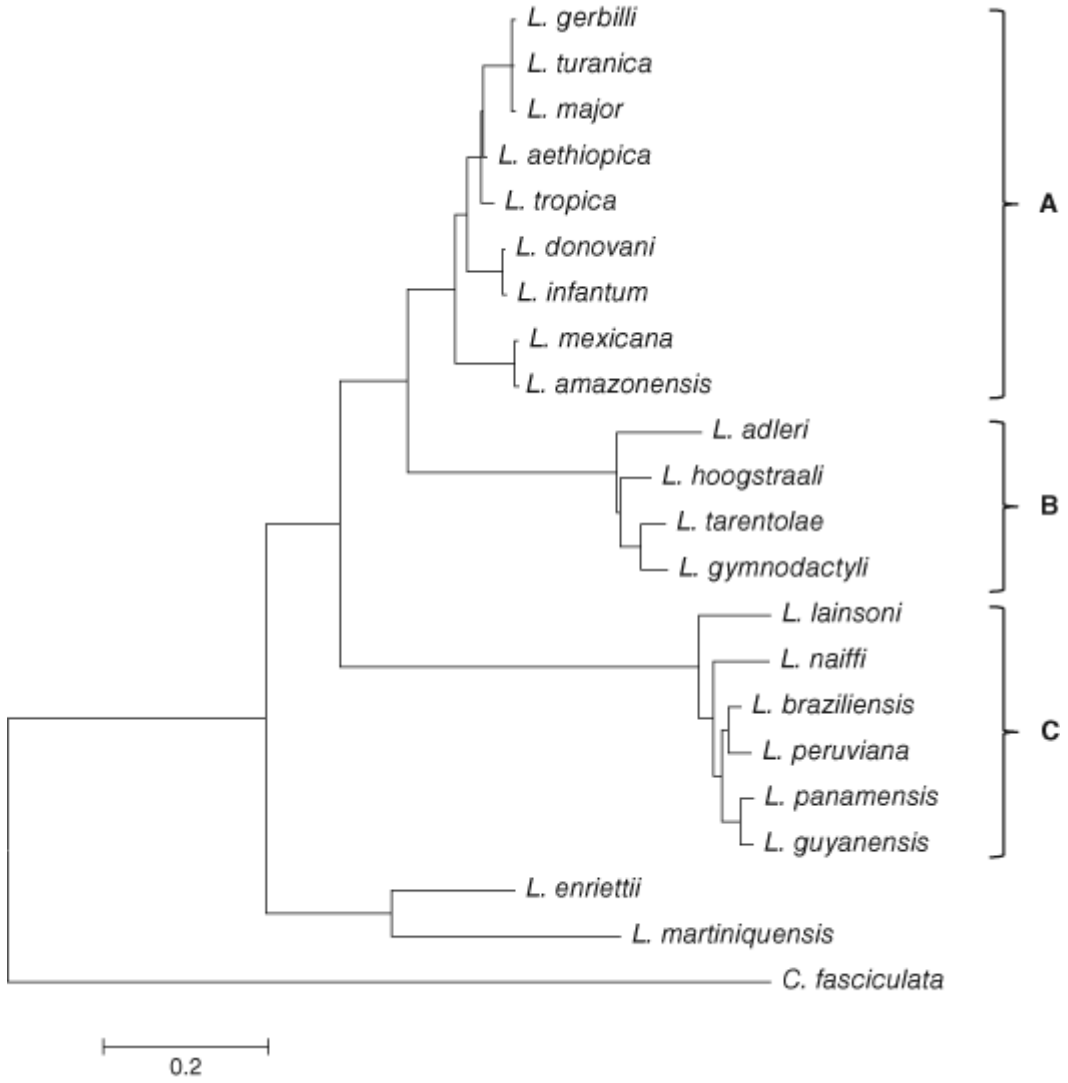


Figure 1.1 Phylogeny of *Leishmania*. Analysis of 21 species of *Leishmania* is shown, including 11 medically important species shown in Table 1.1. Grouping into subgenera (as of 2015) is also shown. A: Subgenus *Leishmania*; B: Sugenus *Sauroleishmania*; C: Subgenus *Viannia*; *Crithidia fasciculata* was used as the outgroup. Source: Bates, 2015.

Leishmaniasis is caused by over 20 different species of the genus *Leishmania*, as seen in Figure 1.1. *Leishmania* parasites are obligate intracellular protozoan parasites that are transmitted via the bite of an infected sand fly to their mammalian hosts, including humans. The *Leishmania* parasites are trypanosomatid protozoans, in which the parasite predominately infects the macrophages of the mammalian host (Weigle and Saravia, 1996). The parasitic disease affects both adults and children (Murray, 2002). The parasite is endemic in countries with a tropical or sub-tropical climate; and there is

variation in the species found across the various geographic locations. The classification of *Leishmania* into its subgenera, as seen in Figure 1.1, is based on the anatomical differences of the sites of parasite development in the gut of their sand fly hosts. The two main subgenera relevant to human disease are the New World *L. Viannia*, and the Old World *L. Leishmania*; New World parasites are found in the Americas and the Old World parasites are found across Asia, Africa and Europe (Pace, 2014). However, new research has proposed the existence of a new subgenus, *Leishmania (Mundinia),* including the species *L. enriettii* and some newly described species causing human disease in Ghana and Thailand (Espinosa et al. 2016). The epidemiologic features of the disease and appropriate control measures depend many factors. These include the geographic location, the infecting species of *Leishmania*, the reservoir host, and the biological aspects of the vectors (Bern et al. 2008). Factors that are associated with an increased risk of the disease progressing include malnutrition and a compromised immune system (Guerin et al. 2002); this means that the disease has a disproportional effect on the poor, and that immunocompromised individuals such as human immunodeficiency virus (HIV) patients have a higher risk of the disease. Leishmaniasis is considered to be one of the most neglected diseases (Yamey, 2002).

Table 1.1 Medically Important *Leishmania* Species and their Main Features

Species	Main disease(s) in humans	Notable features	Geographical distribution	Important mammalian hosts	Important sand fly hosts
Leishmania (*Leishmania*) *major*	Cutaneous (oriental sore)	Rural zoonotic	North Africa, Sahel of Africa, Central and West Asia	Great gerbil (*Rhombomys opimus*), fat sand rat (*Psammomys obesus*)	*Phlebotomus papatasi, Phlebotomus dubosqi, Phlebotomus salehi*
L. (*L.*) *tropica*	Cutaneous (oriental sore)	Urban anthroponotic	Central and West Asia	Humans	*Phlebotomus sergenti*
L. (L.) aethiopica	Cutaneous diffuse cutaneous	Rural zoonotic	Ethiopia, Kenya	Rock hyraxes *Herohyrax brucei* (*Procavia* spp.)	*Phlebotomus longipes, Phlebotomus pedifer*
L.(L.) donovani	Visceral (kala-azar)	Epidemic anthroponotic	Indian subcontinent, East Africa	Humans	*Phlebotomus argentipes, Phlebotomus orientalis, Phlebotomus martini*
L (*L.*) *infantum*	Infantile visceral	Zoonotic peridomestic	Mediterranean basin, Central and West Asia	Domestic dog	*Phlebotomus ariasi, Phlebotomus perniciosus*

Species	Main disease(s) in humans	Notable features	Geographical distribution	Important mammalian hosts	Important sand fly hosts
L. (L.) mexicana	Cutaneous (chiclero ulcer)	Sylvatic zoonotic	Central America	Forest rodents (*Ototylomys phyllotis* and others)	*Lutzomyia olmeca olmeca*
L. (L.) amazonensis	Cutaneous	Sylvatic zoonotic	South America	Forest rodents (*Proechimys* spp. and others)	*Lutzomyia flaviscutellata*
Leishmania (Viannia) braziliensis	Cutaneous mucocutaneous (espundia)	Sylvatic zoonotic	Central and South America	Forest rodents (*Akodon* spp., *Proechimys* spp. and others)	*Lutzomyia wellcomei*, *Lutzomyia complexus*, *Lutzomyia carrerai*
L. (V.) peruviana	Cutaneous (uta)	Upland zoonotic	Peru	Reservoir unknown, ?dog	*Lutzomyia peruensis*, *Lutzomyia verrucarum*
L. (V.) guyanensis	Cutaneous, often metastatic (pian-bois)	Sylvatic zoonotic	South America	Sloth (*Choloepus didactylus*), anteater (*Tamandua tetradactyla*)	*Lutzomyia umbratilis*

Species	Main disease(s) in humans	Notable features	Geographical distribution	Important mammalian hosts	Important sand fly hosts
L. (V.) panamensis	Cutaneous	Sylvatic zoonotic	Central America	Sloth (*Choloepus hoffmanni*)	*Lutzomyia trapidoi*

Source: Bates, 2015

1.2. The life cycle of *Leishmania*

The survival of the *Leishmania* parasite is dependent on successful transmission; zoonotic or anthroponotic; between the sand fly vector and mammalian reservoir (Pace, 2014). Leishmaniasis can be transmitted to humans in sylvatic, domestic or peridomestic cycles (Bern et al. 2008). The mammalian hosts and vectors responsible for transmission of some medically important species of *Leishmania* are shown in Table 1.1. Transmission of the parasite may follow an anthroponotic or zoonotic cycle, and this can vary by region. Anthroponotic transmission often occurs in urban areas via the bite of phlebotomine sand flies (Jacobson, 2013), and accounts for the largest number of human cases of leishmaniasis (Reithinger et al. 2003). In contrast, animals are the main reservoirs in rural areas with a zoonotic life cycle (Jacobson, 2013). Zoonotic transmission of the *Leishmania* parasite occurs in both domestic and sylvatic cycles. In domestic cycles the major reservoir is the dog. Sylvatic cycles occur in areas such as the rain forests of South America, whereas enzootic transmission occurs between wild animals in the deserts of Central Asia. In these cycles humans are a "dead end" host of the infection; however, the animal reservoir hosts can maintain enzootic transmission indefinitely without the human disease. For kala-azar and post kala-azar leishmaniasis (PKDL) humans are believed to be the sole reservoir in these anthroponotic cycles. This is also the case for the accompanying asymptomatic infection (Bern et al. 2008).

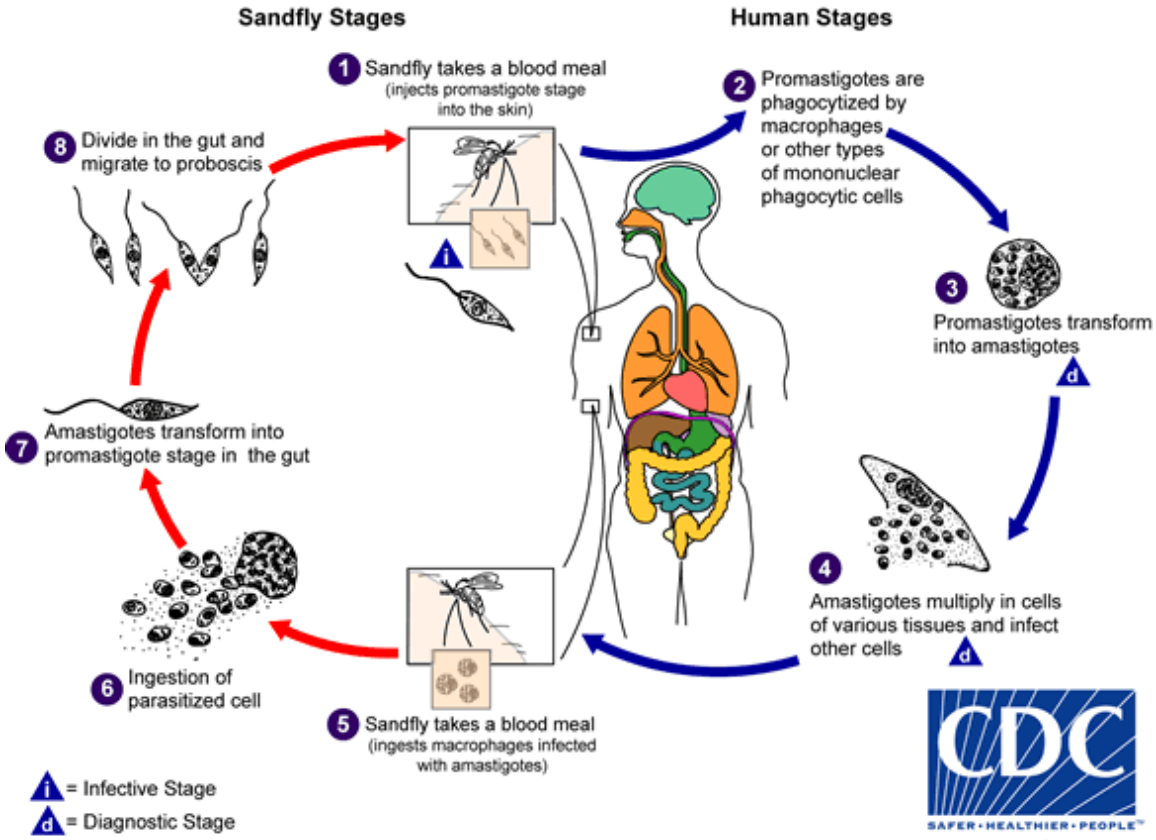


Figure 1.2 Life Cycle of *Leishmania.* Source: Centers for Disease Control and Prevention. (2013*). Parasites – Leishmaniasis: Biology*. [online] Available at: https://www.cdc.gov/parasites/leishmaniasis/biology.html. [Accessed June 30, 2017].

The skin is the point of entry for the *Leishmania* parasite (Murray, 2002) via the bite of the sand fly, as shown in Figure 1.2. There are two major morphological forms in the life cycle of *Leishmania*, as seen in Figure 1.3. One is an extracellular flagellated promastigote, which is 15-20µm long and found within the sand fly (Bates, 2008). The other is an obligate intracellular non-flagellated amastigote. It is 3-5µm long and found within the cell of the mammalian host monocyte-macrophage lineage (Hommel, 1999).

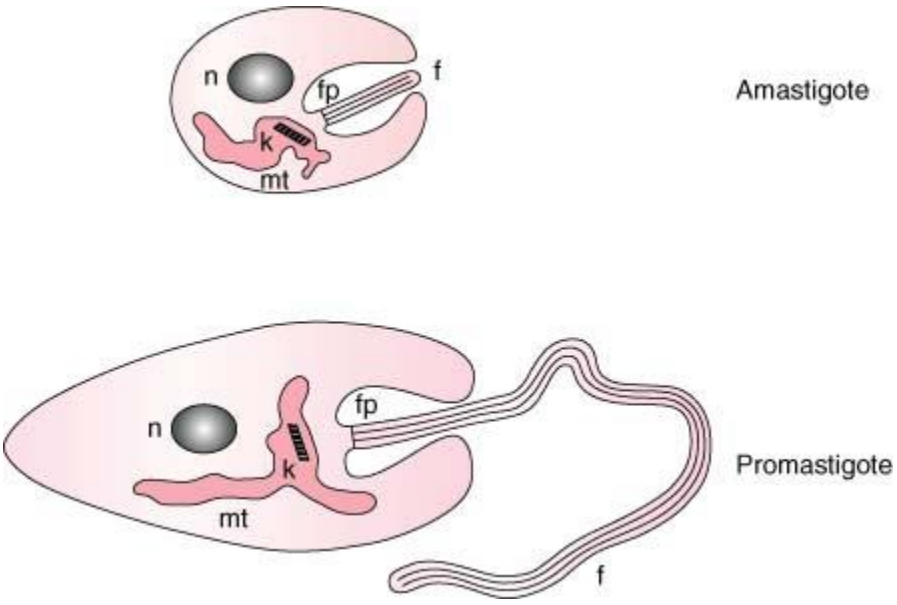


Figure 1.3 Developmental Forms of *Leishmania.* The two developmental form of *Leishmania*. Each form has a nucleus and kinetoplast in the single mitochondrion. The flagellum arises from the flagellar pocket. Source: Bates, 2015.

When infected with the *Leishmania* parasite, the spleen, liver, and bone marrow macrophages are selectively parasitized and support intracellular replication. The lymph nodes and skin are rarely involved (Murray, 2002). Amastigotes develop and multiply in the phagocyte cell until they are released by cell lysis in order to infect other macrophages (Hommel, 1999). Intracellular amastigotes are also known as Leishman-Donovan bodies, particularly in diagnostic tropical medicine. The typical characteristic of Leishman-Donovan bodies is the kinetoplast, a structure that consists of mitochondrial DNA, which can be seen staining separately from the *Leishmania* nucleus in infected clinical specimens. After being ingested by the sand fly from a blood meal the amastigotes re-transform back into flagellated promastigotes. In vitro cultures of samples can be used to grow these promastigotes. The samples can be taken from infected humans, sandflies, or animal reservoirs (Santarém et al. 2014). Specific antibodies against the infection do develop, but they do not provide significant protection and the infection is likely to progress (Murray, 2002).

Genetic exchange of strains of *Leishmania* is believed to occur inside the female sand fly vector. The diversity of *Leishmania* species is thought to have mainly arisen due to the gradual accumulation of divergent mutations rather than by sexual recombination; however, there is a growing number of examples of naturally occurring strains of *Leishmania* that share genotypic markers from two recognised species. This provides circumstantial evidence for the occurrence of sexual recombination (Akopyants et al. 2009). Genetic exchange has been observed in other trypanosmatids which cause disease in humans, such as *Trypanosoma brucei* (Jenni et al. 1986). It has been observed (Akopyants et al. 2009) that *Leishmania* can undergo genetic exchange during growth and development when in the sand fly vector, and infectious-stage hybrid progeny can be transmitted to a mammalian host.

1.3. Vectors of *Leishmania*

The vectors responsible for transmitting the various *Leishmania* parasites are female sand flies. There are over 1000 species of sand fly; of this, 93 are known to spread leishmaniasis (Pace 2014). Sandflies are noiseless 2-3mm long insects in colours ranging from black to white. Unlike other *Diptera* species, their wings are characteristically positioned at an angle to their abdomen in a "V" shape (Killick-Kendrick, 1999). Female sand flies are haematophagous, and are responsible for disease transmission. In the Mediterranean region they are active in the warm summer months (Gálvez et al. 2010), whereas in tropical regions transmission is more related to patterns of rainfall. Most species of sand fly bite outdoors between dusk and dawn; however, there are some species that bite indoors and in the daylight. This alternative behavior impacts upon the methods of insecticide spraying that are utilized to control them (Killick-Kendrick, 1999). *Leishmania* infected sand flies can bite several times on the same host. This behavior increases the chance of transmission of the parasite to

a new host (Ready, 2013). Sand flies are only able to hop vertically in small distances. This means that sleeping higher off the floor makes an individual less likely to be bitten (Hewitt, 1998). The interaction between the type of infecting species of *Leishmania* and the host's immune response affects the expression of the disease (Alexander and Brombacher, 2012).

1.4. Incidence

The annual global incidence of leishmaniasis is over 2 million new cases (Manzano et al. 2011); with 0.2–0.4 million cases of visceral leishmaniasis and 0.7–1.2 million cases of cutaneous leishmaniasis (Manzano et al. 2014). Sporadic or epidemic cases of leishmaniasis occurs when human habitation moves into an area where there is the sylvatic cycle of the disease (Bern et al. 2008).

There has been an increase in serious outbreaks of visceral leishmaniasis over the last 20-30 years in Bangladesh, Sudan, and in particular in India (Murray, 2002). In the leishmaniasis endemic communities in Bihar, India, the transmission rate of *Leishmania* appears to be stable over time. The incidence of one surveillance site showed rates are approximately 2.5 per 1000 people per year (Barnett et al. 2005); however, other areas of India show outbreaks in some communities where the incidence is over 10% (Kumar et al. 1999). Patients with PKDL may maintain the infection between epidemics of kala-azar. Furthermore, in highly infected areas over half the population may have an asymptomatic leishmanial infection. This contributes to the spread of infection (Bern et al. 2007). The most recent epidemic of kala-azar in Sudan, lasting from 1984-1994, is estimated to have killed approximately 100,000 people (Seaman et al. 1996).

1.5. Geographical Distribution

Leishmaniasis is prevalent in 98 countries across the world (Alvar et al. 2012). As seen in figures 1.4 and 1.5, leishmaniasis is a global disease; however, visceral leishmaniasis and cutaneous leishmaniasis have differences in the countries that they affect the most. Human leishmaniasis is found in a wide range of environments; from rain forests to deserts and cities; and across all continents other than Australia and Antarctica. Despite the wide area in which leishmaniasis is found, the disease burden in humans is relatively concentrated (Bern at al. 2008).

Visceral leishmaniasis has a disproportionate impact on countries across South Asia, Brazil, and the Horn of Africa, shown in figure 1.5, with 90% of all cases occurring in India, Nepal, Brazil, Bangladesh and Sudan (Barnett et al. 2005). Even though the disease is endemic in over 60 countries, almost all of the approximately 500,000 new cases of symptomatic visceral leishmaniasis each year occur in the rural areas of these five countries. Recurring unmanaged epidemics in India and Sudan account for the majority of new cases each year (Murray, 2002). As with all leishmaniasis, visceral leishmaniasis predominantly affects poor and rural areas (Barnett et al. 2005). Bihar is one of the poorest and most densely populated states of India, and visceral leishmaniasis has been endemic and epidemic there for decades (Thakur, 2000). Cutaneous leishmaniasis most commonly occurs in South America, Africa, and Southwest Asia (Plourde et al 2012), shown in figure 1.4. The largest focus of cutaneous leishmaniasis worldwide is believed to be in Kabul. Kabul is estimated to have an incidence of 67,000 new cases per annum (Reithinger et al. 2003). This disease is often diagnosed in

military personal who have been in Southwest Asia. Several outbreaks have occurred in troops who have been deployed to Iraq and Afghanistan (Plourde et al 2012).

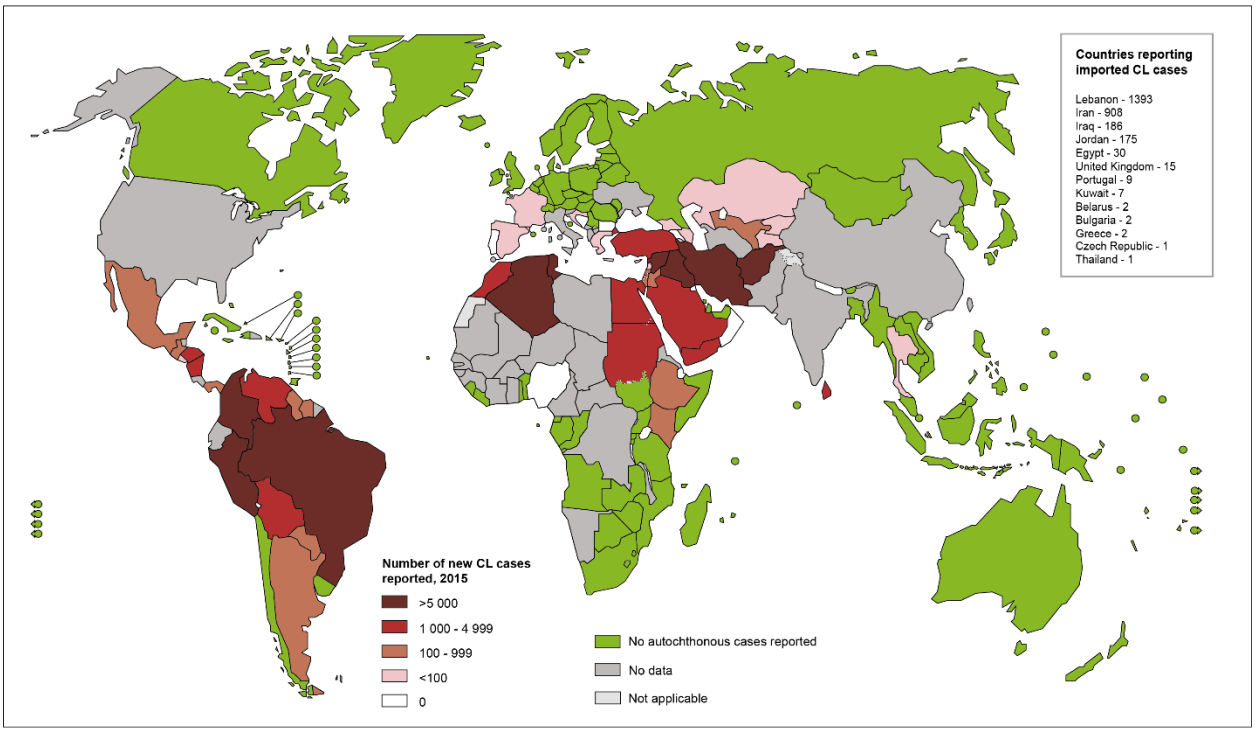


Figure 1.4 Geographical distribution of Cutaneous Leishmaniasis. Status of endemicity of cutaneous leishmaniasis worldwide, 2015. Source: World Health Organization. *Leishmaniasis, Epidemiological Situation*. [online] Available at: http://www.who.int/leishmaniasis/burden/en/ [Accessed 20 Jul. 2017).

Mucosal leishmaniasis in South America is predominant in regions south of the Amazon River and is most commonly caused by *Leishmania braziliensis* (Lainson, 1983); however, it is also common north of the Amazon River, in the Manaus region, where *Leishmania guyanensis* is the most common species of *Leishmania* (Romero et al. 2002).

Due to the limited resources invested into the diagnosis, treatment and control of leishmaniasis there is a strong association between the disease and poverty (Alvar et al. 2006). The main concentration of leishmaniasis is found in poor countries in South America, East Africa, and South East Asia; however, the disease is also endemic to several Mediterranean countries (Pace, 2014). This means the parasite is of importance for not only the local inhabitants but also for travelers.

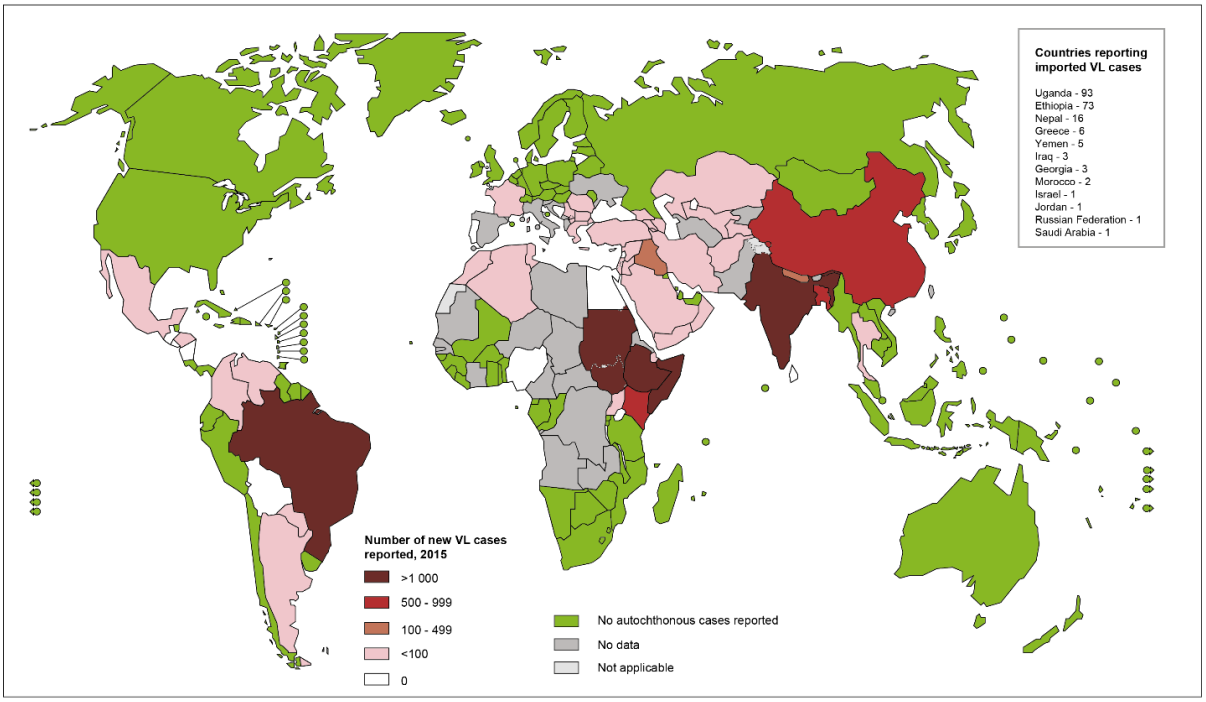


Figure 1.5 Geographical distribution of Visceral Leishmaniasis. Status of endemicity of visceral leishmaniasis worldwide, 2015. Source: World Health Organization. *Leishmaniasis, Epidemiological Situation*. [online] Available at: http://www.who.int/leishmaniasis/burden/en/ [Accessed 20 Jul. 2017).

Climate change as well as other environmental changes have the possibility of affecting geographical range of leishmaniasis and its vectors in the future (Patz et al. 2000).

1.6. Affected Population

In Bihar, India, visceral leishmaniasis often affects older children (Thakur, 2000); however, in Uttar Pradesh state in India there is an equally high risk for those over the age of 15. This is likely due to the fact that the disease has only recently started to affect Uttar Pradesh, and therefore few of the residents would have immunity, making children and adults equally at risk. It was also found that the greater household population density the greater the protective effect. (Barnett et al. 2005). In Bihar, 82% of those affected by the disease were engaged in agriculture and animal husbandry. Furthermore, 78% of patients lived in close proximity to cow sheds, and 22% brought domestic animals into their homes at night. The majority of the patients also lived in mud houses (Thakur, 2000).

The ratio of male to females affected by leishmaniasis seems to vary. In some areas of India the number of men affected can be far greater than that of women. This could be because the women in these regions wear more covering clothing than the men, even at night, and so are less vulnerable to sand fly bites. However, the difference in statistics between males and females may be due to fewer woman seeking or being sent for treatment due to its expense; and so are not accounted for (Thakur, 2000).

Malnutrition, a common consequence of poverty, impairs the immunity to visceral leishmaniasis and so increases vulnerability to the infection (Thakur, 2000). Various other reasons have also led to an increase in immunosuppressed individuals. These include HIV, post-transplant and chemotherapeutic patients, and biological therapies for chronic inflammatory conditions (Pace, 2014). This increase in immunosuppression has resulted in an increase in cases of leishmaniasis in Europe (Ready, 2010).

1.7. The burden of the disease

There are several factors that need to be taken into account when assessing the burden of a disease. The main aspects used to assess the disease burden are the incidence, prevalence, and mortality of the disease. Other measures are important in reflecting an accurate account of the burden of a disease; such as the severity of the disease, disability that may result from it, and what quality of life patients experience due to the disease (Murray et al. 1996). The measure that is most widely used when assessing the burden of a disease is "disability-adjusted life years lost" (DALY) (Mathers et al. 2007).

The disease burden of leishmaniasis is thought to place leishmaniasis as having the second highest mortality and the fourth highest morbidity amongst all tropical diseases (Mathers et al. 2007). Estimating the burden of leishmaniasis is difficult due to many factors; including the clinical and epidemiological diversity of the disease and the geographical distribution and clustering. The lack of reliable data regarding the incidence, duration and impact on various disease syndromes also contributes to the challenge of assessing the disease burden of leishmaniasis (Bern et al. 2008). The majority of the disease burden of leishmaniasis is found in poor countries; however, leishmaniasis is also endemic in several countries within Southern Europe (Pace 2014). Visceral leishmaniasis is an expensive disease to treat, and is often fatal if left untreated (Thakur, 2000). In the high disease endemic villages, visceral leishmaniasis is a major cause of death and impoverishment (Barnett et al. 2005). The mortality of leishmaniasis is second only to malaria amongst all parasitic diseases. In terms of disability adjusted life years (DALYs), leishmaniasis is the third most common cause of morbidity; the two most common being malaria and schistosomiasis. With leishmaniasis children under 15 years of age suffer most of the disease burden (Braga et al. 2013). The impact of the disease on the patients and their families is not limited to its effect on health.

There is social and psychological stigma due to the visible and often scarring lesions and disfigurement (Bern et al. 2005). The scars left by the cutaneous lesions caused by cutaneous leishmaniasis can have a severe social impact due to the strong stigmatization of those affected, especially in cases where the lesions and scars occur on the face or other exposed extremities (Kebede et al. 2013). Significant economic loss is also common (Bern et al. 2005). The impoverishment, disfiguration and social stigma are all non-health related effects of the disease which also add to the burden whilst making assessing it harder (Bern et al. 2008).

The DALY estimates for leishmaniasis are based on three factors: the assumed case-fatality rates; the assumed regional incidence and prevalence, and the disability weights assigned for cutaneous and visceral leishmaniasis. However, there are many uncertainties for all three of these factors due to a lack of documentation for these assumptions. Leishmaniasis is only considered to be a notifiable disease in 33 of the 98 endemic countries. Furthermore, significant underreporting of the disease is widely acknowledged (Desjeux, 2004); however, the magnitude of this has rarely been measured. The underreporting of leishmaniasis varies between the different clinical presentations of the disease; between countries, even in different regions of the same country due to the distance from health care, the availability of treatment, and the local awareness of the disease (Bern et al. 2008). Underreporting of the mortality of leishmaniasis is even more pronounced. This has been shown in various studies. One study in Sudan estimated that 91% of kala-azar deaths go unrecognized (Collin et al 2006); and a study based in a village in India found that as many as 20% of visceral leishmaniasis patients, disproportionately women and those in poverty, died before the disease was recognised (Barnett et al. 2005). Cases of mucosal leishmaniasis are focused in the Western Amazon, particularly south of the Amazon River. This is where

L. braziliensis is the predominant species of *Leishmania.* The patients with the disease in this area often work in areas of primary rainforest; typically involved in work related to the extraction of products from the rainforest. The diagnosis of cutaneous leishmaniasis early in the development of disease and access to the treatment of the disease are crucial to avoid mucosal leishmaniasis and other complications of infection from developing (Guerra et al. 2011). Often patients in these conditions do not receive adequate treatment and so mucosal leishmaniasis is neglected and is an important public health problem in the Brazilian Amazon (Lainson, 1985). Leishmaniasis is also considered an opportunistic disease in patients infected with HIV-1 (Santos et al. 2013).

1.8. Symptoms

Leishmaniasis is a disease known for its clinical diversity (Masood et al. 2012), it has a wide range of clinical manifestations, from self-healing cutaneous lesions to visceral disease (Manzano et al. 2011). Infection may be asymptomatic or may manifest as the cutaneous form, which if untreated may result in disfiguring scars; or the visceral form of the disease which if left untreated can be lethal (Pace, 2014). The cutaneous lesions that leishmaniasis causes tend to heal on their own; however, this may take from months to years, the healing time varying with the species of *Leishmania* that has infected the patient (Reithinger et al. 2007). There is an increasing number of new and atypical clinical variants of the disease (Masood et al. 2012).

The most common form of Leishmaniasis is cutaneous leishmaniasis. This can be caused by both New and Old World species of the parasite. Common infecting species that cause cutaneous leishmaniasis are *Leishmania tropica* and *Leishmania major,* Old World species, and *Leishmania braziliensis* and *Leishmania mexicana*, New World species (Reithinger et al. 2007). The cutaneous leisions caused by the disease can vary in their severity, clinical manifestation, and recovery time (Kebede et al. 2013).

Cutaneous leishmaniasis can be localised or severe and spread across the body (Pereira et al. 2013); however, the cutaneous lesions which characterise the disease typically develop at the site of the insect bite that caused the infection (Kebede et al. 2013), as seen in Figure 1.6.

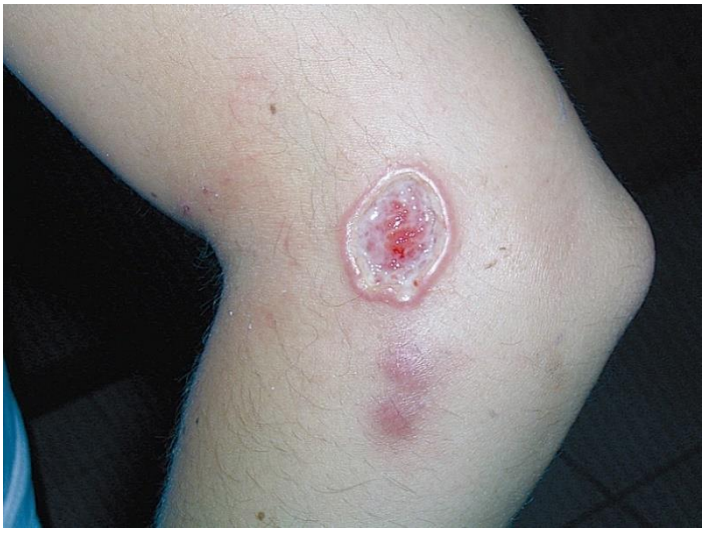


Figure 1.6 Cutaneous Leishmaniasis Lesion. Cutaneous lesion on the arm caused by *L. braziliensis.* There are two subcutaneous nodules below the lesion. Source: Schwartz et al. 2006.

In some cases cutaneous leishmaniasis persists and metastasizes to other areas of the body. This can cause two different clinical presentations of the disease, mucosal leishmaniasis and diffuse cutaneous leishmaniasis (Zangger et al. 1014).

Mucosal leishmaniasis is mainly due to the species *Leishmania braziliensis* (Marsden, 1986), and is associated with inadequate treatment of cutaneous leishmaniasis (Guerra et al. 2011). 1-10% of leishmaniasis infections may evolve mucosal manifestations (Pereira et al. 2013). It often occurs up to months or years after cutaneous leishmaniasis has healed. In addition to forming after cutaneous leishmaniasis is thought to have healed, mucosal forms of the disease can develop from asymptomatic *Leishmania* infections (Ives et al. 2011). The clinical features evolve slowly and commonly affect

the nasal cavity (Guerra et al. 2011). Mucosal leishmaniasis starts with a single cutaneous lesion; however, rather than metastasising to another cutaneous region, the infection metastasises to mucosal tissue. This causes chronic inflammation and disfiguring facial lesions (Zangger et al. 2014). In severe cases, mucosal leishmaniasis can cause destruction of mucosal structures such as the nasal septum and palate, as can be seen in Figure 1.7. This can lead to severe facial mutilation and, rarely, death due to airway involvement (Marsden, 1986).

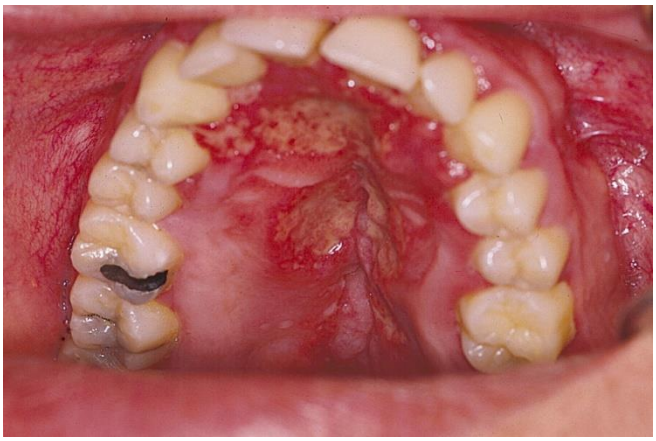
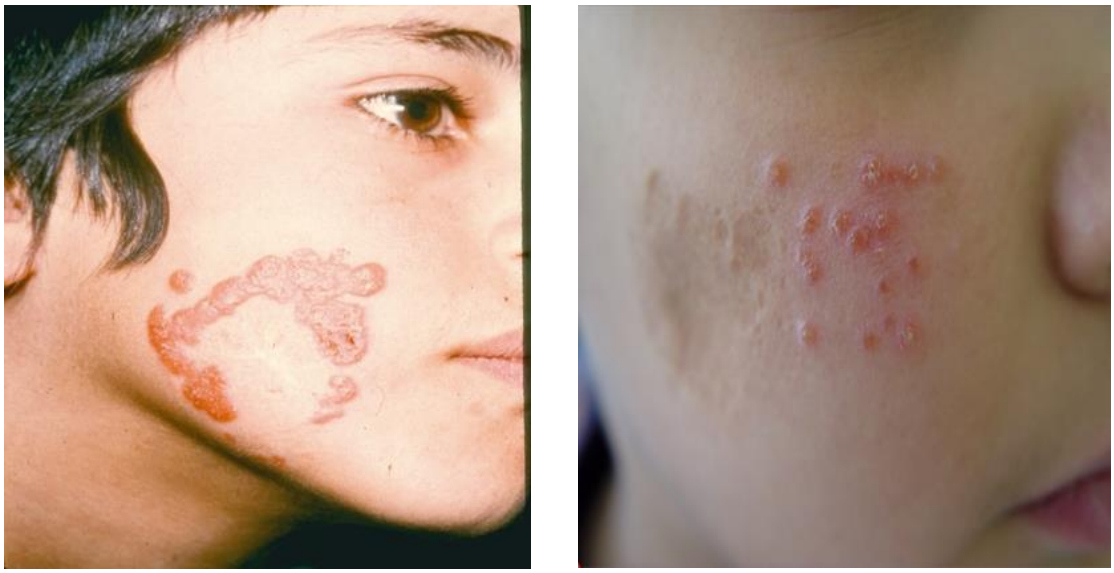


Figure 1.7 Mucosal Leishmaniasis Lesion. A mucosal lesion with ulcerated grey-yellow plaques on the upper palate, without perforation. Source: Schwartz et al. 2006.

Recently, the progression of the disease to mucosal leishmaniasis has also been associated with infections by *Leishmania guyanensis* (Guerra et al. 2011). The parasites genetic involvement in the control of oxidative stress potentially has a role in whether the disease develops metastatic complications. Leishmaniasis is an intracellular infection, and so oxidative destruction is a major mechanism of parasite eradication. The parasites ability to avoid this method of control may be a method of developing latency and metastasis (Hartley et al. 2012). Diffuse cutaneous leishmaniasis occurs when a cutaneous lesion metastasises to another cutaneous site. Patients with diffuse cutaneous leishmaniasis are often anergic in response to the antigens of the *Leishmania* parasite and tend to respond poorly to treatment. The mechanism by which the

Leishmania parasite causes these advanced pathologies of cutaneous leishmaniasis is unknown (Zangger et al. 2014).

First reported in 1923 (Semon & Christopherson, 1923), Leishmaniasis recidivans, also known as lupoid leishmaniasis in the Old World and leishmaniasis recidiva cutis in the New World, is a rare form of cutaneous leishmaniasis. Unusual clinical features and a chronic relapsing nature characterise leishmaniasis recidivans. The disease is an evolving form of cutaneous leishmaniasis, and clinically presents with spreading of the original nodule. This leads to the formation of a plaque (Masood et al. 2012), as seen in Figure 1.8 A. Leishmaniasis recidivans is both clinically and histologically similar to *lupus vulgaris*, complicating the diagnosis of the disease. The time at which leishmaniasis recidivans manifests after the initial cutaneous infection varies. The disease presents in the same area as the scar of the old clinically healed lesion. A clinical course of leishmaniasis recidivans is possibly related to changes in cell mediated immunity leading to localized or diffuse lesions.



A: Leishmaniasis recidivans with active papules on the face

B: Leishmaniasis recidivans showing a scar of on old cutaneous leishmaniasis lesion on the left and active papules on the right

Figure 1.8 Leishmaniasis Recidivans. Source: Sharifi et al. 2010

Visceral leishmaniasis tends to be caused by *Leishmania donovani* and *Leishmania infimum*. This form of the disease has several characteristic symptoms. There is a progressive fever and patients suffer weight loss. Splenomegaly and hepatomegaly are also seen in patients with visceral leishmaniasis, as well as hypergammaglobulinemia and pancytopenia (Bern et al. 2008). Many complications may occur in visceral leishmaniasis. These include immunosuppression and secondary bacterial infections, haemorrhaging and anaemia.

Kala-azar is a chronic systemic form of leishmaniasis. Its clinical characteristics are similar to those of visceral leishmaniasis, and eventually leads to death from bleeding or secondary infections (Collin et al. 2006). If left untreated, kala-azar rapidly progresses and is almost always lethal (Desjeux, 1996). Even when treated, kala-azar has a mortality rate of 10% or higher. Complications including jaundice, severe anaemia and co-infection with HIV are all factors which can increase the risk of mortality (Collin et al. 2004). If kala-azar occurs during pregnancy then foetal wastage and congenital leishmaniasis may also occur (Pagliano et al. 2005). Post-kala-azar dermal leishmaniasis (PKDL) is a chronic rash that is found in kala-azar patients who were thought to be cured in South Asia and Africa (Kaushal et al. 2016). PKDL presents with erythematous or sometimes hypo-pigmented macules, plaques or nodules. In Sudan, up to 60% of kala-azar patients develop PKDL (Zijlstra, 2006). Data for South Asia is lacking, but the cumulative incidence is thought to be around 10%-20% (Kaushal et al. 2006). In Sudan, PKDL has been reported to resolve itself without being treated in the majority of cases where the disease is mild; however, it often requires treatment in South Asia (Zijlstra, 2006).

The large diversity of clinical presentations of leishmaniasis is governed by the parasite and host factors, as well as immuno-inflammatory responses during the course of the

disease (Cordeiro da Silva et al. 2009). The variety of clinical syndromes that leishmaniasis can manifest with provides a challenge for diagnosis and treatment, as well as contributing to the burden of the disease in different ways. The factors that underlie the different clinical presentations of leishmaniasis, cutaneous, visceral, and mucocutaneous, are unknown (Stephenson 2007). Surprisingly, many *Leishmania* infections remain asymptomatic or sub-clinical (Murray, 2002).

When the disease is present in patients with HIV-1 or malnutrition it can alter the clinical course of the disease, as well as complicating therapeutic strategies. Leishmaniasis in HIV infected individuals who are not severely immunosuppressed manifests in a similar way as in immunocompetent individuals (Alvar et al. 2008). In HIV patients with CD4+ T lymphocyte counts <200 cells/µl, leishmaniasis may be more severe and can affect unusual sites including the gastrointestinal tract (Alvar et al. 1997). Without highly active antiretroviral therapy (HAART) the relapse rate after treatment is close to 100% (Alvar et al. 2008).

1.9. Diagnosis

There are various standard diagnostic procedures for cutaneous leishmaniasis. Microscopy or the demonstration of the parasite in culture are both used to detect the parasite in a skin smear or biopsy. However, these techniques are not sensitive enough to confirm all cases of cutaneous leishmaniasis, even when the assays are combined (Kebede et al. 2013). As systemic antibody responses are absent, serology is also insufficient as a diagnostic method for cutaneous leishmaniasis (Herwaldt, 1999). Moreover, these techniques cannot be used to distinguish between the different species and strains of *Leishmania* (Kebede et al. 2013).

Molecular techniques are also used to aid the diagnosis of leishmaniasis. Molecular techniques that can detect the parasite-specific DNA or RNA offer advantages in the sensitivity of the assay and speed of detection (Wilson, 1995). Multilocus enzyme electrophoresis is a classic standardised biochemical method. It is used to distinguish between the different species of *Leishmania*. For clinical diagnosis molecular techniques have recently been introduced. These methods are especially useful when used in areas where different *Leishmania* species co-exist (Foulet et al. 2007). These fast and accurate methods for the identification of the infecting parasite could facilitate the delivery of an appropriate treatment, adding to the attractiveness of molecular methods as a diagnostic strategy (Kebede et al. 2013). The development of a rapid and noninvasive diagnostic technique for visceral disease has been a significant advancement (Murray, 2002). The detection of anti-K39 antibodies by immunochromatographical strip testing can now guide clinical management accurately. The test only requires one drop of blood and can eliminate the need for a conventional diagnosis from splenic or bone marrow. It also eliminates reliance on potentially inaccurate microscopy (Sundar et al. 2002). The use of this technique may also aid in the detection of asymptomatic cases of leishmaniasis (Murray, 2002).

The diagnosis for mucosal leishmaniasis is made by either using the Montenegro skin test, serology and histopathology of the mucosal tissue from the patient, or by the isolation and identification of the parasites (Guerra et al. 2001). The best molecular technique that has been developed to aid the identification of the species of the infecting *Leishmania* parasite is the polymerase chain reaction (PCR) (Mimori et al. 1998), and is often used as a diagnostic method (Guerra et al. 2011). However, low amounts of DNA in the tissue can conceal the presence of different species.

The diagnosis of leishmaniasis recidivans can be complicated by the paucity of the microorganism in the direct smear and tissue specimens (Semon, H., & Christopherson, J. (1923). The clinical presentation of leishmaniasis recidivans resembles that of discoid lupus erythematosus (Masood et al. 2012) and lupus vulgaris, as well as some other gramulomatous disorders. Due to this the differential diagnosis of the disease can be extremely difficult because of the similarities between their clinical features (Bongiorno et al. 2009). This similarity means this is the most important differential diagnostic consideration.

1.10. Treatment

There is currently no vaccine against leishmaniasis. Also there is no consensus regarding what is the best therapeutic option for the treatment of the disease. This is largely due to a lack of properly controlled clinical trials (Gonzlez et al. 2008). The factors that make determining the disease burden of leishmaniasis difficult also contribute to the difficulty in producing a large-scale intervention against the disease. Leishmaniasis has a very diverse geographical distribution, and while the rate of the disease across a region may appear to be low, focal areas can be severely affected (Bern et al. 2005). Since cutaneous leishmaniasis generally heals itself, the treatment depends on many factors. These include the number and site of the lesions, the aetiology of the disease, and personal preferences. The main treatment used is chemotherapy (Plourde et al. 2012). Treatment of leishmaniasis relies on a few first-line drugs. These include miltefosine, paromomycin, and pentavalent antimonials (Manzano et al 2011). Leishmaniasis treatment is primarily based on pentavalent antimonials, seen in Figure 1.9, a treatment developed over 50 years ago. This treatment has poor therapeutic responses and adverse effects are common (Santos et al. 2013). Pentavalent antimonials

are no longer effective in Bihar, India, due to increasing drug resistance (Manzano et al. 2011).

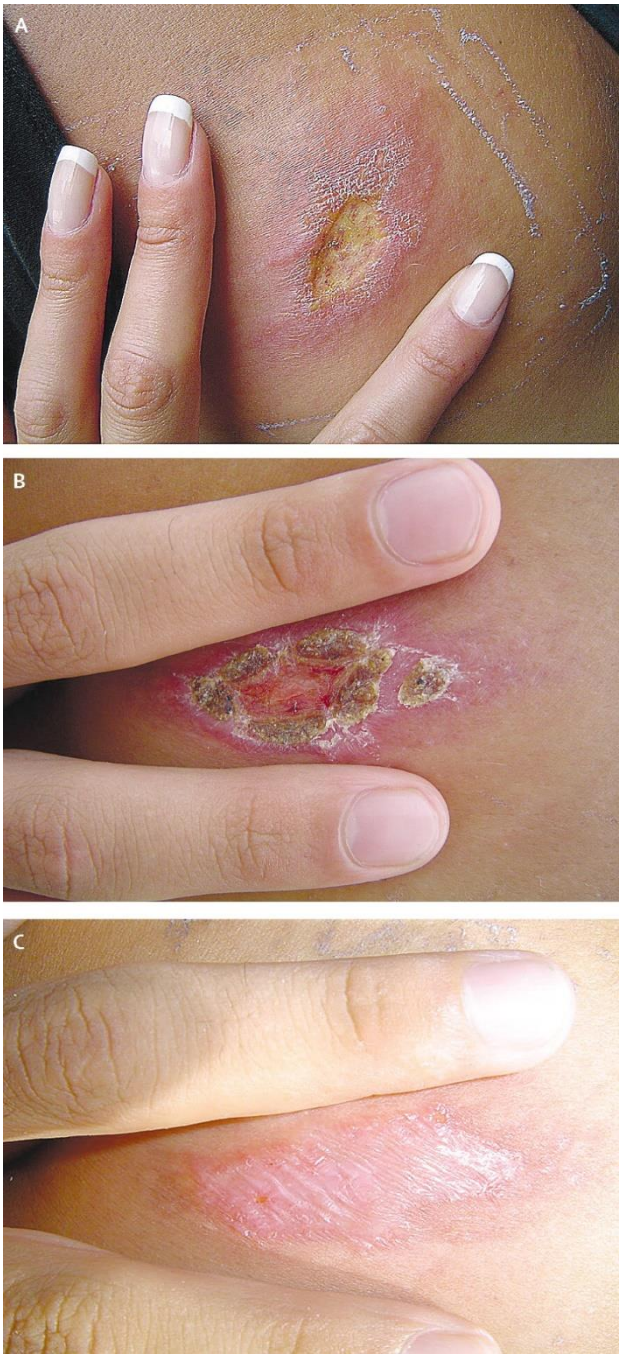


Figure 1.9 Antimonial Treatment of Cutaneous Leishmaniasis. The effect of treatment on a cutaneous lesion caused by *L. braziliensis.* A: Before treatment**;** B: At the end of 21 days of antimonial treatment**;** C: 1 month after end of treatment. Source: Schwartz et al. 2006

The main therapeutic options used for many years for the treatment of cutaneous leishmaniasis have relied on local or systemic administration of pentavalent antimony (Wortmann et al. 2002). Since *Leishmania* parasites are susceptible to heat, radio frequencies can be applied at the sites of the lesions to generate heat. This has also shown to yield a cure rate similar to systemic pentavalent antimony. In addition to the treatments currently available, effective oral treatments would benefit treatment of cutaneous leishmaniasis (Plourde et al. 2012). As previous treatments required injections over periods as long as four weeks (Murray, 2001) the establishment of effective oral treatments is of great importance (Murray, 2002). There is evidence of the benefit of the use of oral triazoles, such as itraconazole and fluconazole, in treating against *L. tropica* and *L. major*, respectively (Gonzlez et al. 2008). Another orally administered drug that has shown to be effective is miltefosine. It was shown to be effective in treating against cutaneous leishmaniasis in South America (Soto et al. 2004), and visceral leishmaniasis in India (Sundar, Jha et al. 2002); however, there is limited data about its efficacy against cutaneous leishmaniasis in Southwest Asia (Plourde et al. 2012). The introduction of new oral treatments in India should be of great benefit as around half of all leishmaniasis cases occur there and drug resistance has rendered treatment by pentavalent antimony largely useless (Murray, 2001).

The only treatment currently available for leishmaniasis is chemotherapy; however, its efficacy is increasingly limited due to the growing drug resistance to first-line drugs; especially antimonials (Manzano et al. 2014). One mechanism by which *Leishmania* is resistant to antimony involves a reduction in its accumulation. This is either by reduced uptake or increased efflux; the latter being mediated by ABC transporters (Brochu et al. 2003). ABC transporters are one of the largest families of proteins and are involved in a broad range of physiological roles which have major medical

consequences (Ouellette et al. 2004). ABC proteins are evolutionarily highly conserved and are found in difference species, ranging from prokaryotes to humans. These proteins use energy from adenosine triphosphate (ATP) hydrolysis to transport different compounds. This includes transporting a variety agents used to treat leishmaniasis; including antimonials and miltefosine, across biological membranes (Manzano et al. 2014).

Due to the limited number of active drugs for leishmaniasis the World Health Organisation (WHO) recommended combination therapy to extend the life expectancy of these compounds. As part of the search for cheaper oral treatments for visceral leishmaniasis new drugs, such as the 8-aminoquinolines, are being developed. The development of antiprotozoal drugs has used the 8-aminoquinoline scaffold extensively; generally as antiplasmodial compounds. Recently, sitamaquine and tafenoquine have been used as alternative leishmanicidal compounds. The introduction of these drugs is at differing stages due to differences in their haematological toxicity (Manzano, 2011). Tafenoquine is an 8-aminoquine analogue of primaquine. It is used in clinical trials for the treatment and prevention of *Plasmodium* infections (Walsh et al. 2004), and is a potential candidate for alternative leishmaniasis therapy. The drug has shown strong leishmanicidal activity *in vitro*. It affects the mitochondrial activity in the *Leishmania* parasites, which leads to an apoptosis-like death process (Carvalho et al. 2010).

In patients with HIV-1, highly active antiretroviral therapy (HAART) is used as a treatment and has shown a strong reduction in opportunistic infections; this includes opportunistic infections caused by parasites (Pozio and Morales, 2005). Despite this, patients that are coinfected with HIV and visceral leishmaniasis could be a reservoir for the development and spread of drug-resistant strains of *Leishmania* (Van Griensven

and Boelaert, 2011). Patients who do not receive HAART have a higher chance of developing the pathology of leishmaniasis. Furthermore, acquired immune deficiency syndrome (AIDS) treatment shows an increased risk of failure. Multiple anti-HIV drugs are used in HAART; including aspartic peptidase inhibitors (PIs). Aspartic peptidases are also found in the Trypanosomatidae family. Soluble extracts of *Leishmania amazonensis* have been shown to be capable of degrading synthetic substrates that are designed for aspartic peptidases. This degradation is eliminated by selective aspartic peptidase inhibitors (Santos et al. 2013).

The HIV PIs nelfinavir and loppinavir are aspartic peptidase inhibitors that have been shown to cause major alterations in several crucial points of the life cycle of *L. amazonensis.* These stages include proliferation, invasion of macrophages, ultrastructural aberrations and the expression of virulence factors (Santos et al. 2009). Other species of *Leishmania* have also shown to be effected by HIV PIs; including *Leishmania major, Leishmania mexicana, Leishmania braziliensis, Leishmania donovani,* and *Leishmania infantum.* There is not full agreement regarding the susceptibility of distinct species of *Leishmania* to HIV PIs. There are inconsistencies between reports, and it is still unknown if they are due to different methodologies, what kind of strains and species are assayed, and the drug origin (Santos et al. 2013).

1.11. Management and prevention

The eradication of leishmaniasis depends of satisfactory and easily available treatment and effective preventative measures (Murray, 2002). There are several control methods that are currently being deployed. These include the spraying of houses with residual insecticides and the culling of dogs. The early detection and treatment of infections is also of great importance in the management of leishmaniasis, including the detection

of PKDL (Guerin et al. 2002). In addition to these measures, newer procedures such as insecticide-impregnated bed nets and dog collars have shown promising effects (Murray, 2002).

A study into kala-azar in areas of south Sudan has found that only half of patients with kala-azar had access to treatment. Due to the absence of roads and vehicles the patients often have to reach health care on foot or be carried on stretchers, on journeys that often last several days. Many of the patients would be too unwell to make the journey and may die whilst attempting to travel to the treatment centers. Furthermore, access to treatment is further limited by the rainy season, impassable rivers, and sometimes by tribal or military conflicts (Collin et al. 2006). An epidemiological study of visceral leishmaniasis in the Indian state Uttar Pradesh found that the disease was commonly thought to be restricted to Bihar, and of little concern to Uttar Pradesh. Some villages knew of the disease, but in others few people had heard of kala-azar, its cause, and the symptoms it presents with. The doctors in the area knew little about visceral leishmaniasis and very rarely considered it in their differential diagnosis. Due to this, many cases of the disease often go unrecognized and untreated. With visceral leishmaniasis becoming an increasing danger in Uttar Pradesh management of disease needs to improve (Barnett et al. 2005).

The lack of development into a vaccine for leishmaniasis is largely due to the low investment into this neglected parasitic disease. Insight has been provided by research from the UK, Brazil and France into the genes involved in causing different forms of leishmaniasis, potentially aiding in the development of drugs and vaccines (Peacock et al. 2007). The high number of asymptomatic cases of leishmaniasis affects the management of the disease. What causes the disease to remain asymptomatic or subclinical indicates that the host-parasite relationship may favour the host (Murray,

2002). Investigation into the circumstances that lead to this could help with the development of a vaccine (Handman, 2001). The particular species of *Leishmania* that has infected the patient, and its resistance patterns, affects the efficacy of the treatment. The main preventative measures are aimed at avoiding sand fly bites. This is effective in reducing the contraction of leishmaniasis. Vector control measures are primarily based on insecticide-treated nets and indoor residual spraying (Killick-Kendrick, 1999). This is thought to greatly reduce the incidence of leishmaniasis (Bern et al. 2008). These measures need to be promoted to travelers who are visiting endemic regions. During the intensive DDT spraying for malaria in the 1950s and 1960s, the reported incidence of visceral leishmaniasis reduced to zero; however, a resurgence of the disease predominantly in northeastern Bihar occurred in the 1970s, with an explosive epidemic in the early 1990s (Barnett et al. 2005). A case study in Nepal found that, in addition to the use of nets arounds beds, the presence of cattle or buffalo helps protect against transmission of the *Leishmania* parasite; and that damp earthen floors was a risk factor (Bern et al. 2000). A study based in communities of Bangladesh identified that the availability of bed nets, even untreated ones, was a strong protective factor against the disease; and that proximity to a previous case of visceral leishmaniasis was the strongest indicator of risk of contracting the disease (Bern et al. 2005). In contrast to the studies in Nepal and Bangladesh, the study in Uttar Pradesh found little significant protective effect from the bed nets or mosquito coils. This could be due to only a few of the villagers using bed nets and statistical limitations of the study. Moreover, many people slept outside during the summer making the nets harder to use and the coils less effective (Barnett et al. 2005). A study in Bangladesh also found that an increase in the presence of cattle correlated with a lower human incidence rate (Bern et al. 2005). In contrast, a study in Sudan found that the presence of cattle increased the risk of

transmission (Bucheton et al. 2002) as did the Uttar Pradesh study. Cattle presence is likely to increase risk of leishmaniasis transmission, as the cattle manure is a preferred food source and habitat for sand fly larvae, and so its presence leads to an increased sand fly density (Dinesh et al. 2001). A possible explanation for the differing effects of cattle presence on infection rate could be sleeping habits. In the Bangladesh study community, less than 5% of the study participants slept outdoors; in contrast with 77% in participants in the study in Uttar Pradesh (Barnett et al. 2005).

In theory it is evident what needs to be done to control leishmaniasis. The rate of transmission needs to be reduced by vector control and the control of infected reservoirs. Resistance in the susceptible population needs to be induced via a vaccine. Many hopes are resting on the development of a vaccine as most transmission control efforts have been unsuccessful, unstable, or too expensive (Murray, 2002). If an effective and affordable vaccine were to be developed it could have a great impact on leishmaniasis. It could mean the successful immunization of animals (Handman, 2001) and increased resistance to the disease in humans (Murray, 2001). The identification of the infecting parasite species by genetic analysis can contribute to the understanding of leishmaniasis (Guerra et al. 2011). Poverty greatly increases the risk of leishmaniasis, and therefore anti-poverty and housing improvement programs could be implemented as part of a long term control strategy for the disease (Thakur, 2000). Leishmaniasis is increasing in the Northern Hemisphere. This is a result of tourism and armed conflicts in tropical regions (Amato et al. 2008).

1.12. Aims of the Study

The aims of this study are to examine the relationship between the *Leishmania* parasite species *Leishmania tropica* and *Leishmania aethiopica*. These species are thought to

be closely related and have a geographical overlap. In this study, PCR for the ITS1 sequence was applied to isolates of both species, and compared to results of the RPL23a sequence. Observing DNA sequences of the two species could help establish the phylogenetic distance between them. The other aim of this study was to investigate the presence of the *Leishmania* Ribonucleic Acid (RNA) Virus (LRV) in *L. tropica* and *L. aethiopica*. The LRV has previously been found in some isolates of *L. aethiopica,* but has not been found in *L. tropica*. The presence of the virus has been linked to metastatic developments in leishmaniasis, and so identification of its presence could aid diagnosis and appropriate treatment of the disease. Whether LRV is found in *L. tropica* could contribute to knowledge about the differences between *L. tropica* and *L. aethiopica,* and so has potential to improve diagnosis, treatment, and management of clinical infections. The presence of LRV was investigated using a double stranded (ds) RNA dot blot.

Chapter Two: Literature Review

2.1. *Leishmania tropica* and *Leishmania aethiopica*

There are at least 20 different species of *Leishmania* that are known to infect humans (Stephenson, 2007). Although host factors play an undeniable role in the course of leishmaniasis, parasite pedigree is the most reliable predictive tool of disease phenotype; which implies that heritable factors of the parasite are the most significant determinants of clinical variation of the disease (Hartley et al. 2012). The identification of the parasite is best done by using polymerase chain reaction (PCR)-based methods (Guerra et al. 2011). The species *Leishmania aethiopica* and *Leishmania tropica* are cutaneous leishmaniasis causing species of *Leishmania.* *L. tropica* and *L. aethiopica* appear to be related species; although their phylogenetic inter-relationship is only partially understood. Geographically, *L. aethiopica* is restricted to Ethiopia and Kenya; whereas *L. tropica* is more widely dispersed, ranging from the Eastern Mediterranean, through the Middle East into eastern India and in north, east and south Africa (Krayter et al. 2015). The predominant animal reservoirs in Ethiopia of *L. aethiopica* are the hyrax species *Procavia capensis* and *Heterohyrax brucei* (Ashford et al. 1973), and the giant pouch rat *Cricetomys sp.* is suspected to be a reservoir for *L. aethiopica* in addition to hyraxes (Alvar et al. 2012). *P. capensis* has also been found to be a reservoir for *L. tropica* in Israel (Svobodova et al. 2006) and Namibia (Grove, 1989). Leishmaniasis is a major health concern in South America, and a wide spectrum of the disease pathologies are present across the country. The infecting species of *Leishmania* affects the presentation of the clinical form of the disease (Alvar et al. 2012).

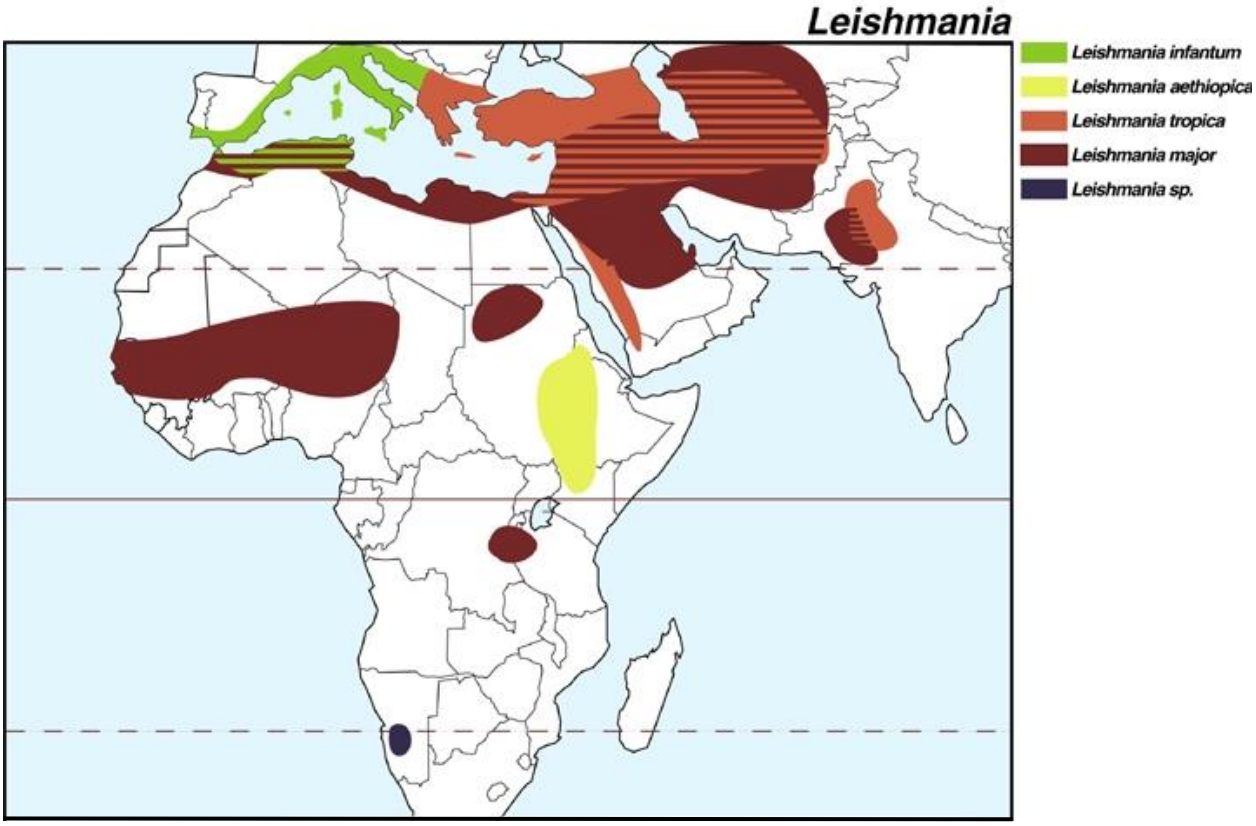


Figure 2.1 The geographical distribution of *Leishmania* species in the Old World including *L. tropica* and *L. aethiopica* in 1994. Source: Colour Atlas of Tropical Dermatology and Venerology

L. tropica is a causative agent of cutaneous leishmaniasis. For a long time *L. tropica* was mistaken for *L. major* and there was only one proven vector. In recent years more research has been made into the parasite (Jacobson, 2013). Different species of *Leishmania* can be found in the same region. Figure 2.1 shows a map of the geographical distribution of several Old World *Leishmania* species, including *L. tropica* and *L. aethiopica.* The map is from 1994 and is somewhat outdated. It shows no presence of *L. tropica* in Africa, where it has since been found; however, accurate distribution maps are difficult to obtain as the surveillance of the disease varies on a county by country basis. A map based on verified cases may not be an accurate representation of the disease. Before identification of species was easily and routinely done by molecular methods, the identification of clinical isolates was presumed based

on the species known to be present in the region or in similar habitats. Typing of clinical isolates was rarely done, and only occurred in larger institutions, and identification performed in the field would often be based on expectations. Improved surveillance and increased access to molecular tools are needed to produce a more accurate map of species distribution.

Leishmania major is the main cause of cutaneous leishmaniasis in Iraq; however, *L. major* and *L. tropica* have both been found to cause the disease in Afghanistan. Differences between the clinical features have been found between the species. The most apparent of these is that *L. tropica* tends to cause more chronic infections and may cause a progression of the disease to viscerotropic leishmaniasis, the systemic form of the disease. This requires special attention (Plourde et al. 2012). Typically, a specific species of *Leishmania* either has anthroponotic transmission or zoonotic transmission; however, *L. tropica* may be transmitted zoonotically although it predominantly has an anthroponotic cycle (Pace, 2014). Strains of *L. tropica* have displayed considerably serological, biochemical, molecular biological and genetic diversity. Cutaneous leishmaniasis caused by *L. tropica* is a public health threat in Morocco (Krayter et al. 2014). There are three species that cause human cutaneous leishmaniasis in Morocco, *L. major, L. infantum,* and *L. tropica.* Of these species, *L. tropica* is the most widely spread geographically (Rhajaoui et al. 2007). Cutaneous leishmaniasis caused by *L. tropica* infections has been increasing in Morocco, leading to it being considered a major public health hazard since 1997 (Alvar et al. 2012). There have also been isolated cases of canine cutaneous leishmaniasis and canine visceral leishmaniasis caused by *L. tropica* reported in Morocco (Krayter et al. 2014).

Cutaneous leishmaniasis is the main presentation of *Leishmania* infection in Ethiopia (Zangger et al. 2014). The incidence of cutaneous leishmaniasis in Ethiopia is

suggested to range from 20,000 to 50,000; however there is no accurate figure for the burden of the disease in the country (Alvar et al. 2012). Recently, there has been an increase in the reported number of cases of cutaneous leishmaniasis in Ethiopia (Negera et al. 2008). Leishmaniasis occurs in both rural and urban areas of Ethiopia (Kebede et al. 2013). The prevalence of the disease varies with location within the country, mostly being sporadic or endemic (Zangger et al. 2014). Four species of *Leishmania* are known to be the causative agents of cutaneous leishmaniasis in Ethiopia: *L. major, L. tropica, L. aethiopica,* and occasionally *L. donovani*, which causes visceral leishmaniasis more often (Krayter et al. 2015). *L. aethiopica* is considered to be the predominant etiological agent of cutaneous leishmaniasis in Ethiopia (Kebede et al. 2013). In addition to localised cutaneous leishmaniasis, *L. aethiopica* also causes mucosal leishmaniasis and diffuse cutaneous leishmaniasis (Ashford et al. 1973). Of these species of *Leishmania* that cause cutaneous leishmaniasis in Ethiopia, *L. aethiopica* is the only species that is restricted to Africa. This geographical restriction could be due to the restricted geographical range of its main sand fly vector species, *Phlebotomus longipes* and *Phlebotomus pedifer* (Alvar et al. 2012). *L. aethiopica* is one of the *Leishmania* parasites that causes cutaneous leishmaniasis and subsequently the mucosal and diffuse cutaneous forms of the disease (Zangger et al. 2014). The increasing cases of leishmaniasis in Ethiopia and the diverse clinical manifestations occurring there highlight the epidemiological significance of the disease in the country (Kebede et al. 2013). In Kenya, *L. aethiopica* has only been reported to cause localised cutaneous leishmaniasis (Alvar et al. 2012).

The *L. aethiopica* species was originally identified as *L. tropica* when it was first isolated from cases of localised and diffuse cutaneous leishmaniasis in humans. These strains were later described and named a new species, *L. aethiopica*, due to the various

criteria. Factors that were involved in the reclassification as a new species include the geographical restriction of human cases; the association between some of the strains and diffuse cutaneous leishmaniasis, previously only known to be caused by New World species; the specificity of the sand fly vectors and hyraxes being the animal reservoir hosts, and the antigenic specificity of the strains (Krayter et al. 2015). Serotyping using indirect haemagglutination was used to show the antigenic specificity of the strains (Bray et al. 1973), and was later corroborated by excreted factor (EF) serotyping (Schnur and Zuckerman, 1977). Over the last 30-40 years many strains of *L. aethiopica* from Kenya and Ethiopia have been serotyped according to the excreted factor that they produce. The results of this has shown all of the strains examined were found to be a EF sub-serotype that has so far been exclusive to the *L. aethiopica* species of *Leishmania*, the EF sub-serotype B_1. This has antigenically separated the strains from all other species of *Leishmania* (Krayter et al. 2015).

A close relationship between *L. aethiopica*, *L. tropica* and *L. major* has been suggested by phylogenetic studies on different cellular components of the parasites (Krayter et al. 2015). A close relationship between *L. aethiopica* and *L. tropica* was indicated by studies based on electrophoretic motilities of enzymes (MLEE) comparing enzyme profiles of various strains of *Leishmania* (Le Blancq et al. 1986). However, MLEE studies on the mobility profiles of other enzymes showed a closer relationship with *L. major*, with *L. aethiopica* and *L. major* forming a monophyletic group (Pratlong et al. 2009).

Analysis of the length of polymorphisms of microsatellite-containing regions has recently become an important method for studying populations and genetics of different species of *Leishmania* (Tóth et al. 2000). Microsatellites are tandemly repeated short stretches of nucleotide motifs. They are typically one to six base pairs long, and are

ubiquitously distributed throughout the genomes of eukaryotic organisms. The rate of mutation for microsatellites is five to six orders of magnitude higher than the mutation rate of the bulk DNA. The microsatellite loci present high variability, largely due to allelic repeat length variations (Kebede et al. 2013). Screening for the length variation of individual loci is easily done after amplification with PCR primers that anneal specifically to their flanking regions (Sampaio et al. 2003). *Leishmania* parasites are relatively rich in microsatellites (Rossi et al. 1994). The short sequences are dispersed throughout the genomes of the parasites, and are highly polymorphic and codominant (Kebede et al. 2013). Microsatellite loci of the Trypanosomatidae family have been shown to be stable under laboratory conditions, and are detectable directly in biological samples even when there is a low amount of the parasite DNA (Macedo et al. 2001). Moreover, the results from microsatellite analysis are easy to store in databases and compare between laboratories (Ochsenreither et al. 2006).

The use of multilocus microsatellite typing (MLMT) has proven to be a useful method for identifying different strains of *Leishmania.* MLMT has an advantage over other methods for identification and characterization as it is reproducible and can be transferred between laboratories (Schönian et al. 2011). It has been found to be a useful method for the study of population genetics and epidemiological investigations of different *Leishmania* species (Bulle et al. 2002). MLMT has been used to reveal geographical and hierarchic population structures for *L. major*, *L tropica* and the *L. donovani* complex (Schönian et al. 2011).

There are sixteen microsatellite markers that have been designed for *L. tropica* (Schwenkenbecher et al. 2004). MLMT of *L. tropica* has shown it to have extensive genetic variation. The application of MLMT to strains of *L. tropica* found in Morocco showed that species separated into two genetically distinct clusters (Schwenkenbecher

et al. 2006). Strains of the parasite tended to cluster to their geographic origin. Possible explanations to explain the genetic diversity existing in a single focus include: the introduction of separate and distinct genetic species from different regions to a previously unaffected area; mutations occurring in a single original species that are associated with genetic exchange; and recent hybridisation and recombination resulting in distinctly separate progenies (Krayter et al. 2014), as has been reported for *L. infantum* (Rogers et al. 2014). Mutations alone are unlikely to account for the distinct separation of *L. tropica* strains found in certain areas. Although genetic clustering of parasite strains most often associates to the geographic region, this is not always the case. This could be due to individuals being infected while in another country, but being diagnosed as a local case when they return to their home country. The application of MLMT to *L. tropica* found in Morocco revealed the parasites separating into two separate groups, each primarily associated with a particular geographical focus but with strains from each population occasionally found in the population of the other focus. This indicates that the genetic clustering of the parasites is not exclusively due to geographical or temporal factors (Krayter et al. 2014).

The close relationship between *L. tropica* and *L. aethiopica* was confirmed by the successful application of the 12 microsatellite markers to strains of *L. aethiopica*; the markers were originally thought to species specific to *L. tropica.* The Bayesian and distance based methods of analysis showed strains of *L. aethiopica* to be clustered amongst the African strains of *L. tropica;* whereas factorial correspondence analysis showed the two species to be clearly separated (Krayter et al. 2015).

It is thought that there are only a few species-specific genes that are important in determining which form of leishmaniasis develops from the infection. This is from a study where the genomes of three species of *Leishmania* that cause different forms of

the disease were compared. The study found that the species differed in only 200 of the >8000 genes in the genome. This indicates that these few genes are responsible for determining the differences in the form of the disease that develops or that the parasite genome only has a small involvement in the clinical presentation of the disease (Stephenson, 2007). Common factors to all *Leishmania* species may potentially be used as drug targets or candidates for vaccines (Stephenson 2007).

2.2. Use of DNA Sequences for Identification and Phylogenetics of *Leishmania*

The detection and identification of parasitic species is often central for studies into the understanding of many factors of the parasite; including the biology, immunology, pathology, and epidemiology. This knowledge underpins the instigation and implementation of measures to control against diseases that afflict many people with social and economic consequences (Snounou, 1996). Microscopy can be used to detect the presence of a parasite, and in some cases can identify the species of a parasite. Microscopy has many advantages, mainly its relatively low cost and its availability for most field conditions; however, alternative methods are required for high sensitivity of detection and accurate identification (Snounou, 1996). These higher levels of sensitivity and accuracy can be obtained through the detection of parasite nucleic acids (Barker, 1990). The need and use of molecular biological techniques has culminated in the use of the polymerase chain reaction (PCR) (Saiki et al. 1998).

A method used for classification an organism does not necessarily mean it can be used for the identification of the organism, and vice versa. The level of phylogenetic classification used has a differing role in identification. Classification on the level of evolutionary comparisons would not work for identification of different species. The DNA sequence used needs to be of a resolution appropriate for the level of identification and classification required. A homologous gene, or homolog, is a gene that is inherited

in two species from a common ancestor. Homologous genes can be orthologs or paralogs. An orthologous gene is one that has diverged after a speciation event, but the gene and main function is conserved. When a gene duplicates within a species, the resulting duplicate genes are paralogs of each other; even though they might differentiate in sequence and function over time. When used for either identification or classification it is important that orthologues are compared.

PCR is potentially the most sensitive method for specific detection of a particular DNA sequence in a sample. This is because in theory successful amplification only needs one copy of the DNA sequence to be present. The only limitation of the PCR method is in the amount of the sample that can be screened (Snounou, 1996). PCR can be used for identification and classification of a species of a parasite by targeting different DNA sequences; and the sequence of DNA amplified is dependent on the primers used. Microsatellites provide an insight into the genetics of the parasite on a very small scale. These can be used to observe the phylogenetic pattern of different parasite isolates within a single species; however, they are often species-specific and so cannot be used to compare the relationships between different species. On the other hand, ribosomal RNA can be used to study phylogeny on a broad, evolutionary scale, and so would not be able to identify different species of the same parasite. The target sequence for PCR depends on the scale of phylogeny and identification that is desired.

Leishmania parasite isolates can be identified by sequencing the PCR product generated by using ITS1 specific primers; and performing sequence alignments against the *Leishmania* genome database. Species-specific primers could provide a quicker, cost effective, and highly useful tool for typing and diagnosis of *L. aethiopica*. This could aid case detection and the determination of the appropriate therapeutic measures to take, in addition to the implementation of control measures. Furthermore, a restriction

enzyme digestion step is not required for this method as is needed in restriction fragment length polymorphism (RFLP), and so it provides an added advantage in accelerating species identification (Kebede et al. 2013). Although this would aid in the identification of *L. aethiopica*, it would not be able to identify different species, and would not provide insight into the relationship between *L. aethiopica* and other species.

2.3. *Leishmania* RNA Virus

An RNA virus was detected to be present in some South American species of *Leishmania* parasites several decades ago; however, the role of the virus in the virulence and metastasis of leishmanial infections was only described recently. The nucleic acid of LRV1 acts as a potent innate immunogen in some species of *Leishmania* parasites. The resulting inflammatory cascade increases disease severity, parasite persistence, and possibly anti-leishmanial drug resistance. LRV1 has mainly been found in clinical parasite isolates that are prone to infectious metastasis development; this has been found in both human cases and animal models. LRV1 presence in infections prone to metastasis development suggests that there is an association between the viral pathogen and metastatic complications of the disease, such as mucocutaneous leishmaniasis (Hartley et al. 2012).

Infection with some species of *Leishmania* including *L. braziliensis, L. guyanensis* and *L. aethiopica* have shown inflammatory metastatic forms of leishmaniasis, causing not only cutaneous leishmaniasis but mucosal leishmaniasis and disseminated leishmaniasis also. There is a lack of knowledge as to the factors that underlie this exacerbated clinical presentation (Zangger et al. 2014). Over ten different species of *Leishmania* parasite cause leishmaniasis in South America (Reithinger et al. 2007), with seven different species causing cutaneous leishmaniasis in humans (Guerra et al. 2011). *Leishmania braziliensis* and *Leishmania guyanensis* are the two primary etiological

agents of the disease in Brazil. (Pereira et al. 2013). Mucosal leishmaniasis and other metastatic complications of *Leishmania* infections such as diffuse cutaneous leishmaniasis also occur in other parts of the world other than South America, such as Ethiopia (Zangger at al. 2014). Metastatic complications can occur and mucosal leishmaniasis can develop even in formally asymptomatic leishmaniasis infections (Hartley et al. 2012). Mucosal leishmaniasis primarily affects the nasal cavity, as well as other mucosal structures, and can cause perforation or destruction of the septum. *L. braziliensis* is recognised as the most important etiological agent for mucosal leishmaniasis in the New World; however there are isolated cases of the disease being caused by several different species such as *Leishmania panamensis, Leishmania amazonensis,* and *L. guyanensis* in the Amazon region. Previously it was thought that *L. guyanensis* infection causing mucosal leishmaniasis was very rare; however, this could be due to limited studies in areas where *L. guyanensis* is endemic (Guerra et al. 2011). The genetic variability of the infecting *Leishmania* parasite does not account for all of the variability in the clinical presentation of the disease (Schonian et al. 2000). The range of outcomes is illustrated in Figure 2.2.

The progression of leishmaniasis to other forms of the disease appears to be due to many aspects of the infection (Reithinger et al. 2007); with the presentation of the disease likely reflecting the polygenetic factors of the host and parasite (Zangger et al. 2014). One of these factors is the parasite's resistance to oxidative stress (Acestor et al 2006). In addition to the parasite features, host factors such as the age and gender of the patient, as well as their nutritional and immune status (Reithinger et al. 2007), and a range of genetic susceptibilities are able to affect the progression of the disease (Sakthianandeswaren et al. 2009). The clinical variation in the manifestation of a *Leishmania* infection appears to be largely influenced by the parasite pedigree; however

unique genes between species of the parasite are relatively scarce (Smith et al. 2007). There are some species-specific genes between the genomes of *L. major*, *L. mexicana*, *L. infantum* and *L. braziliensis;* however, the pattern from these differences is not sufficient to explain the symptomatic grouping (Rogers et al. 2011).

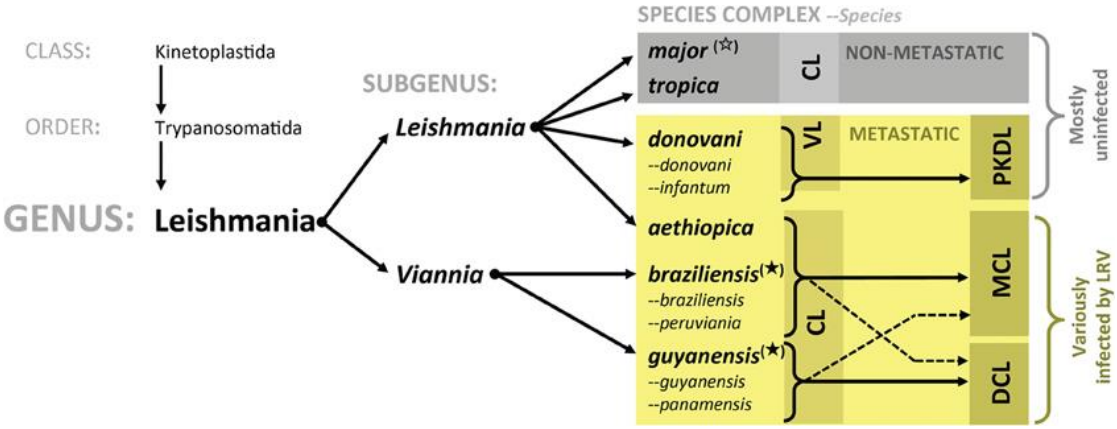


Figure 2.2 Leishmanial phylogeny and LRV presence. Intrinsic parasite factors for some species of *Leishmania* that underlie clinical disparities in metastatic leishmaniasis. Source: Hartley et al. 2012.

★ LRV found in numerous isolates within group

☆ LRV found only in a single, perhaps exceptional, isolate in this group

CL, Cutaneous leishmaniasis; **DCL**, Disseminated cutaneous leishmaniasis; **VL**, Visceral leishmaniasis; **PKDL**, Post Kala Azar dermal leishmaniasis; **MCL**, Mucocutaneous leishmaniasis; **LRV**, Leishmania RNA virus

Although previously believed that infection with certain species of *Leishmania* would only lead to the development of specific presentations of the disease, it is now known that several factors influence the various clinical development of leishmaniasis. Figure 2.2 shows the clinical disease that is most often found with infections by each species. Mucosal leishmaniasis is almost exclusively caused by infections of the *L. (Viannia)* subtype of *Leishmania*. This is a further indication that leishmanial metastasis is caused in part by a component of the parasite. The presence of LRV in the parasites of this subgenus potentially contributes to the destructive inflammation of the metastatic form

of the disease. This could be by either acting alongside other metastatic factors or by preying on the host's toll like receptor (TLR) hypersensitivity (Hartley et al. 2012). The presence of LRV may prove to be a driving factor of metastatic potential in *L. (Viannia)* parasite infections (Ronet et al. 2011). Mucocutaneous leishmaniasis development is associated with a persistent pro-inflammatory immune response; and so the immune status of the patient appears to influence the development of the disease (Blackwell, 1999). The onset of a destructive hyper-inflammatory immune response is a common occurrence in most cases of metastatic *Leishmania* infections. It is characterized by a flooding of activated immune cells, swelling, and the destruction of the local tissue (Ronet et al. 2011). The hyper-inflammatory immune response common to mucosal leishmaniasis infections may be instigated by a parasite factor (Hartley et al. 2012). Furthermore, co-infection with HIV also increases the risk of mucosal leishmaniasis (Alvar et al. 2008). Recently, the presence of LRV in the infecting parasite has been associated with the progression of the disease to mucosal leishmaniasis (Guerra et al. 2011), where in *L. guyanensis* mouse infections it was reported that inflammation and the severity of the infection was associated with a high burden of the virus infecting the parasites; suggesting that the LRV presence could be contributing to the severity of the disease in metastasizing leishmaniasis (Ives et al. 2011).

The *Leishmania* RNA virus (LRV) is a symbiotic component of the *Leishmania* parasites found in South America (Pereira et al. 2013). LRV has mainly been found in South America in the *Leishmania* species *L. braziliensis* and *L. guyanensis* (Zangger et al. 2014). The *Leishmania* RNA virus is a member of the *Totiviridae* family. Other viruses that affect protozoa and fungi are also members of this family. LRV is a double stranded RNA virus that has been found to persistently infect different *Leishmania* lineages. LRV has been categorised into LRV1 and LRV2 (Pereira et al. 2013). LRV2

has been identified in the Old World species *Leishmania major* (Scheffter et al. 1995). LRV1 has also been found in some strains of *L. braziliensis* and *L. guyanensis.* These strains of *Leishmania* circulate in specific areas of South America (Saiz et al. 2011). The viral particles are comprised of a capsid protein; an RNA-dependent RNA polymerase (RdRp), and a segmented double stranded RNA genome 5.3kb in length (Zangger et al. 2014). Like many *Totivridae* viruses LRV1 is neither infectious nor shed, and so it is viewed as a long term, evolutionary endosymbiont. The effects of the virus on the mammalian host arise indirectly via the parasite rather than through direct infection of the mammalian host by the virus (Hartley et al. 2014). LRV was first described over two decades ago (Stuart et al. 1992); however it has only recently been reported to have an influence on the development of mucosal leishmaniasis.

As LRV presence may be one of the factors determining the severity of the disease in leishmaniasis it provides reason to screen different species of *Leishmania* for the presence of the virus. There have been instances where LRV has been reported outside of South America, also infecting other *Leishmania* species such as in certain *L. aethiopica* strains in Africa and a *L. major* strain in central Asia. This could indicate that LRV was present before the divergence of the *Leishmania* subgenera. Sequencing of the LRV found in the *L. aethiopica* parasites, LRV-*Lae,* and the LRV2 found in *L. major* has shown that these viruses are members of the same *Totiviridae* family as the LRV1 viruses that were found in South America (Zangger et al. 2014).

High loads of LRV1 in the infecting parasite have been associated with parasite persistence, metastasis, and evasion of the host immune response. The recognition of LRV1 within metastasizing *Leishmania* parasites by the host promotes inflammation and subverts the immune response against the parasite infection, leading to the persistence of the parasitic infection (Ives et al. 2011). In a mouse model of *L.*

guyanensis infection the presence of the *Leishmania* RNA virus has been shown to be responsible for a hyper-inflammatory response. The mice infected with LRV1-bearing strains of *L. guyanensis* exhibited higher levels of parasites in the blood and greater footpad swelling (Ives et al. 2011). This was driven by the host's Toll-like Receptor 3 (TLR3) recognizing the viral RNA, leading to an exacerbation of the disease. When TLR3 recognizes the viral RNA it induces pro-inflammatory cytokines and chemokines. These are hallmarks of human mucosal leishmaniasis (Zangger et al. 2014). The recognition of LRV1 by the host occurs early on in the *Leishmania* infection; this suggests that the LRV1 dsRNA is released from dead parasites which were unable to survive within the macrophage of the host (Ives et al. 2011). Furthermore, a study infecting macrophages in vitro with LRV1 positive *L. guyanensis* (Ives et al. 2011) and LRV2 positive *L. aethiopica* (Zangger et al. 2014) showed a higher release of cytokines, phenotypes that were dependent on TLR3. The investigation of several strains of *Leishmania* parasites is needed to fully determine if the presence of the virus favours the development of mucocutaneous leishmaniasis and increases the rate of disease relapse (Scott 2011). Metastasis does occur in the absence of LRV; however, LRV1 mediated hyper-inflammatory immune responses could explain the differences between the clinical outcomes observed in different species and strains of *Leishmania* (Pereira et al. 2013).

Studies have also shown that LRV1 presence in *L. braziliensis* (Adaui et al. 2016) and *L. guyanensis* (Bourreau et al. 2016) clinical isolates correlates with drug treatment failure. The immunological hallmarks of a mucocutaneous infection of leishmaniasis are similar to those of the reaction to LRV. These include the production of type 1 interferons; impairment of the host cells ability to eliminate parasites via oxidative stress, and a bias towards a chronic Th1 inflammatory state (Hartley et al. 2012).

LRV1 presence in leishmaniasis has been investigated using a nested retro-transcription polymerase chain reaction in Brazil. In the areas of Rio de Janeiro that are endemic for leishmaniasis there was no LRV1 detected, even with mucosal involvement. LRV1 has been detected in cutaneous legions caused by *Leishmania guyanensis* in the northern region. These samples were obtained from patients who were presenting with disease reactivation after receiving clinical treatment for their primary legions. In areas of Rio de Janeiro where severe leishmaniasis is primarily caused by *Leishmania braziliensis* there is no association with *Leishmania* LRV1 infection (Pereira et al. 2013).

Although there are many parasite and host factors affecting the development of mucosal leishmaniasis, it appears that LRV1 has a role in exacerbating the pathogenesis of the disease. Investigation into the role of LRV in the development of mucosal leishmaniasis could lead to improved diagnosis of the risk of disease development; and aid the development of new and improved treatments (Ives et al. 2011). Since LRV presence amplifies the virulence of *Leishmania* infections its presence potentially provides a unique target for diagnosis and clinical intervention of mucosal leishmaniasis (Hartley et al. 2012). Some species of *Leishmania* have been found to express a potent RNA-mediated interference (RNAi) activity (Lye et al. 2010). The parasitic genes that control RNAi are of interest as it is a possibility that this defense mechanism recognises the nucleic acid of LRV1 and targets the foreign RNA (Hartley et al. 2012). In the RNAi pathway, double stranded RNA is converted into small interfering RNAs (siRNAs). SiRNA then triggers the degradation of a messenger RNA (mRNA) with a complementary sequence (Wilson and Doudna, 2013). Functional RNAi is not present in most of the *L. Leishmania* subgenera, but can be found in major metastatic leishmaniasis causing parasites of the *L. Viannia* group, including *L. braziliensis* and *L. guyanensis* (Lye et al. 2010) where LRV1 has been found to be

present. Active RNAi often controls RNA viruses in many organisms; however, LRV1 has been found to survive in its presence (Brettmann et al. 2016). Further investigation into the possibility that the retention or loss of RNAi in *Leishmania* parasites is related to an evolution of the viral interaction (Hartley et al. 2012). It is also possible that RNAi could be used to generate LRV1 free isogenic isolates (Brettmann et al. 2016).

Chapter Three: Materials and Methods

3.1. Laboratory Work

3.1.1 The Parasites

Materials

The *Leishmania* parasites used in this study were provided by the Lancaster University Cryo-Bank. The parasite isolates used are listed in Table 3.1. 25 isolates of *Leishmania* parasites were used in this study, a mixture of *L. tropica* and *L. aethiopica*, as shown in Table 3.1. An isolate of *L. major* (MRHO/SU/1959/LV39) and of *L. guyanensis* (MHOM/BR/1975/M4147) were also used as controls.

Table 3.1 Table of parasite isolates used with WHO code, country of origin, and source

Isolate	Original ID	WHO Code	Country of Origin	Other Information
LV1	L. aethiopica	MHOM/ET/XX/L86	Ethiopia	Human CL
LV13	L. aethiopica	MHOM/ET/70/L96	Ethiopia	Human CL
LV15	L. aethiopica	MPRV/ET/70/L97	Ethiopia	Rock hyrax
LV25	L. aethiopica	MPRV/ET/71/L111	Ethiopia	Rock hyrax
LV26	L. aethiopica	ILNP/ET/72/L122	Ethiopia	*Phlebotomus longipes*
LV27	L. aethiopica	ILNP/ET/72/L123	Ethiopia	*Phlebotomus longipes*
LV28	L. aethiopica	ILNP/ET/72/L124	Ethiopia	*Phlebotomus longipes*
LV29	L. aethiopica	MHOM/ET/72/L127	Ethiopia	Human CL
LV377	L. aethiopica	MHOM/ET/XX/L93	Ethiopia	Human CL

LV472	L. aethiopica	MHTX/KE/XX/HR3	Kenya	Rock hyrax
LV546	L. aethiopica	MHOM/ET/72/L100;LRC-L147	Ethiopia	Human DCL
LV697	L. aethiopica	XXXX/ET/XX/5.104	Ethiopia	Unknown
LV142	L. tropica	MHOM/IQ/65/LRC-L32	Iraq	Human LR
LV357	L. tropica	MHOM/SU/60/LRC-L39	USSR	Human CL
LV369	L. tropica	MHOM/SA/68/11	Saudi Arabia	Human CL
LV444	L. tropica	MHOM/NA/71/VS	Namibia	Human CL
LV487	L. tropica	IROS/NA/71/RossiI	Namibia	*Phlebotomus rossi*
LV488	L. tropica	IROS/NA/71/RossiII	Namibia	*Phlebotomus rossi*
LV489	L. tropica	IROS/NA/71/RossiIIIa	Namibia	*Phlebotomus rossi*
LV490	L. tropica	IROS/NA/71/RossiIIIb	Namibia	*Phlebotomus rossi*
LV508	L. tropica	MHOM/IQ/XX/IN	Iraq	Human CL
LV509	L. tropica	MHOM/IQ/XX/NA	Iraq	Human CL
LV510	L. tropica	MHOM/IQ/XX/SA	Iraq	Human CL
SU23	L. tropica	MHOM/TR/98/SU23	Tunisia	Human CL

CL = Cutaneous Leishmaniasis; DCL = Diffuse Cutaneous Leishmaniasis; LR = Leishmaniasis Recidivans

3.1.2 Culture

Materials

The following materials were used for in vitro culture of *Leishmania* promastigotes: BioWhittaker® Medium 199 from Lonza; Dulbecco's Phosphate Buffered Saline from SIGMA; BME vitamins from SIGMA; Foetal Bovine Serum from Thermo-Fisher, and Gentamicin (10mg/ml) from PAA – The Cell Culture Company.

The culture medium for the *Leishmania* parasites (M199 complete) was prepared as follows. To 500ml of M199 base medium 10% Foetal Bovine Serum (FBS) was added,

5ml BME vitamins, and 1.2ml of gentamicin solution. The mixture was filter sterilised by passage through a 0.2µ filter unit under vacuum suction, and stored at 4°C.

The parasites were cultured in 10ml volumes of M199 complete culture medium using 25 cm^2 tissue culture flasks and incubated at 26°C. The parasite cultures were regularly checked by microscopy and passaged at 1:20 dilution approximately once per week. For harvesting 7-10ml volumes were placed into sterile polypropylene capped test tubes and centrifuged at 1500g for 10 minutes; after which the supernatant was discarded. The parasite pellet was resuspended in 2ml Phosphate-buffered saline (PBS) and then two 1ml aliquots placed into microfuge tubes, centrifuged at 10,000g and the supernatant discarded. The pellets were snap-frozen and stored at -80°C until required for DNA extraction.

3.1.3 Preparation of *Leishmania* DNA for PCR

Materials

The following materials were used to prepare DNA: QIAGEN DNeasy® Blood and Tissue Kit Cat. No. 69504; Phosphate Buffered Saline from SIGMA.

The *Leishmania* pellets were directly resuspended in 200µl PBS after removal from -80°C and stored on ice. The DNA of the *Leishmania* was prepared using the DNeasy kit. 20µl proteinase K and 4µl RNAse A were added to the resuspended pellet and incubated for 2 minutes at room temperature. 200µl of AL buffer was then added, mixed by vortexing, and incubated at 56°C for 10 minutes. 200µl of 100% ethanol was then added and mixed into the sample. The mixture was carefully transferred to the surface of a spin column and centrifuged at 8000g for 1 minute, under which conditions the DNA binds to the column matrix. The flow through was discarded and 500µl AW1 buffer added to wash the bound DNA. The spin column was again centrifuged at 8000g

for 1 minute and the flow-through was discarded. 500µl AW2 buffer was added to the spin column and centrifuged at 14000g for 3 minutes for a second wash. The spin column was then transferred to a microfuge tube and 100µl AE buffer added for DNA elution. After incubating at room temperate for 1 minute or longer, the column was centrifuged at 8000g for 1 minute. The amount of DNA recovered was measured using a NanoDrop Spectrophotometer.

3.1.4 Polymerase Chain Reaction

Materials

DNA polymerase and the PCR buffer 5X from the QIAGEN HotStar HiFidelity Polymerase Kit were used in this method.

DNA amplification was done by Polymerase Chain Reaction (PCR). The main target of the PCR was ribosomal RNA ITS-1 sequence; however, the RPL23a IGS was also used in some of the early experiments. The primers used for ITS-1 were LEF and LER, and the primers used for RPL23a IGS were PS1 and PS2 (Table 3.2).

Primers	Sequences
LEF	5' TCC GCC GCA AAG TTC ACC GAT A 3'
LER	5' CCA AGT CAT CCA TCG CGA CAC G 3'
P1	5' GCG CCA ACA AGA CTG AGA T 3'
P2	5' CGT CAC CTT GAC GAC CTT G 3'

Table 3.2. Primer names and sequences for PCR amplification.

For PCR 10µM dilutions of the primers were prepared. The final volume to be used in the PCR was 25µl, this was composed of molecular grade dH_2O; 5µl HotStar HiFidelity PCR Buffer 5X; 1µl of Primer 1 (LEF/PS1); 1µl of Primer 2 (LER/PS2); 0.5µl HotStar HiFidelity DNA polymerase, and the DNA to be tested. 100ng/µl of DNA was used, and depending on the volume of DNA used the volume of water was adjusted to make

up the 25µl final volume. The most common volumes used were 2µl DNA and 15.5µl molecular grade dH_2O.

After the reaction mix was prepared accordingly in a PCR-grade tube, the ingredients were gently mixed and briefly spun in a microfuge. The tubes were then placed in the thermocycler. The PCR cycling parameters are detailed in Table 3.3.

Conditions					
Hotstart	Cycling		Number of cycles	Final extension	Hold
	Temperature /°C	Time / seconds			
95°C for 5 minutes	95	30	35	72°C for 10 minutes	4°C
	57	30			
	72	60			

Table 3.3. PCR Cycling Parameters

3.1.5 Agarose Gel Electrophoresis

Materials

The following materials were used in this method: molecular biology grade agarose from SIGMA; TAE 1X buffer was made from TAE 50X buffer from QIAGEN; 6X DNA loading dye from Thermo Scientific, and GelRed Nucleic Acid Stain from Biotium.

The agarose gel was made by mixing and heating 1.5% agarose (0.75g) in 50ml TAE 1X buffer. After cooling slightly the agarose was poured into the gel mould and left to polymerise for around 30 minutes with a loading comb in place. Once set, the comb was removed and the gel was placed in a gel tank with TAE 1X buffer filling the tank and just covering the gel. 5µl of the 100bp DNA ladder was loaded on the gel. 2µl DNA from the PCR was mixed on parafilm with 1µl 6X loading dye and 3µl dH_2O and was loaded. The gel was run at 90V for approximately 40 mins. After this the gel was

stained with GelRed for 30 minutes whilst being agitated. The gel was imaged on a BioRad Chemidoc system.

3.1.6 PCR Purification

Materials

All materials needed for this method except the microfuge tubes came from the GeneJet PCR Purification Kit from Thermo Scientific.

The GeneJet PCR Purification Kit was used to purify the PCR products. A 1:1 volume (25µl) of Binding Buffer was added to the PCR mixture. For the ITS-1 PCR products an equal volume (25µl) of isopropanol was also added since the ITS-1 DNA fragment is ≤500bp. This was transferred to a GeneJet purification column. All centrifugations were carried out at 10,000g for 60 seconds. The supernatant was discarded and 700µl of Wash Buffer was added to the column. This was centrifuged; the supernatant discarded, and centrifuged again. The column was then transferred to a new microfuge tube and 50µl of Elution Buffer was added and left for at least 1 minute. The column was then centrifuged again and the microfuge tube kept. The DNA concentration was measured on the NanoDrop. Another agarose gel was also run, and a sample checked to ensure that a clean product had been produced.

3.1.7 Preparation of Samples for Sequencing

Sequencing of the PCR samples was done externally by Source Bioscience.

Each reaction requires 5µl of sample, but 20µl was prepared to allow for repeats. 1ng/µl per 100 bp of DNA was required. The ITS-1 product is 400bp and the RPL23a IGS product is 698bp. This meant 80ng/µl DNA was required for the ITS-1 PCR products and 139.6ng/µl DNA required for the RPL23a IGS PCR products. The volume of DNA

needed to produce the needed amount of DNA was calculated using the ng/µl value from the NanoDrop spectrophotometer.

5µl of each primer was also needed per reaction, at a concentration of 3.2pmol/µl. 10nmol/l primers were used, and the required concentration made by mixing 10µl of primer stock and 21.25µl dH_2O.

3.1.8 Dot Blot

Materials

The following materials were used in this method: nitrocellulose membrane from the BIO-RAD Trans-Blot® Turbo™ RTA Transfer Kit; PBS was from SIGMA; dried skimmed milk used was from TESCO; to make the TBS: Tris from ALDRICH, sodium chloride from Fisher Scientific, and hydrochloric acid from SIGMA-ALDRICH were used; to make the TBS-T Tween®20 from SIGMA was used; anti-Mouse IgG HPR conjugate antibody from PROMEGA, and ECL reagent from BIO-RAD Clarity™ Western ECL Substrate; the J2 anti-dsRNA antibody was from SCICONS.

The preparation of the parasites for the dot blot started with the parasite pellets from the −80°C freezer. The pellet was resuspended in cold PBS to reach a dilution of $3.125x10^8$ cell equivalents/ml and kept on ice. A 2.5 fold dilution was prepared by taking 10µl of this first dilution and diluting it by mixing with 15µl PBS. A second dilution was prepared from this the same way. The resulting concentrations were the equivalent of $3.125x10^8$/ml, $1.25x10^8$/ml, and $5x10^7$/ml. 2µl spots were used in the experiment, and therefore each contained $6.25x10^5$, $2.5\ x10^5$, and $1\ x10^5$ promastigote equivalents, respectively. When not being used the lysate samples were kept at −80°C.

The blot was performed using a nitrocellulose membrane. The nitrocellulose was handled wearing gloves and using forceps. A grid was drawn outlining where different parasites and concentrations were to be spotted, as in Figure 3.1.

	positive control	negative control	Parasite 1	Parasite 2	Parasite 3
$3.125x10^8$/ml					
$1.25x10^8$/ml					
$5x10^7$/ml					

Figure 3.1. Dot blot nitrocellulose membrane layout

The membrane was left to air dry. Once the membrane was dried it was soaked for 60 minutes in 5% powdered milk in 20ml TBS-T to block non-specific sites. Next the membrane was incubated for 30 minutes with the antibody J2 (mouse-anti-dsRNA). The J2 antibody was prepared at a 1:3000 dilution with 20ml TBS-T. The membrane was then washed in TBS-T four times, 5 minutes each. Next the membrane was incubated with the rabbit anti-mouse-conjugated secondary antibody for 30 minutes. The secondary antibody was in a 1:2000 dilution with 1% milk TBS-T. The membrane was then washed in TBS-T again; first for 15 minutes, then for 5 minutes two times. The final wash was 5 minutes in TBS. All washes and incubations were on a rocker.

The membrane was incubated in 2mls ECL reagent for 1 minute. Excess solution was removed from the surface by covering the membrane in cling film. Within 10 minutes the membrane was read on the Chemidoc. The main exposure time used was 8 seconds. The membrane was post-stained with Ponceau red to detect the total protein present. This was left incubating for 30-60 minutes on a rocker. The membrane was rinsed with

dH_2O and read on the Chemidoc. When testing the different *Leishmania* parasites for the presence of the viral RNA a positive and negative control was used each time. The positive control was *L. guyanensis* M4147 and the negative control was *L. major* LV39.

3.2. Data Analysis

3.2.1 Processing PCR Sequencing Results

Materials

Forward and reverse sequences were obtained for each PCR product sequenced. These were initially checked using Chromas Lite 2.6.4 (Technelysium Pty Ltd) for suitable quality – good peak separation and base calling with minimal ambiguity. The reverse complement of the reverse sequences was found using the Bioinformatic website, which should match to the forward sequence. The forward sequence and the reverse/reverse complement were compared using the DNA version of Clustal Omega from the European Bioinformatics Institute website. If the two sequences were the same, they were combined to generate a consensus DNA sequence for the particular parasite sample. Finally the DNA sequence was identified to be either *L. tropica* or *L. aethiopica* using the nucleotide BLAST programme on the National Centre for Biotechnology Information website by searching for similar sequences already discovered.

Worked example

LV39 RPL23a

Primers:

PS1: GCGCCAACAAGACTGAGAT

PS2: CGTCACCTTGACGACCTTG

RC: CAAGGTCGTCAAGGTGACG

Forward sequence from Source Bioscience

NNNNNNNNNNNNNNNGNNNNNGNNNTGNNGGTCAANANCCTNNTCCGTCCGGATGGTCTGAA
GAAGGCGTACATCCGCCTGTCTGCGGCCCACGATGCTCTGGACACTGCCAACAAGATCGGTC
TTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAACAACGAA
AAAGAAGTGCTACGGTAACTTTGGTGCACCAGAGGCTGGCGACGGCTCTTAGGGTGGTTTAA
TGAGGTGTTACCTCTATGGTAAACTGAAGTGATCAGCTGCGGTATCTTTGTGCGCGTCGAGG
GTGCGCTGGTTTCTGCATCTCCGAGTTTGTGATTCTCTCTGAAGATGTATTGCAAAAAGTGC
GCTGCGGCGCTGCTTTTCTACTCCTGCGCTGCTGTTCCTTCAAATGTGTCGACATGTCCGAC
TTTCTGCAGCTGCTCACCTCCACTTTCATCTCTCCCTCTTCCTTTTTTTTTCTGTGTGTCCA
TGCGTCTCGTGTGCGTCTCAACTTGCGACAACTTTTCTTGCGCCCTTCTGTGGAATACCATC
TGAACAGCTTTAGGGCTTCATTCGCAACGGATCATGCCTCCTGCTCAGAAGACCGCCAAGAA
GGCCGCGCCAAAGGATGCGAAGGCGACCAAGGNCNNNNNGGTGACGANNCCNNGNGACNGAG
ANNANNNTCGGCNANGNNNNNNGGCGGTCTTCTGANCAGGAGGNTGATCGTTGCNAAGAAAC
CTAAAGCTGTTCNNNGGNATCNCANAANGGNGCAGGAAAANTTGTCNCAGNTNNNNNCNNCG
NGANNNNNNNNNCAGAAAAAAAAGANANGNGNNNNNAAAGNGNGNNNNNNCTNCNAAGTCGN
ANTGTNNCNNNNNNNNNNCANNNNNANTGANNNNNNNCCNCNNNNNTNCATNANTNTNNNNN
NNANANNNANNNNNANNNNNGNAANCNNNCCNCGNCNCNNNNNNANNCN

Reverse sequence from Source Bioscience

NNNNNNNNNNNNNNNNNNNNNCGANGNNGGTCTTCNGANNGGNGGCNCGATCCGTTGCGANT
GAAGCCCTAAAGCTGTTCAGATGGTATTCCACAGAAGGGCGCAAGAAAAGTTGTCGCAAGTT
GAGACGCACACGAGACGCATGGACACACAGAAAAAAAAAGGAAGAGGGAGAGATGAAAGTGG
AGGTGAGCAGCTGCAGAAAGTCGGACATGTCGACACATTTGAAGGAACAGCAGCGCAGGAGT
AGAAAAGCAGCGCCGCAGCGCACTTTTTGCAATACATCTTCAGAGAGAATCACAAACTCGGA
GATGCAGAAACCAGCGCACCCTCGACGCGCACAAAGATACCGCAGCTGATCACTTCAGTTTA
CCATAGAGGTAACACCTCATTAAACCACCCTAAGAGCCGTCGCCAGCCTCTGGTGCACCAAA
GTTACCGTAGCACTTCTTTTTCGTTGTTGATAAAATGAAATTATCTCAAGTAATCGCATCAT
CCAAATTCTGCTTAGACAAGACCGATCTTGTTGGCAGTGTCCAGAGCATCGTGGGCCGCAGA
CAGGCGGATGTACGCCTTCTTCAGACCATCCGGACGGATCAGGGTGTTGACCTTCACGGCCT
TCACCTGGTACAGCTTGCGCATTGCCTTCTTGATCTCANTCTGNTTGGCGCNAAANNNNTAN
CNNGNNNNNGNNNNNNCGTGNNGNCNNNNTCCNTCGGATGGTCTGAANAAGNGTACNTCCCC
TGTCTGCGNNCACNATGCTCTGGACNNNGCAAAANANCGGNNNGNNCAANCANAATTTNGAT
GANGNNATTCCTNNNANAANTTNNTTTNNNNNNANAAAAANAAANGNNNNNNNNNNNNNTG
GNNNNNNNCGNGNNNANNNNNNNNNGNNNNNNTNNNNNGGNNNNNCNNNGCNNNNNANNNNN
NNTNNNNNNNTNNNNNCNNNNNNNNNNNNGNNNNNNNNNTNCNNANTNNNANNNNNNNNNN
NNNNNANNNNNNNNGGNNGNNNNNNNCNNNNNNNNNNNNTNNNNCANNNGN

Reverse complement of Reverse from the Bioinformatic website

GCGCCAANCAGANTGAGATCAAGAAGGCAATGCGCAAGCTGTACCAGGTGAAGGCCGTGAAG
GTCAACACCCTGATCCGTCCGGATGGTCTGAAGAAGGCGTACATCCGCCTGTCTGCGGCCCA
CGATGCTCTGGACACTGCCAACAAGATCGGTCTTGTCTAAGCAGAATTTGGATGATGCGATT
ACTTGAGATAATTTCATTTTATCAACAACGAAAAAGAAGTGCTACGGTAACTTTGGTGCACC
AGAGGCTGGCGACGGCTCTTAGGGTGGTTTAATGAGGTGTTACCTCTATGGTAAACTGAAGT
GATCAGCTGCGGTATCTTTGTGCGCGTCGAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTGT
GATTCTCTCTGAAGATGTATTGCAAAAAGTGCGCTGCGGCGCTGCTTTTCTACTCCTGCGCT
GCTGTTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTGCTCACCTCCACTTTCATC
TCTCCCTCTTCCTTTTTTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTGCGACA
ACTTTTCTTGCGCCCTTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTCANTCGCAACGG
ATCGNGCCNCCNNTCNGAAGACC

CLUSTAL O(1.2.1) multiple sequence alignment by the DNA and RNA Clustal Omega from the European Bioinformatics Institute website

```
RC      GCGCCAANCAGANTGAGATCAAGAAGGCAATGCGCAAGCTGTACCAGGTGAAGGCCGTGA
F       ------------------------------------------------------------


RC      AGGTCAACACCCTGATCCGTCCGGATGGTCTGAAGAAGGCGTACATCCGCCTGTCTGCGG
F       ---------------TCCGTCCGGATGGTCTGAAGAAGGCGTACATCCGCCTGTCTGCGG
                       ******************************************

RC      CCCACGATGCTCTGGACACTGCCAACAAGATCGGTCTTGTCTAAGCAGAATTTGGATGAT
F       CCCACGATGCTCTGGACACTGCCAACAAGATCGGTCTTGTCTAAGCAGAATTTGGATGAT
        ************************************************************

RC      GCGATTACTTGAGATAATTTCATTTTATCAACAACGAAAAAGAAGTGCTACGGTAACTTT
F       GCGATTACTTGAGATAATTTCATTTTATCAACAACGAAAAAGAAGTGCTACGGTAACTTT
        ************************************************************

RC      GGTGCACCAGAGGCTGGCGACGGCTCTTAGGGTGGTTTAATGAGGTGTTACCTCTATGGT
F       GGTGCACCAGAGGCTGGCGACGGCTCTTAGGGTGGTTTAATGAGGTGTTACCTCTATGGT
        ************************************************************

RC      AAACTGAAGTGATCAGCTGCGGTATCTTTGTGCGCGTCGAGGGTGCGCTGGTTTCTGCAT
F       AAACTGAAGTGATCAGCTGCGGTATCTTTGTGCGCGTCGAGGGTGCGCTGGTTTCTGCAT
        ************************************************************

RC      CTCCGAGTTTGTGATTCTCTCTGAAGATGTATTGCAAAAAGTGCGCTGCGGCGCTGCTTT
F       CTCCGAGTTTGTGATTCTCTCTGAAGATGTATTGCAAAAAGTGCGCTGCGGCGCTGCTTT
        ************************************************************

RC      TCTACTCCTGCGCTGCTGTTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTGCT
F       TCTACTCCTGCGCTGCTGTTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTGCT
        ************************************************************

RC      CACCTCCACTTTCATCTCTCCCTCTTCCTTTTTTTTTCTGTGTGTCCATGCGTCTCGTGT
F       CACCTCCACTTTCATCTCTCCCTCTTCCTTTTTTTTTCTGTGTGTCCATGCGTCTCGTGT
        ************************************************************

RC      GCGTCTCAACTTGCGACAACTTTTCTTGCGCCCTTCTGTGGAATACCATCTGAACAGCTT
F       GCGTCTCAACTTGCGACAACTTTTCTTGCGCCCTTCTGTGGAATACCATCTGAACAGCTT
        ************************************************************

RC      TAGGGCTTCANTCGCAACGGATCGNGCCNCCNNTCNGAAGACC-----------------
F       TAGGGCTTCATTCGCAACGGATCATGCCTCCTGCTCAGAAGACCGCCAAGAAGGCCGCGC
        ********** ************  *** **       *   *

RC      ---------------------------------------
F       CAAAGGATGCGAAGGCGACCAAGGNCNNNNNGGTGACG
```

Combined Consensus Sequence

GCGCCAANCAGANTGAGATCAAGAAGGCAATGCGCAAGCTGTACCAGGTGAAGGCCGTGAAG
GTCAACACCCTGATCCGTCCGGATGGTCTGAAGAAGGCGTACATCCGCCTGTCTGCGGCCCA
CGATGCTCTGGACACTGCCAACAAGATCGGTCTTGTCTAAGCAGAATTTGGATGATGCGATT
ACTTGAGATAATTTCATTTTATCAACAACGAAAAAGAAGTGCTACGGTAACTTTGGTGCACC
AGAGGCTGGCGACGGCTCTTAGGGTGGTTTAATGAGGTGTTACCTCTATGGTAAACTGAAGT
GATCAGCTGCGGTATCTTTGTGCGCGTCGAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTGT
GATTCTCTCTGAAGATGTATTGCAAAAAGTGCGCTGCGGCGCTGCTTTTCTACTCCTGCGCT
GCTGTTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTGCTCACCTCCACTTTCATC
TCTCCCTCTTCCTTTTTTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTGCGACA
ACTTTTCTTGCGCCCTTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTCATTCGCAACGG
ATCATGCCTCCTGCTCAGAAGACCGCCAAGAAGGCCGCGCCAAAGGATGCGAAGGCGACCAA
GGNCNNNNNGGTGACG

Consensus = 459 nt

Identification from National Centre for Biotechnology Information website

99% identical to L. major FV1

3.2.2. Phylogenetic Trees

Materials

The phylogenetic trees were generated using the Molecular Evolutionary Genetics Analysis (MEGA) software. The *L. donovani* and *L. infantum* ITS1 sequences were found using the nucleotide BLAST on the National Centre for Biotechnology Information website. The sequences were aligned using the DNA Clustal Omega programme from the European Bioinformatics Institute website.

The ITS1 DNA sequences from the PCR were aligned using *L. major, L. donovani,* and *L. infantum* as different outgroups, and entered into the MEGA software. Using the software the best model of sequence evolution was determined to be Jukes-Cantor, and a Maximum Likelihood Tree was generated with bootstrap method of 500 replications. This was repeated for RPL23a DNA sequences.

3.2.3. Distance Pairwise Analysis

Materials

The distance pairwise data was generated using the MEGA software. The sequences were aligned using the DNA and RNA Clustal Omega from the European Bioinformatics Institute website.

The ITS1 DNA sequences were aligned and entered into the MEGA software. Pairwise Distance using the sequences was generated in MEGA with the Nucleotide Substitutions in a p-distance model. This was done for the comparison of *L. tropica* and *L. aethiopica* data, and for a *L. tropica* and *L.* major comparison, and a *L. donovani* and *L. infantum* comparison. This was repeated for RPL23a DNA sequences.

Chapter Four: Results

4.1. DNA Sequencing

4.1.1 Polymerase Chain Reaction

There were two main aims to this study and these provided two separate sets of results. The first stage of the study was to compare the ITS1 DNA sequences of various *L. tropica* and *L. aethiopica* parasite isolates produced using the polymerase chain reaction (PCR). Two control isolates were used, one *L. major* and one *L. guyanensis*, to develop the experimental method and which are known to be distinct from the closely related *L. tropica* and *L. aethiopica* parasite isolates. These isolates were also to be used as controls in the viral RNA dot blot experiments. Another sequence was also examined, the RPL23a intergenic sequence (between two tandemly repeated RPL23a genes), for the first four parasite isolates to be examined. These provided further technical controls, and also acted to demonstrate reproducibility, as they were compared to the RPL23a sequences generated in previous work.

The parasites were cultured in vitro as promastigotes, pellets produced, DNA extracted and then PCR reactions performed, using the P1 and P2 primers for the RPL23a sequence and the LEF and LER primers for the ITS1 sequence. Agarose gel electrophoresis was performed to check the PCR products, such as the example shown in Figure 4.1. If this indicated suitable quality (clear band of the expected size: ~400 bp for ITS-1; ~700 bp for RPL23a) the product was adjusted to the correct concentration and sent for sequencing by Source Bioscience. The resulting sequences were analysed and used to generate a consensus sequence for each isolate as described in Materials and Methods. Full sequence data are recorded in Appendix I. In some cases the sequencing was successful at the first attempt. However, some required additional attempts to resolve poor amplification or ambiguity in the sequence data. For three

isolates amplification was successful but clear sequence data could not be obtained for ITS-1 (LV142, SU23 and LV29). Table 4.1 shows a summary for each of the 23 isolates examined. This includes their original species identification based on criteria such as geographical origin, their identification by RPL23a sequence data, and their identification by ITS-1 sequence data.

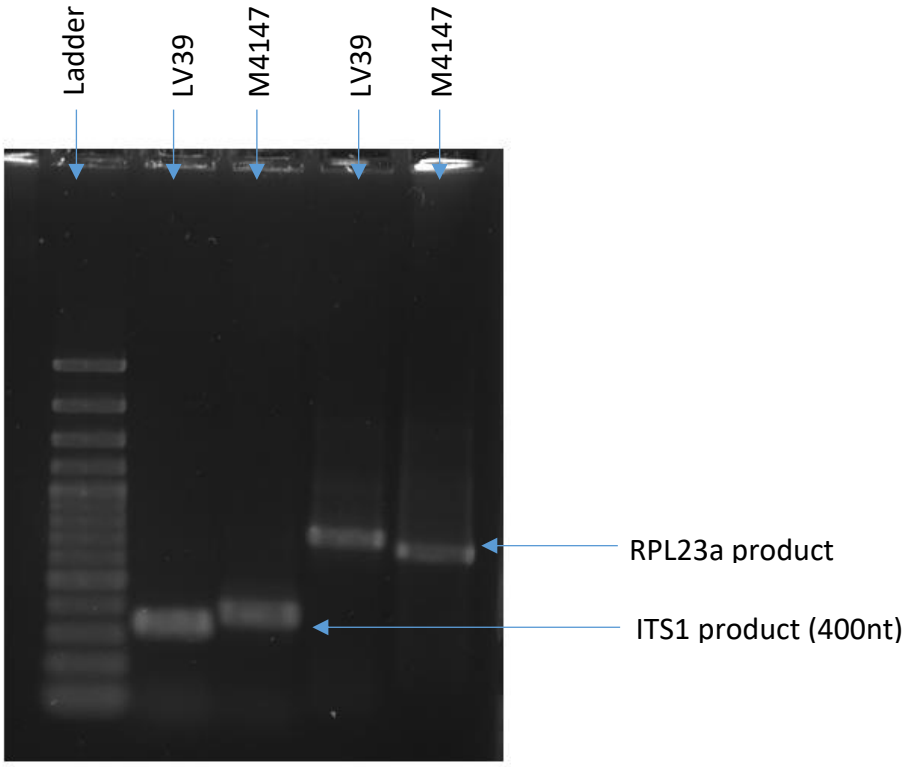


Figure 4.1 Agarose Gel Electrophoresis for RPL23a and ITS1 PCR products from *L. major* LV39 and *L. guyanensis* M4147. Products were examined on a 1.5% agarose gel and visualized with Gel Red. Products of the correct size were obtained as illustrated and their identity confirmed by sequencing and BLAST searching.

The results for the *L. tropica* and *L. aethiopica* parasite isolates tested show a number of interesting features. With one exception (LV28) there is correspondence between the two molecular identification methods, which gives some confidence in their use. For LV28, subsequent to this work being done the ITS-1 and RPL23a PCRs were repeated on the same DNA samples (Prof Bates, personal communication) and this

confirmed the *L. tropica* result for ITS-1 but indicated a mixed sequence for RPL23a. The results also show that three *Leishmania* isolates that were originally identified as *L. aethiopica* (LV697, LV13 and LV1), when tested were found to actually be *L. tropica* for both the RPL23a and ITS1 sequences. PCRs for the RPL23a sequence for LV357, LV25 and LV456 were repeated in this study, in addition to PCR for the RPL23a product for LV39 and M4147, and confirmed earlier results.

Isolate	Original ID	RPL23a ID	ITS1 ID
LV357	L. tropica	**L. tropica (457)**	**L. tropica (308)**
LV508	L. tropica	L. tropica (457)	**L. tropica (308)**
LV697	L. aethiopica	L. tropica (458)	**L. tropica (313)**
LV142	L. tropica	L. tropica (457)	-
SU23	L. tropica	L. tropica (458)	-
LV13	L. aethiopica	L. tropica (457)	**L. tropica (313)**
LV1	L. aethiopica	L. tropica (459)	**L. tropica (306)**
LV369	L. tropica	L. tropica (458)	**L. tropica (308)**
LV509	L. tropica	L. tropica (458)	**L. tropica (308)**
LV487	L. tropica	L. tropica (457)	**L. tropica (308)**
LV444	L. tropica	L. tropica (457)	**L. tropica (308)**
LV489	L. tropica	L. tropica (457)	**L. tropica (308)**
LV488	L. tropica	L. tropica (457)	**L. tropica (308)**
LV490	L. tropica	L. tropica (457)	**L. tropica (308)**
LV27	L. aethiopica	L. aethiopica (460)	**L. aethiopica (324)**
LV377	L. aethiopica	L. aethiopica (460)	**L. aethiopica (327)**
LV546	L. aethiopica	**L. aethiopica (460)**	**L. aethiopica (326)**
LV29	L. aethiopica	L. aethiopica (460)	-
LV472	L. aethiopica	L. aethiopica (460)	**L. aethiopica (325)**
LV28	L. aethiopica	L. aethiopica (460)	**L. tropica (308)**
LV26	L. aethiopica	L. aethiopica (460)	**L. aethiopica (327)**
LV15	L. aethiopica	L. aethiopica (460)	**L. aethiopica (330)**
LV25	L. aethiopica	**L. aethiopica (460)**	**L. aethiopica (327)**

Table 4.1: Table showing the original identification, the RPL23a PCR identification, and the ITS1 identification performed in this study for 23 *L. tropica* and *L. aethiopica* parasite isolates. The results in **bold** are from this study; the remainder were from a study conducted by Godwin Kwakye-Nuako during his MSc studies at Lancaster (unpublished data).

4.1.2. Phylogeny

Maximum likelihood phylogenetic trees were generated with the MEGA program using the ITS1 sequences generated by PCR, which are recorded in Appendix I. Figure 4.2 shows a phylogenetic tree produced using a Jukes-Cantor with Gamma distribution model of sequence evolution, selected by the software as the best fitting model from those available. The outgroup used in Figure 4.2 was the sequence from the *L. major* LV39 isolate used in this study. Both *L. donovani* and *L. infantum* were also tested as alternative outgroups and gave essentially identical results, which are shown in Appendix III, but the *L. major* outgroup provided a tree with higher bootstrapping values. The phylogenetic tree in Figure 4.2 shows a single strong branch point with 99% bootstrap value separating all of the parasite isolates identified as *L. aethiopica* in this study. There is a more complex branching among the rest of the isolates within the *L. tropica* group. LV28, the isolate that was identified as *L. aethiopica* originally and with the RPL23a sequence but as *L. tropica* with the ITS1 sequence is amongst the *L. tropica* isolates.

Phylogenetic trees were also generated using RPL23a sequences previously found for *L. tropica* and *L. aethiopica,* recorded in Appendix II, as shown in Figure 4.3. The outgroup used in Figure 4.3 was *L. major*, and *L. donovani* and *L. infantum* were also tried as outgroups, see Appendix III. As with the ITS-1 sequence dataset all three produced similar trees but the bootstrapping was higher on the *L. tropica* outgroup. Again there is a strong branch point separating the isolates identified as *L. tropica* and *L. aethiopica* with the RPL23a sequence. There is branching within both the *L. tropica* and *L. aethiopica* groups, but the branching is more extensive within the *L. tropica*

isolates. However, these branch points are different than the branch points amongst the *L. tropica* isolates in Figure 4.2.

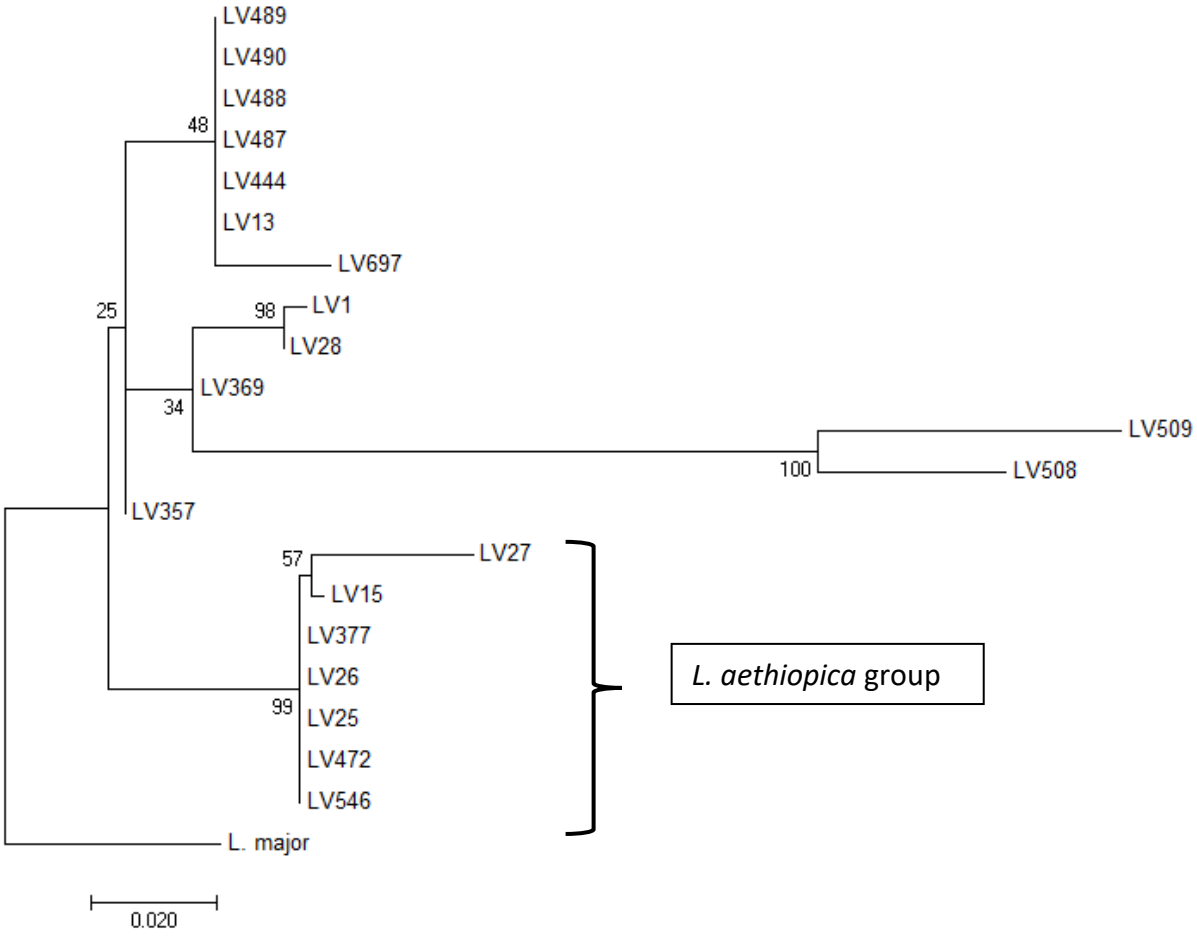


Figure 4.2: Phylogenetic tree of ITS 1 sequences for *L. tropica* and *L. aethiopica* with *L. major* as an outgroup. The scale represents 0.02 amino acid substitutions per site.

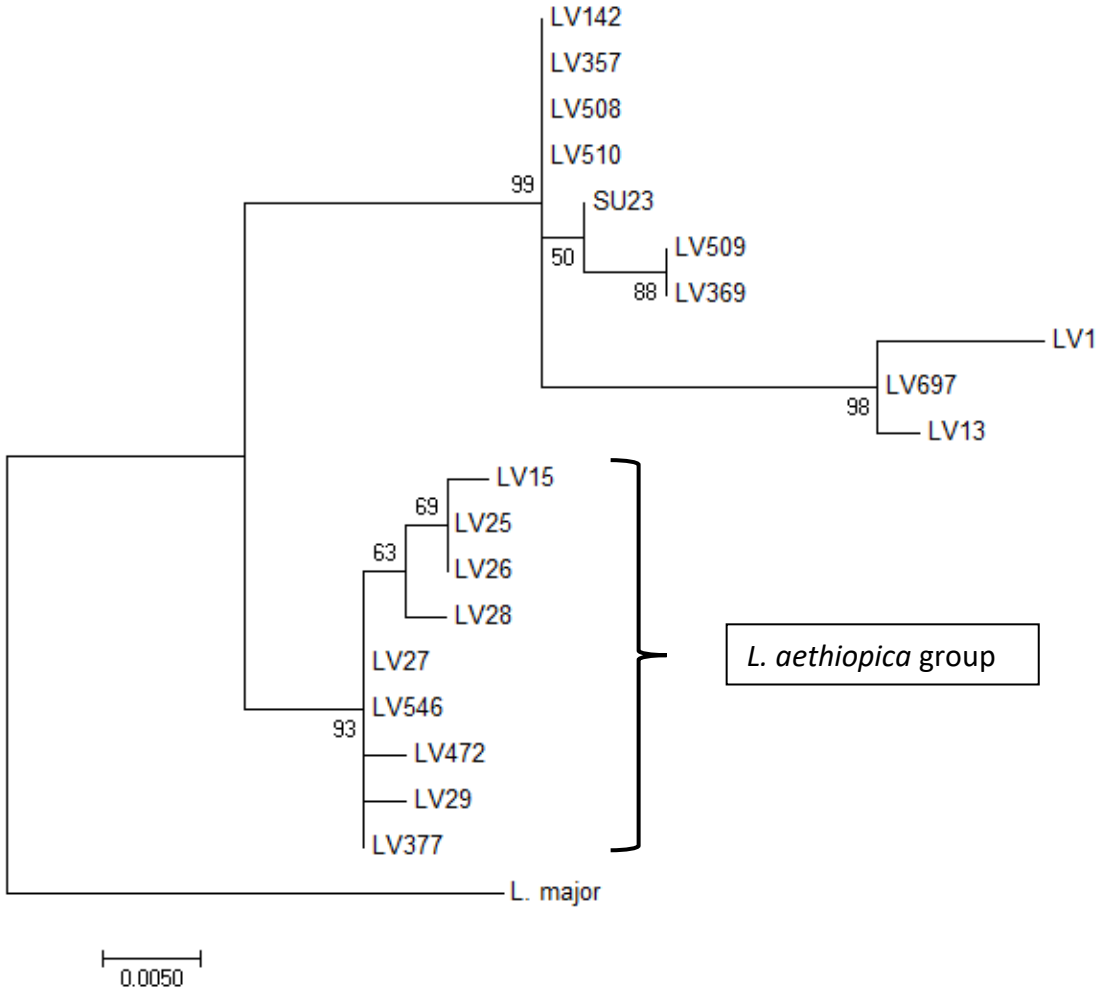


Figure 4.3: Phylogenetic tree of RPL23a sequences for *L. tropica* and *L. aethiopica* with *L. major* as an outgroup. The scale represents 0.02 amino acid substitutions per site.

4.1.3. Distance Pairwise

In order to explore the differences between and within the *L. aethiopica* and *L. tropica* groups in more detail, pairwise comparisons were made between all the sequences to determine their percentage identity. In these analyses 100% identity is indicated by a pairwise distance of 0.0, the value rising as the sequences become more dissimilar. For comparison Table 4.2 shows the Distance Pairwise Comparison for ITS1 sequences of *Leishmania* species *L. infantum* and *L. donovani*, and *L. tropica* and *L. major*. *L. tropica* and *L. major* are known to be different species from both molecular and

biological characteristics, whereas the position of *L. infantum* and *L. donovani* is less clear but with most researchers regarding them as very closely related species. Table 4.3 shows a Distance Pairwise Comparison for all the *L. tropica* and *L. aethiopica* isolates ITS1 DNA sequences anaylsed in this study. The distance pairwise results for *L. tropica* isolates against other *L. tropica* isolates can be seen to produce mostly low values; lower than both distance pairwise results for the *Leishmania* species pairs in Table 4.2. The distance results for LV508 and 509 do not fit this however. The distance results for these two *L. tropica* isolates are higher when compared to the other *L. tropica* parasites, and are higher than the result for *L. infantum* and *L. donovani* in Table 4.2. However, the distance result when comparing LV508 and LV509 to each other is very low. Table 4.3 shows that the *L. aethiopica* isolates compared to other *L. aethiopica* isolates have low distance values, lower than both the distance pairwise values for the *Leishmania* pairs in Table 4.2. The only exception for this is LV28, which has higher distance values against the other *L. aethiopica* isolates, and low values when compared to the *L. tropica* isolates. The distance values in Table 4.3 for the *L. aethiopica* isolates apart from LV28 compared against the *L. tropica* isolates are higher than against the other *L. aethiopica* parasites. The difference value is higher than that of *L. infantum* and *L. donovani* in Table 4.2; however they are still lower than the distance value for *L. tropica* and *L. major*.

***Leishmania* species**	**Distance Pairwise**
L. infantum ***L. donovani***	0.059
L. tropica* LV357** ***L. major	0.192

Table 4.2. Distance Pairwise Comparison for *L. tropica* and *L. major*, and *L. donovani* and *L. infantum* ITS1 Sequences

Table 4.3. Distance Pairwise Comparison for *L. tropica* and *L. aethiopica* isolates ITS1 Sequences

L. tropica with *L. tropica*
L. aethiopica with *L. aethiopica*
L. tropica with *L. aethiopica*

Distance Pairwise for *L. tropica* and *L. aethiopica* ITS1 Sequences																			
		LV1	**LV13**	**LV357**	**LV369**	**LV444**	**LV487**	**LV488**	**LV489**	**LV490**	**LV508**	**LV509**	**LV697**	**LV15**	**LV25**	**LV26**	**LV27**	**LV28**	**LV377**	**LV472**
L. tropica	**LV1**																		
	LV13	0.036																	
	LV357	0.032	0.032																
	LV369	0.047	0.047	0.014															
	LV444	0.022	0.036	0.040	0.054														
	LV487	0.022	0.036	0.040	0.054	0.000													
	LV488	0.022	0.036	0.040	0.054	0.000	0.000												
	LV489	0.022	0.036	0.040	0.054	0.000	0.000	0.000											
	LV490	0.022	0.036	0.040	0.054	0.000	0.000	0.000	0.000										
	LV508	0.137	0.137	0.112	0.126	0.137	0.137	0.137	0.137	0.137									
	LV509	0.151	0.147	0.126	0.140	0.147	0.147	0.147	0.147	0.147	0.072								
	LV697	0.047	0.011	0.043	0.058	0.040	0.040	0.040	0.040	0.040	0.133	0.140							
L. aethiopica	**LV15**	0.119	0.115	0.101	0.115	0.112	0.112	0.112	0.112	0.112	0.176	0.176	0.112						
	LV25	0.122	0.119	0.104	0.119	0.115	0.115	0.115	0.115	0.115	0.180	0.180	0.115	0.004					
	LV26	0.122	0.119	0.104	0.119	0.115	0.115	0.115	0.115	0.115	0.180	0.180	0.115	0.004	0.000				
	LV27	0.133	0.129	0.115	0.129	0.126	0.126	0.126	0.126	0.126	0.180	0.180	0.126	0.032	0.032	0.032			
	LV28	0.004	0.032	0.029	0.043	0.018	0.018	0.018	0.018	0.018	0.133	0.147	0.043	0.115	0.119	0.119	0.129		
	LV377	0.122	0.119	0.104	0.119	0.115	0.115	0.115	0.115	0.115	0.180	0.180	0.115	0.004	0.000	0.000	0.032	0.119	
	LV472	0.126	0.122	0.108	0.122	0.122	0.122	0.122	0.122	0.122	0.183	0.183	0.119	0.011	0.007	0.007	0.040	0.122	0.007
	LV546	0.126	0.122	0.108	0.122	0.122	0.122	0.122	0.122	0.122	0.183	0.183	0.119	0.011	0.007	0.007	0.040	0.122	0.007	0.000

An alternative way of displaying this data is shown in Figure 4.4. This shows the values for the Distance Pairwise Comparison of *L. tropica* and *L. aethiopica* isolate ITS1 sequences to the *L. tropica* isolate LV1. Figure 4.4 illustrates the grouping of most of the *L. tropica* isolates together and below the threshold for *L. donovani* and *L. infantum*, with the exception of LV508 and LV509 as noted above. The *L. aethiopica* isolates are grouped together and clearly distinct from the L. tropica group, with the exception of LV28.

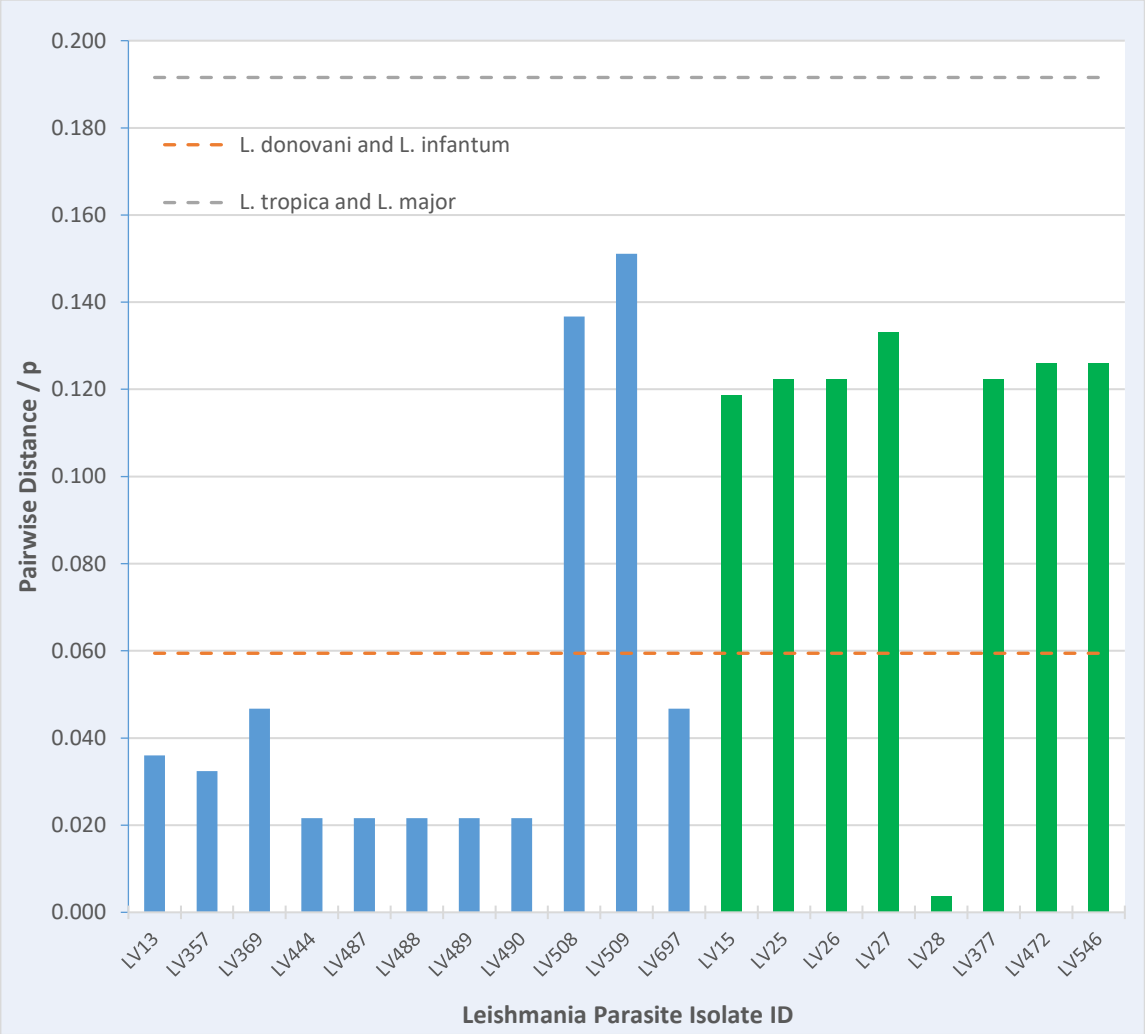


Figure 4.4. Graph of Pairwise Distance for ITS1 *L. tropica and L. aethiopica* Isolates against LV1 (*L. tropica*) with Pairwise Distance for *L. infantum* and *L. donovani*, and *L. tropica* and *L. major*.

Similar analyses were performed for the RPL23a dataset. Table 4.4 shows the Distance Pairwise Comparison for the RPL23a sequences of *L. infantum* and *L. donovani,* and *L. tropica* and *L. major* and Table 4.5 shows this for the *L. tropica* and *L. aethiopica* isolate.

In Table 4.5, the distance pairwise results for *L. tropica* isolates against other *L. tropica* isolates can be seen to be mostly low values. All the values are lower than both distance pairwise results for the *Leishmania* species pairs in Table 4.4, with the exception of LV13 and LV697. The distance results for the LV13 and LV697 *L. tropica* isolates are higher when compared to the other *L. tropica* parasites, and are higher than the result for *L. infantum* and *L. donovani* in Table 4.4. When comparing LV13 and LV697 to each other the distance pairwise has a very low value. The distance results for these isolates compared to the *L. aethiopica* isolates are higher than when they were compared to the *L. tropica* isolates.

The distance values in Table 4.5 for the *L. aethiopica* isolates compared against the *L. tropica* isolates are higher than when comparing like against like, and higher than that of *L. infantum* and *L. donovani* in Table 4.4; however, they are still lower than the distance value for *L. tropica* and *L. major.* LV13 and LV697 have the highest difference values out of the *L. tropica* isolates when being compared against the *L. aethiopica* parasites.

Table 4.5 shows that *L. aethiopica* isolates compared to other *L. aethiopica* isolates have low distance values, lower than both the distance pairwise values for the *Leishmania* pairs in Table 4.4. All of the results from the distance pairwise comparison in Table 4.5, including those for LV13 and LV697, are lower than the distance pairwise for *L. tropica* and *L. major* in Table 4.5.

Leishmania species	Distance Pairwise
L. infantum	
L. donovani	0.006
***L. tropica* LV357**	
L. major	0.050

Table 4.4. Distance Pairwise Comparison for *L. tropica* and *L. major*, and *L. donovani* and *L. infantum* RPL23a Sequences

Table 4.5. Distance Pairwise Comparison *L. tropica* and *L. aethiopica* isolates RPL23a Sequences

Distance Pairwise for *L. tropica* and *L. aethiopica* RPL23a Sequences																	
		LV1	**LV13**	**SU23**	**LV142**	**LV357**	**LV369**	**LV697**	**LV508**	**LV510**	**LV15**	**LV25**	**LV26**	**LV27**	**LV28**	**LV29**	**LV377**	**LV472**
L. tropica	**LV1**																	
	LV13	0.019																
	SU23	0.004	0.023															
	LV142	0.002	0.017	0.006														
	LV357	0.002	0.017	0.006	0.000													
	LV369	0.004	0.023	0.000	0.006	0.006												
	LV697	0.027	0.008	0.023	0.025	0.025	0.023											
	LV508	0.002	0.017	0.006	0.000	0.000	0.006	0.025										
	LV510	0.002	0.017	0.006	0.000	0.000	0.006	0.025	0.000									
L. aethiopica	**LV15**	0.029	0.042	0.029	0.027	0.027	0.029	0.046	0.027	0.027								
	LV25	0.027	0.040	0.027	0.025	0.025	0.027	0.044	0.025	0.025	0.002							
	LV26	0.027	0.040	0.027	0.025	0.025	0.027	0.044	0.025	0.025	0.002	0.000						
	LV27	0.023	0.038	0.023	0.021	0.021	0.023	0.042	0.021	0.021	0.006	0.004	0.004					
	LV28	0.027	0.040	0.027	0.025	0.025	0.027	0.044	0.025	0.025	0.006	0.004	0.004	0.004				
	LV29	0.025	0.040	0.025	0.023	0.023	0.025	0.044	0.023	0.023	0.008	0.006	0.006	0.002	0.006			
	LV377	0.023	0.038	0.023	0.021	0.021	0.023	0.042	0.021	0.021	0.006	0.004	0.004	0.000	0.004	0.002		
	LV472	0.025	0.040	0.025	0.023	0.023	0.025	0.044	0.023	0.023	0.008	0.006	0.006	0.002	0.006	0.004	0.002	
	LV546	0.023	0.038	0.023	0.021	0.021	0.023	0.042	0.021	0.021	0.006	0.004	0.004	0.000	0.004	0.002	0.000	0.002

L. tropica with *L. tropica*
L. aethiopica with *L. aethiopica*
L. tropica with *L. aethiopica*

Figure 4.5 is a graph showing the Distance Pairwise Comparison of *L. tropica* and *L. aethiopica* isolate RPL23a sequences to the *L. tropica* isolate LV1. The distance results for the LV13 and LV697 *L. tropica* isolates are higher when compared to the other *L. tropica* parasites, and are higher than the result for *L. infantum* and *L. donovani*. The difference values are higher than that of *L. infantum* and *L. donovani* in Table 4.4; however, they are still lower than the distance value for *L. tropica* and *L. major*.

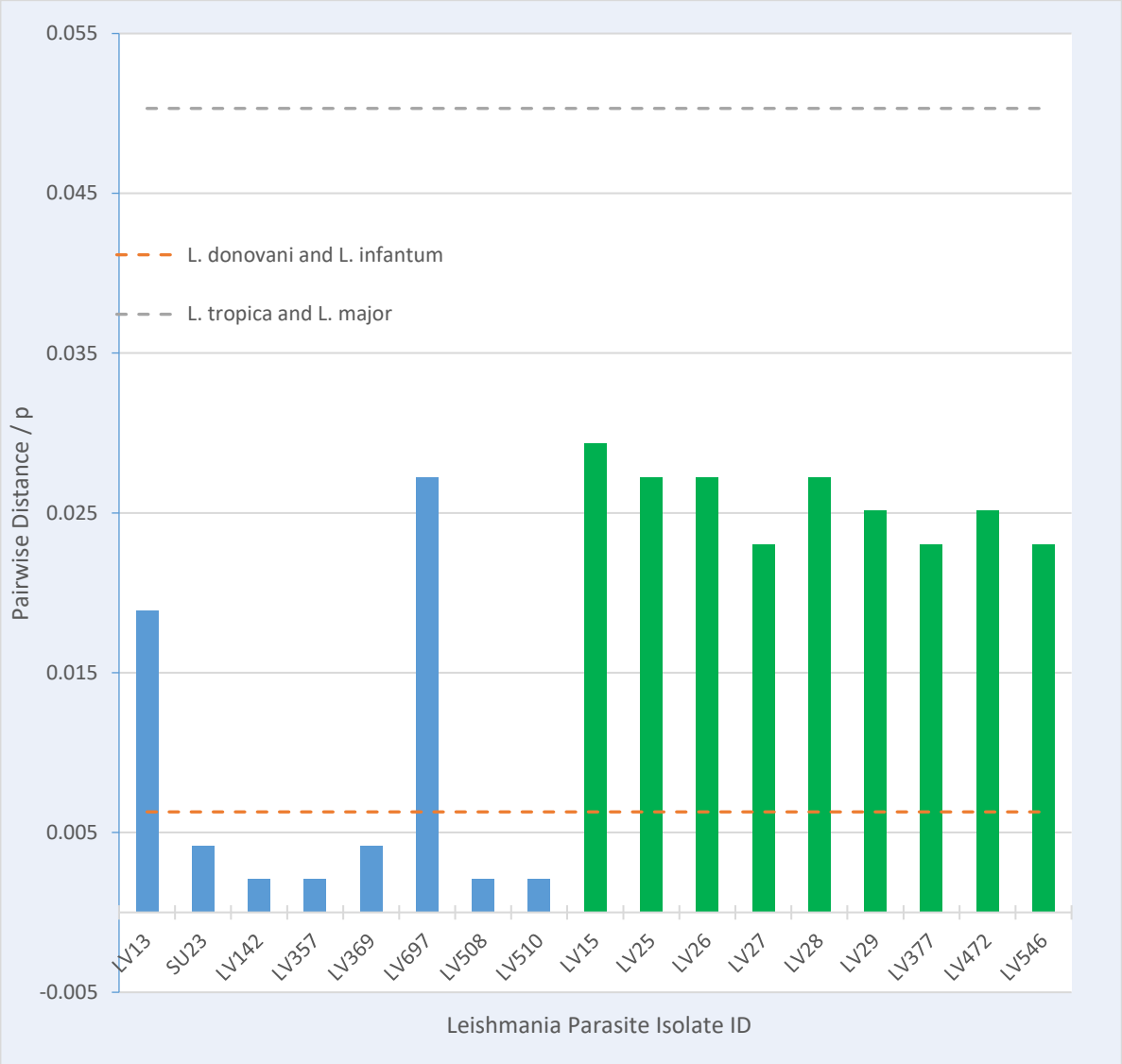


Figure 4.5. Graph of Pairwise Distance for RPL23a *L. tropica* and *L. aethiopica* Isolates against LV1 (*L. tropica*) with Pairwise Distance for *L. infantum* and *L. donovani*, and *L. tropica* and *L. major*.

4.2. Dot Blot for Viral dsRNA

Dot blots were used to identify the presence of the dsRNA virus in different isolates of *Leishmania.* Isolates were tested in batches of two to five with a positive and negative control each time. The positive control was *L. guyanensis* M4174 and the negative control was *L. major* LV39. A dot blot using the controls at different concentrations is shown in Figure 4.6.

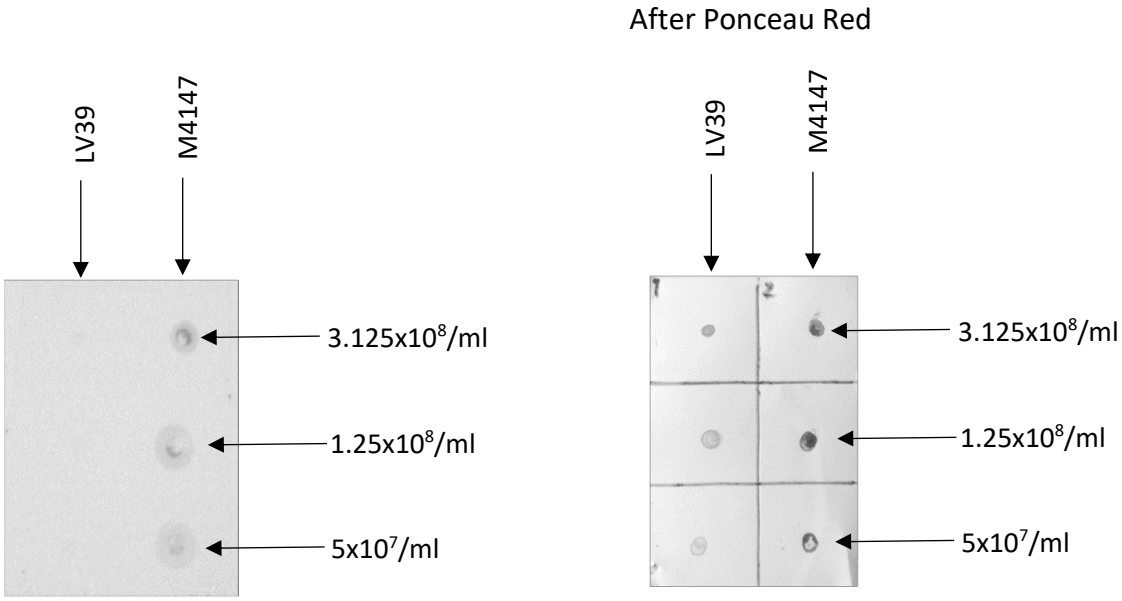


Figure 4.6 Dot blot of positive, M4147, and negative, LV39, control isolates M4147 at three concentrations. Membrane after Ponceau Red post stain on the right.

The image shows the positive and negative controls at three concentrations. The image of the gel before the Ponceau red stain shows the difference between the two parasites. The image on the left shows the Ponceau red stain and shows the presence of the negative control, supporting the accuracy of negative results. The positive and negative controls were used in all of the dot blots performed to increase the reliability of the experiments as it shows the dot blot has worked properly. The results were tested with different concentrations of the anti-mouse IgG HPR conjugate antibody and the anti-

dsRNA MAB J2 (Ig2a) antibody to find the concentrations that best showed the positive control without over saturating the negative control.

The collection of *L. aethiopica* and *L. tropica* parasite isolates were tested for the presence of the RNA virus in groups of two to four. Figure 4.7 illustrates a typical experiment and shows four *Leishmania* isolates being testing for the virus alongside the positive and negative control.

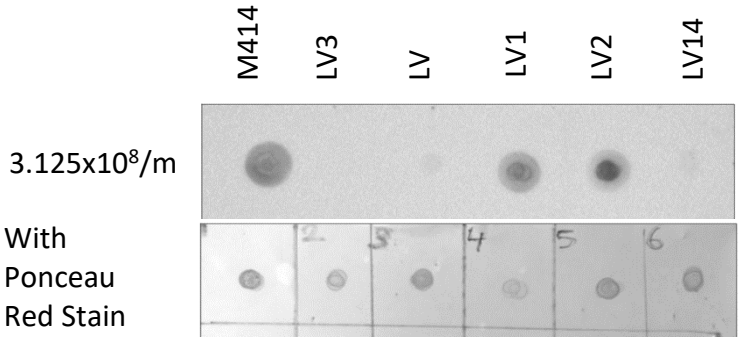


Figure 4.7 Dot blot showing positive and negative controls with samples testing positive and negative. Post stain with Ponceau red is shown below.

Figure 4.7 shows the positive and negative controls and four parasite isolates being tested for the virus. Two of the isolates are positive and two are negative. The Ponceau red stain shows that all isolates were present and that the negative results were not due to the protein being washed off the membrane. As shown in Figure 4.7, some results show up very faintly on the dot blot, LV1 and LV142 in the case of the dot blot shown; however, these are counted as a negative result as they are distinctly different from the positive control. The dot blots for all *L. tropica* and *L. aethiopica* isolates tested can be seen in Appendix IV. All positive results were repeated after all the *Leishmania* parasite isolates were tested. An overview of the results of the dot blot for the RNA virus is shown in Table 4.6.

Isolate	Original ID	RPL23a ID	ITS1 ID	dsRNA Virus
LV357	L. tropica	L. tropica (457)	L. tropica (308)	Negative
LV510	L. tropica	L. tropica (457)		
LV508	L. tropica	L. tropica (457)	L. tropica (308)	Negative
LV697	L. aethiopica	L. tropica (458)	L. tropica (313)	Negative
LV142	L. tropica	L. tropica (457)		Negative
SU23	L. tropica	L. tropica (458)		Negative
LV13	L. aethiopica	L. tropica (457)	L. tropica (313)	Negative
LV1	L. aethiopica	L. tropica (459)	L. tropica (306)	Negative
LV369	L. tropica	L. tropica (458)	L. tropica (308)	Negative
LV509	L. tropica	L. tropica (458)	L. tropica (308)	Negative
LV487	L. tropica	L. tropica (457)	L. tropica (308)	Negative
LV444	L. tropica	L. tropica (457)	L. tropica (308)	Negative
LV489	L. tropica	L. tropica (457)	L. tropica (308)	Negative
LV488	L. tropica	L. tropica (457)	L. tropica (308)	Negative
LV490	L. tropica	L. tropica (457)	L. tropica (308)	Negative
LV27	L. aethiopica	L. aethiopica (460)	L. aethiopica (324)	Positive
LV377	L. aethiopica	L. aethiopica (460)	L. aethiopica (327)	Negative
LV546	L. aethiopica	L. aethiopica (460)	L. aethiopica (326)	Negative
LV29	L. aethiopica	L. aethiopica (460)		Negative
LV472	L. aethiopica	L. aethiopica (460)	L. aethiopica (325)	Negative
LV28	L. aethiopica	L. aethiopica (460)	L. tropica (308)	Negative
LV26	L. aethiopica	L. aethiopica (460)	L. aethiopica (327)	Positive
LV15	L. aethiopica	L. aethiopica (460)	L. aethiopica (330)	Positive
LV25	L. aethiopica	L. aethiopica (460)	L. aethiopica (327)	Negative

Table 4.6 – Table of all *leishmania* isolates with original, RPL23a and ITS1 IDs and stating the presence of the viral dsRNA.

The percentage of positive and negative results of the viral dsRNA presence for both *L. tropica* and *L. aethiopica* is shown in Figure 4.8.

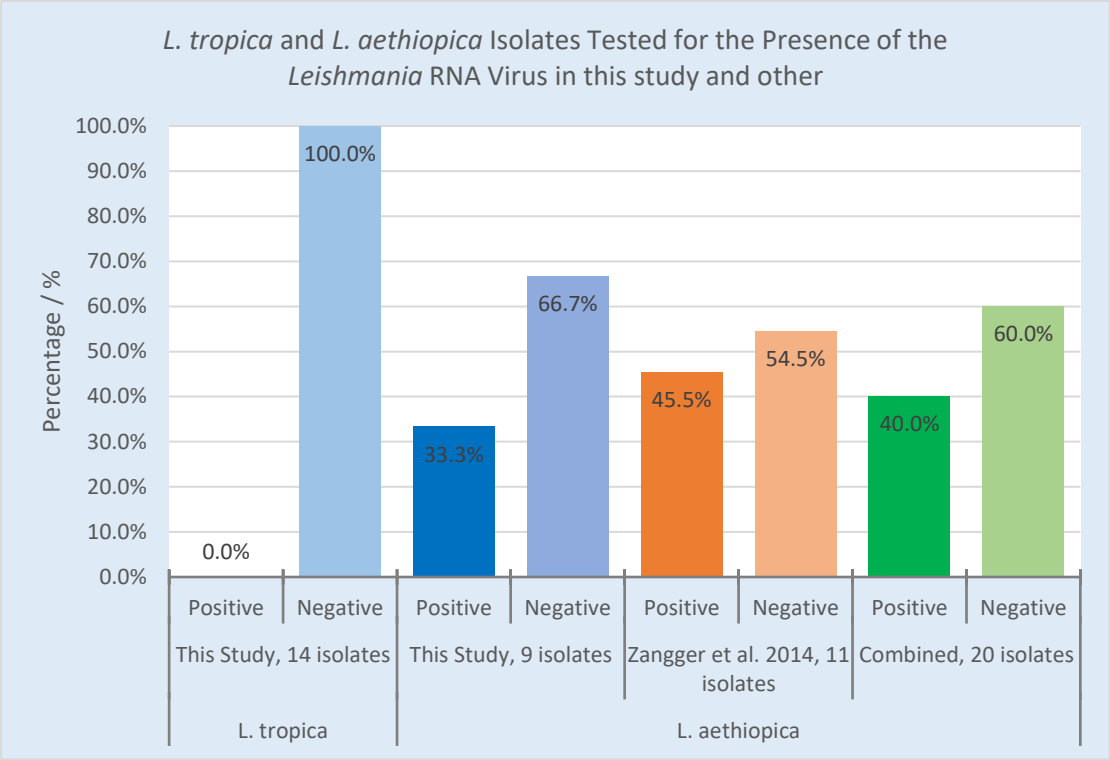


Figure 4.8. The percentage of *L. tropica* and *L. aethiopica* parasites that were found positive and negative for the *Leishmania* RNA Virus in this study and others.

The graph shows that 100% of the *L. tropica* parasite isolates tested were negative for the virus. The *L. aethiopica* parasite isolates on the other hand had positive and negative results, with 33.3% testing positive for the virus. Figure 4.8 also shows the percentage of *L. aethiopica* that have previously been found positive or negative for LRV in a study by Zangger et al. in 2014. In that study 45% of *L. aethiopica* parasite isolates tested were found positive for the presence of LRV. The graph in Figure 4.8 also shows that the overall percentage of *L. aethiopica* isolates tested that were found to contain the virus is 40%.

Chapter Five: Discussion

The main aim of this study was to investigate the relationships between *Leishmania aethiopica* and *L. tropica* using a molecular approach with DNA sequence analysis and a biological approach regarding the presence of *Leishmania* viruses. For the molecular approach PCR was used to sequence the ITS1 products of *L. aethiopica* and *L. tropica* parasite isolates, and the results were presented in phylogenetic trees. The phylogenetic tree in Figure 4.2 generated from the ITS1 primer PCR results from this study shows a strong branch point separating the parasite isolates identified as *L. aethiopica.* The results of the PCR with ITS1 primers supported the findings from a PCR performed with RPL23a primers and indicated that three parasite isolates that were originally identified as *L. aethiopica* are actually *L. tropica.* The phylogenetic tree of the RPL23a primer PCR product in Figure 4.3 also shows a strong branch point between the parasite isolates identified as *L. tropica* and *L. aethiopica.* This provides further support that *L. tropica* and *L. aethiopica* are two clear biological entities.

Within the *L. tropica* and *L. aethiopica* groups there is further branching; however, there is little correlation for this branching generated by the ITS1 and RPL23a sequences. The bootstrap values for the in-group branching vary. This is not surprising because these sequences chosen for their ability to discriminate at the species level are not generally used for population level analysis. Microsatellite sequencing would provide greater insight and accuracy into the branching within the species; however, these sequences are often species specific and would not provide the phylogenetic analysis needed for this study.

The phylogenetic patterns for both the ITS1 and RPL23a PCR products support each other's identification of the isolates as *L. tropica* and *L. aethiopica;* however, this does

not fully determine that *L. tropica* and *L. aethiopica* are two different species of *Leishmania.* The bootstrap values on the phylogenetic trees can provide some indication of the difference between the *L. tropica* and *L. aethiopica* isolates, but does not indicate if they are sufficiently different to be classed as different species. It is unclear where to draw the line to classify them as different species, and the percentage difference between the nucleotides of isolates identified as *L. tropica* and *L. aethiopica* would provide additional insight. Tests for distance pairwise were performed to help determine this.

Distance pairwise tests were performed on isolates from pairs of *Leishmania* species whose relationship is more established to give calibration to these data. The pairs used were *L. tropica* and *L. major*, and *L. donovani* and *L. infantum.* The species *L. tropica* and *L. major* are known to cause very similar clinical diseases, yet are significantly different biologically, with different sand fly vectors and reservoirs. The *Leishmania* species *L. donovani* and *L. infantum* meanwhile are thought to be much more closely related. The use of these pairs of *Leishmania* in the distance pairwise test for the *L. tropica* and *L. aethiopica* parasite isolates provides examples of the distance between two closely related species and two more distant species.

The distance pairwise for the ITS1 results can be seen in Table 4.3 and Figure 4.4. The distance pairwise results in Table 4.3 show that when comparing an *L. tropica* isolate to the other *L. tropica* isolates the distance values are generally low, apart from those for the isolates LV508 and LV509. With the exception of those two isolates, the distance values are lower than those for both *L. infantum* vs *L. donovani* and *L. tropica* and *L. major* in Table 4.2. These results indicate that the isolates are the same species of *Leishmania.* The distance pairwise results show that when comparing an *L.*

aethiopica isolate to other *L. aethiopica* isolates the distance values are low, with the exception of LV28. These values are lower than both of the distance pairwise values for the control *Leishmania* pairings in Table 4.2. This would indicate that these isolates are of the same species. The distance pairwise results for the ITS1 sequence of *L. tropica* and *L. aethiopica* isolates shows that there is a higher distance value when comparing an *L. aethiopica* against an *L. tropica* relative to when comparing like with like. These distance values are higher than that of the *L. infantum* against the *L. donovani* in Table 4.2. This contributes to the impression that the isolates are from different species. The exception for this is the *L. aethiopica* isolate LV28.

All of the distance values are below that for *L. tropica* and *L. major;* this includes the isolates that contrast with the rest of the results, LV28, LV508 and LV509. The *L. tropica* isolates LV508 and LV509 were found to be very similar to each other when compared, and even though they had a higher difference from the other *L. tropica* isolates they had an even higher difference when compared to the *L. aethiopica* isolates. This indicates that they are more like *L. tropica* than *L. aethiopica.* The two isolates are from Iraq and possibly taken from the same area at the same time, and so it is possible that their difference from the *L. tropica* isolates is due to a microsatellite expansion that they have in common. The *L. aethiopica* isolate LV28 on the other hand has a lower difference from the *L. tropica* isolates than the other *L. aethiopica* isolates. This indicates that the isolate might be an *L. tropica.* This would support the findings from the identification and phylogenetic tree of the PCR products.

The pairwise distances for the RPL23a sequence of *L. tropica* and *L. aethiopica* parasite isolates is shown in Table 4.5 and Figure 4.5. When comparing an *L. aethiopica* isolate to the other *L. aethiopica* isolates the pairwise distance value is very low; lower than both *L. infantum* vs *L. donovani* difference and *L. tropica* and *L. major* difference in

Table 4.4. This would indicate that the isolates are of the same species. This is also the case when comparing an *L. tropica* isolate to the other *L. tropica* isolates, with the exception of the isolates LV13 and LV397. These isolates were originally identified as *L. aethiopica* but were identified as *L. tropica* from the PCR of the RPL23a and ITS1 products. The pairwise distance when comparing the two isolates together is very low. The distance value when comparing the *L. aethiopica* isolates against the *L. tropica* isolates is higher than when comparing isolates identified as the same species, and higher than the pairwise distance value for *L. infantum* and *L. donovani*. This includes the LV13 and LV397 isolates. This indicates that *L. tropica* and *L. aethiopica* are different species.

The distance pairwise results for both the ITS1 and RPL23a products generally support the phylogenetic results. The results indicate that the *Leishmania* species *L. tropica* and *L. aethiopica* are more significantly different than those of *L. donovani* and *L. infantum*, but are more similar than *L. tropica* and *L. major*. The results for both sequences have some outliers, but only LV28 contradicts expectations.

The *L. aethiopica* isolate LV28 has been identified as *L. tropica* through the PCR of the ITS1 sequence. The isolate also grouped as *L. tropica* in the phylogenetic tree of the sequence, and the results of the distance pairwise test show the isolate to be closer to the *L. tropica* isolates than the other *L. aethiopica* isolates. All these results would indicate that the isolate has been misidentified; however, previous PCR work on the isolate with the RPL23a sequence identifies it as an *L. aethiopica.* The most likely explanation for this would be a mix up with the culture of the isolate at some point. Since this study, the isolate has been retested. A repeat of the PCR with the ITS1 primers showed the isolate as a *L. tropica* again. A repeat of the PCR for the RPL23a sequence provided inconclusive results, indicating a mixture. Earlier work on the

isolate showed it to be *L. aethiopica.* The possible explanations for the results for this isolate are misidentification when the isolate was originally collected; a mix up of the culture at some point between its collection and this study; or a problem with the experimental method. These results indicate that there has been a mix-up or contamination of the isolate at some point. It should be noted that these isolates were originally deposited in the cryobank in the 1970s, at that time held in the Liverpool School of Tropical Medicine. To resolve the identification earlier or alternative (from another cryobank) samples of the isolate need to be tested.

Other results from this study that need to be examined are the isolates that were originally identified as *L. aethiopica* but the results consistently returned as *L. tropica.* These isolates, LV1, LV13 and LV697, were identified as *L. tropica* by the PCR of both the ITS1 and RPL23a sequences. There are a few possible explanations for these results. Firstly, it is also possible that these isolates were misidentified when first collected. All the isolates came from Ethiopia, and so it is possible that they were taken at the same time as other *L. aethiopica* isolates and were presumed to be the same; as PCR sequencing on samples was not possible at the time. Another significant possibility is that the isolates were originally *L. aethiopica* but at some point between collection and these studies there was a mix up or contamination of the cultures with *L. tropica* samples. There are other possible explanations, such as the isolate being a hybrid of the two species; however, this is less likely. One study used a fluorescence resonance energy transfer (FRET)-based real-time PCR assay to differentiate between *L. tropica, L. aethiopica* and *L. major* (Nath-Chowdhury et al. 2016). The three species were readily differentiated based on their melting curve characteristics. The study identified LV13 as *L. aethiopica.* This indicates that the isolate was originally accurately identified as *L. aethiopica* and suggests that the sample used in this study

may have been subject to a mix up or contamination of the culture, although this is not for certain. A study by Zelazny in 2005 which evaluated the use of the 7SL RNA gene sequence for the identification of *Leishmania* species identified LV1 as an *L. aethiopica;* however, the results of the PCR found *L. aethiopica, L. tropica, L. major* and *L. mexicana* sequences to be the same length, and the *L. tropica* and *L. aethiopica* to be very close on the phylogenetic tree. A study in 2011 by Odiwuor used PCR of the ITS1 sequence on Old World species of *Leishmania.* In this study, LV1 was identified as *L. tropica.* These results support the ITS1 results of this study and the RPL23a findings. This could indicate that the sample was originally miss identified as *L. tropica,* although this cannot be said for certain.

Misidentification of *L. tropica* isolates as *L. aethiopica* upon collection could be due to the assumed species present at the site. The two species of *Leishmania* are now known to have some overlap in geographical incidence. One possible explanation for the presence of *L. tropica* in areas of Africa where *L. aethiopica* is found is the impact of climate change on the distribution of species. In the 2014 report of the Intergovernmental Panel on Climate Change (IPCC) it is stated that climate change will affect human health through the exacerbation of already existing health problems (Woodward et al. 2014). One way in which *Leishmania* may be affected by climate change is through its vectors. Vector borne diseases are particularly susceptible to climate change (Woolhouse et al. 2001). This is because the species distribution is related to climatic variables (Carvalho et al. 2015), and so vector species with a distribution based on climatic variables may experience a shift in distribution when those variables change (Parham et al. 2015). Leishmaniasis is a climate sensitive disease (Ready, 2008), and sand flies behaviour and distribution being affected by temperature, rainfall and humidity is a large factor in this (Carvalho et al. 2015). It has

been observed that cutaneous leishmaniasis has expanded beyond its natural ecoregion, particularly in North Africa and regions of the Middle East, areas where significant environmental changes have occurred (Bounoua et al. 2013). Cutaneous leishmaniasis has spread from northwards from the Algerian arid zones and towards the semi-arid areas. Zoonotic cutaneous leishmaniasis in this region caused by *L. major* is the dominant infection (Alvar et al. 2012). Tt has been suggested that climate change and desertification observed in the northern Sahara has had a role in this expansion of the disease (Mcdowell et al. 2011). Cutaneous leishmaniasis is also rapidly spreading across Morocco (Bounoua et al. 2013). Climate change is known to have an impact on vector borne diseases and is thought to have had an impact on the incidence of zoonotic cutaneous leishmaniasis caused by *L. major;* it is possible that changes in the climate has had an impact on the distribution of cases of *L. tropica*. Furthermore, increased understanding of the impact of climate change on vector distribution is vital for the understanding of the eco-epidemiology of leishmaniasis; and for the success of surveillance and control implementations (Carvalho et al. 2015).

Leishmania viruses were originally described some time ago, but have received more recent attention as a potential contributor to pathology of leishmaniasis. Currently it is not clear whether these are restricted to certain species of *Leishmania*. In this study the presence of LRV in the *L. tropica* and *L. aethiopica* isolates was tested for using a dot blot with a monoclonal antibody specific for double-stranded RNA. The results of the dot blots are summarized in Table 4.6. The dot blot assay found that none of the *L. tropica* isolates were positive for the presence of the virus, and the species has not previously been found to be positive. Three of the *L. aethiopica* isolates tested in this study tested positive for the presence of LRV. Other *L. aethiopica* isolates have previously been tested and some found positive for the virus by Zangger in 2013. One

of the *L. aethiopica* isolates tested in this study has previously been tested for LRV, and was found to be negative for the virus in both studies. The *Leishmania* parasites found positive for the virus in this study were not tested previously. Figure 4.8 shows the percentage of *L. aethiopica* isolates that have been found to be positive for the presence of LRV in this study and those previously performed. The combined results show that the RNA virus is present in 40% of *L. aethiopica* isolates tested so far.

LRV being found present in *L. aethiopica* but not *L. tropica* parasites is evidence of a further difference between the two, supporting that they are different species. The LRV results indicates that there might be a biological difference between *L. tropica* and *L. aethiopica.*

The aims of this study were to look into the relationship between *L. tropica* and *L. aethiopica*, as well as to look for the presence of the *Leishmania* RNA Virus in these species. The results of the PCR on the ITS1 sequences reinforce findings that *L. tropica* and *L. aethiopica* are two different species. This is also shown in the phylogenetic trees for both the ITS1 and RPL23a products where the *L. tropica* and *L. aethiopica* isolates split into two distinct groups. This is further supported by the distance pairwise results for the two sequences. The results of this test showed that the difference between the *L. tropica* and *L. aethiopica* isolates is greater than when comparing like with like. The results also showed that the two species are more different than *L. donovani* and *L. infantum*, supporting the different species concept; however, the results also showed that the species are more similar than *L. tropica* and *L. major*. This would indicate that the species are closely related.

The results of the dot blot contributed to evidence of the *Leishmania* RNA Virus being present in the *L. aethiopica* species. The combined results of this study and others

indicate that it is present in approximately 40% of the species, although more isolates should be tested to support this. The presence of LRV was not detected in the *L. tropica* species. The presence of the virus in *L. aethiopica* isolates but absent from all *L. tropica* isolates tested introduces another difference between the two and adds support to the two being different species.

Both the PCR sequencing and the dot blot for the viral RNA were effective for answering the aims of this study. The PCR was a relatively quick way to identify and classify the parasite species, and the method provided data that could be used in several evaluations. PCR is a useful technique for the sequencing of DNA. It has a high sensitivity for the detection of a specific DNA sequence in a sample. In this study, PCR was used for identification and classification of *Leishmania* parasite isolates. The results of the PCR were used in several ways in this study. Sequences were identified, made into phylogenetic tress with bootstrapping and different outgroups; pairwise distance evaluated against two pairs on known species; and all assays compared to the sequences for the RPL23a sequence. The PCR successfully helped to identify the isolates as *L. tropica* or *L. aethiopica* and phylogenetic trees were generated from the sequences, and demonstrated two data sets. The PCR successfully aided in classifying the species, and also in determining if they should be classified as different species. Comparing the PCR of the ITS1 sequence to the PCR of the RPL23a sequence and analysing both sets of data provided additional substance to the results as the data sets generally supported each other.

The sequence used in this study, ribosomal DNA internal transcribed spacer 1 (ITS1), is a commonly used sequence when working with *Leishmania*. The wide use of the sequence in molecular phylogeny and taxonomy is due to several favorable features of

the gene. The sequence can be amplified with PCR using universal primers. The gene undergoes rapid concerted evolution which promotes inter-genomic homogeneity of the ITS paralogues. Due to the high copy number of rDNA clusters, the sequence can be detected even in small quantities of DNA. The gene has a high degree of variation, even between closely related species (Baldwin et al. 1995), making it an appropriate target for the PCR in this study. Despite the advantages of the ITS1 gene, there are also drawbacks in its use. Although the gene is very good for pure identification, the ITS1 gene can be misleading for phylogenetic purposes. ITS1 is from a multigene family, and a change in one copy can spread throughout the genome. Because of this, large changes can occur. An ideal phylogenetic marker evolves at a steady rate through time, and so the amount of change or degree of difference between two markers directly correlates to how far and how long they have been separated. This is the basis that a reliable phylogenetic tree is built on. The large changes from random events that can occur in the ITS1 gene can be compromising when generating a good map of phylogeny.

The sequences for the PCR used in this study did not provide highly accurate insight in group branching. A microsatellite sequence approach would be more appropriate for this; however those tend to be species-specific and would not have helped in determining the classification of the *L. tropica* and *L. aethiopica* isolates in respect to each other. Microsatellite sequencing could add insight into the *L. tropica* isolates LV508 and LV509, and the length of their sequences and results from the distance pairwise indicate that there might have been a microsatellite expansion.

The PCR method and the sequences used were appropriate for achieving the aims of this study. All results of the PCR support that *L. tropica* and *L. aethiopica* are different

species with exception of the *L. aethiopica* isolate LV28. Furthermore, the results for the *L. aethiopica* isolate LV28, and the misidentified isolates, demonstrate the importance and value of the PCR method. If the results are due to a prior culture mix up, it exhibits how important it is to ensure the sample is accurate, and how PCR is an effective and accessible method to achieve this. Although the PCR provided various data that supports *L. tropica* and *L. aethiopica* being different species, it cannot definitively determine that they are so.

One of the main steps that would need to be taken next would be to re-examine the isolates that returned unexpected or contradictory results, LV1, LV13, LV697 and LV28, with LV28 taking the highest priority. All of these isolates should be re-assayed with PCR of both the ITS1 and RPL23a sequences. The PCR should be run for both recent and older samples of the isolates. If possible, other laboratories with the isolates could be contacted. This could help indicate if the isolates have been contaminated or mixed at some point.

The dot blot method used in this study was successful for detecting LRV in the *Leishmania* parasite isolates tested. It was only used to check for the presence of the virus in *L. tropica* and *L. aethiopica* isolates used in the PCR. This study did not assess the amount of the virus that was present, and the method does not provide insight into the ability of the parasite to gain or lose the virus in culture or over the course of infection. These are possible areas for future research into LRV. The dot blot in this study found that a third of the *L. aethiopica* isolates tested had presence of LRV, which supports previous findings by Zangger (2013); and found all of the *L. tropica* isolates tested to be negative for the presence of the virus. These findings provide further evidence of a difference between *L. tropica* and *L. aethiopica*, supporting the results of

the PCR that they are two species. This shows that not only was the dot blot an appropriate method for the aim of the study to detect LRV, but also contributed to the aim of study to investigate the relationship between *L. tropica* and *L. aethiopica.*

The results for the dot blot have the helped to increase knowledge of which species carry LRV. As the virus has strong links to an increased pathology of leishmaniasis understanding which species of *Leishmania* can carry the virus is important. The knowledge could be used to provide increased care needed in areas affected by this species due to increased risk of mucosal complications.

An area of further research could be in the relationship between the *Leishmania* RNA virus and the *Leishmania* parasites. Further research could help determine whether the presence of LRV is a benefit or a detriment to the parasite, or if it simply acts as a silent partner. There is a high possibility that the presence of the virus in the *Leishmania* has some cost to the parasite; however, if the cost is small then the virus may potentially be a benefit for the parasite. LRV has links to increased pathology of leishmaniasis. It is possible that the increase in lesions on the host attracts the sand fly vectors, and so improving the transmission of the *Leishmania* parasite. There have been many reports that parasite presence in a host can lead to transmission in various ways. It has been shown that mosquitoes have a greater feeding success when exposed to mice infected with malaria due to the impairment of the host's defensive behavior (Day and Edman, 1983). It has been observed that female sand flies were more attracted to hamsters that were infected with *L. infantum* possibly due to the odour of the host (O'Shea et al in 2002). Coleman and Edman (1988) studied the feeding of sand flies on cutaneous lesions caused by *Leishmania.* They concluded that there was a positive correlation between the probing and feeding and the location of the lesions, although the initial

landing of the sand fly was random. A possible future study to investigate a correlation between the presence of the virus and increased transmission would be observing the feeding of the sand flies and transmission of the parasite with mice, comparing the results for *Leishmania* with and without the presence of LRV.

If an increase in pathology does lead to an increase in transmission, the presence of LRV in *Leishmania* would be advantageous to the parasite. It has already been seen that LRV is only found in some *Leishmania* species. A continuation of this study would be to test other species of *Leishmania* for presence of the *Leishmania* RNA virus. This would help to develop an understanding of why some species have the virus and some do not, in addition to providing a greater understanding of the role of the virus in the pathology of the disease. Furthermore, if more *Leishmania* species are tested for presence of LRV then the pattern of how the virus is distributed could be analysed. RNAi is only expressed in some species of *Leishmania.* The species that do express RNAi activity include *L. braziliensis* and *L. guyanensis*, species that LRV1 is present in. RNAi usually controls RNA viruses infecting the organism, yet it appears that LRV1 is able to survive in its presence. Despite this, further research into the interaction of RNAi and LRV in species of *Leishmania* where both are present has the possibility to lead to the use of RNAi to generate LRV-free isogenic isolates.

Another potential area to investigate would be the impact on immunity in areas affected by *Leishmania* species with LRV. As the virus is thought to increase the pathology of the disease, this could potentially lead to an increase in immunity in the populations of these areas. This could be investigated by documenting cases of infection and immunity in areas where strains of *Leishmania* that are known to be infected with LRV are

prevalent and comparing with regions were species that have not yet been found to contain the virus are dominant.

In addition to investigating any beneficial aspect of the presence of the virus for the parasite, other aspects of the relationship between the virus and the parasite need to be explored. Areas of their relationship that could be looked into include how the parasite acquires the virus and the potential to lose it. *Totiviridae* viruses, such as the Leishmania RNA Virus, like most others of the family, are typically neither infectious nor shed, and so are generally only inherited vertically or during genetic exchange. This raises the question of how the parasites gain the virus in the first place. The diversity of species of *Leishmania* are thought to have arisen primarily through the gradual accumulation of divergent mutations, rather than through sexual recombination. However, despite its presumed infrequency in nature and experimentally, genetic exchange does occur. There have been many examples of genetic hybridization in field isolates involving most *Leishmania* species, and it is possible that they have occurred though mating events. In this case, it could be possible for one of the parasites to have the virus, and so pass it to any offspring. A possible future study would be a comparison of *Leishmania* species that have been found to have the presence of LRV and the species that are known to exhibit genetic exchange. It is also possible that LRV can also be horizontally transmitted, but that it occurs rarely or at a lower rate than has been detected. Another point of interest is the potential loss of the virus. It has been observed (Ro et al. 1997) that an isolate with LRV can be cultured to produce a strain that no longer has the virus, although this was achieved by drug selection and not obtained through culture alone. In culture conditions the virus can only undergo vertical transmission. The culture medium is high in nutrients, and so the parasites grow and replicate faster than they would normally. Due to this the growth rate of the parasites

could be greater than the replication rate of the virus. There are other areas around the infection of LRV that could be investigated. The fluctuation and amount of virus present over the course of an infection could be examined. As LRV appears to be mostly limited to vertical transmission, it is likely that there is a limit to how much it will replicate. If there is no common spreading mechanism, the virus replicating to the point it is detrimental to its host will harm the virus too.

For *L. aethiopica,* half of isolates from fresh clinical samples tested were positive for the virus, and less than half when testing isolates from a cryo-bank. An area for further study would be testing more fresh clinical *Leishmania* isolates for LRV as this could provide additional insight into the relationship between the parasite and the virus. The *Leishmania* parasite could be attempting to get rid of the virus, or is it purely random whether the parasite has the virus or not. Moreover, knowing which species of *Leishmania* may have the virus could also mean that the areas where the species is prevalent need more clinical care and preventative measures against the parasite.

In summary, this work has shown *L. tropica* and *L. aethiopica* to be different species. The results of the PCR for the ITS1 product were used to identify the isolates, map them phylogenetically and were compared to the results of the RPL23a sequences. The results were also used in a distance pairwise assay to observe the significance of the difference between the two species. All of these results support *L. tropica* and *L. aethiopica* as different species. The results of the dot blot showed that the Leishmania RNA Virus is present is some *L. aethiopica* isolates but has not been found in *L. tropica.* These results are consistent with previous findings, and also support that *L. tropica* and *L. aethiopica* are different species.

There are many areas for future investigation related to this study. The priority is to repeat the PCR for LV28 using different sequences with new and old isolates to try and determine whether the isolate has been misidentified in the past or has become contaminated more recently. In addition to this, key areas for future research include testing more species for the presence of LRV; to examine biological differences between *L. tropica* and *L. aethiopica* investigating the relationship between LRV presence, the rate of transmission, and immunity in the affected population; and the spreading mechanism of LRV between parasites. A more complete list of which species of *Leishmania* can carry the virus will help improve the understanding of the role of LRV and how it interacts with the *Leishmania* parasites. As the virus is linked to increased pathology, the knowledge could also be used to promote aid in the areas that are affected by *Leishmania* species that carry LRV. The results of the dot blot showing *L. aethiopica* isolates can contain LRV but no evidence that *L. tropica* can carry the virus indicates that there may be a possible biological difference. If this is found then it could conclusively determine whether *L. tropica* and *L. aethiopica* are indeed different species. Developing a greater understanding of the spreading mechanism of LRV could help develop treatment or prevention against the highly pathogenic disease the virus is associated with. Examining the relationship between LRV and the rate of transmission of leishmaniasis will help understand whether the virus is overall beneficial for the *Leishmania* parasite, and could explain its presence. Finally, investigating the effect of LRV presence in *Leishmania* on the level of immunity in the affected population would increase understanding of the effect of the viral presence.

References

Acestor, N. G., Masina, S., Ives, A., Fasel, N., Walker, J., & Saravia, N. **(2006).** Resistance to oxidative stress is associated with metastasis in mucocutaneous leishmaniasis. *Journal of Infectious Diseases, 194*(8), 1160-1167.

Akopyants, N. S., Dobson, D. E., Beverley, S. M., Kimblin, N. L., Secundino, N., Patrick, R., . . . Sacks, D. **(2009).** Demonstration of genetic exchange during cyclical development of Leishmania in the sand fly vector. *Science, 324*(5924), 265-268.

Alexander, J., & Brombacher, F. (2012). T helper1/T helper2 cells and resistance/susceptibility to Leishmania infection: Is this paradigm still relevant? *Frontiers In Immunology, 3*, Frontiers In Immunology, 2012, Vol.3.

Alvar, **J**, Canavate, C, Gutierrez-Solar, B, Jimenez, M, Laguna, F, Lopez-Velez, R, Moreno, J. **(1997).** Leishmania and human immunodeficiency virus coinfection: The first 10 years. *Clinical Microbiology Reviews, 10*(2), 298.

Alvar, Jorge, Yactayo, Sergio, & Bern, Caryn. **(2006).** Leishmaniasis and poverty. (Report). *Trends in Parasitology, 22*(12), 552-557.

Alvar J, Aparicio P, Aseffa A, (2008). The Relationship between Leishmaniasis and AIDS: The Second 10 Years. *Clinical Microbiology Reviews, 21*(2), 334-59

Alvar, Vélez, Bern, Herrero, Desjeux, Cano . . . Kirk. **(2012).** Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS ONE, 7*(5), PLoS ONE, 2012, Vol.7 (5).

Amato, Tuon, Bacha, Neto, & Nicodemo. **(2008).** Mucosal leishmaniasis: Current scenario and prospects for treatment. *Acta Tropica, 105*(1), 1-9.

Ashford, **R.W.,** Bray, M.A., Hutchinson, M.P., & Bray, R.S. **(1973).** The epidemiology of cutaneous leishmaniasis in Ethiopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 67*(4), 568-601.

Baldwin, Sanderson, Porter, Wojciechowski, Campbell, & Donoghue. **(1995).** The its Region of Nuclear Ribosomal DNA: A Valuable Source of Evidence on Angiosperm Phylogeny. *Annals of the Missouri Botanical Garden, 82*(2), 247-277.

Barnett, **P.,** Singh, S., Bern, C., Hightower, A., & Sundar, S. **(2005).** Virgin soil: The spread of visceral leishmaniasis into Uttar Pradesh, India. *The American Journal of Tropical Medicine and Hygiene, 73*(4), 720-5.

Bates, P. (2005). Leishmania. *Encyclopedia of Life Sciences*11, 1-10.

Bates, P. (2008). Leishmania sand fly interaction: Progress and challenges. *Current Opinion in Microbiology, 11*(4), 340-344.

Bern, C., Joshi, S., Jha, A., Thakur, G., & Bista, M. **(2000).** Use of the recombinant K39 dipstick test and the direct agglutination test in a setting endemic for visceral leishmaniasis in Nepal. *American Journal of Tropical Medicine and Hygiene, 63*(3-4), 153-157.

Bern, Caryn, Hightower, Allen W., Chowdhury, Rajib, Ali, Mustakim, Amann, Josef, Wagatsuma, Yukiko, Maguire, James H. **(2005).** Risk factors for Kala-Azar in Bangladesh. (RESEARCH). *Emerging Infectious Diseases, 11*(5), 655.

Bern, C., Haque, R., Chowdhury, R., Ali, M., Kurkjian, K., Vaz, L., Maguire, J. **(2007).** The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. *The American Journal of Tropical Medicine and Hygiene, 76*(5), 909-14.

Bern, **C.,** Maguire, J., Alvar, J., & Brooker, S. **(2008).** Complexities of Assessing the Disease Burden Attributable to Leishmaniasis. *PLoS Neglected Tropical Diseases, 2*(10), E313.

Blackwell, J. **(1999).** Tumour Necrosis Factor alpha and Mucocutaneous Leishmaniasis. *Parasitology Today*, *15*(2), 73-75.

Bongiorno, M. R., Pistone, G., & Aricò, M. **(2009).** Unusual clinical variants of cutaneous leishmaniasis in Sicily. *International Journal of Dermatology, 48*(3), 286-289.

Braga, A., Toledo Junior, A., & Rabello, A. **(2013).** Factors of poor prognosis of visceral leishmaniasis among children under 12 years of age. A retrospective monocentric study in Belo Horizonte, State of Minas Gerais, Brazil, 2001-2005. *Revista Da Sociedade Brasileira De Medicina Tropical, 46(*1), 55-9.

Bray, R.S., Ashford, R.W., & Bray, M.A. **(1973).** The parasite causing cutaneous leishmaniasis in Ethiopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 67*(3), 345-348.

Brochu, C., Roy, G., Messier, N., Ouellette, X., Jingyu Wang, M., Wang, & Saravia. **(2003)**. Antimony uptake systems in the protozoan parasite Leishmania and accumulation differences in antimony-resistant parasites. *Antimicrobial Agents and Chemotherapy, 47*(10), 3073-3079.

Bucheton, Kheir, El-Safi, Hammad, Mergani, Mary . . . Dessein**. (2002).** The interplay between environmental and host factors during an outbreak of visceral leishmaniasis in eastern Sudan. *Microbes and Infection, 4*(14), 1449-1457.

Bulle, B., Millon, L., Bart, JM, Gallego, Montserrat, Gambarelli, Francoise, Portus, Montserrat . . . Piarroux, Renaud**. (2002).** Practical Approach for Typing Strains of Leishmania infantum by Microsatellite Analysis. *Journal of Clinical Microbiology, 40*(9), 3391.

Semon, H., & Christopherson, J. (1923). Case of Leishmaniasis of the Skin. *Journal of the Royal Society of Medicine, 16*(Dermatol_Sect), 8-11.

Carvalho, Luis, Luque-Ortega, Juan Roman, Manzano, Jose Ignacio, Castanys, Santiago, Rivas, Luis, & Gamarro, Francisco. **(2010)**. Tafenoquine, an Antiplasmodial 8-Aminoquinoline, Targets Leishmania Respiratory Complex III and Induces Apoptosis. *Antimicrobial Agents and Chemotherapy, 54*(12), 5344-51.

Carvalho, B., Rangel, E., Ready, P., & Vale, M. (n.d.). **(2015).** Ecological Niche Modelling Predicts Southward Expansion of Lutzomyia (Nyssomyia) flaviscutellata (Diptera: Psychodidae: Phlebotominae), Vector of Leishmania (Leishmania) amazonensis in South America, under Climate Change. *PLoS ONE, 10*(11), E0143282.

Coleman, R., & Edman, J. (1988). Feeding-site selection of Lutzomyia longipalpis (Diptera: Psychodidae) on mice infected with Leishmania mexicana amazonensis. *Journal of Medical Entomology, 25*(4), 229-233.

Collin, S., Davidson, R., Ritmeijer, Koert, Keus, Kees, Melaku, Yosef, Kipngetich, Sammy, & Davies, Clive. **(2004).** Conflict and kala-azar: Determinants of adverse outcomes of kala-azar among patients in southern Sudan. (Major Article). *Clinical Infectious Diseases, 38*(5), 612.

Collin, S., Coleman, P., Ritmeijer, K., & Davidson, R. **(2006).** Unseen Kala-azar deaths in south Sudan (1999–2002. *Tropical Medicine & International Health, 11*(4), 509-512.

Cordeiro-da-Silva, A., Silvestre, R., Santarém, N., Teixeira, L., Cunha, J., & Schallig, H. **(2009).** Evaluation of Leishmania species reactivity in human serologic diagnosis of leishmaniasis. *American Journal of Tropical Medicine and Hygiene, 81*(2), 202-208.

Day, J., & Edman. (1983*).* Malaria renders mice susceptible to mosquito feeding when gametocytes are most infective. *Journal of Parasitology, 69(*1), 163-170.

Desjeux, P. **(1996).** Leishmaniasis. Public health aspects and control. *Clinics in Dermatology, 14*(5), 417-23.

Desjeux, P. (2004). Leishmaniasis: Current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases, 27*(5), 305-318.

Dinesh, Ranjan, Palit, Kishore, & Kar. **(2001).** Seasonal and nocturnal landing/biting behaviour of Phlebotomus argentipes (Diptera: Psychodidae). *Annals of Tropical Medicine and Parasitology, 95*(2), 197-202.

Espinosa, O., Serrano, M., Camargo, E., Teixeira, M., & Shaw, J. **(2016).** An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as Leishmania and Endotrypanum. Parasitology, 1-13.

Field, V., Gautret, P., Schlagenhauf, G., Burchard, E., Caumes, M., Jensenius, F., . . . Loutan. **(2010).** Travel and migration associated infectious diseases morbidity in Europe, 2008. *BMC Infectious Diseases, 10*(1),

Foulet, Francoise, Botterel, Francoise, Buffet, Pierre, Morizot, Gloria, Rivollet, Daniele, Deniau, Michele . . . Bretagne, Stephane. **(2007).** Detection and Identification of Leishmania Species from Clinical Specimens by Using a Real-Time PCR Assay and Sequencing of the Cytochrome b Gene. *Journal of Clinical Microbiology, 45*(7), 2110-2115.

Gálvez, Descalzo, Miró, Jiménez, Martín, Dos Santos-Brandao . . . Molina**. (2010).** Seasonal trends and spatial relations between environmental/meteorological factors and leishmaniosis sand fly vector abundances in Central Spain. *Acta Tropica, 115*(1), 95-102.

Gelanew, Amogne, Abebe, Kuhls, Hailu, & Schönian**. (2010).** A clinical isolate of Leishmania donovani with ITS1 sequence polymorphism as a cause of para-kala-azar dermal leishmaniasis in an Ethiopian human immunodeficiency virus-positive patient on highly active antiretroviral therapy. *British Journal of Dermatology, 163*(4), 870-874.

Gonzlez, U., Pinart, M., Reveiz, L., & Alvar, J. **(2008).** Interventions for Old World cutaneous leishmaniasis. *Cochrane Skin Group*, (4), Cochrane Database of Systematic Reviews, 2008, Issue 4.

Grove, S. (1989). Leishmaniasis in South West Africa Namibia to Date. *South African Medical Journal, 75*(6), 290-292.

Guerin, Olliaro, Sundar, Boelaert, Croft, Desjeux . . . Bryceson**. (2002).** Visceral leishmaniasis: Current status of control, diagnosis, and treatment, and a proposed research and development agenda. *The Lancet Infectious Diseases, 2*(8), 494-501.

Guerra, J., Prestes, S., Silveira, H., Coelho, L., Gama, P., Moura, A. . . . Carvalho, E. **(2011).** Mucosal Leishmaniasis Caused by Leishmania (Viannia) braziliensis and Leishmania (Viannia) guyanensis in the Brazilian Amazon (Mucosal Leishmaniasis in the Brazilian Amazon). *PLoS Neglected Tropical Diseases, 5*(3), E980.

Handman E. (2001). Leishmaniasis: current status of vaccine development. *Clinical Microbiology Reviews, 14*(2), 229.

Hartley, M., Ronet, C., Zangger, H., Beverley, S., & Fasel, N**. (2012).** Leishmania RNA virus: When the host pays the toll. *Frontiers In Cellular And Infection Microbiology, 2*, 99.

Herwaldt, B. (1999). Leishmaniasis. *The Lancet, 354*(9185), 1191-1199.

Hewitt, Reyburn, Ashford, & Rowland. **(1998).** Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: Vertical distribution of cases in apartment blocks. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 92*(3), 273-274.

Hommel, M. (1999). Visceral leishmaniasis: Biology of the parasite. *Journal of Infection, 39*(2), 101-111.

Ives, A. F., Ronet, C. M., Prevel, F., Ruzzante, G., Fuertes-Marraco, S., Zangger, H. . . . Launois, P. **(2011).** Leishmania RNA virus controls the severity of mucocutaneous leishmaniasis. *Science, 331*(6018), 775-778.

Jacobson, R. (2003). Leishmania tropica (Kinetoplastida: Trypanosomatidae) - a perplexing parasite. *Folia Parasitologica, 50*(4), 241-250.

Jenni, L., Marti, S., Schweizer, J., Betschart, B., R. W. F. Le Page, J. M. Wells, A. Tait, P. Paindavoine, E. Pays, and M. Steinert. **(1986).** "Hybrid Formation between African Trypanosomes during Cyclical Transmission." *Nature 322,* no. 6075: 173-175.

Kaushal, H., Bras-Gonçalves, R., Avishek, K., Kumar Deep, D., Petitdidier, E., Lemesre, J. . . . Salotra, P. **(2016).** Evaluation of cellular immunological responses in mono- and polymorphic clinical forms of post-kala-azar dermal leishmaniasis in India. *Clinical & Experimental Immunology, 185*(1), 50-60.

Kebede, N., Oghumu, S., Worku, A., Hailu, A., Varikuti, S., & Satoskar, A. **(2013).** Multilocus microsatellite signature and identification of specific molecular markers for Leishmania aethiopica. *Parasites & Vectors 6*(1), 160.

Killick-Kendrick, R. (1999). The biology and control of Phlebotomine sand flies. *Clinics in Dermatology, 17*(3), 279-289.

Krayter, Alam, Rhajaoui, Schnur, & Schönian. **(2014).** Multilocus Microsatellite Typing reveals intra-focal genetic diversity among strains of Leishmania tropica in Chichaoua Province, Morocco. *Infection, Genetics and Evolution, 28*, 233-239.

Krayter, L. F., Schönian, G., & Schnur, L. **(2015).** The genetic relationship between leishmania aethiopica and leishmania tropica revealed by comparing microsatellite profiles. *PLoS ONE, 10*(7), E0131227.

Kumar, R, Kumar, P, Chowdhary, R K, Pai, K, Mishra, C P, Kumar, K . . . Sundar, S. **(1999).** Kala-azar epidemic in Varanasi district, India. *Bulletin of the World Health Organization, 77*(5), 371-4.

Lainson, R. (1983). The American leishmaniases: Some observations on their ecology and epidemiology. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 77*(5), 569-596.

Lainson, R. (1985). Our present knowledge of the ecology and control of leishmaniasis in the Amazon region of Brazil. *Revista Da Sociedade Brasileira De Medicina Tropical, 18*(1), 47-56.

Le Blancq, Cibulskis, & Peters. (1986). Leishmania in the Old World: 5. Numerical analysis of isoenzyme data. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 80*(4), 517-524.

Lye, L., Owens, K., Shi, H., Murta, S., Vieira, A., Turco, S. . . . Ullman, B. **(2010).** Retention and Loss of RNA Interference Pathways in Trypanosomatid Protozoans (RNA Interference in Leishmania braziliensis). *PLoS Pathogens, 6*(10), E1001161.

Macedo, A. M., Pimenta, J. R., De Aguiar, R. S., Melo, A. I., Pena, S. D., Chiari, E. P., . . . Oliveira, R. **(2001).** Usefulness of Microsatellite Typing in Population Genetic Studies of Trypanosoma cruzi. *Memorias Do Instituto Oswaldo Cruz, 96*(3), 407-413.

Manzano, J., Lecerf-Schmidt, F., Lespinasse, M., Di Pietro, A., Castanys, S., Boumendjel, A., & Gamarro, F. **(2014).** Leishmania. *Journal of Antimicrobial Chemotherapy, 69*(3), 664-672.

Manzano, Carvalho, García-Hernández, Poveda, Ferragut, Castanys, & Gamarro. **(2011).** Leishmania. *Journal of Antimicrobial Chemotherapy, 66*(11), 2562-2565.

Marsden, P. (1986). Mucosal leishmaniasis ("spundia" Escomel, 1911). *Transactions of the Royal Society of Tropical Medicine and Hygiene, 80*(6), 859-876.

Masood, S., Naveed, S., & Alvi, R. **(2012).** Infiltrated leishmaniasis recidivans cutis on the face: A rare clinical presentation. *Tropical Doctor, 42*(2), 120-121.

Mathers, C., Ezzati, M., Lopez, A., & Brooker, S**. (2007).** Measuring the Burden of Neglected Tropical Diseases: The Global Burden of Disease Framework (Review). *PLoS Neglected Tropical Diseases, 1*(2), E114.

Mcdowell, M., Rafati, S., Ramalho-Ortigao, M., & Ben Salah, A. **(2011).** Leishmaniasis: Middle East and North Africa Research and Development Priorities. *Plos Neglected Tropical Diseases, 5*(7),

Mimori, Sasaki, Nakata, Gomez, Uezato, Nonaka . . . Saya**. (1998).** Rapid identification of Leishmania species from formalin-fixed biopsy samples by polymorphism-specific polymerase chain reaction. *Gene, 210*(2), 179-186.

Murray, C., Lopez, Alan D, World Bank, & World Health Organization. **(1996).** *The global burden of disease: A comprehensive assessment of morality and disability from disease, injuries, and risk factors in 1990 and projected to 2020: Summary* (Global burden of disease and injury series). Harvard School of Public Health.

Murray, Henry W. (2001). Clinical and Experimental Advances in Treatment of Visceral Leishmaniasis. *Antimicrobial Agents and Chemotherapy, 45*(8), 2185.

Murray, H. (2002). Kala-Azar — Progress against a Neglected Disease. *The New England Journal of Medicine, 347*(22), 1793-1794.

Nath-Chowdhury, M., Sangaralingam, M., Bastien, P., Ravel, C., Pratlong, F., Mendez, J. . . . Ndao, M. **(2016).** Real-time PCR using FRET technology for Old World cutaneous leishmaniasis species differentiation. *Parasites & Vectors, 9*(1), Parasites & Vectors, May 3, 2016, Vol.9(1).

Negera, Gadisa, Yamuah, Engers, Hussein, Kuru . . . Aseffa. **(2008).** Outbreak of cutaneous leishmaniasis in Silti woreda, Ethiopia: Risk factor assessment and causative agent identification. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 102*(9), 883-890.

Ochsenreither, Sebastian, Kuhls, Katrin, Schaar, Matthias, Presber, Wolfgang, & Schonian, Gabriele. **(2006).** Multilocus Microsatellite Typing as a New Tool for Discrimination of Leishmania infantum MON-1 Strains. *Journal of Clinical Microbiology*, *44*(2), 495.

Odiwuor SOC, Saad AA, De Doncker S, Maes I, Laurent T, El Safi S, Mbuchi M, Buscher P, Dujardin JC, Van der Auwera G. **(2011).** Universal PCR assays for the differential detection of all Old World Leishmania species. *European Journal of Clinical Microbiology and Infectious Diseases, 30*(2), 209-18.

Ouellette, Drummelsmith, & Papadopoulou. **(2004).** Leishmaniasis: Drugs in the clinic, resistance and new developments. *Drug Resistance Updates*, *7*(4), 257-266.

Pace, D. (2014). Leishmaniasis. *Journal of Infection, 69,* S10-S18.

Pagliano, P., Carannante, N., Rossi, M., Gramiccia, M., Gradoni, L., Faella, F., & Gaeta, G. **(2005).** Visceral leishmaniasis in pregnancy: A case series and a systematic review of the literature. *Journal of Antimicrobial Chemotherapy, 55*(2), 229-233.

Parham, P., Waldock, J., Christophides, G., Hemming, D., Agusto, F., Evans, K., . . . Michael, E. **(2015)**. Climate, environmental and socio-economic change: Weighing up the balance in vector-borne disease transmission. *Philosophical Transactions Of The Royal Society B-Biological Sciences, 370*(1665), 20130551-20130551.

Patz, Graczyk, Geller, & Vittor. **(2000).** Effects of environmental change on emerging parasitic diseases. *International Journal for Parasitology*, *30*(12), 1395-1405.

Peacock, C., Seeger, K., Harris, D., Murphy, L., Ruiz, J. C., Quail, M. A.... Berriman, M. **(2007).** Comparative genomic analysis of three Leishmania species that cause diverse human disease. *Nature Genetics, 39,* 839-847. DOI: 10.1038/ng2053

Pereira, L., Maretti-Mira, A., Rodrigues, K., Lima, R., Manoel Paes de Oliveira-Neto, Elisa Cupolillo . . . Márcia Pereira de Oliveira. **(2013).** Severity of tegumentary leishmaniasis is not exclusively associated with Leishmania RNA virus 1 infection in Brazil. *Memórias Do Instituto Oswaldo Cruz., 108*(5), 665-667.

Plourde, M., Coelho, A., Keynan, Y., Larios, O., Ndao, M., Ruest, A. . . . Kamhawi, S. **(2012).** Genetic Polymorphisms and Drug Susceptibility in Four Isolates of Leishmania tropica Obtained from Canadian Soldiers Returning from Afghanistan (Leishmania tropica from Afghanistan). *PLoS Neglected Tropical Diseases, 6*(1), E1463.

Pozio, & Morales. (2005). The impact of HIV-protease inhibitors on opportunistic parasites. *Trends in Parasitology, 21*(2), 58-63.

Pratlong, F., Dereure, J., Ravel, C., Lami, P., Balard, Y., Serres, G. . . . Dedet, J. **(2009).** Geographical distribution and epidemiological features of Old World cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains. *Tropical Medicine & International Health, 14*(9), 1071-1085.

Ready, P. (2008). Leishmaniasis emergence and climate change. *Revue Scientifique Et Technique-Office International Des Epizooties, 27*(2), 399-412.

Ready, P. (2010). Leishmaniasis emergence in Europe. *Eurosurveillance, 15*(10), 29-39.

Ready, P. (2013). Biology of Phlebotomine Sand Flies as Vectors of Disease Agents. *Annual Review of Entomology, 58*, 227-250.

Reithinger, R., Mohsen, M., Aadil, K., Sidiqi, M., Erasmus, P., & Coleman, P. **(2003).** Anthroponotic Cutaneous Leishmaniasis, Kabul, Afghanistan. *Emerging Infectious Diseases, 9*(6), 727-729.

Reithinger, Dujardin, Louzir, Pirmez, Alexander, & Brooker. **(2007).** Cutaneous leishmaniasis. *The Lancet Infectious Diseases, 7*(9), 581-596.

Rhajaoui, M., Nasereddin, A., Fellah, H., Azmi, K., Amarir, F., Al-Jawabreh, A. . . . Abdeen, Z. **(2007).** New Clinicoepidemiologic Profile of Cutaneous Leishmaniasis, Morocco. *Emerging Infectious Diseases, 13*(9), 1358-1360.

Ro, YT, Scheffter, SM, & Patterson, JL. **(1997).** Hygromycin B resistance mediates elimination of Leishmania virus from persistently infected parasites. *The Journal of Virology, 71*(12), 8991-8.

Rogers, M., Hilley, James D., Dickens, Nicholas J., Wilkes, Jon, Bates, Paul A., Depledge, aniel P. . . . Mottram, Jeremy C. **(2011).** Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania.(Report). *Genome Research, 21*(12), 2129-2142.

Rogers, M., Downing, T., Smith, B., Imamura, H., Sanders, M., Svobodova, M. . . . Didelot, Xavier. **(2014).** Genomic Confirmation of Hybridisation and Recent Inbreeding in a Vector-Isolated Leishmania Population (Sexual Reproduction in Leishmania). *10*(1), E1004092.

Romero, Ishikawa, Cupolillo, Toaldo, Guerra, Paes . . . Shaw. **(2002).** The rarity of infection with Leishmania (Viannia) braziliensis among patients from the Manaus region of Amazonas state, Brazil, who have cutaneous leishmaniasis. *Annals of Tropical Medicine and Parasitology, 96*(2), 131-136.

Ronet, C., Beverley, S., & Fasel, N. **(2011).** Muco-cutaneous leishmaniasis in the New World: The ultimate subversion. *Virulence, 2*(6), 547-52.

Rossi, Wincker, Ravel, Blaineau, Pagés, & Bastien. **(1994).** Structural organisation of microsatellite families in the Leishmania genome and polymorphisms at two (CA) n loci. *Molecular & Biochemical Parasitology, 65*(2), 271-282.

Saiz, M., Llanos-Cuentas, A., Echevarria, J., Roncal, N., Cruz, C., Tupayachi Muniz, D. . . . Patterson. **(1998).** Short report: Detection of Leishmania virus in human biopsy samples of leishmaniasis from Peru. *American Journal of Tropical Medicine and Hygiene, 58*(2), 192-194.

Sakthianandeswaren, Foote, & Handman. **(2009).** The role of host genetics in leishmaniasis. *Trends in Parasitology, 25*(8), 383-391.

Sampaio, Paula, Gusmao, Leonor, Alves, Cintia, Pina-Vaz, Cidalia, Amorim, Antonio, & Pais, Celia. **(2003).** Highly Polymorphic Microsatellite for Identification of Candida albicans Strains. *Journal of Clinical Microbiology, 41*(2), 552.

Santarém, N., Cunha, J., Silvestre, R., Silva, C., Moreira, D., Ouellette, M., & Cordeiro-Da-Silva, A. **(2014).** The impact of distinct culture media in Leishmania infantum biology and infectivity. *141*(2), 192-205.

Santos, L., Marinho, F., Altoé, E., Vitório, B., Alves, C., Britto, C. . . . Ratner, Adam J. **(2009).** HIV Aspartyl Peptidase Inhibitors Interfere with Cellular Proliferation, Ultrastructure and Macrophage Infection of Leishmania amazonensis (HIV PIs on L. amazonensis). *PLoS ONE, 4*(3), E4918.

Santos, BS., Branquinha, MH., Pedroso e Silva, Concei M., Santos, Andr; L. S., & D'Avila-Levy, Claudia M. (2013). Leishmania. *Journal of Antimicrobial Chemotherapy*, *68*(2), 348-353.

Scheffter, Ro, Chung, & Patterson. **(1995).** The Complete Sequence of Leishmania RNA Virus LRV2-1, a Virus of an Old World Parasite Strain. *Virology, 212*(1), 84-90.

Schönian, Akuffo, Lewin, Maasho, Nylén, Pratlong . . . Presber**. (2000).** Genetic variability within the species Leishmania aethiopica does not correlate with clinical variations of cutaneous leishmaniasis. *Molecular & Biochemical Parasitology*, *106*(2), 239-248.

Schönian, G., Kuhls, K., & Mauricio, I. **(2011).** Molecular approaches for a better understanding of the epidemiology and population genetics of Leishmania. *Parasitology, 138*(4), 405-425.

Schnur, L., & Zuckerman, A. (1977). Leishmanial excreted factor (EF) serotypes in Sudan, Kenya and Ethiopia. *Annals of Tropical Medicine and Parasitology, 71*(3), 273-94.

Schwartz, Hatz, & Blum. (2006). New world cutaneous leishmaniasis in travellers. *The Lancet Infectious Diseases, 6*(6), 342-349.

Schwenkenbecher, Fröhlich, Gehre, Schnur, & Schönian. **(2004).** Evolution and conservation of microsatellite markers for Leishmania tropica. *Infection, Genetics and Evolution, 4*(2), 99-105.

Schwenkenbecher, Wirth, Schnur, Jaffe, Schallig, Al-Jawabreh . . . Schönian. **(2006).** Microsatellite analysis reveals genetic structure of Leishmania tropica. *International Journal for Parasitology, 36*(2), 237-246.

Scott, P. (2011). Leishmania--a parasitized parasite. *The New England Journal of Medicine, 364*(18), 1773-4.

Seaman, J., Mercer, A., & Sondorp, E**. (1996).** The Epidemic of Visceral Leishmaniasis in Western Upper Nile, Southern Sudan: Course and Impact from 1984 to 1994. *25*(4), 862-871.

Sharifi, I., Fekri, A., Aflatoonian, M., Khamesipour, A., Mahboudi, F., Dowlati, Y. . . . Modabber, F. **(2010).** Leishmaniasis recidivans among school children in Bam, South-east Iran, 1994-2006. *International Journal of Dermatology, 49*(5), 557-61.

O'Shea, B., Rebollar-Tellez, E., Ward, R.D., Hamilton, J.G.C., El Naiem, D., & Polwart, A. **(2002).** Enhanced sandfly attraction to Leishmania-infected hosts. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 96*(2), 117-118.

Smith, Peacock, & Cruz. (2007). Comparative genomics: From genotype to disease phenotype in the leishmaniases. *International Journal for Parasitology, 37*(11), 1173-1186.

Soto, Arana, Toledo, Rizzo, Vega, Diaz . . . Sindermann**. (2004).** Miltefosine for new world cutaneous leishmaniasis. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 38*(9), 1266-72.

Stephenson, J. (2007). Leishmania. *JAMA, 298*(3), 277.

Stuart, K., Weeks, R., Guilbride, L., & Myler, P. **(1992).** Molecular Organization of Leishmania RNA Virus 1. *Proceedings of the National Academy of Sciences of the United States of America, 89*(18), 8596-8600.

Sundar, S., Sahu, M., Mehta, H., Gupta, A., Kohli, U., Rai, M. . . . Murray, H.W**. (2002).** Noninvasive management of Indian visceral leishmaniasis: Clinical application of diagnosis by K39 antigen strip testing at a kala-azar referral unit. (Major Article). *Clinical Infectious Diseases, 35*(5), 581.

Sundar, S., Jha, T., Thakur, C., Engel, J., Sindermann, H., Fischer, C. . . . Berman, J**. (2002).** Oral Miltefosine for Indian Visceral Leishmaniasis. *The New England Journal of Medicine, 347*(22), 1739-1746.

Svobodova, Milena, Votypka, Jan, Peckova, Jitka, Dvorak, Vit, Nasereddin, Abedelmajeed, Baneth, Gad . . . Warburg, Alon. **(2006).** Distinct transmission cycles of Leishmania tropica in 2 adjacent foci, northern Israel. (RESEARCH). *Emerging Infectious Diseases, 12*(12), 1860.

Thakur, C. (2000). Socio-economics of visceral leishmaniasis in Bihar (India). *Transactions of the Royal Society of Tropical Medicine and Hygiene, 94*(2), 156-157.

Tóth, G., Gáspári, Z., & Jurka, J. **(2000).** Microsatellites in different eukaryotic genomes: Survey and analysis. *Genome Research, 10*(7), 967-81.

Van Griensven, & Boelaert. (2011). Combination therapy for visceral leishmaniasis. *The Lancet, 377*(9764), 443-444.

Walsh, D., Wilairatana, P., Tang, D., Heppner, D., Brewer, T., Krudsood, S. . . . Looareesuwan, S. **(2004).** Randomized trial of 3-dose regimens of tafenoquine (WR238605) versus low-dose primaquine for preventing Plasmodium vivax malaria relapse. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 39*(8), 1095-103.

Weigle, & Saravia. (1996). Natural history, clinical evolution, and the host-parasite interaction in New World cutaneous leishmaniasis. *Clinics in Dermatology, 14*(5), 433-450.

Wilson, S. (1995). DNA-based methods in the detection of Leishmania parasites: Field applications and practicalities. *Annals Of Tropical Medicine And Parasitology, 89,* 95-100.

Woodward, Smith, Campbell-Lendrum, Chadee, Honda, Liu . . . Haines. **(2014).** Climate change and health: On the latest IPCC report. *The Lancet, 383*(9924), 1185-1189.

Woolhouse, M., Dye, C., Kovats, R., Campbell-Lendrum, D., McMichel, A., Woodward, A., & Cox, J. **(2001).** Early effects of climate change: Do they include changes in vector-borne disease? *Philosophical Transactions of the Royal Society B: Biological Sciences, 356*(1411), 1057-1068.

Wortmann, G., Miller, R., Oster, C., Jackson, J., & Aronson, N. **(2002).** A randomized, double-blind study of the efficacy of a 10- or 20-day course of sodium stibogluconate for treatment of cutaneous leishmaniasis in United States military personnel. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America, 35*(3), 261-7.

Yamey, G. (2002). The world's most neglected diseases. *British Medical Journal*, 325(7357), 176.

Zijlstra, E. (2016). The immunology of post-kala-azar dermal leishmaniasis (PKDL). *Parasites & Vectors, 9*(1), 464.

Zangger, H., Hailu, A., Desponds, C., Lye, L., Akopyants, N., Dobson, D. . . . Bates, P**. (2014).** Leishmania aethiopica Field Isolates Bearing an Endosymbiontic dsRNA Virus Induce Pro-inflammatory Cytokine Response (Leishmania RNA Virus in L. aethiopica). *8*(4), E2836.

Zelazny, A. M., Fedorko, D. P., Li, L. H., Fischer, S. A., & Neva, F. **(2005).** Evaluation of 7SL RNA gene sequences for the identification of Leishmania spp. *American Journal of Tropical Medicine and Hygiene*, 72(4), 415-420.

WEBSITES

Centers for Disease Control and Prevention. (2013*).* *Parasites – Leishmaniasis: Biology*. [online]

Available at: https://www.cdc.gov/parasites/leishmaniasis/biology.html. [Accessed June 30, 2017].

World Health Organization. *Leishmaniasis, Epidemiological Situation*. [online] Available at: http://www.who.int/leishmaniasis/burden/en/ [Accessed 20 Jul. 2017)

BOOKS

Schaller, K F., Bahmer, F. A., Büttner, W., Lieske, H., Wassilwv, S.W., Weyer, F., **(1994)** "Protozoan Dermatoses." Colour Atlas of Tropical Dermatology and Venerolog. Berlin: Springer

Appendix

Appendix I - List of ITS-1 sequences

DNA sequences produced from PCR with the ITS1 primers on *L. tropica* and *L. aethiopica* isolates, with *L. major* and *L. guyanensis.*

***L. major* LV39**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCCCAAAAAACATATACAACTCGGGGAGGCTTAT
TCTATATATATATAGTATAGGCTTTTCCCACATACACAGCAAACTTTTATACTCAAA
ATTTGCAGTAAAAAAGGCCGATCGACGTTGTAGAACGCACCGCCTATACACAAAAGC
AAAAATGTCCGTTTATACAAAAAAATAGACGGCGTTTCGGTTTTTGGCGGGAGGGAG
AGAGAGGGGGGTGCGTGCGCGTGGATAACGGCTCACATAA

***L. guyanensis* M4147**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCAAAAAACATACAACTCCGGGGAGGCTTGTGTT
TTCTAGCAAGCCTTTCCCACAGATACGCAATACAATCTATATATATATGTATATAGA
CACAACATACAGTAGAAAAAGGCCGATCGACGTTAACATATCGCGTATACAACAAAA
AAGTTCGTTCTACGGCTTTTTTTTTGGCGGCGTGCGTGGATAACGGCTCACATAA

***L. tropica* LV1**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCAAAAAAAACATATACAAAACTCGGGGAGGCCTATATTATACA
TTATAGGCCTTTCCCACACATACACAGCAAACTTTTTATACTAGAAGTTTGCAGTAAACAAA
AGGCCGATCGACGTTATAACGCACCGCCTATACACAAAAGCAAAAATGTCCGTTTATACAAA
TATACGGCGTTTCGGTTTTGTTGGCGGGGGGTGCGTGTGTGGATAACGGCTCACATAA

***L. tropica* LV13**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCAAAAAAAAACATATACAAAACTCGGGGAGGCCTATATATTA
TACATTATATAGGCCTTTCCCACACATACACAGCAAACTTTTATACTCGAAGTTTGCAGTAA
ACAAAAGGCCGATCGACCTTATAACGCACCGCCTATACACAAAAGCAAAAATGTCCGTTTAT
ACAAATATACGGCGTTTCGGTTTTGTTGGCGGGGGGTGCGTGTGTGTGGATAACGGCTCACA
TAA

***L. aethiopica* LV15**

ATATTTNTTCAATAGAGGAAGCAAAAGTCGTAACAANGTAGCTGTAGGTGAACNNGCAGCTG
GATCATTTTCCGATGATTACACCCAAAAAAAAACATATACAAAACTCGGGCAGGCCTATTAT
ATATATTATTATAGGCCTTTCCCACATACACACAGCAAACTTTTATACTCGAAGTTTGCAGT
AAAGAAAAGGCCGATCGACGTTATATAACGCACCGCGCGCCTATACACACAAAAGCAAAAAC
GTCCGTTTATACAAATATACGGCGTTTCCGTCTTTTGTTGGGGGGGGCGGGGGTGCGTGTGT
GTGGATAACGGCTCACATAA

***L. aethiopica* LV25**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCAAAAAAAAACATATACAAAACTCGGGCAGGCCTATTATATA
TATTATTATAGGCCTTTCCCACATACACACAGCAAACTTTTATACTCGAAGTTTGCAGTAAA
GAAAAGGCCGATCGACGTTATATAACGCACCGCGCGCCTATACACACAAAAGCAAAAACGTC
CGTTTATACAAATATACGGCGTTTCCGTCTTTTGTTGGCGGGGGCGGGGGTGCGTGTGTGTG
GATAACGGCTCACATAA

***L. aethiopica* LV26**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCAAAAAAAAACATATACAAAACTCGGGCAGGCCTATTATATA
TATTATTATAGGCCTTTCCCACATACACACAGCAAACTTTTATACTCGAAGTTTGCAGTAAA
GAAAAGGCCGATCGACGTTATATAACGCACCGCGCGCCTATACACACAAAAGCAAAAACGTC
CGTTTATACAAATATACGGCGTTTCCGTCTTTTGTTGGCGGGGGCGGGGGTGCGTGTGTGTG
GATAACGGCTCACATAA

***L. aethiopica* LV27**

TTTCTTCAATAGAGGAAGCAAAAGTAGTAACAAGGTAGCAGTAGGTGAACCTGCAGC
AGGATCATTTTCCGATGACTACACCCAAAAAAAACATACAAAACTCGGGCAGGCCTA
TTATATATATTATTATANGCCTTTCCCNCNTACACACAGCAAACTTTTATACTCCAA
GTTTGTAGTAAAAAAAAGGCCGATCGACGTTATATAACGCACCGCGCGCCTATACAC
ACAAAAGCAAAAACGTCCGTTTATACAAATATACGGCGTTTCCGTCTTTTGTTGGTG
GGGGCGGGGGTGCGTGTGTGTGGATAACGGCTCACATAA

***L. aethiopica* LV28**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCAAAAAAAACATATACAAAACTCGGGGAGGCCT
ATATTATACATTATAGGCCTTTCCCACACATACACAGCAAACTTTTTATACTCGAAG
TTTGCAGTAAACAAAAGGCCGATCGACGTTATAACGCACCGCCTATACACAAAAGCA
AAAATGTCCGTTTATACAAATATACGGCGTTTCGGTTTTGTTGGCGGGGGGTGCGTG
TGTGGATAACGGCTCACATAA

***L. tropica* LV357**

TTTTTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCCAAAAAAAAACATATACAAAACTCGGGGAGGCCTATTATAT
ACATTATAGGCCTTTCCCACATACACAGCAAACTTTTATACTCGAAGTTTGCAGTAAACAAA
AGGCCGATCGACGTTATAACGCACCGCCTATACACAAAAGCAAAAATGTCCGTTTATACAAA
TATACGGCGTTTCGGTTTTGTTGGCGGGGGGTGCGTGTGTGTGGATAACGGCTCACATAA

***L. tropica* LV369**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCCCCCAAAAAAAACATATACAAAACTCGGGGAGGCCTATTAT
ATACATTATAGGCCTTTCCCACATACACAGCAAACTTTTATACTCGAAGTTTGCAGTAAACA
AAAGGCCGATCGACGTTATAACGCACCGCCTATACACAAAAGCAAAAATGTCCGTTTATACA
AATATACGGCGTTTCGGTTTTGTTGGCGGGGGGTGCGTGTGTGGATAACGGCTCACATAA

***L. aethiopica* LV377**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCCAAAAAAAAACATATACAAAACTCGGGCAGGC
CTATTATATATATTATTATAGGCCTTTCCCACATACACACAGCAAACTTTTATACTC
GAAGTTTGCAGTAAAGAAAAGGCCGATCGACGTTATATAACGCACCGCGCGCCTATA
CACACAAAAGCAAAAACGTCCGTTTATACAAATATACGGCGTTTCCGTCTTTTGTTG
GCGGGGGCGGGGGTGCGTGTGTGTGGATAACGGCTCACATAA

***L. tropica* LV444**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCAAAAAAACATATACAAAACTCGGGGAGGCCTATATTATACA
TTATAGGCCTTTCCCACACATACACAGCAAACTTTTATACTCGAAGTTTGCAGTAAACAAAA
GGCCGATCGACCTTATAACGCACCGCCTATACACAAAAGCAAAAATGTCCGTTTATACAAAT
ATACGGCGTTTCGGTTTTGTTGGCCGGGGGGTGCGTGTGTGTGGATAACGGCTCACATAA

***L. aethiopica* LV472**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCAAAAAAAAACATATACAAAACTCGGGCAGGCCTATTATATA
TATTATTATAGGCCTTTCCCACATACACACAGCAAACTTTTATACTCGAAGTTTGCAGTAAA
GAAAAGGCCGATCGACGTTATATAACGCACCGCGCGCCTATACACACAAAAGCAAAAACGTC
CGTTTATACAAATATACGGCGTTTCCGTCTTTTGTTGGCGGGGGCGGGTGCGTGTGTGTGGA
TAACGGCTCACATAA

***L. tropica* LV487**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCCAAAAAAACATATACAAAACTCGGGGAGGCCT
ATATTATACATTATAGGCCTTTCCCACACATACACAGCAAACTTTTATACTCGAAGT
TTGCAGTAAACAAAAGGCCGATCGACCTTATAACGCACCGCCTATACACAAAAGCAA
AAATGTCCGTTTATACAAATATACGGCGTTTCGGTTTTGTTGGCCGGGGGGTGCGTG
TGTGTGGATAACGGCTCACATAA

***L. tropica* LV488**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCCAAAAAAACATATACAAAACTCGGGGAGGCCT
ATATTATACATTATAGGCCTTTCCCACACATACACAGCAAACTTTTATACTCGAAGT
TTGCAGTAAACAAAAGGCCGATCGACCTTATAACGCACCGCCTATACACAAAAGCAA
AAATGTCCGTTTATACAAATATACGGCGTTTCGGTTTTGTTGGCCGGGGGGTGCGTG
TGTGTGGATAACGGCTCACATAA

***L. tropica* LV489**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCCAAAAAAACATATACAAAACTCGGGGAGGCCT
ATATTATACATTATAGGCCTTTCCCACACATACACAGCAAACTTTTATACTCGAAGT
TTGCAGTAAACAAAAGGCCGATCGACCTTATAACGCACCGCCTATACACAAAAGCAA
AAATGTCCGTTTATACAAATATACGGCGTTTCGGTTTTGTNGGCCGGGGGGTGCGTG
TGTGTGGATAACGGCTCACATAA

***L. tropica* LV490**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGC
TGGATCATTTTCCGATGATTACACCCAAAAAAACATATACAAAACTCGGGGAGGCCT
ATATTATACATTATAGGCCTTTCCCACACATACACAGCAAACTTTTATACTCGAAGT
TTGCAGTAAACAAAAGGCCGATCGACCTTATAACGCACCGCCTATACACAAAAGCAA
AAATGTCCGTTTATACAAATATACGGCGTTTCGGTTTTGTTGGCCGGGGGGTGCGTG
TGTGTGGATAACGGCTCACATAA

***L. tropica* LV508**

TTTTTTTTCAATAGAAGAAGCAAAAGTNGTAACAAGGTAGGTGTAGGTGAACCCGCA
GCTGTTTCTTTTTCCGGAGCCCACACCCCAAAAAAAAACATATACAAAACTCGGGGA
GGCCTATTATATACATTATANGCCTTTTTCCCATACACACCACACTTTTTTATTCTA
AGTTTGCTTTAAACAAAAGGCCGAGCGACGTTATAACACACCGCCCATACACAAAAA
AAAAAAAGTCCGTTTATACAAATATACAGCGTTTCTTTTTTGTTGGGGGGGGGGGCG
TGTGTGGGTAANGGCTCACATAA

***L. tropica* LV509**

TTTTTACAAAAGAAGAAGCAAAAGTAGTAACAAGGTAGGTGTAGGTGAACNCGCAGC
TGGTTCATTTTGTGAAGATCACACCCCAAAAAAAAACATATACAAAACTCGGGGAGG
CCTATTATATACATTATANGCCTTTTTCCCATACACACCACACTTTTTTATTCTAAA
TTTGTTTTATACAAAAGGCGGATCGACATTATAACACACCGCCCCTACACAAAAAAA
AAAAAGTCCGTTTATACAAATATACAGCGTTTCTGTTTTGTTTTGGGGGGGGGCGTG
TGTGGATAACGGCTCACATAA

***L. aethiopica* LV546**

TTTCTTCAATAGAGGAAGCAAAAGTCGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGATGATTACACCCAAAAAAAAAACATATACAAAACTCGGGCAGGCCTATTATAT
ATATTATTATAGGCCTTTCCCACATACACACAGCAAACTTTTATACTCGAAGTTTGCAGTAA
AGAAAAGGCCGATCGACGTTATATAACGCACCGCGCGCCTATACACACAAAAGCAAAAACGT
CCGTTTATACAAATATACGGCGTTTCCGTCTTTTGTTGGCGGGGGCGGGTGCGTGTGTGTGG
ATAACGGCTCACATAA

***L. tropica* LV697**

TTTTTTCAATAGAGGAAGCAAAAGTNGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGAT
CATTTTTCGATGATTACACCCAAAAAAAAAACATATACAAAACTCGGGGAGGCCTATATATTA
TACATTATATAGGCCTTTCCCACACATACACAGCAAACTTTTATACTCGAAGTTTGCAGTAA
ACAAAAGGCCGATCGACCTTATAACGCACCGCCTATACACAAAAGCAAAAATGTCCGTTTAT
ACAAATATACGGCGTTTCGGTTTTGTTGGGGGGGGGGGCGGGGGTGTGGATAACGGCTCACA
TAA

Appendix II - List of RPL23 sequences

RPL23a DNA sequences for *L. tropica* and *L. aethiopica, L. major, L. donovani, L. infantum* and *L. guyanensis* isolates. The sequences were generated by Godwin Kwakye –Nuako; *L. major* LV39, *L. guyanensis* M4147, *L. aethiopica* LV25 and LV546 and *L. tropica* LV357 were repeated in this study.

***L. donovani* LV9**

TCTCGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTCATCA
GCAAAAACGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCCGGCAAAGCTCTTAGG
GTGGTGTAATGGGCTGTTAGCGCTACGGTACACTGAAGCGATCAGCTGCGGTATCTG
TGTGAGCGTCAAGGGTGCGCTGGTTTCTACATCTCCGAGTTTTTGATTCGCTCTGAG
GATGTATCGCAAAAAGCACGCTGCGGTGCTGCTTTACTACTCCTGCGCTGCTGCTCC
TTCAAATGCGTCGACATGTCCGATTTTCTGCAGCTACTCACCTCCACTTTCATCTCT
CCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGCCTTGTGTGCGTCTCAACTTGCG
ACAACTTTTCTTTCGCGTCTCTGTGGAATAACATCTGAACAGCTTTAGGGCTTCATT
CGCAACGGATCATGCCTCCTG

***L. infantum* LV111**

TCTCGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTCATCA
GCAAAAACGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCCGGCAAAGCTCTTAGG
GTGGTGTAATGGGCTGTTAGCGCTACGGTACACTGAAGCGATCAGCTGCGGTATCTG
TGTGAGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTTTGATTCGCTCTGAG
GATGTATCGCAAAAAGCACGCTGCGGTGCTGCTTTACTACTCCTGCGCTGCTGCTCC
TTCAAATGCGTCGACATGTCCGATTTTCTGCAGCTACTCGCCTCCACTTTCATCTCT
CCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGCCTTGTGTGCGTCTCAACTTGCG
ACAACTTTTCTTTCGCGTCTCTGTGGAATAACATCTGAACAGCTTTAGGGCTTCATT
CGCAACGGATCATGCCTCTTG

***L. major* LV39**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACGAAAAAGAAGTGCTACGGTAACTTTGGTGCACCAGAGGCTGGCGACGGCTCTT
AGGGTGGTTTAATGAGGTGTTACCTCTATGGTAAACTGAAGTGATCAGCTGCGGTAT
CTTTGTGCGCGTCGAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATTGCAAAAAGTGCGCTGCGGCGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTGCTCACCTCCACTTTCATC
TCTCCCTCTTCCTTTTTTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTG
CGACAACTTTTCTTGCGCCCTTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTCA
TTCGCAACGGATCATGCCTCCTG

***L. guyanensis* M4147**

TAAACGCAGAACTTGACTGGGGTGCAGCTACTTTTGGGATTATTTTCTTATTCCCTT
TTTTTTCAGTTCAACAACAAAAAGTGAGGGGCAAGGCGCGCTCTGTGGCTCTCCAGA
GGCGGACAAGGGCTCTGTGAGAGGGGGCGCCCTGGCTCGTGTGCAACCCTTAGGGTT
CACGGAGGTGTGACCAGCTGCAGGCATCGCGGTTTGCGTCGAGGGTGCACTACTGTC
TGCGTCTCGGAGCGCTGGGTTTTGTTTCTGTGAGGGCGTAGGAGGAAAGACGGTGCT
TTGCAGTGCTGCTTTTCTTTTTCCGCGCTTCGGCTACGCCAAATGAGCTGTGAGATG
TTCCGCTTTGTGCCGACCGTTACCACCACTTCCCCCCTTTTCTCCTGCCCTGTTCTT
CTTTGCTTGGGACAATCTGCTTCGCACGCCTCTACACGTGCGATAACGCGCTCTTCT
CGCGCGCCTCTGTCCAATATGGCCTGCCCAACTGAAGCAGTGTTGTTTTCCACC

***L. tropica* LV357**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTCT
GAAGATGTATCGCAAAAAGTGCGTTATGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATT
TCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTGCG
ACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCATT
CGCAACGGATCATGCCTCCTG

***L. tropica* LV142**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTCT
GAAGATGTATCGCAAAAAGTGCGTTATGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATT
TCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTGCG
ACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCATT
CGCAACGGATCATGCCTCCTG

***L. tropica* LV369**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCT
TAGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTA
TCTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCTGAGTGTGGGATTCTCTC
TGAAGATGTATCGCAAAAAGTGCGTTGTGGTGCTGCTTTTCTACTCCTGCGCTGCTG
TTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCAT
TTCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCGACTTGC
GACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCAT
TCGCAACGGATCATGCCTCCTG

***L. tropica* LV508**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTCT
GAAGATGTATCGCAAAAAGTGCGTTATGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATT
TCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTGCG
ACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCATT
CGCAACGGATCATGCCTCCTG

***L. tropica* SU23**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCT
TAGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTA
TCTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTC
TGAAGATGTATCGCAAAAAGTGCGTTATGGTGCTGCTTTTCTACTCCTGCGCTGCTG
TTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCAT
TTCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCGACTTGC
GACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCAT
TCGCAACGGATCATGCCTCCTG

***L. tropica* LV509**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCT
TAGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTA
TCTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCTGAGTGTGGGATTCTCTC
TGAAGATGTATCGCAAAAAGTGCGTTGTGGTGCTGCTTTTCTACTCCTGCGCTGCTG
TTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCAT
TTCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCGACTTGC
GACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCAT
TCGCAACGGATCATGCCTCCTG

***L. tropica* LV510**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTCT
GAAGATGTATCGCAAAAAGTGCGTTATGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATT
TCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTGCG
ACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCATT
CGCAACGGATCATGCCTCCTG

***L. aethiopica* LV546**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV472**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTCTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV1**

ACAAGATCGGGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAACAAAAAGAAGTGCAACGGTATTTTTGGTTCACCAGAGGCTGGCGAAGGCTC
TTAGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGT
ATCTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCTGAGTGTGGGATTCTCT
CTGAAGATGTATCGCAAAAAGTGCGTTGTGGTGCTGCTTTTCTACTCCTGCGCTGCT
GTTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCA
TTTTTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTG
CGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCA
TTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV697**

ACAAGATCGGGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCT
TAGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTA
TCTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTC
TGAAGATGTATCGCAAAAAGTGCGTTATGGTGCTGCTTTTCTACTCCTGCGCTGCTG
TTCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCAT
TTCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTTGC
GACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCAT
TCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV13**

ACAAGATCGGGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTACCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTGTGTGCGCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTCT
GAAGATGTATCGCAAAAAGTGCGTTATGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATT
TCTCCCTCTTCCTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCGACTTGCG
ACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGTTTTAGGGCTTCATT
CGCAACGGATCATGCCTCCTG

***L. aethiopica* LV15**

TCTCGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGCTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
CCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV25**

TCTCGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGCTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV26**

TCTCGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGCTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV27**

TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV28**

TCTCGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCGCCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV29**

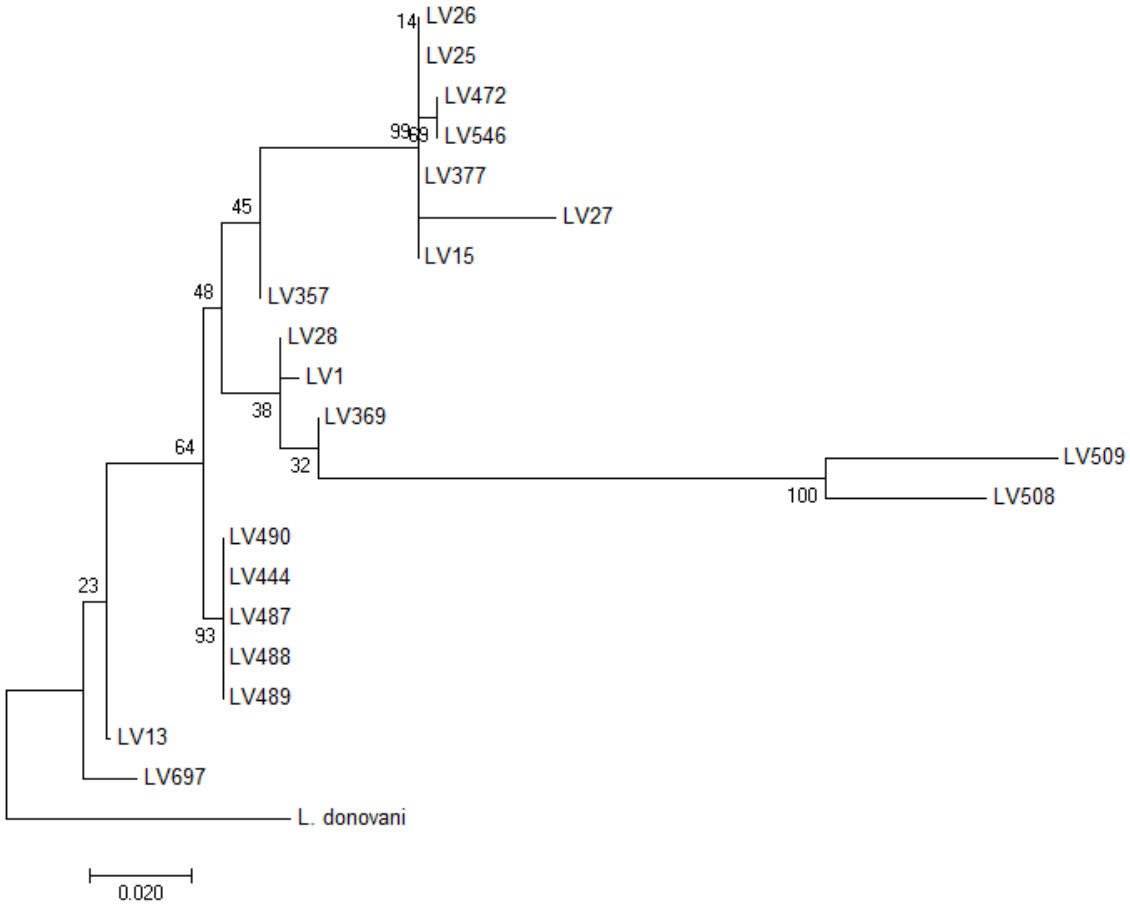
TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAGGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

***L. aethiopica* LV377**

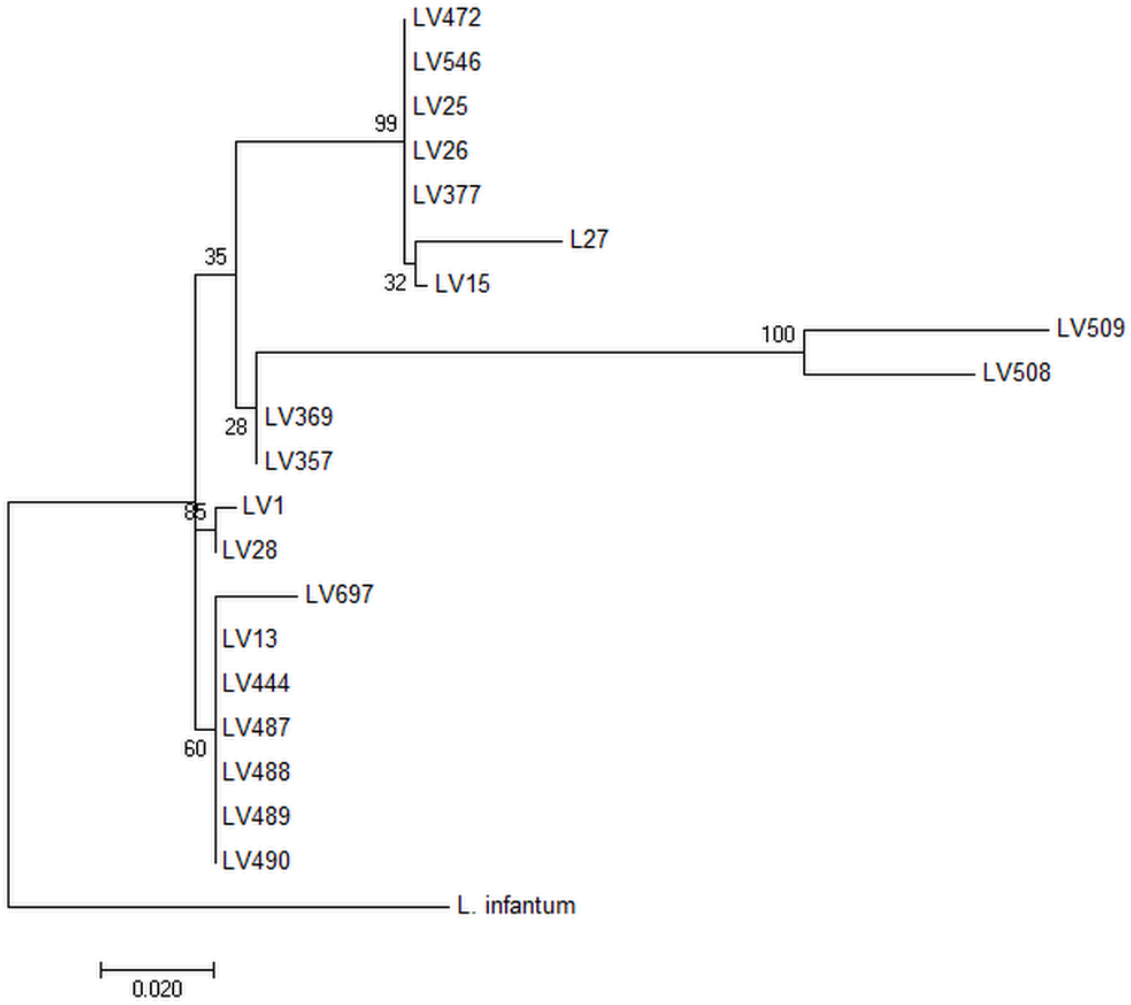
TCTTGTCTAAGCAGAATTTGGATGATGCGATTACTTGAGATAATTTCATTTTATCAA
CAACAAAAAAGAAGTGCAACGGTATCTTTGGTTCACCAGAGGCTGGCGAAGGCTCTT
AGGGTGGTTTAATGAGCTGTTGCCTCTATGGTACACTGAAGCGATCAGCTGCGGTAT
CTTTGTGCGCGTCAAGGGTGCGCTGGTTTCCGCATCTCCGAGTTTGTGATTCTCTCT
GAAGATGTATCGCAAAAAGCGCGTTGCGGTGCTGCTTTTCTACTCCTGCGCTGCTGT
TCCTTCAAATGTGTCGACATGTCCGACTTTCTGCAGCTACTCACCTCCACTTTCATC
TCTCCCTCTTCCTTGTTGTTTTCTGTGTGTCCATGCGTCTCGTGTGCGTCTCAACTT
GCGACAACTTTGCTTGCGCGCCTCTGTGGAATACCATCTGAACAGCTTTAGGGCTTC
ATTCGCAACGGATCATGCCTCCTG

Appendix III – Phylogenetic Trees

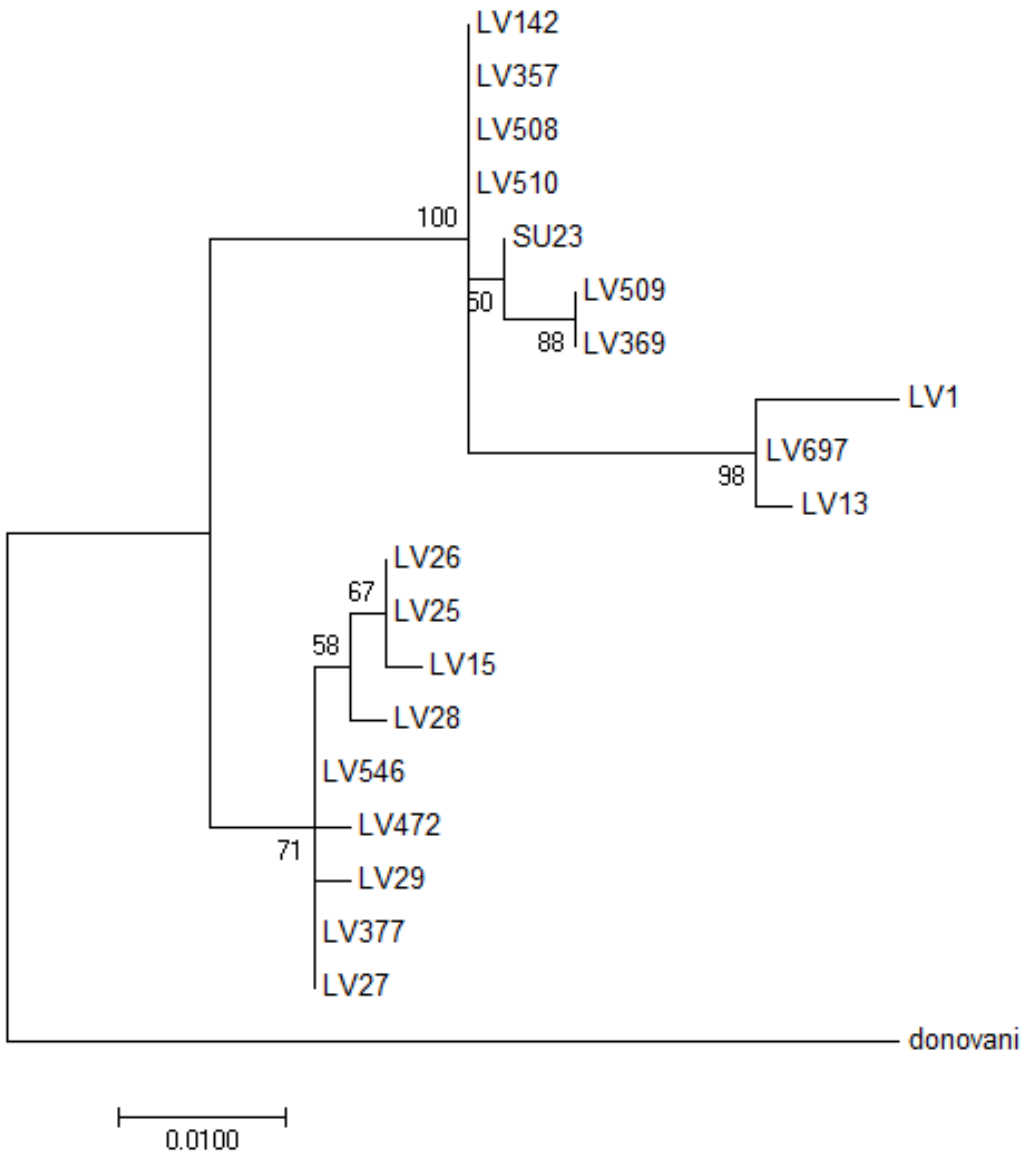
Phylogenetic tree with bootstrapping of ITS 1 sequence for *L. tropica* and *L. aethiopica* with *L. donovani* as an outgroup.



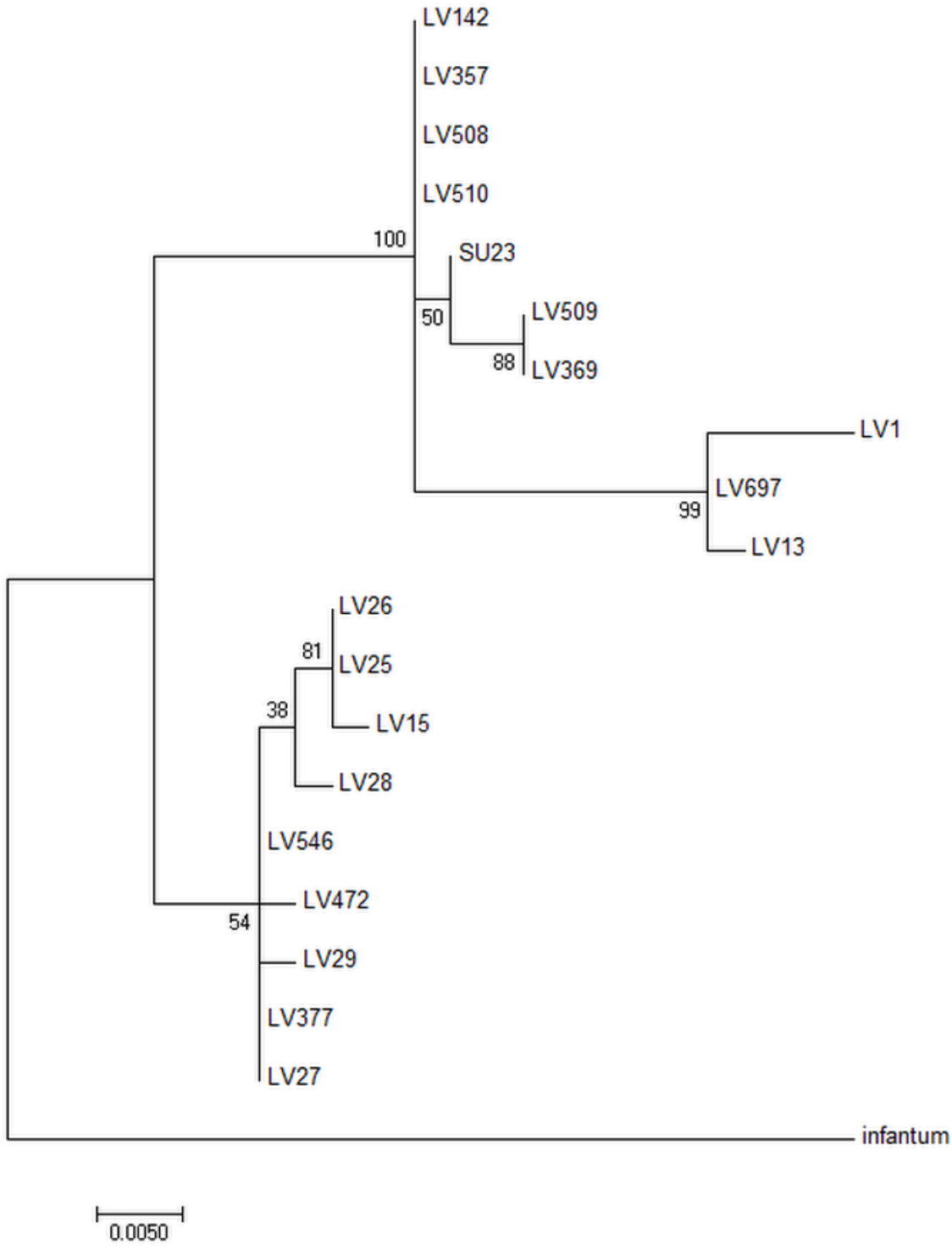
Phylogenetic tree of ITS 1 sequence for *L. tropica* and *L. aethiopica* with *L. infantum* as an outgroup.



Phylogenetic tree with bootstrapping of RPL23a sequence for *L. tropica* and *L. aethiopica* with *L. donovani* as an outgroup.

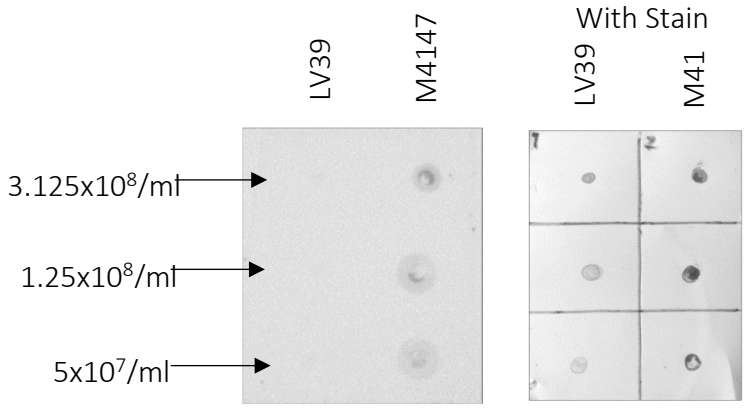


Phylogenetic tree with bootstrapping of RPL23a sequence for *L. tropica* and *L. aethiopica* with *L. infantum* as an outgroup.

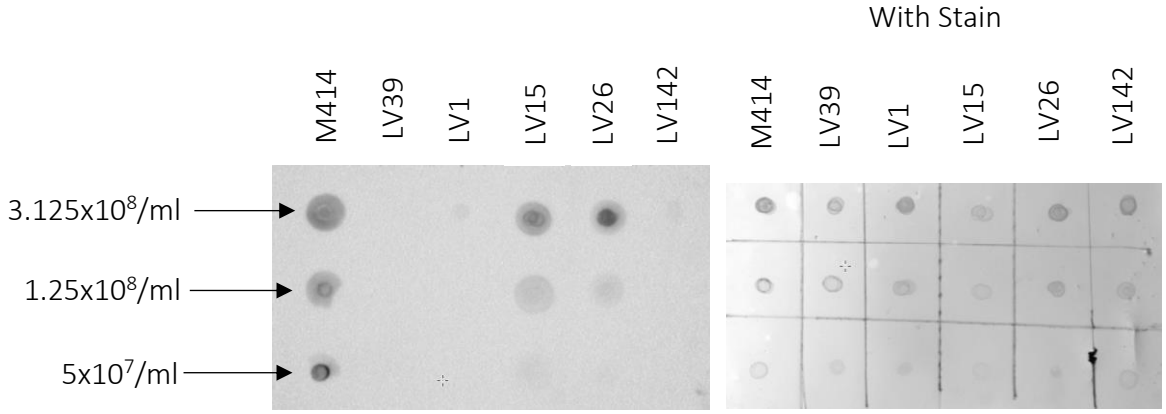


Appendix IV – Dot Blots

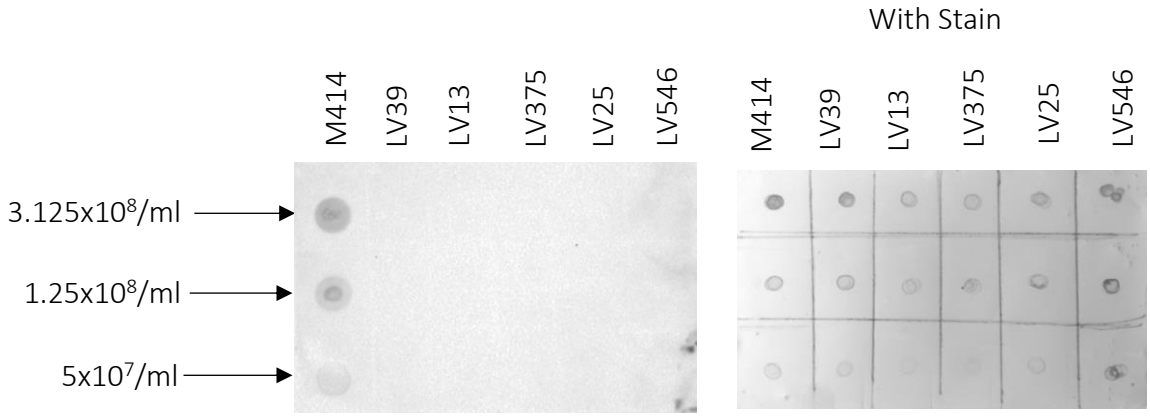
L. major LV29 and *L. guyanensis* M4147 controls before and after Ponceau Red staining



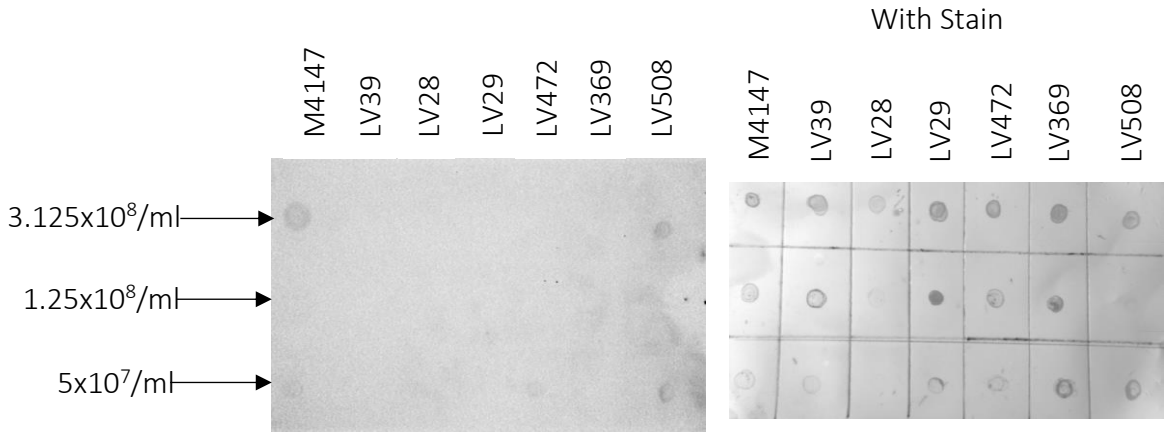
LV1, LV15, LV26 and LV142 with positive and negative controls before and after Ponceau Red staining



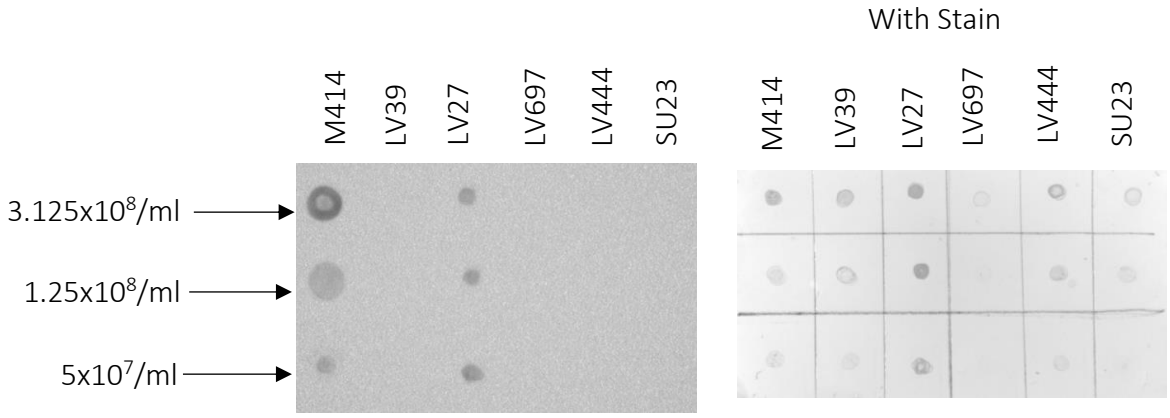
LV13, LV375, LV25 and LV546 with positive and negative controls before and after Ponceau Red stainingLV13, LV375, LV25 and LV546 with positive and negative controls before and after Ponceau Red staining



LV28, LV29, LV472, LV369 and LV508 with positive and negative controls before and after Ponceau Red staining



LV27, LV29, LV697, LV444 and SU23 with positive and negative controls before and after Ponceau Red staining



LV377 and LV489 with positive and negative controls before and after Ponceau Red staining