# Investigation of a New Focus of Cutaneous Leishmaniasis in Ghana

**By**

**Godwin Kwakye-Nuako**
**BSc, MSc, MPhil**

**PhD Thesis**

**In Biomedical and Life Sciences**

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## DECLARATION

I declare that the content of this thesis is my own work, except were clearly stated, and has not been submitted in substantially the same form for the award of a higher degree elsewhere.

# DEDICATION

To my wife and children:

Charlotte Omane Kwakye-Nuako

Nana Akua Pokua Kwakye-Nuako

Mame Yaa Kwakye-Nuako

Barima Yaw Kwakye-Nuako

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## Abbreviations

| | |
|---|---|
| **°C** | Degree Celsius |
| **6GPDH** | 6- Glucose Phosphate Dehydrogenase |
| **ACL** | Anthroponotic Cutaneous Leishmaniasis |
| **AD** | Anno domini |
| **AIDS** | Acquired Immunodeficiency Syndrome |
| **Au/Pd** | Gold/Palladium |
| **BME** | Basal Medium Eagle |
| **CL** | Cutaneous Leishmaniasis |
| **DALYs** | Disability-Adjusted Life Years |
| **DAT** | Direct agglutination tests |
| **DCL** | Diffuse Cutaneous Leishmaniasis |
| **dd$H_2O$** | Deionise water |
| **DL** | Disseminated Leishmaniasis |
| **DMSO** | Dimethyl Sulfoxide |
| **DNA** | Deoxyribonucleic Acid |
| **$ED_{50}$** | Doses for 50% maximal effect (Half maximal inhibitory dose) |
| **EDTA** | Ethylenediaminetetraacetic Acid |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **EM** | Electron microscopy |
| **FBS** | Fetal Bovin Serum |
| **FGT** | Formol gel test |
| **GH5** | Ghana *Leishmania* isolate number 5 |
| **gp** | Glycoprotein |
| **H&E** | Hematoxylin and Eosin |
| **HBSS** | Hanks Balanced Salt Solution |

| | |
|---|---|
| **HIV** | Human Immunodeficiency Virus |
| **$IC_{50}$** | Half maximal inhibitory concentration |
| **IFA** | Indirect immunofluorescence antibody |
| **IFAT** | Immunofluorescent Agglutination Test |
| **ILSb** | Intralesional antimonials |
| **ITS1** | Internal Transcribed Spacer 1 |
| **KAtex** | Latex agglutination test |
| **kDNA** | kinetoplast Deoxyribonucleic Acid |
| **L-AMB** | Liposomal Amphotericin B |
| **LCF** | *Leishmania* Chemotactic Factors |
| **LCL** | Localized Cutaneous Leishmaniasis |
| **M199** | Medium199 |
| **MBCL** | Methylbenzethonium chloride |
| **MCL** | Mucocutaneous Leishmaniasis |
| **ML** | Maximum-Likelihood |
| **MP** | Maximum Parsimony |
| ***Msp1*** | Endonuclease (restriction enzyme) from *Moraxella* species |
| **NJ** | Neighbour-Joining |
| **NNN** | Novy MacNeal Nicolle |
| **NTDs** | Neglected Tropical Diseases |
| **OD** | Optical Density |
| **$OsO_4$** | Osmium Stain |
| **PCR** | Polymerise Chain Reaction |
| **pH** | (pi) Measure of acidity or alkalinity |
| **PKDL** | Post-kala-azar dermal leishmaniasis |
| **PM** | Peritrophic Matrix |

| | |
|---|---|
| **POC** | Point-of-care |
| **PSG** | Promastigote Secretary Gel |
| **RDTs** | Rapid Diagnostic Tests |
| **RE** | Reticuloendothelial |
| **RFHT** | Radiofrequency Heat Therapy |
| **RFLP** | Fragment Length Polymorphism |
| **rK or rKE** | recombinant Kinesin protein |
| **rK39** | recombinant Kinase 39 |
| **RNA** | Ribonucleic acid |
| **RNS** | Reactive Nitrogen Species |
| **ROS** | Reactive Oxygen Species |
| **RPL23a** | Ribosomal Protein L23a |
| **RPMI** | Roswell Park Memorial Institute |
| **rRNA** | ribosomal Ribonucleic Acid |
| **SbV** | Sodium stibogluconate |
| **SEM** | Scanning Electron Microscopy |
| **SH** | Sulph-hydryl |
| **SL RNA** | Spliced Leader RNA |
| **SLME** | Spliced Leader Mini-Exon |
| **SV** | Stomodeal Valve |
| **TAE** | Tris-acetate |
| **TDR/WHO** | Tropical Diseases Program/World Health Organization |
| **TEM** | Transmission Electron Microscopy |
| **THP1** | Human leukemia cell lines |
| **TRALd** | Rapid Antibody Test *Leishmania donovani* |
| **™** | Trade mark |
| **UA** | Uranyl Acetate |

| | |
|---|---|
| **UK** | United Kingdom |
| **USA** | United States of America |
| **VL** | Visceral Leishmaniasis |
| **VTL** | Viscerotropic Leishmaniasis |
| **WHO** | World Health Organisation |
| **WR** | Walter-Reed |
| **ZCL** | Zoonotic Cutaneous Leishmaniasis |

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**Abstract**

Leishmaniasis is a disease of significant public health importance, which burdens a number of countries around the world, particularly in the tropics and subtropics. An outbreak of suspected cutaneous leishmaniasis (CL) has been witnessed in the Ho district of the Volta region in the south-eastern part of Ghana since 1999, where chronic ulcers typical of CL are being diagnosed. In this part of Ghana leishmaniasis has remained endemic to date. To add to the improvement of the level of understanding of the diseases in Ghana; the identity of the parasite, vector incrimination, non-invasive and field friendly diagnosis, and compound susceptibility

tests were investigated. Patients presenting with cutaneous lesions suggestive of CL were selected where skin aspirates were collected from the sites of active lesion(s). Portions of the aspirates were cultured in M199 medium and DNA extracted from the promastigotes generated, while portions of the aspirates were inoculated onto FTA cards. PCR and PCR-RFLP were directly performed on the isolated DNA and the FTA cards. The pattern of bands produced from the patient samples were a complete deviation from DNAs of all the positive controls of *Leishmania* species. The sequenced PCR products and the further phylogenetic analysis revealed close relatedness to *Leishmania enriettii* species. The *Leishmania* species (GH5) responsible for the CL cases in that part of Ghana were successfully isolated into culture for the first time and proved to be distinct from the known species but closely related to non-pathogenic *Leishmania enriettii*. The transmission and the scanning electron micrograph evidence of the parasite confirmed their *Leishmania* identity. A peroxidoxin-based simple field friendly antigen detection test device was found diagnostically sensitive to Ghana species (GH5) and the other species of *Leishmania* used as controls in the diagnostic investigation. In the compound susceptibility test, the species isolated from Ghana (GH5) was found to be relatively resistant to cryptolepine, at concentrations to which the control species *Leishmania mexicana* was susceptible.

# Chapter One

## 1.0 General Introduction and Literature Review

### 1.1 The public health importance of leishmaniasis

Transmission of parasitic diseases and their morbidity and mortality have commonly been associated with the developing world, more especially in communities located in the tropical and the sub-tropical regions. Among such diseases are the neglected tropical diseases (NTDs), of which leishmaniasis is a typical member (Montalvo *et al*., 2012; Yamey and Torreele, 2002; Alvar *et al*., 2006, 2012; Hotez *et al*., 2004, 2006).

Leishmaniasis is a disease of significant public health importance with the etiological agents causing morbidity and/or mortality among its victims (Herwaldt, 1999). It is ranked among the top priority of parasitic diseases listed by the World Health Organisation (WHO), placed second to malaria (Bhargava and Singh, 2012). Leishmaniasis has recently demonstrated geographical expansion, invading many places where it was previously not endemic (Faiman *et al*., 2013). The concept of leishmaniasis being endemic only in tropical and sub-tropical regions seems to be fast eroding with the recent report of the identification of etiological agents found in animals in Australia, Europe and in the USA (Rose *et al*., 2004; Muller *et al*., 2009; Reuss *et al*., 2012). The disease in human hosts has now extended its range from 88 to 98 countries, covering 3 territories and 5 continents (Alvar *et al*., 2012; Roberts *et al*., 2015).

Histopathological evidence, isolation and in vitro culture have clearly demonstrated the evidence of leishmaniasis in Australia some years ago (Rose *et al*., 2004; Herwaldt, 1999; Handman, 2001). Whether this is perhaps due to the changing

climate and/or the adaptation of parasites to a changing environment is not clear, or leishmaniasis may have been present undetected for some time. Climatic models which seek to predict the spreading of leishmaniasis to a naive environment have been studied. Using potential vectors and reservoirs, models have predicted that transmission of the disease will expand to non-endemic areas like USA and Canada, where climate changes will enhance availability of suitable habitat for the vectors and the reservoirs in a near distant future (Gonzalez *et al*., 2010). This will place humans in such habitats at a higher risk of leishmaniasis. Additionally, increasing global travelling, immigration and military interventions in the endemic areas have substantially contributed to the emergence of new cases in leishmaniasis naïve areas (Roberts *et al*., 2015).

An upsurge in the vectors of cutaneous leishmaniasis (CL) spreading northward across Europe due to climate change has been forecast (Watson *et al*., 2005). Additional man-made risk factors increasing the frontiers of leishmaniasis include massive migration, deforestation, urbanization, immunosuppression, malnutrition and treatment failure (Desjeux, 2001). Environment modifications due to construction of dams, which could impact on temperature changes, soil humidity and vegetation with resultant changes in the composition and density of vectors as well as changes in populations of reservoir, can all contribute to the establishment of new foci of leishmaniasis. The development of agro-industrial projects is also contributing factors to leishmaniasis exacerbation. Intrusion into the habitats of vectors and reservoirs for new settlements and/or other interest, with non-immune populations, facilitates the outbreak of leishmaniasis (Kimutai *et al*., 2009). The possibility of introducing parasites to non-endemic areas through increased travelling of humans, and in some cases their dogs, could enhance the natural spread of visceral and cutaneous

leishmaniasis from endemic regions to non-endemic neighbouring areas where vectors are available but no disease (Desjeux, 2001; Ready, 2010). A recent study has also revealed that low socio-economic status is a significant risk factor for the disease (Kolaczinski *et al*., 2008).

The true incidence of the disease is almost certainly underestimated or unknown (McDowell *et al*., 2011; Desjeux, 2004), due mainly to the fact that most of the victims are poor and have no access to facilities where proper records could be documented. Additionally, for the reasons discussed above the dynamics of disease burden are changing fast. Despite the lack of reliable evidence, the World Bank has estimated disease burden among the regions of the world (**Table 1.1**).

| **Region** | **DALYs** |
|---|---|
| East Asia/Pacific | 48,000 |
| Europe/Central Asia | 6,000 |
| South America | 37,000 |
| Middle East/North Africa | 48,000 |
| South Asia | 1.3 million |
| Sub-Saharan Africa | 312,000 |

**Table 1.1** The burden of leishmaniasis disease expressed in disability-adjusted life years (DALYs). Estimated by the World Bank (Kedzierski *et al*., 2006).

The global reported incidences of CL and VL are ≈210,000 and ≈58,000 cases, respectively, but the estimated incidences allowing for under-reporting are much higher, up to ≈1.2 million cases of CL and 400,000 cases of VL (Alvar *et al*., 2012). It is estimated that about 350 million people are at risk of infection (PAHO-WHO report, 2013).

### 1.2 Clinical forms of leishmaniasis

Leishmaniasis is caused by various species in the protozoan genus *Leishmania*, and presents in a variety of clinical syndromes, with three major forms (McCall, *et al*., 2013). These are cutaneous, mucocutaneous and visceral leishmaniasis (**Table 1.2**), but within each are several types depending on various factors such as the outcome of treatment and immunological status of the victim. Including new emerging *Leishmania* species, there are more than 20 species causing human leishmaniasis (Marco *et al*., 2006; Toz *et al*., 2013).

| **Syndrome** | | **Species** |
|---|---|---|
| Cutaneous leishmaniasis | Common | *L. major*<br>*L. tropica*<br>*L. amazonensis*<br>*L. mexicana*<br>*L. braziliensis*<br>*L. aethiopica* |
| | Rare | *L. infantum*<br>*L. donovani*<br>*L. peruviana* |
| Mucocutaneous leishmaniasis | Common | *L. braziliensis* |
| | Rare | *L. panamensis*<br>*L. guyanensis*<br>*L. amazonensis* |
| Visceral leishmaniasis | Common | *L. donovani*<br>*L. infantum*<br>*L. infantum chagasi* |
| | Rare | *L. tropica*<br>*L. amazonensis* |

**Table 1.2** Important *Leishmania* species and their resultant syndromes **(**McCall *et al.,* 2013; Bates, 2007).

The species listed in **Table 1.2** are divided into Old World or New World depending on the geographical origin. Species of the Old World include *L. donovani, L. infantum, L. tropica, L. major* and *L. aethiopica*. New World species include *L.*

*infantum chagasi*, *L. mexicana*, *L. amazonensis*, *L. brazilensis*, *L. peruviana*, *L. guyanensis* and *L. panamensis*. The five most important species to humans are: *L. tropica*, *L. major*, *L. donovani*, *L. mexicana* and *L. braziliensis*.

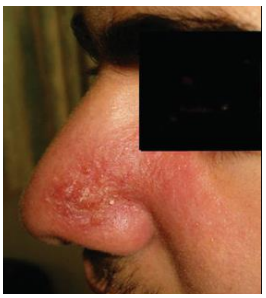
### 1.2.1 Cutaneous leishmaniasis

The cutaneous form of leishmaniasis has been known for many centuries. In the Old World CL was mentioned in the first century AD, whereas in the New World CL was described in Peru and Ecuador since 400 - 900 AD (Peters, 1988; Lainson and Shaw, 1987). In the 16$^{th}$ and 17$^{th}$ century, "Aleppo evil" and "Dehli boil" were used to describe cases of CL in the Middle East and the Indian subcontinent, respectively, when skin lesion biopsies were found to contain protozoa (Borovsky, 1938; Grevelink and Lemer, 1996). CL lesions later earned various names such as; Baghdad sore, Rose of Jericho, Chiclero's ulcer, uta, and forest yaws (Grevelink and Lemer, 1996), depending on their geographical origin. CL has since expanded it frontiers including the evolution of new foci caused by new species. This has been linked with risk factors including population surge and displacement, urbanization, anthropogenic environmental modifications, drug resistance, and new agricultural practices among many influences (Ashford, 2000; Daszak *et al*., 2001; Patz *et al*., 2000; Jeddi *et al*., 2011).

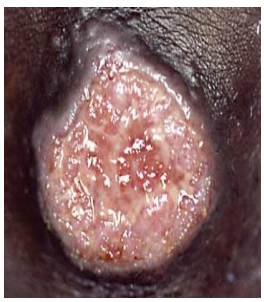
CL is a disfiguring and stigmatizing disease that starts with erythematous papules, which usually transform to nodules and finally become crusted ulcerated lesions (Roberts *et al*., 2015). They occur at the site of the bite of the sand fly vector, due to parasite replication in the dermis. CL is reported to occur in 82 countries, but 75% of the cases are recorded in only 10 of these (Alvar *et al*., 2012). The typical etiological agents are *L. tropica*, *L. major*, *L. aethiopica* and *L. mexicana*, there are some new emerging species of the parasite, and a few cases have been recorded for *L.*

*donovani* and *L. infantum* (Rhajaoui *et al*., 2007; Desbois *et al*., 2014; Dedet, *et al*., 1995; Sukmee *et al*., 2008). In certain geographical locations two CL epidemiological forms are distinguished. These are Zoonotic CL (ZCL), for example caused by *L. major*, and anthroponotic CL (ACL), for example caused by *L. tropica*.

Various subtypes of CL exist, ranging from localized cutaneous leishmaniasis (LCL) to more severe and disseminated cutaneous leishmaniasis (DL) or diffuse cutaneous leishmaniasis (DCL) (Zijlstra, 2014). LCL is the classical cutaneous leishmanial ulcer, caused by a spectrum of species, but mainly *L. major* and *L. tropica* in the Old World. The lesions produced by *L. major* or *L. tropica* are often wet or dry, respectively, occurring mostly on an unclothed part of the body, primarily on the face, arms or neck where the sand fly vector can easily bite (Shoaib *et al*., 2007; Mujtaba and Khalid, 1998). The species *L. braziliensis* and *L. mexicana* are mainly responsible for LCL in the New World. After an incubation period typically of 1 week to 3 months, the LCL lesion starts as a red (erythematous) papule, which subsequently broadens to a plaque or nodule form, and which further develops to a circumscribed ulcer characterised by a violaceous border, granulomatous and crusted, with hypertrophic margins (Grevelink and Lemer, 1996) within where the parasite lives (**Figure 1.1**).



nodules and popule



ulcerated

**Figure 1.1** Examples of various forms of cutaneous lesions.

The sores created in LCL are usually self-resolving, but leaving hypo- or hyper-pigmentation. They can become superinfected with bacteria or fungi, which can influence and compromise diagnosis (Killick-Kendrick *et al*., 1985).

DCL is an anergic variant of LCL, where lesions disseminate to different parts of the body resembling lepromatous leprosy, with multiple small and painless papules developing around the old lesion scars (Grevelink and Lemer, 1996; Calvopina *et al*., 2006) (**Figure 1.2**). Heavily parasitized non-ulcerative lesions of DCL can erupt during failed drug treatment of LCL (Calvopina *et al*., 2006).

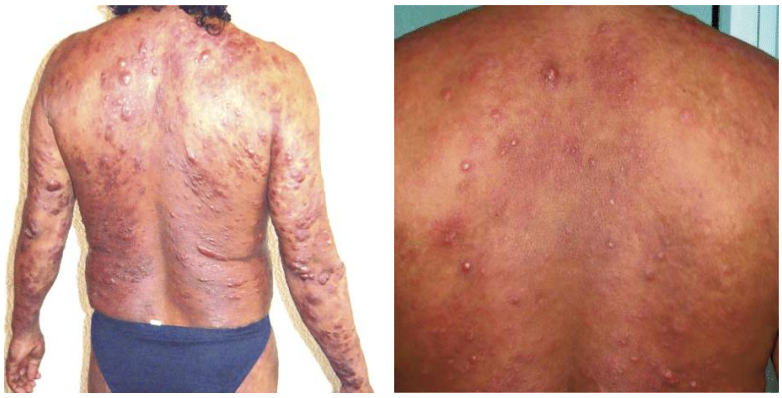


**Figure 1.2** Multiple lesions indicative of DCL (Calvopina *et al*., 2006).

DL is characterised by the presence of more than 10-800 mixed-type lesions (e.g., acneiform, papular, nodular, and/or ulcerated), located in more than two body parts (head, trunk, arms, and legs) (Couppie *et al*., 2004; Zijlstra, 2014), which distinguishes it from LCL and DCL. These lesions are presumed to have disseminated from a single initial lesion within 3 days to 8 weeks, and can be caused by *L. braziliensis*, *L. amazonensis* and *L. guyanensis* (Couppie *et al*., 2004; Turetz *et al*., 2002). DCL is rarer than DL, with DCL caused by *L. amazonensis*, *L. mexicana* and *L. pifanoi* in the New World, and *L. aethiopica* in Old World (Zijlstra, 2014).

### 1.2.2 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL) is a disfiguring and mutilating naso-oropharyngeal form of leishmaniasis. MCL is characterised by mucosal lesions mainly affecting the nasal septum, palate, pharynx, tonsils, gums, and/or lip (Grevelink and Lemer, 1996), and mainly occurs after the healing of previous LCL caused by *L. braziliensis* in the New World, though some cases have been reported due to *L. guyanensis* and *L. amazonensis* (Lucas *et al*., 1998; Santrich *et al*., 1990). Due to the nasal involvement, early symptoms involve nasal stuffiness and mild difficulty breathing, mucosal erythema and oedema (Showler and Boggild, 2015). The mucosal lesions are mostly found on the oral cavity, nose, pharynx, larynx, or the eyes and may occur with LCL, but when such lesions result during VL or PKDL, they are due to *L. donovani* or *L. infantum* (Zijlstra, 2014). The ensuing deformations from MCL have earned names such as "tapir's nose," "parrot's beak," and "camel's nose" (Grevelink and Lemer, 1996).

MCL is reported to occur in about 5% of untreated leishmaniasis cases due to *L. braziliensis* and related parasites (Stebut, 2015). At the start of MCL the nasal septum often becomes inflamed, infiltrated and consequently perforates. Risk factors include sex (males mostly affected), large and/or multiple primary lesions (LCL), inadequate treatment of the primary cutaneous lesions and persistent lesions lasting longer than 1 year. Among MCL infected individuals, nearly 50% percent of them have experienced mucocutaneous lesions within 2 years, and 90% within 10 years, of the initial cutaneous lesions, depending on the species and the region of acquisition (Marsden *et al*., 1984; Showler and Boggild, 2015). This demonstrates that the activation of MCL after the initial cutaneous lesion is rare, in some cases this has occurred 35-50 years after the initial cutaneous lesion, but more typically within 1-5 years (Mansueto *et al*., 2014; Schleucher *et al*., 2008). In Bolivia, Peru, Brazil and

Ecuador for instance, between 2-15% MCL cases have been recorded in infections by *L. braziliensis* and *L. peruviana* species (Showler and Boggild, 2015; Reveiz *et al*., 2013). Fatality as a result of MCL is often linked to negative effects associated with acute respiratory pneumonia. Malnutrition has also been linked to MCL fatality due to exertion in swallowing (Stebut, 2015; Showler and Boggild, 2015).

### 1.2.3. Visceral leishmaniasis

VL is the most frequently fatal form of leishmaniasis (McCall, *et al*., 2013), and has been ranked second and fourth in mortality and morbidity, respectively, among all the tropical diseases (Alvar *et al*., 2012; Zijlstra, 2014). However, in addition to its deadly form, many infections go unnoticed, with the proportion of the asymptomatic to symptomatic cases estimated at 18:1 (Badaro *et al*., 1986). There are various species of *Leishmania* which have been implicated in causing VL, commonly these are *L. donovani* and *L. infantum* (McCall, *et al*., 2013). Their ability to cause VL is presumed to be attributable to some genes they carry.

Following inoculation into the skin the causal agent disseminates and multiplies in the reticuloendothelial (RE) system, affecting visceral organs in particular the spleen and liver, also lymph nodes, to cause pathology (Canton *et al*., 2012; Ashford, 2000). The clinical symptoms associated with VL include irregular fever (similar to that cased by malaria) and malaise, followed by wasting, anaemia and emaciation, and prostration of the abdomen due to hepatosplenomegaly, all of which contribute to fatality (Bern *et al*., 2008; Murray *et al*., 2005).

The clinical symptoms are not diagnostic by themselves, so demonstration of the parasite in the Giemsa stained aspirates from the spleen, bone marrow or other affected organs can be used to make a diagnosis. Immunodiagnostic tests such as the rK39 strip test, or other serological tests such as IFAT and ELISA, have also been

used for diagnosis (Figueiredo *et al*., 2010). In addition, PCR amplification of certain gene targets has been applied to VL samples to help in diagnosis and to identify the agents of the disease (Alvar *et al*., 1997). The treatment of VL is usually based on the parenteral administration of pentavalent antimonials or amphotericin B formulations, with pentamidine and miltefosine as alternatives, although there have been records of some treatment failures, increased parasite resistance and toxic side effects to some of these drugs (Castilho *et al*., 2003; Martins *et al*., 2015).

Sometimes drug treated VL leads to complications in a form of dermal leishmaniasis (**Figure 1.3**). This complication is referred to as Post-kala-azar dermal leishmaniasis (PKDL), a skin rash (Zijlstra *et al*., 2003). It develops as a post-treatment florid cutaneous presentation, which have been recorded to develop in as many as 60% of VL patients (Brooks *et al*., 2004; Zijlstra, 2014). It has been postulated that antileishmanial treatment towards VL forces the parasites to harbour in the dermis, which will delay VL relapse, but fosters PKDL. PKDL is recognised by the appearance of the rash, its typical distribution, and the temporal relationship to VL caused mainly by *L. donovani* (Zijlstra, 2014). PKDL is not always associated with chemotherapy, and in such cases the pathogenesis of PKDL has been speculated to be the attempt of the immune system to clear latent dermal parasites. This type of VL leads to the reactivation of the residual parasite in the skin of the patients, producing numerous papular lesions (**Figure 1.3**) and could serve as reservoir to enhance disease transmission (Antinori *et al*., 2007).

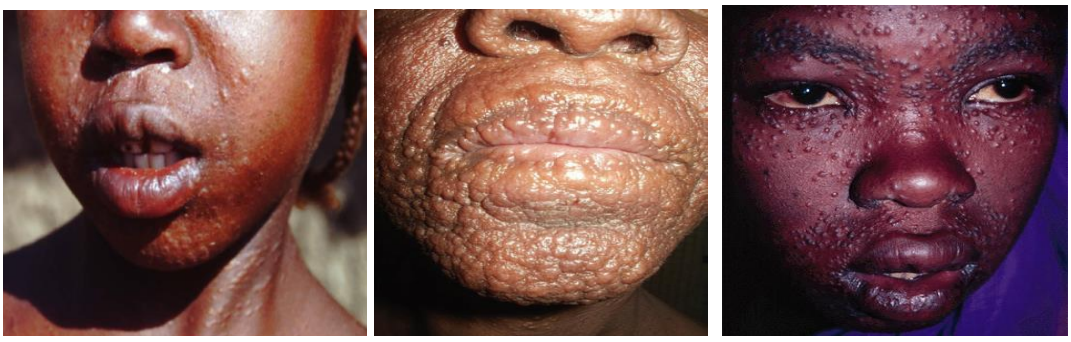


**Figure 1.3** Examples of PKDL showing numerous papular lesions. (Zijlstra, 2014; CDC, Atlanta, USA; Niamba *et al*., 2007).

Further characteristic features associated with PKDL include painful macules, papulo-erythematous eruption, dispersed papules, nodules and plaques which spread from the face to other part of the body (Stark *et al*., 2006; Rihl *et al*., 2006; Antinori *et al*., 2007). The papular and/or nodular rash accounts for approximately 51% cases of PKDL reported (Bittencourt *et al*., 2003). PKDL could show up 6-60 months (5 years) after an apparently successful treatment of VL (Osman *et al*., 1998; Bittencourt *et al*., 2003). It has been found to occur between one and twenty years after recovery from VL in India, in addition to developing during/within months after treatment of VL in the Sudan where the demonstrable signs persist for decades in some Sudanese patients (Salotra *et al*., 2003).

### 1.2.4 *Leishmania*-Human Immunodeficiency Virus co-infection

The Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) pandemic is altering the natural history and the epidemiology of leishmaniasis across the globe (**Figure 1.4**), especially resulting in increasing VL cases in endemic areas (Herrador, *et al*., 2015). HIV infection increases the risk of developing VL by a factor between 100 and 1000 in endemic areas, drastically reducing the therapeutic response and increasing the chances of relapse (Singh *et al*., 1992; WHO Report, 2007; Herrador, *et al*., 2015). The clinical manifestation of all forms of leishmaniasis

and HIV infection act synergistically via their effects on the immune system. Leishmaniasis predisposes to HIV infection due to the immunosuppression it causes (WHO Report, 2007). The first *Leishmania*-HIV associated cases were reported as far back as 1985 and the co-infected cases have been increasing since then especially across the southern Europe (WHO Report, 2007; Lopez-Velez *et al*., 2001).

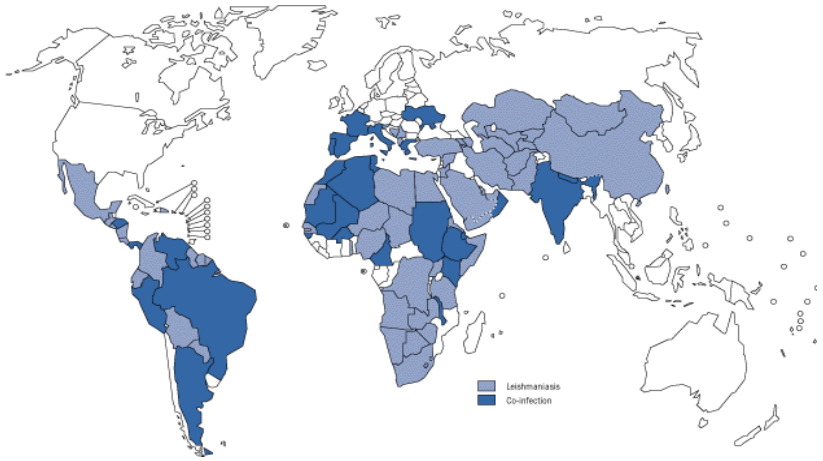



**Figure 1.4** The global outlook of leishmaniasis and *Leishmania*-HIV co-infection (Desjeux and Alvar, 2003).

It is estimated that 35 of 88 countries where leishmaniasis is endemic have *Leishmania*-HIV co-infection, presenting greater public health concern, especially in Brazil, eastern Africa and the India (Ready, 2010; Sinha *et al*., 2005; Kedzierski *et al*., 2006). The Mediterranean region has recorded the highest number of *Leishmania*-HIV co-infections, accounting for 1440 out of the first 1700 cases documented by WHO, from 33 countries (Singh, 2014). Cumulatively, 2000 cases had been observed towards the end of 2001 among European countries such as France, Italy, Portugal and Spain (WHO Report, 2007). In these countries up to 70% of all adult VL cases could be associated with HIV infection, and 9% of all AIDS patients could have newly acquired or reactivated visceral leishmaniasis (Cruz *et al*., 2002). *Leishmania*-HIV co-infection is now being recognised as a significant problem in certain places on the continent of Africa. In Burkina Faso, of 80 HIV patients studied, 74 were confirmed

to have contracted leishmaniasis (Guiguemde *et al*., 2003). Other cases of *Leishmania*/HIV co-infections are being reported more frequently in various parts of Africa (Niamba *et al*., 2007) and it is anticipated that the number of co-infections will not be restricted to traditional endemic areas (Lartey *et al*., 2006). Despite the lack of an official surveillance system in West Africa on *Leishmania*-HIV, a number of cases have been reported in Mali and Senegal (Lartey *et al*., 2006). It has been recorded that 70% of Sudanese and Ethiopian adults having VL were co-infected with HIV (Desjeux, 2001). This has therefore raised alarm as AIDS becomes a leishmaniasis-defining infection (Singh, 2006).

PKDL is commonly found in East Africa and Indian sub-continent in VL endemic foci (Celesia *et al*., 2014). There is documented evidence that PKDL is uncommon in HIV-positive patients, however, recent reports have found PKDL cases associated with HIV in Sudan, Ethiopia, India as well as in Europe, Asia, the Americas and the Middle-East (Burza *et al*., 2014; Santos-Oliveira *et al*., 2011; Bittencourt *et al*., 2003; Antinori *et al*., 2007; Alsina-Gibert *et al*., 2006; Ritmeijer *et al*., 2006). In India and Sudan 10-20% and nearly 50% of HIV patients healed of VL reported PKDL, respectively (Salotra *et al*., 2003). Additionally, sporadic cases of PKDL have been reported in China and the Mediterranean, especially Spain where PKDL in HIV-infected individuals was found among intravenous drug users due to needle sharing (Osman *et al*., 1998; Zijlstra, 2014). The severity of PKDL is more pronounced in HIV-positive than the HIV-negative patients (Ritmeijer *et al*., 2006). The lesions in PKDL-HIV co-infection are multiple, florid, non-ulcerating nodular lesions with copious amounts of parasites.

In HIV-*Leishmania* infections where there is lesion involvement, there is a high parasite load and their circulation in the peripheral blood and a profusion of parasites

in dermal lesions with evidence of active excretion of parasites. This is evidence that HIV-infected patients with leishmaniasis would play an important role in transmission and epidemiology. Hence, early detection, diagnosis and treatment with antileishmanial and antiretroviral therapy is important to reduce transmission (Zijlstra, 2014). Sensitive diagnostic methods are required to identify the disease and to plan intervention. PCR-based methods used on the peripheral blood and on bone marrow aspirates from *Leishmania*-HIV co-infected patients have yielded between 72-100% and 82-100% sensitivity, respectively, and the latex agglutination test using urine samples yielded a sensitivity of 85.7-100% (WHO Report, 2007; Pizzuto *et al*., 2001). The use of amphotericin B deoxycholate, miltefosine or paromomycin has produced good treatment results after successful diagnosis (WHO Report, 2007).

### 1.3 *Leishmania* parasites and their life cycle

The genus *Leishmania* is a member of the subphylum Kinetoplastida, a group of protozoan parasites characterised by a structure called the kinetoplast, a DNA-rich organelle situated near the basal body, close to the base of the flagellum (**Figure 1.5**) (Rodgers *et al*., 1990). The group possess additional characteristic features such as the presence of a flagellar pocket and a paraxial rod alongside the axoneme, but they are also distinguished by their host distribution, life cycle, medical, and veterinary importance (Roberts and Janovy Jnr., 1996).

The *Leishmania* parasite was first described by William Boog Leishman and Charles Donovan in their separate investigations in 1903, and recognised since as responsible for leishmaniasis (Herwaldt, 1999). There are over 20 *Leishmania* species causing the various forms of the disease, transmissible by over 30 species of sand flies and possibly other emerging vectors, which, together with a range of mammalian reservoir hosts, presents a complex variety of interactions (Ashford, 2000; Herwaldt,

1999; Shaw, 1994; Desjeux, 1996). However, among the potential combinations of parasite-hosts-vectors, there is some kind of specificity where in a given natural environment particular combinations are maintained (Ashford, 1996; 2000). Another level of complexity is provided by combinations of clinical syndromes in a particular case, for example, CL-DCL, CL-MCL, PKDL-HIV-VL, PKDL-VL, with variable clinical manifestation, diagnosis and therapeutic response (Canton *et al*., 2012; Herwaldt, 1999). In contrast to this functional complexity, across the various species of *Leishmania* the two main forms are essentially morphologically identical (Ashford, 2000).

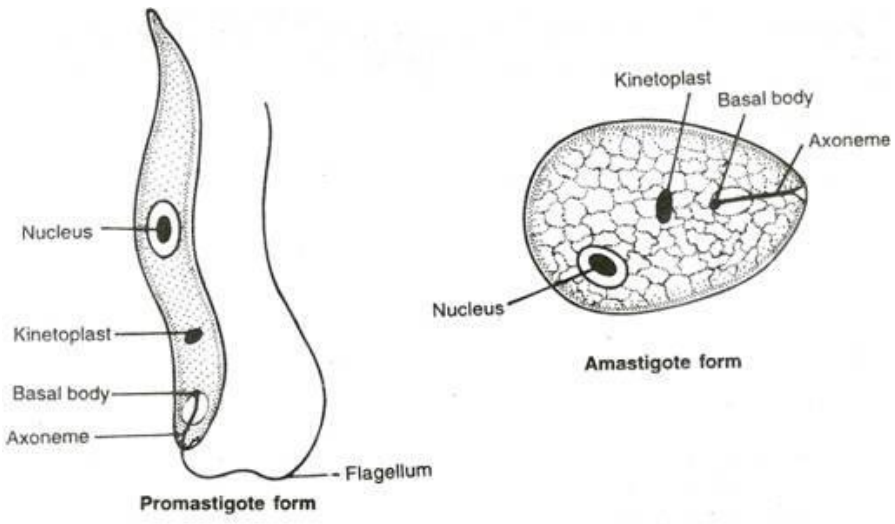



**Figure 1.5** The two major morphological forms of *Leishmania* parasites.

The life cycle of *Leishmania* is sustained by interactions involving the parasites, the sand fly vector, and the vertebrate host (**Figure 1.6**). The intracellular amastigotes are about 3-5µm in size and live in macrophages, and are responsible for the clinical symptoms and pathology of disease. They are introduced into the sand fly gut during blood feeding, where the macrophages get ruptured releasing the amastigotes, which then transform into promastigote forms between 5-15µm in size.

The non-motile amastigotes are ovoid in shape with the absence of a functional flagellum (**Figure 1.5**). In the cell body of the amastigote are found a centrally placed nucleus and kinetoplast anterior to the nucleus (Bates, 2007). The amastigote has only one developmental form.

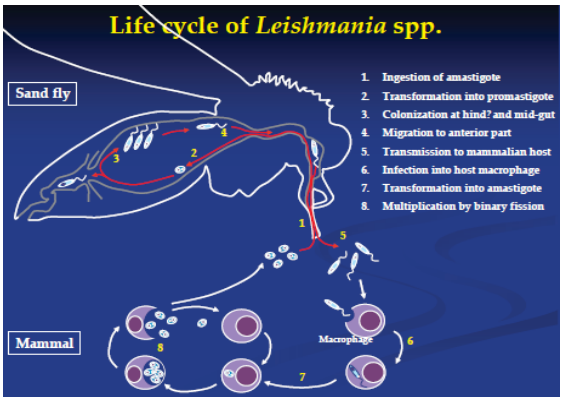



**Figure 1.6** Overview of the life cycle of *Leishmania* in vector and mammalian hosts.

Several different types of promastigote can be distinguished (**Figure 1.7**), and these occur at different places in the sand fly midgut (**Figure 1.8**). The motile promastigote on the other hand is large and elongated with anteriorly positioned elaborate flagellum emanating from the flagella pocket, with anteriorly positioned kinetoplast, and central nucleus posterior to the kinetoplast.

The promastigotes in the gut of sand fly host undergo series of developmental stages involving procyclic, nectomonad, haptomonad, leptomonad and metacyclic promastigote forms (**Figure 1.7, 1.8**). The resultant infective forms produced at the end of development in the vector are the metacyclic promastigotes (Bates, 2007). The sand fly vector acquires an infection through ingesting the amastigote form from the mammalian host during blood feeding, which then become encased in the peritrophic matrix (PM) in the posterior midgut (**Figure 1.9**), produced by the midgut epithelium in the presence of blood meal (Pimenta *et al*., 1997).

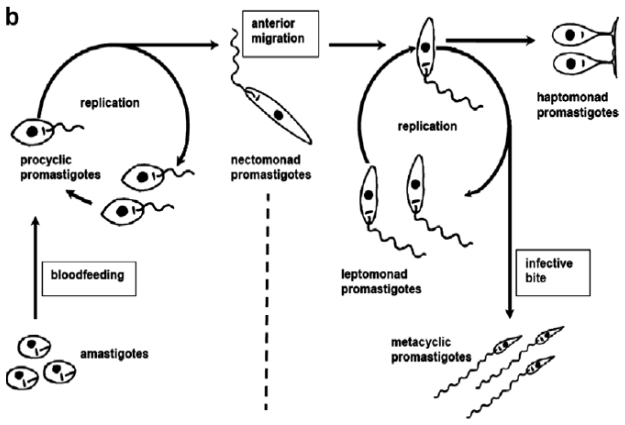



**Figure 1.7** Different developmental forms of *Leishmania* (Bates, 2007).

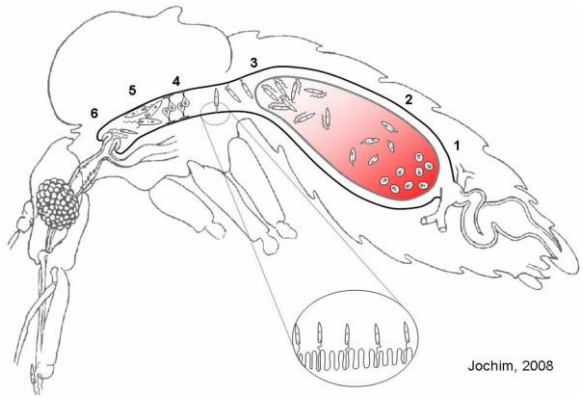



**Figure 1.8** Location of parasite forms in the sand fly midgut (Jochim, 2008). 1 amastigote, 2 procyclic promastigote, 3 nectomonad promastigote, 4 haptomonad promastigote, 5 leptomonad promastigote, 6 metacyclic promastigote.

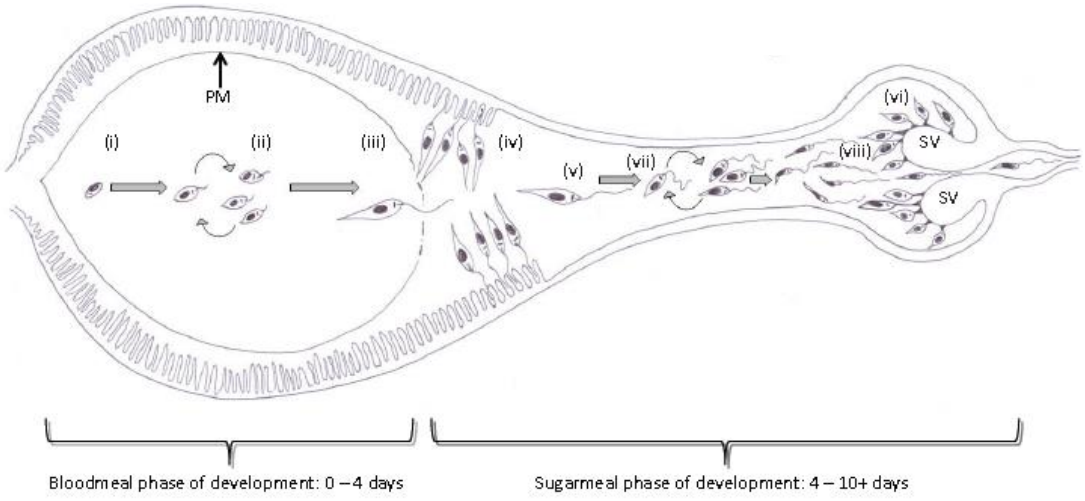


**Figure 1.9** Movement of *Leishmania* parasites in the sand fly gut. Two barriers that are negotiated are the peritrophic matrix (PM) and stomodeal valve (SV) (Ready and Rogers, 2013; Ready, 2000).

The secretion of the PM – a composite of proteins, glycoproteins and chitin fibrils - is a characteristic of haematophagous insects, and is believed to offer protection during the amastigote transformation to the early promastigote stage (procyclic) (Shao *et al*., 2001). The location of the promastigotes for further transformation is dependent on the subgenus of the parasite. The *Leishmania* (*Viannia*) species, of which *L. braziliensis* is an example, migrate posteriorly, attaching to the hind gut of the vector for development, a phenomenon refer to as peripylarian development, though there is still involvement of the mid- and the foreguts later (**Figure 1.10**). This contrasts with *Leishmania* (*Leishmania*) species, which undergo suprapylarian development where the promastigotes are confined to the midgut and foregut (Lainson *et al*., 1979a).

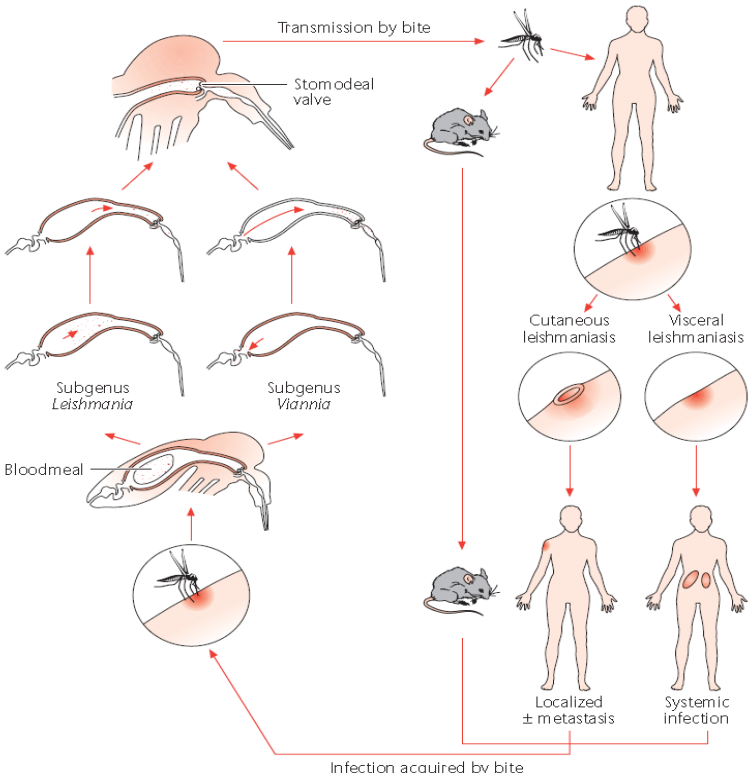


**Figure 1.10** Comparison of suprapylarian and peripylarian development of *Leishmania* (Bates 2001).

In the gut of the vector the promastigotes adapt to the gut conditions and attach to the gut walls to survive being defecated together with the remnants of the blood approximately 3 days after the blood meal, to sustain development and perpetuate transmission (Ashford, 2000, Bates, 2006). In suprapylarian development the first stage is the procyclic promastigote, which transforms from the amastigotes, divides and multiplies rapidly within 2-5 days after blood meal in the PM. They are ellipsoidal in shape, have a short body of 6-8μm, with a short flagellum and characteristically form rosettes (Bates and Rogers, 2004). The procyclic promastigote gives rise to highly motile nectomonad forms, which are approximately 12-20μm in body length, within 2-3 days after blood feeding. The nectomonad transformation is initiated in the PM. The large and long, slender, non-dividing, anteriorly migratory nectomonad forms then move to the thoracic mid-gut (stomodeal valve) to establish infection in an appropriate sand fly vector (Bates and Rogers, 2004). Though significant, the procyclic and nectomonad promastigotes do not guarantee the parasite establishment in the gut of the vector, especially if the parasite fails to attach to the gut walls (Lawyer *et al*., 1990a; Bates and Rogers, 2004). An important function of the nectomonad non-dividing cell population is to establish infection in the vector by attaching their flagella to the microvilli of the gut, and they also migrate forward to colonise the midgut near the stomodeal valve (Warburg *et al*., 1986a; Gossage *et al*., 2003).

Within the 3-7 days after blood meal, the next developmental stage occurs, which involves the transformation of nectomonad to dividing leptomonad promastigotes, and these populate the gut and sustain infectivity in both the stomodeal

valve and the foregut in the presence of a gel-like substance called Promastigote Secretary Gel (PSG) (Rogers *et al*., 2002). The leptomonad parasites produce PSG and become embedded within it, which facilitates transmission of infective promastigotes (Rogers *et al*., 2004; Bates and Rogers, 2004). The leptomonad forms are about 6-8μm in body length and are derived from the nectomonad population. The non-dividing metacyclic promastigotes of about 5-8μm length differentiate from the leptomonad forms. They are the mammalian infective forms, and have a short body length, long flagellum, high motility, and are free swimming (Rogers *et al*., 2002; Gossage *et al*., 2003).

To effect transmission to mammalian hosts, the infected sand fly bites and releases the metacyclic promastigotes together with a collection of pharmacological, immunomodulatory, and immunogenic molecules with effects on the host, during salivation that occurs at the moment of blood feeding (Secundino *et al*., 2012). Upon feeding, the vector expels between 1,000 and 100,000 promastigotes into the host (Rogers *et al*., 2004; Kimblin *et al*., 2008). The sand fly vectors are pool feeders, and they regurgitate the infectious metacyclic promastigotes into the pool of blood in the skin of the vertebrate host during blood feeding. These metacyclic promastigotes are released from PSG in the anterior midgut and stomodeal valve, which gets dissolved by contact with the influx of the blood (Rogers *et al*., 2002). The metacyclic promastigotes are released into the hosts' skin at the point of injury or bite.

Although the main host cells are macrophages, neutrophils are believed to play an important early role in establishing infection. Metacyclic promastigotes are taken up by neutrophils that infiltrate at the point of injury, which act as carriers, the neutrophil and its contents being engulfed by the macrophages to internalise the parasite (Laskay *et al*., 2003). This migration of neutrophils to the site of infection is

induced by the promastigote factors called *Leishmania* chemotactic factors (LCF) with strong chemotactic action towards the neutrophil (van Zandbergen *et al*., 2003). The changes in microenvironment of the cell (macrophages) and temperature stimulates the metacyclic promastigote to transform to the aflagellate amastigote form, which resides in the macrophage phagolysosomal system. Release of amastigotes enables infection of more macrophages, which perpetuates the infection and provides a source of parasites that could be ingested by a vector to maintain the life cycle. The vector can only access the parasite in the skin during blood feeding. The parasites in the visceral organs or other internal sites cannot be accessed by the vector. As pool feeders, the vectors feed by agitating and cutting the skin to create tissue damage and make a wound with their mouthparts. This would release macrophages or free amastigotes into the wound for uptake by the vector (Bates, 2007).

### 1.4 Diagnosis and *Leishmania* species identification

The diagnosis of leishmaniasis has traditionally relied on direct microscopic examination of Giemsa stained smears of the prepared appropriate clinical samples and detection of amastigotes (Leishman-Donovan bodies). Samples include bone marrow aspirates, spleen aspirates, lesion scrapings, lesion aspirates, or biopsies, collected from sites of the patients depending on the form of the disease (CL, MCL, VL). Microscopy can be supplemented by in vitro culture of clinical samples if tissue culture facilities are available, and examination for promastigotes growing in culture. The growing of promastigotes in culture can facilitate species identification through application of other molecular or immunological tools. However, these can be burdensome, tiresome, capital intensive and time-consuming and require specialised facilities and individual expertise, frequently absent in endemic areas. Nonetheless, the need for diagnosis is crucial, which emphasizes the need for facilities and trained

personnel to be available with expertise in methods of diagnosis and parasite identification.

*Leishmania* species are morphologically very similar, and require alternative identification methods to tell them apart. The microscopic observation of parasites in Giemsa stained tissue smears, and/or culture of promastigotes from tissue (Bensoussan et al., 2006; Magill, 2005; Vega-Lopez, 2003; Marfurt *et al*., 2003) are not ideal diagnostic tools, lacking in both sensitivity and specificity. In epidemiological investigations dissection of vectors followed by microscopy to detect the parasites has been used. Such methods demonstrate the availability of the parasite but do not reveal the parasite identity to the species level. Electron microscopy can reveal the ultrastructure of the parasite, though it would not disclose the identity of the parasite. It can be applied in either the vector or mammalian hosts, and can yield useful information about the biology of the parasite. For instance, scanning electron microscopy of vectors infected with *Leishmania major* and *L. mexicana* revealed a dense matrix surrounding these parasites (Stierhof *et al*., 1999), which was later characterised as PSG.

In vitro culture techniques to isolate the parasite is a useful tool, on the other hand, it requires a complicated laboratory setup, is time-consuming, and has a risk of contamination (Marfurt *et al*., 2003; Berman, 1997; Bensoussan *et al*., 2006). Such methods alone cannot identify specific species responsible for clinical manifestations. In recent times, molecular techniques for identification have been demonstrated to be more sensitive than biological methods for the detection of *Leishmania* parasites and can be used to identify parasites to the species level (Vergel *et al*., 2006). It is important to properly identify a particular species of *Leishmania* responsible for a particular form of infection to achieve effective species-directed treatment (Showler

and Boggild, 2015). Different molecular methods can be used to detect and identify *Leishmania*, both at the species and complex levels (Noyes *et al*., 1998). PCR has been for some time now the preferred choice amongst identification methods due to advantages such as being rapid, highly sensitive, specific and more versatile (Belli *et al*., 1998). When the appropriate target is amplified by PCR, the product can later be sequenced and the results analysed phylogenetically.

Such phylogenetic analysis has revealed the relationships among *Leishmania* species with regards to the differences in the natural history of their vertebrate hosts, vector specificity, clinical manifestations and geographical distribution (Boite *et al*., 2012). Controversies like disputed origins, relationships among the species, and the similar clinical presentations and have often been solved by phylogenetic analysis (Marcili *et al*., 2014). These analyses have helped to place the species into their taxa, though taxonomy disputes keep emerging in the genus *Leishmania*. For instance, the taxonomic status of *L. hertigi*, *L. equatoriensis* and *L. enriettii* have previously been questioned (Momen and Cupolillo, 2000), until recently when some answers have been provided by phylogenetic analysis (Marcili *et al*., 2014). Moreover, *L. hertigi* and *L. equatoriensis* have by molecular methodologies been placed in a closely related genus, however, phylogenetic investigations have put the two different species together, but different from true *Leishmania* (Cupolillo *et al*., 2000). Thus, evolutionary relations among the genus can be represented by phylogenetic trees, with each branch representing lineage-connected species. This is due to the fact that, as evolution advances, certain species become altered over time and can undergo speciation into separate branches, which either persist or undergoes extinction. Molecular data such as DNA sequences and/or protein have been used reconstruct phylogenies by inferring the evolutionary relationships among present-day species.

This relies on the assumption that closely related species would usually have high levels of sequence conservation, contrary to more distantly related species and genera, which would exhibit more divergence. Among the trypanosomatids, the sequences of a suitable target gene can discriminate between *Leishmania* species (Marcili *et al*., 2014).

### 1.5 Treatment of leishmaniasis

The identification of the various species of the *Leishmania* parasite is important to enable appropriate treatment, with treatment guidelines shifting towards a species-based approach, enabled by molecular diagnostics. However, in addition to species-directed therapeutics, several other factors should be considered including: the risk of mucocutaneous spread (following CL), the risk of PKDL (following VL), the extent and location of lesions (CL), host immune status, treatment toxicity, patient preference, and reliability of follow-up (Showler and Boggild, 2015). Various drugs are available to treat leishmaniasis (**Table 1.3**).

| **Class of Drug** | **Mechanism of action** |
|---|---|
| **Antimonials**<br>Sodium Stibogluconate | Not really clear; assumed to inhibit Sulph-hydryl (SH) dependent enzymes. |
| **Diamidine**<br>Pentamidine | Interaction with kinetoplast DNA, inhibition of topoisomerase II, interference with aerobic glycolysis. |
| **Antifungals**<br>Amphotericin B<br><br><br><br>Ketoconazole | Binding with ergosterol of cell membrane of the parasites to form micopores leading to cellular content leakage.<br>Inhibiting the conversion of lanosterol to ergosterol; impairment of membrane function. |
| **Others**<br>Miltefosine<br>Paromomycin<br>Allopurinol | Triggers apoptosis (programmed cell death).<br>Acts on ribosomes, leads to protein synthesis inhibition.<br>Inhibits cell growth (prototype of pyrazolopyrimidine). |

**Table 1.3** Some classes of anti-leishmanial drugs and their mode of action. (Mohapatra, 2014, Department of Microbiology, Vardhaman Mahavir Medical College and Safdarjung Hospital, New Delhi, India)

A good treatment response normally takes between 4-6 weeks, although a follow-up until at least a year or more is desirable to monitor any relapse which could lead to MCL or PKDL, depending on the causative species involved (Blum *et al*., 2014; Murray, 2012). Most of the treatment regimens involving these synthetic commercial drugs, most of which have been on the market for more than 5 decades, have demonstrated various cure rates and adverse effects.

For CL cases, paromomycin cream is topically applied to the local area of infection, to promote wound care and/or healing and prevention of secondary infection. Alternative therapies for CL are cryotherapy, thermotherapy and intralesional antimonials (ILSb) (Showler and Boggild, 2015). Topical application normally excludes sensitive areas like the genitalia, eye lids, and lips. Different responses to varying lesions caused by diverse species have been achieved in CL cases. Between 20-100% wound healing with re-epithelialisation is mostly achieved within 45 days to 1 year with CL resulting from *L*. *major*, whereas *L*. *tropica* lesions take up to 3 years to achieve 100% healing (Salah *et al*., 1995; Bailey and Lockwood, 2007). For *L*. *mexicana*, cure rates of 88% have been achieved within 3 months of treatment, and even for relapse, 68% cure is achieved with 6 months of treatment (Herwaldt *et al*., 1992; Soto *et al*., 2013). The combined treatment of cryotherapy-intralesional antimonials produced between 89-91% cure rate in Old World lesions, as well as 70-80% cure rate in New World lesions, and was significantly better than monotherapy (Showler and Boggild, 2015; Soto *et al*., 2013). Paromomycin in combination with other compounds has achieved greater cure rates in CL from the Old World (Kim *et al*., 2009). For example, 15% paromomycin-0.5% gentamicin in an

ointment formulation (The Walter-Reed formulation WR 279,396 - not commercially available yet) cured between 80-94% of ulcers resulting from *L. major* infection (Salah *et al*., 2009; Showler and Boggild, 2015). Topical application of 15% paromomycin-12% MBCL (methylbenzethonium chloride) and 15% paromomycin-0.5% gentamicin, to ulcers caused by *L. braziliensis*, *L. mexicana* and *L. panamensis* produced estimated cure rates of 79-91.4% in South America (Krause and Kroeger, 1994; Arana *et al*., 2001). Due to temperature sensitivity of the amastigote forms, thermotherapy in the form of radiofrequency heat therapy (RFHT), administered locally, has achieved between 70-98% cure rates in both Old and New World lesions, over a short application period without any recorded relapses (Reithinger *et al*., 2005; Safi *et al*., 2012; Velasco-Castrejon *et al*., 1997; Valencia *et al*., 2013). The immunomodulator imiquimod in combination with pentavalent antimonials, has been used to achieve fast wound healing and reduced scars in CL resulting from New World species (Arevalo *et al*., 2007; Miranda-Verastegui *et al*., 2005).

In the cases where local treatment has failed or there is mucosal or visceral involvement, systemic treatment is recommended, with all their attendant adverse effects. The parenteral administration of sodium stibogluconate (SSG) among the pentavalent antimonials, has since in the 1940s been used as first-line treatment for VL (Kala-azar), despite its adverse effects in the patients (Vikrant *et al*., 2015). Miltefosine has been used to treat various types of leishmaniasis including MCL and VL, and is comparable to pentavalent antimonials in the level of efficacy and tolerance. It has been found efficacious for CL in both Old and New Worlds with cure rates of between 49-94% in patients including Dutch military (Soto *et al*., 2001, 2004; van Thiel *et al*., 2010). There are a number of liposomal amphotericin B (L-AMB or Ambisome) formulations on the market which have demonstrated effectiveness and

have been approved as a first-line drug for treating VL, with a high cure rate following intravenous administration for 1-5 days depending on the dosage. L-AMB formulations have been used in the USA and Canada (Solomon *et al*., 2011; Wortmann *et al*., 2010; Solomon *et al*., 2013; Harms *et al*., 2011), and have been successful following failed antimonial treatment. Ambisome has been used to treat a number of patients diagnosed with a first episode of VL in India (Burza *et al*., 2014). Others patients with *L. braziliensis*, *L. mexicana*, and *L. major* infections have been successfully treated with ketoconazoles and fulconazoles (Emad *et al*., 2011; Sousa *et al*., 2011). As a first-line drug for the treatment of *L. panamensis* and *L. guyanensis*, pentamidine has achieved a cure rate of 90% in an endemic population in certain parts of South America (Soto-Mancipe *et al*., 1993).

### 1.6 The sand fly vectors of leishmaniasis

The proven incriminated vectors of *Leishmania* are all female sand flies (Diptera: Psychodidae: Phlebotominae) of various species, and are regarded as definitive hosts. Female sand flies are small Diptera, approximate length of 2-3mm, silver grey to black in colour, whose wings characteristically fold into V-shape on resting (Kato *et al*., 2010) (**Figure 1.11**).

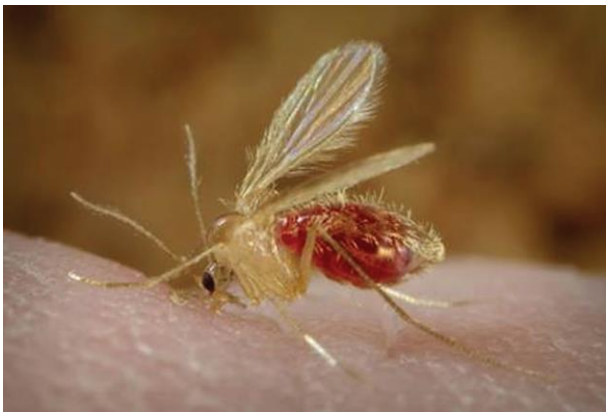


**Figure 1.11** Female sand fly taking a blood meal (Stebut, 2015).

Sand flies are silent and frail flyers, and display a characteristic hopping motion when on their hosts during feeding. They are mainly active during the night, and inhabit houses, caves and crevices in the day (Kato *et al*., 2010).

Approximately 900 species of sand fly have been described, and these are placed in five major genera in both the New and the Old world namely, *Phlebotomus* (94 species) and *Sergentomyia* (258 species) in the Old World, and *Lutzomyia* (379 species), *Brumptomyia* (23 species) and *Warileya* (5 species) in the New World. However, the proven vectors of *Leishmania* are approximately 70 species in two genera only, *Phlebotomus* and *Lutzomyia*, (Kato *et al*., 2010, Bates, 2007). each vector species is capable of supporting development of a specific species of *Leishmania* and transmitting them accordingly (Bates, 2007). In other words, the sand fly-*Leishmania* relationship is species-specific. The establishment and complete development of the parasite in the vector require various barriers to be overcome. The promastigote form of the parasite should be able to attach to the gut walls of the vector to prevent been expelled by gut movement, and be able to multiply, transform and differentiate into the infective form ready for transmission to mammalian host (Sacks, 2001; Killick-Kendrick, 1999). The promastigote factor PSG should be produced to facilitate transmission of the infective form of the promastigote (Rogers, 2012). These are crucial for completion of the developmental life cycle of the parasite, without which, the status of the incrimination of the sand fly as a vector is in doubt. These vectorial competencies have been demonstrated by a number of sand fly species, including *Phlebotomus papatasi* and *P. duboscqi*, which are the principal natural vectors transmitting *L. major*, *P. sergenti*, the vector of *L. tropica*, and *P. argentipes*, the vector of *L. donovani*, all in the Old World. In the New World incriminated vectors include *Lutzomyia longipalpis* and *Lu. verrucarum*, which transmit *L. infantum* and *L.*

*peruviana*, respectively (Kamhawi *et al*., 2004). Other medically important sand flies are shown in **Table 1.4**, including their geographical location and susceptible *Leishmania* species.

Additionally, there has been speculation on midges as vectors of *Leishmania* in Australia, in an area where the most common sand fly was *Sergentomyia queenslandi* vicinity (Dougall *et al*., 2011). None of these were found infected, whereas a high proportion of *Forcipmyia* midges contained kangaroo *Leishmania* parasites. This speculated new vector in Australia is in addition to the previously known 18 sand fly species, belonging to the genera *Phlebotomus* or *Sergentomyia,* which feed on small mammals and reptiles (Lewis and Dyce, 1982; Dougall *et al*., 2011).

When considering suspected vectors, it is necessary to identify the circulating sand fly or vector species in *Leishmania* endemic and surrounding areas for vector incrimination, risk predictions and disease expansion in future. Vector identification has been largely based on morphology, such as the structures of spermathecae, cibarium, pharynx, and terminal genitalia, thoracic pigmentation, antennae, and leg segments. This requires microscopy, a high degree of skill and expertise (Bauzer *et al*., 2007; Kato *et al*., 2010). However, simpler, fast, convenient, sensitive and highly specific molecular methods have been developed as data accumulates on the vectors, especially sand flies (Kato *et al*., 2005; 2007; 2008). These methods can potentially identify both the vector and infected vectors when combined with parasite detection, which can help with vector incrimination. These molecular methods require basic PCR, applied to various different target genes, depending on diversity of population.

| **Sand fly species** | **Geographical location** | **Susceptible *Leishmania* species** |
|---|---|---|

| | | |
|---|---|---|
| *P. guggisbergi* | Africa | *L. major* |
| *P. papatasi* | | |
| *P. duboscqi* | | |
| *P. longipes* | | *L. aethiopica* |
| *P. pedifer* | | |
| *P. orientalis* | | *L. donovani* |
| *P. martini* | | |
| *P. perniciosus* | | |
| *P. sergenti* | Asia | *L. tropica* |
| *P. papatasi* | | |
| *P. argentipes* | | *L. donovani* |
| *Lu. flaviscutellata* | Caribbean | *L. amazonensis* |
| *Lu. umbratilis* | | *L. guyanensis* |
| *Lu. whitmani* | | |
| *Lu. anduzei* | | |
| *Lu. ayrozai* | | *L. naiffi* |
| *Lu. paraensis* | | |
| *P. ariasi* | Europe | *L. donovani* |
| | | *L. infantum* |
| *P. perniciosus* | | *L. gerbilli* |
| *P. papatasi* | Middle East | *L. major* |
| | | *L. Arabica* |
| *Lu. anthophora* | North America | *L. mexicana* |
| *Lu. longipalpis* | South America | *L. infantum* |
| *Lu. evansi* | | |
| *Lu. olmeca* | | *L. mexicana* |
| *Lu. faviscutellata* | | *L. amazonensis* |
| | | *L. pifanoi* |
| *Lu. wellcomei* | | *L. braziliensis* |
| *Lu. ubiquitalis* | | *L. lainsoni* |
| *Lu. trapidoi* | | *L. panamensis* |
| *Lu. ylephiletor* | | |
| *Lu. gomezi* | | |
| *Lu. panamensis* | | |
| *Lu. verrucanum* | | *L. peruviana* |
| *Lu. peruensis* | | |
| *Lu. ayacuchensis* | | |
| *Lu. whimani* | | *L. shawi* |

**Table 1.4** Geographical location of some medically important sand flies and their susceptible *Leishmania* species (Ashford, 2000; Rogers, 2012).

The potential vectors in a new disease endemic area can sometimes be predicted, since sand fly evolution has partly been linked with vector-parasite

coevolution in natural habitats (Ready, 2013). These techniques require detection of DNA of the parasite in their vectors, however, the amplification of DNA in the vector alone is not conclusive of vector incrimination (Seblova *et al*., 2014). The dissection of the field vectors and finding the parasite is not conclusive either, unless there is compelling evidence of the development of the parasite to the infective metacyclic promastigote form.

To supplement the identification of the vectors in and around the endemic area as well as detecting the parasite in the vectors, there are other considerations for vector incrimination as set out by Ready (2013). It is necessary to isolate and/or type promastigotes from several wild female flies containing digested blood meals (the timing of the blood meal digestion is dependent on the vector(s) in question), and demonstrate the location of the infective forms in the stomodeal valve and/or midgut in the field or colony flies. Moreover, the suspected flies in the endemic focus should be attracted to and be able to bite both human and reservoir hosts, with all the disease players experiencing common seasonality and ecology in the focus. The experimental transmission and infection should successfully be achieved from a natural host species or equivalent laboratory model. Additionally, mathematical modelling should demonstrate that reduced biting densities of the vector will lead to declined disease incidence. Fulfilling these conditions among others would amount to vector incrimination. However, not all vectors which have been implicated have fully satisfied these conditions. For instance, *Lu*. *wellcomei* and *Lu*. *complexa* are closely related, and *Lu*. *complexa* occurs abundantly in a CL focus, nonetheless, the former is considered the more important vector of *L*. *braziliensis* than the latter (Ready *et al*., 1991). The subgenus *Sergentomyia* is not considered to be a human vector, notwithstanding their ability to feed on human blood, in addition to apparently found

to have been infected with *L. major* in some parts of Africa and Middle-East (Mutinga *et al*., 1994; Parvizi and Ready, 2008).

In the northern part of Africa, an important vector is *Phlebotomus papatasi*, and *P. sergenti* has also been incriminated in some transmission sites (El-Buni *et al.*, 2000). But the situation can be complex, for example in Tunisia, where various different sand flies species have been implicated in the foci, as a result of diverse vegetation and the widespread distribution of *Leishmania* parasites. Sand fly species such as *P. perfiliewi*, *P. perniciosus*, *P. longicuspis*, and *P. alexandri* have been implicated in addition to *P. papatasi* and *P. sergenti* (Ghrab *et al*., 2006).

### 1.7 Emerging foci of the disease outside Africa

In the last two decades it has become apparent that leishmaniasis has expanded its range, in both tropical and subtropical regions, with infections being seen in new regions across the globe. This has involved both known species and the emergence of new species (Dedet *et al*., 1995; Desbois *et al*., 2014). Some of these are closely related species appearing in different locations around the world (**Figure 1.12**).

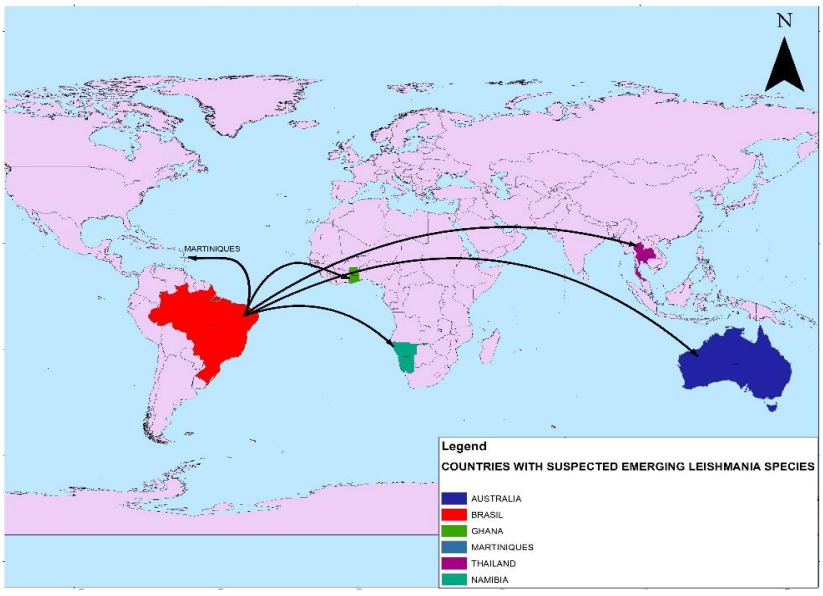


**Figure 1.12** Map showing new foci of leishmaniasis (Benedict Nartey, Geography Department, University of Cape Coast, Ghana).

### 1.7.1 Leishmaniasis in Australia

Australia and Oceania have long been considered leishmaniasis free zones (Handman, 2001; Herwaldt, 1999), the closest occurrence being in East Timor, which reported their first case about a decade and a half ago (Chevalier *et al*., 2000). In Australia the reported clinical form of the disease is CL, and to date autochthonous cases have only been found in animals. The first indigenous and locally acquired CL cases were in red kangaroos appearing as chronic and self-limiting cutaneous lesions found mostly on tails and ears (Rose *et al*., 2004). The mammalian forms of the parasite were demonstrated in H&E (Hematoxylin and Eosin) stained samples from these body parts confirming the presence of amastigotes, and parasites were isolated and used to establish promastigote cultures. The cutaneous lesions were similar to those produced by known agents of CL, starting as papules, progressing to raised edges and ulceration, and characteristically became more severe during the tropical wet season. DNA sequence analysis of a conserved locus found between 98 – 99.3% identity with *Leishmania* reference isolates, but 100% identity with none. Analysis of further variable genetic loci revealed sequence of the isolate which were strikingly different from the reference *Leishmania* isolates, whose percentage identity ranged from 50 – 70%, which suggests the presence of a novel *Leishmania* species.

In addition to the animal CL cases reported in Australia, there have been reports of human CL, however, all these appear to be imported and not indigenous cases. All the human leishmaniasis cases in Australia to date appear to be examples of the infections acquired in the Middle East or Central America (Storer and Wayte, 2005; Davies *et al*., 2003; Choi and Lerner, 2001; Maguire *et al*., 1998). For instance, four Afghani refugees presented at a clinic in Australia with lesions whose biopsy samples

proved positive for *Leishmnia* (Storer and Wayte, 2005). They were patients between the ages of 2-15 years, some of which were siblings, and who had the disease between 5 months to 1 year. The lesions were from one to more on each patient, located on the lower limbs, upper limb and the trunk. In one of the cases, there was evolution of a small plaque that recurred at the edge of a previously treated and healed region on the limb, characteristic of Old World *L. tropica* infection (Davies *et al*., 2003), which responded to treatment. All the lesions responded very well to treatment with much reduced scarring under different treatments such as ketoconazole (topical, oral and intradermal injection), cryotherapy and sodium stibogluconate (intra-lesion injection) (Storer and Wayte, 2005). CL cases are sometimes not treated due to their self-limiting nature, however, treatment is warranted depending on the immune response of the host (immuno -competent or -compromised), site of infection (mouth, ear and neck areas), multiple lesions (more lesions on affected part) and the tendency for dissemination (mucosal spread) (Herwaldt, 1999; Storer and Wayte, 2005; Davies *et al*., 2003; Choi and Lerner, 2001; Stark *et al*., 2008). In another example, a patient imported *L. braziliensis* CL to Australia (Konecny and Stark, 2007). PCR diagnosis on a skin biopsy was negative, however, when the promastigotes were isolated following culture from a similar biopsy and were analysed using PCR-RFLP, diagnosis and identification of the species was made. Increasing cases of leishmaniasis have been reported in Australia on account of travellers returning from endemic areas (Schwartz *et al*., 2006; Ju *et al*., 2004; Maguire *et al*., 1998).

The identification of additional species in imported leishmaniasis has continued in Australia. Twenty (20) leishmaniasis cases involving 17 CL, 2 VL and 1 PKDL have been reported recently (Stark *et al*., 2008). Of the 20 cases, 11 were migrants and 11 were travellers for different reasons, who in one way or the other migrated or

visited disease endemic areas such as Afghanistan, Pakistan, South America, Greece and Africa, whose ages ranged between 8 to 59 years, in male to female ratio of 17:3. Various *Leishmania* species were involved, 13 isolates from the Old World and 7 from the New World, in the following breakdown: *L. tropica* (*n* = 7), *L. braziliensis* (*n* = 5), *L. major* (*n* = 3), *L. infantum* (*n* = 2), *L. mexicana* (*n* = 2), and *L. donovani* (*n* = 1). These infections raise the possibility of local transmission should there be suitable vectors susceptible to these *Leishmania*. The species were identified by parasite isolation into culture followed by molecular diagnostic methods. All the patients were successfully treated with liposomal Amphotericin B (Stark *et al*., 2008).

These cases of imported leishmaniasis (Davies *et al*., 2003; Choi and Lerner, 2001) have been introduced into a country where biting midges have been recently implicated as a vector (Dougall *et al*., 2011). It indicates that an influx of refugees, international tourism and military interventions, to and/or from endemic foci, can impact on the spread of the disease. Midges or other suitable vectors could help the disease to become established in Australia, and reservations have already been expressed about the reported leishmaniasis cases (Rodriguez-Morales *et al*., 2009).

Many forms of leishmaniasis are zoonotic, so potential reservoirs could influence the disease epidemiology in Australia, and leishmaniasis infections in any form should be considered with almost concern. In Brazil, canine leishmaniasis is the main reservoir of human visceral leishmaniasis (Gramiccia and Gradoni, 2005), and has been reported in Australia, with both diagnosed cases of visceral and cutaneous forms (Cleare *et al*., 2014). A case of dog leishmaniasis infected with *L. infantum* has been diagnosed 5 years after it had been imported into Australia, and prior to quarantine at the Australian territory had previously lived in UK and travelled back and forth between Portugal and UK (Best *et al*., 2014). Leishmaniasis infections in

dogs could pose risks in Australia, since the incubation period of the infection can last for 7 years, and become subclinical infections showing seropositivity between 29.4-66% (Miro *et al*., 2008; Mettler *et al*., 2005; Trotz-Williams and Gradoni, 2003). Even assuming there are no vectors for *L. infantum* transmission, possible dog-to-dog transmission has been suggested (Schantz *et al*., 2005). All these factors will influence understanding of the risk of transmission, disease prevalence and monitoring in Australia.

### 1.7.2 Leishmaniasis in Thailand and the Caribbean

These two new leishmaniasis areas are considered together because of the unexpected similarity in their *Leishmania* species. In the past, cases of VL have been sporadically recorded in Thailand, first reported from patients who had visited an endemic area in the 1980s (Suttinont *et al*., 1987). However, more recently, cases started being reported in non-travelling patients both in the Northern and Southern parts of Thailand (Thisyakorn *et al*., 1999; Kongkaew *et al*., 2007). These particular regions had no previous reported cases or known vector(s) of the disease (Sukmee *et al*., 2008). The first reported case of indigenous leishmaniasis in Thailand, though diagnosed from clinical samples, however, was not identified to the species level (Bualert *et al*., 2012), until it was again reported in 2008 in a VL patient, and suggested to be a novel agent of *Leishmania*, later being named *L. siamensis* (Leelayoova *et al*., 2013). The first in vitro culture and characterisation of this novel species as *L. siamensis* came from a HIV patient suffering from both dermal and visceral leishmaniasis in 2010 (Bualert *et al*., 2012). Recently, veterinarians in Florida, USA, have identified *Leishmania siamensis* in a horse (Leelayoova *et al*., 2013; Sukmee *et al*., 2008). A similar agent causing CL in domestic animals and livestock appears to be circulating in Europe, yet

to be reported in humans. In a case report in a cow, *L. siamensis* was implicated in CL in Switzerland, based on sequence analysis of diagnostic PCR products from the internal transcribed spacer 1 (ITS1) of ssrRNA gene (Lobsiger *et al*., 2010). To the previously identified parasites in animals such as dogs, cats and horses in Europe (Otranto *et al*., 2009; Koehler *et al*., 2002; Muller *et al*., 2009), CL in cattle is now added.

The emergence of *L. siamensis* as an etiological agent for both CL in animals and VL in humans is widespread (Reuss *et al*., 2012; Sukmee *et al*., 2008; Suankratay *et al*., 2010). The cutaneous lesions of the animals commonly appear on both upper and the lower extremities, typical of CL (Reuss *et al*., 2012.). Despite reports of animal CL in South America and in some parts of Europe implicating known and properly identified species, reports in some other European countries have implicated *L. siamensis* as the agent from the molecular analysis (Muller *et al*., 2009; Lobsiger *et al*., 2010). A horse in the USA with no travel history, had its lesion physically and microscopically examined after its sample was submitted for sequence analysis by ITS1 yielded 310bp and was found to have 99% identity with *L. siamensis*, responsible for VL in Thailand (Reuss *et al*., 2012). After generating a PCR product of 350bp by the amplification of ITS1, the subsequent sequence analysis showed 98% identity to four horses under study in Germany and Switzerland, and to the *Leishmania* parasites from Thai *L. siamensis* species, but yielded much less identity of between 63-73% to most known species from the Old World and New World responsible for CL (Muller *et al*., 2009).

The interesting scenario surrounding uniqueness of this new species is that it intriguingly caused both CL and VL of humans and animals. Isoenzyme and EM

analysis have uncovered a novel *Leishmania* species from a HIV patient from Martinique Island, thought to be an opportunistic infection due to the immunocompromised status of the victim (Dedet *et al*., 1995), and now becoming one of the new *Leishmania* species emerging in recent times. The confirmation that it could be a possible emerging *Leishmania* species came when the same species was isolated from immunocompetent CL patient from the same Martinique Island, thereby invalidating the perception that the previous isolation was an opportunistic species. These two independent isolations have been found to be the same based on the same genes (Noyes, *et al*., 2000). Previously, there was evidence of a similar species of *Leishmania* parasite from the island of Martinique in the 1950s, 1960s and 1970s, in CL patients, though not with robust molecular methods of identification but rather with the demonstration of the amastigotes in skin smear (Noyes *et al*., 2000), which is conclusive evidence of the presence of the parasite.

### 1.7.3 Leishmaniasis in Sri Lanka

Sri Lanka had been free from any form of leishmaniasis until recently when cases were reported in returnee workers from the Old World (Naotunne *et al*., 1990), after which the first autochthonous case of CL was reported in 1992 (Athukorale *et al*., 1992). In the decade thereafter, there were consistent reported cases going up to more than 400 cases in 2001, with doubts though on the vectors of CL in Sri Lanka (Siriwardena *et al*., 2003, 2007; Karunaweera *et al*., 2003), just around which time an emerging focus was found in Ghana (Fryauff *et al*., 2006). There has since been an increasing number of CL cases in the northern and southern part of Sri Lanka, with annual reported cases of approximately 1000 (Surendran *et al*., 2005; Ranawaka *et al*., 2010; Rajapaksa *et al*., 2007; Sandanayaka *et al*., 2014). Surprisingly, the Sri Lankan CL were caused by *L. donovani*, (Karunaweera *et al*., 2003) the same species as the

cause of visceral leishmaniasis in India, Nepal, Bangladesh and East Africa, with the vector in India and Sri Lanka foci reportedly being the same (Surendran *et al*., 2005). The CL cases in Sri Lanka were initially blamed on the influx of refugees from India to the island, but it was surprising that the Sri Lankan leishmaniasis cases were all CL, none VL, which is the known leishmaniasis form in India and caused by *L. donovani*. Interestingly, the *L. donovani* causing CL in Sri Lanka were distinct from the *L. donovani* isolates from India and Bangladesh, which cluster with *L. donovani* and *L. infantum* from Europe but distant from *L. tropica* and *L. major*, responsible for CL cases in the Old World, although the Sri Lankan *L. donovani* were quite close to the isolates from India, Nepal and Bangladesh (Siriwardena *et al*., 2007). However, a single nucleotide substitution identified at position 976 in the 6-phospogluconate dehydrogenase gene of both Sri Lankan (*L. donovani* zymodeme MON-37) and Indian (*L. donovani* zymodeme MON-2) *L. donovani*, have put them apart (Ranasinghe *et al*., 2012; Karunaweera *et al*., 2003; Siriwardana *et al*., 2007). The location and the nature of the cutaneous lesions caused by Sri Lankan *L. donovani* has also raised some concerns. In the 86 (74.1%) CL cases confirmed to have been caused by *L. donovani* zymodeme MON-37, of the 116 lesions were mostly located on the upper extremities (90.7% lesions), with very few on the lower extremities (9.3% lesions), with lesion durations ranging from 2 weeks to 4 years (Nawaratna *et al*., 2007). Seventy (70) of these patients (81.4%) had single lesions, the typical presentation (Rajapaksa *et al*., 2007), with the remaining 16 (18.6%) having from 2-5 lesions. Of interest were characteristic dry (69 out of 86 – 80%) or wet (17 out of 86 – 19.8%) lesions produced by the *L. donovani*, which are features more usually associated with *L. tropica* (dry lesion) and *L. major* (wet lesion) in the same endemic focus (Shoaib *et al*., 2007; Mujtaba and Khalid, 1998). Probably the exclusive dry and wet lesions ascribed to *L.*

*tropica* and *L. major* need revision. However, the different types of lesion occurring in the same focus and supposedly caused by one species of *Leishmania* raise concerns. CL has been reported in 8 out of 9 provinces in Sri Lanka (Rajapaksa *et al*., 2007; Nawaratna *et al*., 2007) and uncertainty surrounds the attribution of the Sri Lankan CL on the refugee influx from India. However, since *L. donovani* is responsible for VL in other parts of the world, there could be a possibility of VL emerging as a significant problem in Sri Lanka, which will impact on CL treatment (Siriwardena *et al*., 2007). The first autochthonous VL case was recorded in the north central province in 2006 (Abeygunasekara *et al*., 2007), also caused by *L. donovani* zymodeme MON-37 (Ranasinghe *et al*., 2012; Siriwardana *et al*., 2007), in addition to 3 recent cases (Ranasinghe *et al*., 2011). On the other hand, CL from *L. donovani* in Sri Lanka could have some benefit, owing to the fact that self-resolving infections might confer some form of immunity. This might have contributed to the low number of reported cases of VL so far, by some form of leishmanization (McCall *et al*., 2013; Noazin *et al*., 2008).

There have also been few reports of MCL in Sri Lanka (Rajapaksa *et al*., 2005; Rathnayake *et al*., 2010; Karunaweera and Rajapaksa, 2009; Karunaweera, 2009). Most of the MCL cases have been linked with contact with jungle and among military stationed in the forest in the north of the country. It has been suggested that genetic variations among the Sri Lankan *L. donovani* might have resulted in different pathologies (Ranasinghe *et al*., 2011). These variable clinical forms within the same geographical focus with an unknown vector, presents an epidemiological challenge.

These increasing numbers of CL cases have necessitated interventions, which require identification and characterisation of the vector species as well as the pursuit of unknown reservoirs. On the reservoirs, diagnosis has been performed using microscopy of Giemsa stained smears and PCR, on samples prepared from dogs and

rodents collected from areas where infected persons have been confirmed. These methods, which have confirmed CL in humans over the years in Sri Lanka, could detect parasites in only 2 out of 151 dogs examined and in none of 47 rodents. Therefore, it is possible that anthroponotic transmission is occurring in this focus. However, the evidence that animals are not reservoir hosts is inconclusive so far (Nawaratna *et al*., 2009). Regarding vector control, possible insecticide-mediated interventions have received growing attention. However, this would require vector identification and characterisation to establish a successful vector control programme. *P*. *argentipes* was assumed to be a possible vector of Sri Lankan leishmaniasis, but *Sergentomyia* species have also been found in Sri Lanka (Surendran *et al*., 2005). Zoophagic and anthropophagic *P*. *argentipes* have been reported in certain parts of Sri Lanka. Within the *P*. *argentipes* complex, characteristic morphologies, such as the length of the sensilla chaetica, have been linked to VL transmission (Ilango, 2000). In India, where VL is endemic *P*. *argentipes* were found to have short sensilla chaetica, whereas the same flies from non-VL endemic areas have long sensilla chaetica (Ilango, 2000). One vector investigation confirmed the presence of the three members of the argentipes complex in Sri Lanka (*P*. *annandalei*, *P*. *argentipes*, *P*. *glaucus*), but their zoophagic or anthropophagic nature were not fully confirmed (Gajapathy *et al*., 2011). The identity of the vector of CL in Sri Lanka remains to be established.

### 1.7.4 Leishmaniasis in Europe

Leishmaniasis has been long established in the Mediterranean region of Europe (**Figure 1.13**). Histological evidence has shown that leishmaniasis has been present in Europe at least since the 14$^{th}$ century, following identification of *L*. *infantum* in an Italian named Eleonora from Toledo, from her bones by molecular and serological methods (Nerlich *et al*., 2012). However, new foci of leishmaniasis, especially of CL

have gradually been reported in previously non-endemic areas of Europe (Rhajaoui *et al*., 2007; Antoniou *et al*., 2008). Several reports of leishmaniasis both in animals and humans have been described in locations north of the Mediterranean basin in recent times, including from Germany, France and Italy (Faber *et al*., 2012; Naucke *et al*., 2008; Pratlong *et al*., 2004).


**Figure 1.13** Leishmaniasis mapping in Europe (http://www.crouchvalevets.co.uk/).

In France, new cases of human and animal VL and CL due to *L. infantum* have been reported lately, since it was first reported in the 1920s, with dogs regarded as the reservoir host (Lachaud *et al*., 2013; Marty *et al*., 2007). There was a reported case of visceral leishmaniasis in a 5-year-old child from Vosges, a region of France bordering Germany, as far back as 1931, after which sporadic autochthonous cases of both dog and human visceral leishmaniasis were recorded in Paris and several parts of northern France (Bogdan *et al*., 2001). Similarly, zoonotic cases of VL and CL due to *L. infantum* have also been reported in northern Italy since the 1970s to date, even in immunocompetent individuals, the spread being attributed to climatic changes (Desjeux, 1991; Maroli, *et al*., 2008). The cutaneous form of leishmaniasis due to *L. infantum* was reported from Switzerland, in a 20-year-old male who had never

travelled to any disease endemic area, except to other European countries including Spain (Bogdan *et al*., 2001). There has also been a report of an elderly man who travelled from Netherlands to France for holiday and who was diagnosed later with cutaneous leishmaniasis thought to be acquired while in Jura (Faber *et al*., 2012). Two cases of autochthonous kala-azar were reported in Austria, the first in a 36-year-old woman, and the second in a 3-year-old girl born to a Nigerian father and Ghanaian mother whose parents had lived in Austria for 15 years and never travelled to any known endemic area during the time of the infection. Both diagnoses were confirmed by serological tests and parasite isolation into culture (Kollaritsch, *et al*., 1989).

Portugal has been known endemic for leishmaniasis with both VL and CL in both animals and humans some seven decades ago, due mainly to *L. infantum*. A prevalence of 6.31% was found in 18 districts of mainland Portugal when a survey was conducted on some 4000 dogs infected in 2009 (Cortes *et al*., 2012). Infection has also been reported in domestic cats with a seroprevalence of 2.8% (Cardoso *et al*., 2010). Human infections are possible due to the close proximity of cats, dogs and humans. There appears to have been an increase in adult human cases, especially in immunocompromised individuals, in three main endemic foci from north to the south with one regional focus recording as many as 8.3 human cases/100,000/year (Campino and Maia, 2010).

The disease in Spain has been expressed as hypoendemic, with a hospitalization rate of 0.41 cases per 100,000 inhabitants, a total of 2,028 over a 12 year period to 2008 (Gil-Prieto *et al*., 2011). These hospitalized VL cases showed a case fatality rate of 3.3%, and about 1/3 of them were HIV-positive. Additionally, a high prevalence of >10% canine leishmaniasis has been recorded in different parts of Spain, which indicates a high infection risk to humans (Alvar *et al*., 2004). A significant number of

*Leishmania*-HIV co-infected cases have previously been recorded in 1994 in adult males of ages 25-35 years, of which 74% were found to be intravenous drug users, but gradually decreased (Alvar *et al*., 1997, 2008; Lopez-Velez *et al*., 2001; Cruz *et al*., 2002). These make the risk of transmission of the infections to other EU countries significant, since Spain is one of the most highly visited tourist destinations (Cruz *et al*., 2002). Although rarer, a substantial number of CL cases have been reported in Spain, representing 2% of all leishmaniasis cases (Alvar *et al*., 2008).

Leishmaniasis cases have also been recorded in Germany. Following establishment of monitoring centres for leishmaniasis in 2000, a total of 70 cases were reported, 43 cutaneous or mucocutaneous/mucosal and 27 visceral, all of which were acquired elsewhere, the largest group being infections in tourists who had visited the Mediterranean region (Harms *et al*., 2003). Until recently, there were no true autochthonous human cases of leishmaniasis reported in Germany, however, a case was reported of a 15-month old child who had never visited an endemic focus, diagnosed with visceral leishmaniasis, and for whom non-sand fly routes of infections such as congenital transmission or blood transfusion were ruled out. The patient had only visited southern Germany for a 3-week vacation two months before the onset of illness, though the site is frequently visited by tourists returning from Mediterranean countries (Bogdan *et al*., 2001), by which it could speculate on the possible location for the contraction of the infection. This indicates the possibility of indigenous transmission of the disease in Germany. Animal *Leishmania* infection has also been observed in Germany, with the demonstration of the parasite in a horse which never travelled to any endemic area in 2000 (Koehler *et al*., 2002).

Neither the vectors of leishmaniasis or the disease have become established in the Netherlands (Teske *et al*., 2002; Slappendel, 1988). However, an exceptional

autochthonous transmission of VL was recorded in 1997 in the Netherlands. This infection was observed in 12-month old dog, but the bitch that delivered the dog was supposedly infected in Spain and brought to the Netherlands well before the dog was identified as having visceral leishmaniasis, even though placental transmission was ruled out (Diaz-Espineira and Slappendel, 1997). Interestingly, similar sporadic autochthonous foci of VL in dogs have been reported in vector-free areas of Ohio and Oklahoma (Swenson *et al*., 1988), as well as in some sand fly-free European countries including England (Harris, 1994). Moreover, VL has been demonstrated in a Dutch dog that had never travelled to a leishmaniasis endemic area except a short stay in a non-endemic area in France, although the owners travelled frequently without the dog to an endemic area in Spain (Slappendel, 1988). This infection was attributed to the possibility of sand flies imported in vehicles, other objects or by mechanical dog to dog contact as well as non-conventional means such as via brown ticks, which have previously been implicated (Bravo *et al*., 1993). In many occasions the owners of the dogs have never gone out of the country during all the period they possessed the dogs. This suggests that vertical transmission in dogs via the placenta, acknowledged in humans, though not in dogs, might have occurred in Europe (Slappendel, 1988).

In relation to the geographical mapping (**Figure 1.13**), a new autochthonous pattern of leishmaniasis, emergence of new species coupled with tourism, travelling, and returnees from military deployment have contributed to the widened geographic distribution of the disease being reported in areas in which it was previously non-endemic. This requires health professionals in both endemic and non-endemic areas to be vigilant to the possibility of the disease, especially the visceral form, in individuals presenting with pyrexia of unknown origin and hepatosplenomegaly. Likewise, advice and reference centres could be established to monitor the frequency and origin of the

disease as well as to advise physicians, and to share and improve travel health information for travellers to and from disease-endemic areas.

Leishmaniasis infections in animals beside that of humans are of grave concern due to close associations between some of these animals and humans. The observation of known and new species of *Leishmania* in *Leishmania* naive and non-endemic areas and the resulting changing transmission dynamics of leishmaniasis could be influenced by possible unknown risk factors (Gonzalez *et al*., 2010). It is therefore vital to explore the knowledge gaps in the understanding of the life cycle of the parasite in both vertebrate and invertebrate hosts.

The vector sand flies are widespread between latitudes 50$^{o}$N and 40$^{o}$S, and complete their life cycle in an area less than 1500m from their breeding places with a lifespan of about two weeks (Slappendel, 1988). There has been a high prevalence of *P. perniciosus* reported in Switzerland, which are proven vectors of *L. infantum* (Knechtli and Jenni, 1999). *Phlebotomus mascittii* was reported to have been found in different places in Germany and close to the borders of France and Switzerland, however, its vectorial role of transmitting *Leishmania* species remains to be demonstrated (Naucke *et al*., 2000). With the emergence of both imported and autochthonous cases being observed in Europe and the presence of the vectors, even though the vectorial role of some are yet to be determined, they could serve as vectors for the parasite in most countries in Europe.

### 1.8 Leishmaniasis in Africa

In the northern part of Africa, various forms of the infection have been reported in many countries, caused by the known characterised species of *Leishmania*. Cutaneous and visceral leishmaniasis have both been reported from certain parts of Morocco, and which are causing a possible epidemic in a naive population in certain foci where the

disease has not been previously demonstrated (Ben Salah *et al*., 1995). In most endemic areas of Morocco there have been reports of both human and canine VL caused by different species of *Leishmania* parasite (Rhajaoui *et al*., 2007).

Visceral leishmaniasis cases have been reported from all parts of Libya, for close to a century, with children as the predominant victims. The high prevalence has been ascribed to massive infrastructural and environmental changes that have taken place over these years (Kimutai *et al*., 2009). Cutaneous leishmaniasis cases have also been widespread in the country (El-Buni *et al*., 2000), with several cases being reported on the Libyan-Tunisian border (El-Buni *et al*., 2000). The disease in Tunisia, on the other hand, have mainly been different forms of CL in which *L. tropica* and *L. infantum* have chiefly been implicated. Non-mutilating and non-disfiguring treatment-responsive mucocutaneous leishmaniasis due to *L. major*, have been reported in Tunisia. Three epidemio-clinical forms, cutaneous, zoonotic cutaneous and chronic cutaneous leishmaniasis caused by *L. infantum*, *L. major*, *L. tropica*, respectively, have sporadically been identified across the entire expanse of Tunisia (Kharfi *et al*., 2003).

Over one hundred (100) strains of the *Leishmania* have been identified in Algeria, with both cutaneous and visceral forms reported since 1965. The prevalence of the visceral form stands at 5 cases in 100,000 with a death rate of 6%, with 1,121 and 2000 cases of VL and CL, respectively recorded at one point (Harrat *et al*., 1996). A very high frequency of about 37% of dogs with infections has been recorded in the big cities in Algeria (Harrat and Belkaid, 2003). In Egypt, a low prevalence of VL and CL forms of the infection have been documented, mostly in rural settings, and very few cases have been reported from big cities. In one survey, as low as 16 cases of

leishmaniasis were identified out of 100 suspected patients enrolled, the rest have mostly been imported from Sudan (Kimutai *et al*., 2009).

Sudan has been proposed as the origin of VL, especially in the north of the country with consistently high annual incidence of thousands of reported cases (Zink *et al*., 2006). The spread of the disease between the northern and the southern parts have been attributed to the shuttling of nomadic tribes, causing outbreaks in the non-immune populations (Zijlstra and el-Hassan, 2001). Reported sporadic cases of autochthonous CL have been followed by three outbreaks with high prevalence involving all age groups in Darfur, Kordofan and the capital Khartoum (el-Hassan and Zijlstra, 2001). Medecins Sans Frontieres (MSF), which have been providing humanitarian health services in Sudan, have treated 51,000 cases of primary VL, relapsed VL, and post-kala-azar dermal leishmaniasis (PKDL) in 2000, achieving 90.8% cure rate in the treated patients (Ritmeijer and Davidson, 2003).

VL, CL and PKDL have been recognized both in humans and animals in many parts of Kenya since the start of the century. Turkana, Baringo, Kitui, West Pokot, Machakos, Mwingi, Meru, Wajir, Mandera, Keiyo and Marakwet have all been considered endemic districts (Ngure *et al*., 2009). In one major outbreak, cases rose from 300 to over 2,000 in a year, including mixed infection of human cases with *L. donovani* and *L. major* in a dual focus of VL and CL (Tonui, 2006; Ngure *et al*., 2009). Some of the sporadic outbreaks in Kenya have been blamed on returnee soldiers from military assignment and movement of nomads from the north of Africa (Ryan *et al*., 2006). Cutaneous and diffuse cutaneous leishmaniasis (DCL) have been recorded in the country, caused by agents such as *L. major, L. aethiopica and L. tropica* with *Phlebotomous duboscqi*, *P. guggisbergi*, *P. pedifer* implicated as possible vectors (Jacobson, 2003; Ryan *et al*., 2006).

Leishmaniasis-HIV co-infection, especially VL-HIV co-infection, in Ethiopia, has been blamed on extensive agricultural development and migration of people to seek jobs in the big cities (Berman, 2006; Berhe *et al*., 2001). The annual incidence of VL in Ethiopia stands at 1000 to 2000, among which approximately 40% are co-infected with HIV, in addition to a complex non-self-healing DCL and localized cutaneous leishmaniasis (LCL) due to *L. aethiopica*, *L. tropica* and *L. major* (Gadisa *et al*., 2007). A 14% incidence rate of PKDL has been confirmed in Ethiopia, with prevalence of 27% in HIV-positive individuals and 13% in HIV-negative individuals (Ritmeijer *et al*., 2001; Zijlstra *et al*., 2003).

Other countries in the east and the Horn of Africa have also contributed to the increasing cases of leishmaniasis, among which are Djibouti, Somalia and Eritrea. Despite none or low reported cases of cutaneous and mucocutaneous leishmaniasis, the coast of Somalia has reported some cases of visceral leishmaniasis in recent times (Ryan *et al*., 2006). Even though it lies within the leishmaniasis belt in the horn of Africa, Eritrea has not reported cases of the disease. Nonetheless, an internally solicited report by WHO has revealed the existence of VL and CL cases, of which 95% were within the last 5 years as at 2009, including leishmaniasis-HIV co-infection (Ghebrat, 2009 for WHO).

Each form of leishmaniasis was recorded a little over a decade and half ago in Uganda but, despite the huge reported cases across the eastern part of Africa, these seem to have been confined to one semi-arid lowland area in the country. This area has termite mounds, possible vector breeding and resting sites, and is regarded as a high risk area where people sit and take care of their livestock (Kolaczinski *et al*., 2007).

All forms of leishmaniasis have been reported in the West African sub-region since 1911 to date (Boakye *et al*., 2005), causing a lot of debilitating effects on its victims, who have little or no access to treatment. A high prevalence of both HIV and CL have been recorded, and in addition DCL masquerading as leprosy in the context of HIV co-infection was recently reported (Niamba *et al*., 2007). A survey conducted in 2000 confirmed 10 out of 74 CL patients were co-infected with HIV, after which there has been consistent HIV-*Leishmania* (CL) co-infection reported (Guiguemde *et al*., 2003). Since the first report in 1911, there have been cases of CL and VL on the border of West African with Algeria (Desjeux 1991). VL and CL have both been reported in Cameroon since the 1930s, mostly in the northern and eastern parts, though there has been under-reporting. Additionally, a recent survey confirmed 9 VL out of the 49 individuals who had symptoms suggestive of leishmaniasis (Kimutai *et al*., 2009). While MCL cases have hardly been reported frequently in any country in the west African sub-region, a few cases of cutaneous leishmaniasis with mucous membrane involvement have been recorded in Senegal (Boakye *et al*., 2005).

### 1.9 Leishmaniasis in Ghana

Leishmaniasis in Ghana had not been a subject of importance until recently when the first reported cases were recorded. It is not clear whether it is an emerging or re-emerging infection in the south-eastern part of the country, which shares a border with Togo where visceral leishmaniasis cases have previously been reported (Kimutai *et al*., 2009).

From 1999 to 2003, the number of recorded suspected cases of CL, the only form of leishmaniasis known in Ghana, has risen from 2,426 suspected cases to 6,450, mainly found in 116 villages (Fryauff *et al*., 2006). The number of cases has decreased from 105 in 2004, to 14 cases in 2005, with subsequent reported cases

remaining very low to zero between 2008 and 2009. However, this is probably not a true reflection of the CL situation, and more likely due to a decline in case searching activity and self-reporting, since an active search in 2012 could find cases in the endemic areas.

A report of *Leishmania*-HIV co-infection in a patient has been documented in Ghana in an entirely different location in the country outside the recent focus, the Volta region of Ghana. This case was a patient from New Longoro-Bamboi in the Brong Ahafo region of central Ghana (Lartey *et al*., 2006). This could be due to an undetected spread of the disease towards the north of the country. It was found in a 38-year-old HIV-positive female patient presenting at the Fevers Unit of the Korle Bu Teaching Hospital, Accra, Ghana, to be registered on the antiretroviral therapy. The histopathological analysis of punch biopsies from this patient revealed many free small cells, ovoid in shape, confirmed as *Leishmania* by Giemsa staining under the light microscope, among other cells of the body (Lartey *et al*., 2006). There is the likelihood of this patient harbouring a high number of *Leishmania* parasites, and such individuals could aid parasite transmission by acting as reservoirs, thus resulting in increased risk of future epidemics.

No reservoir has been implicated for CL infection in Ghana. Elsewhere in the sub-region of West Africa, many mammals have been found as reservoirs of all the *Leishmania* species responsible for the various forms of the disease. Among such mammals are the rodents *Mastomys erythroleucus, Tatera gambiana, Arvicanthis niloticus* and *Mastomys erythroleucus,* and dogs found as reservoir hosts in the Gambia (Boakye *et al*., 2006). The rodents *Psammomys obesus, Gerbillus gerbillus, Meriones libycus, M. shawi* and other *Meriones spp.* have been found to serve as

animal reservoir hosts of *L. major*, a possible agent for the infection in Ghana (Ghrab *et al*., 2006; Fryauff *et al*., 2006).

Like the reservoir, the vector of the disease in Ghana is still unknown, in spite of the effort put into recent vector studies. The dissection and microscopic examination of thousands of individual female sand flies captured during an epidemiological survey in the Ho District revealed no infections in the vectors (personal communication, Mba-Tihssommah Mosore). There is a suspicion that *P. duboscqi* is the primary vector but this species was the least abundant of 17 different sand fly species collected and constituted less than 0.5% of the total catch (Boakye *et al*., 2005; Fryauff *et al*., 2006). Another suspected potential vector, *P. rodhaini*, captured for the first time in Ghana, were of very low numbers of less than 0.5%, and whose vectorial role is questionable due to their seasonal abundance (Fryauff *et al*., 2006). In recent times, molecular analysis using minicircle primers have implicated *Sergentomyia* species as potential vector (unpublished data and personal communication, Mba-Tihssommah Mosore). However, the detection of DNA in a vector is not conclusive evidence of vector incrimination (Sadlova *et al*., 2013) though some have strongly argued for this (Slama *et al*., 2014), but others disagree (Seblova *et al*., 2014).

### 1.10 Rationale/Justification of the study

Cutaneous leishmaniasis (CL) is a potential cause of high levels of morbidity to many people, mainly in poor tropical and sub-tropical communities around the world where the disease is endemic. It has left many of its victims deformed and disfigured and stigmatised due to the dreadful scars they leave behind after healing. It has the capacity to slow economic development as a result of loss of man hours, and to slow down the academic performance of its victims of school going children (personal

communication from school attendance book). One of the agents of CL, *L. tropica*, has also been reported to cause visceral leishmaniasis (viscerotropic leishmaniasis - VTL), a fatal form of the disease elsewhere (Mebrahtu *et al*., 1989; Oren *et al*., 1991; Magill *et al*., 1993; Reed, 1996).

Without satisfactory and convenient diagnosis, the victims who mostly live in poverty, are left unattended and wrongly managed. Those affected could facilitate the transmission dynamics of the disease by serving as reservoirs hosts. To suitably manage the disease, factors influencing the transmission and affecting the frequencies and distributions should be clearly defined. These call for careful investigations, to make available data to serve as a platform for further studies. Moreover, accurate understanding of the role of the epidemiological factors would help in monitoring and intervention in the transmission of the disease. However, before any treatment intervention, the specific agents causing the disease should undoubtedly be identified, since erroneous treatment will have adverse consequences.

The cutaneous form of leishmaniasis appears to be an emerging/re-emerging disease in the Volta region of Ghana. The limited available epidemiological information indicates a relatively high prevalence amongst the affected populations with over 30% of schoolchildren showing evidence of infection in one study (Boakye *et al*., 2005). However, uncertainty still surrounds the identity of the species responsible for this disease focus. One study had reported *Leishmania major* but another reported an unknown species, although a limited sequence data was used in both cases for identification (Fryauff *et al*., 2006; Villinski *et al*., 2007).

An important constraint in the previous studies is that the species of the parasite have not been isolated and cultured to date, which is required for reliable identification and to perform detailed studies on the relationship of Ghanaian

*Leishmania* to other isolates/species. Further to establishing identity of the parasite, neither the vector nor possible reservoir hosts are known. Progress on all these fronts is required both to help reveal the extent of the problem and for the design and implementation of future control measures. Leishmaniasis is also an infection exacerbated by HIV infection and vice versa, so has wider public health implications beyond the parasitic infection alone, especially in countries like Ghana where there are reported HIV/AIDS cases.

Additionally, the control of leishmaniasis remains a serious problem given the fact that there are currently no vaccines and available antileishmanial treatment agents are toxic or expensive and frequently ineffective (Croft and Coombs 2003; Croft and Yardley 2002). Compounding an already difficult situation is the fact that an estimated 5-70 % of the patients in some endemic areas sometimes do not respond to standard antiparasitic drugs. Therefore, there is an urgent quest for novel, effective and safe treatments by the scientific community for this seemingly neglected disease (Pandey *et al*., 2004; Minodier and Parola, 2007). Furthermore, the availability of indigenous treatment intervention would greatly help the poor endemic communities who suffer from the disease. To this end, local herbal treatments would be convenient and affordable for the inhabitants living in such endemic communities.

These circumstances have therefore left several gaps yet to be explored, regarding the epidemiology of leishmaniases in Ghana. Specifically these are: the prevalence of the disease in the population of endemic focus; identifying new foci in the nearby nations bordering Ghana and in the country where some vectors have previously been collected, especially in the northern part of the country; the repertoire of specific species of the *Leishmania* parasite(s) causing CL in the current focus; vectors and the vectorial role of sand flies collected from the endemic focus and other

parts of the country; the nature of animal reservoirs; and natural and man-made factors that might have contributed to CL and its spread in the endemic focus. Advances on these would all provide very useful data on the disease in Ghana. These records would be indispensable in planning any programme of intervention against this emerging or re-emerging public health problem, would enable better appreciation of the risk of outbreaks, and help to limit the impact of the disease on the health of the population who have little or no access to health care.

### 1.11 Aims of the project

In a broader context this work seeks to explore cutaneous leishmaniasis in the Volta region of Ghana, which presumably is caused by a new species of *Leishmania* or a known species in a new epidemiological setting, and to assess the susceptibility of the parasites to compounds in indigenous plant(s) used by the local inhabitants in the endemic communities in Ghana. It would further explore other insects (e.g. biting midges) as potential vector(s) other than sand flies and a field-friendly diagnostic test.

#### 1.11.1 Specific objectives

1. To isolate *Leishmania* parasites from patients and establish in vitro cultures.

2. To prepare DNA from such cultures and apply molecular biology techniques to identify the parasite species responsible or confirm the presence of a new species.

3. To analyse the morphology and phylogenetic relationships of Ghanaian *Leishmania*.

4. To evaluate a simple field-friendly rapid diagnosis test of cutaneous leishmaniasis that might be suitable for use in the endemic community.

5. To examine the anti-*Leishmania* effects of extracts from *Cryptolepis sanguinolenta* on *Leishmania* parasites in culture.

6. To investigate other possible potential vectors apart from sand flies.

# Chapter Two

## 2.0 General Materials and Methods

### 2.1 Study Sites

The field sites for this study were in the Ho district and its environs (**Figure 2.1**). This district is found in the middle zone of the Volta region in the south-eastern part of Ghana. This is an atypical area for leishmaniasis in Africa, which is usually found in semi-arid regions in this continent. The Volta region is mainly secondary rain forest interspersed with subsistence farming, more similar to *Leishmania*-endemic regions of Central and South America than to typical African foci. It shares borders on the north, south and west with Hohoe, North-Tongu and Akatsi and Kpando districts, respectively. The Ho district borders on the east with Togo.

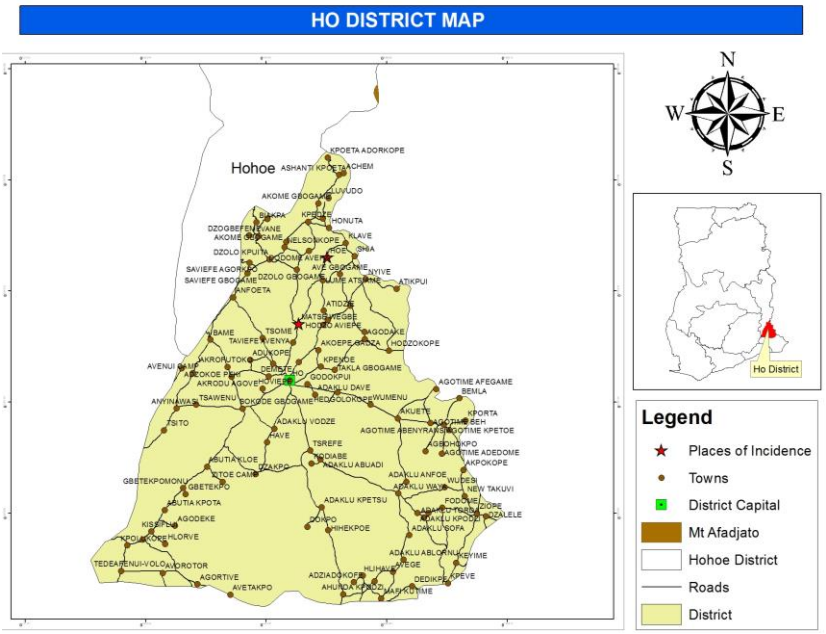


**Figure 2.1** Map of Ho district showing endemic area. (R. O. Amoako-Sakyi, Department of Geography and Regional planning, University of Cape Coast, Ghana).

The Ho district covers an area of about 2564 $km^2$. It has two main seasons, the wet and the dry seasons, normally raining from mid-May to November, and is generally dry in the remaining months. It has an estimated average annual rainfall between 1300-1400mm. The Ho district is relatively low lying, with an altitude of 150-200ft. The estimated population of the district stands at 235,331, of which 113,436 are made of males and the females constitute 121,895 (Ghana Statistical Service, 2002). The major to minor occupations of the inhabitants are farming, trading and pottery, woodcarving, kente weaving, and cattle rearing in that order. Most of the houses are constructed from mud, bricks and raffia. Other communities have their houses constructed from stone walls coated with soil mixed with straw, and thatch roofs. The search for the diseases as part of this study was specifically conducted in the communities in the Ho district, where there has recently been reported new incidence and old cases of leishmaniasis over the years.

The region is situated 3-4 hours drive from Accra, and 6 hours from Cape Coast, where laboratory work was performed. Therefore, a number of field visits were made. The materials needed for performing primary isolations and molecular identification of the parasite were taken to the field site, aspirations performed and the samples then brought back to the lab for culture and analysis.

### 2.2 Study participants

Patients seen with skin lesions on the body in the endemic communities were recruited into the study, after informed consent was received from the patients and/or their guardians. The study was assessed and approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board, Ghana, CPN 062/11-12, and all samples were taken by medical physicians. Patients without skin lesions and those

with lesions that did not resemble CL according to the clinical manifestation and after taking the history were excluded from the study. A total of 50 people were included in the study, although many more were interviewed.

### 2.3 Case Search

The case search was initially carried out using methods described as “passive” and “active” case search approaches adopted from Mosleh *et al*. (2008). The passive case search approach involved CL patients who sought treatment for skin lesions at the designated health facilities in the communities and were then possibly referred to the district or regional hospitals directly for care of skin lesions. This approach was discontinued since none of the patients reported to any of the health facilities. The active case search approach was the main search approach used. This search method was performed by visiting from house to house in the endemic area to identify CL cases who did not seek medical care. During these visits, clinical guides were used to identify the CL lesions and the detected cases were then sampled for laboratory investigations.

The endemic communities were visited upon reports of suspected leishmaniasis. The health centres were the first point of call, although none or very few of such centres were able to report of a case to be recruited. Community members were asked whether they knew of any case of the disease, which is referred to as “Agbamekanu” in their local dialect. This offered the investigators/research team the opportunity to go to the houses of the infected individuals. Chiefs were also called upon and their subordinates were detailed to search through the communities for possible cases in their territories. Though somewhat neglected by the community, district and regional health authorities, because the patients do not report to the

facilities, the community members reported a continuous incidence of cases of the disease every year.

In selecting a suspected CL case, most cutaneous lesions were of interest as far as this study was concerned, especially those with ulceration, raised edges or violaceous borders, ≥ 2mm in diameter and slightly painful (Davies *et al*., 1997; Herwaldt, 1999; Klaus and Frankenburg, 1999; Piscopo and Mallia, 2006). The descriptions of the start of lesion by the patients were also taken into account. Lesions on the exposed parts of the body, or compatible localized cutaneous lesions erupting primarily from exposed areas of skin or any part of the body that had endured for a week and more were considered. Any lesions which had apparently been unaffected by treatments such as topical application of antibiotics, antifungal agents and by application of local herbs, which could not be attributed to any other skin disease, were considered as well. Lesions which did not exhibit these characteristics were excluded. All the lesions that fit the selecting descriptions were considered for sampling.

**2.4 Sample collection**

*Leishmania* amastigotes are often present in low numbers in cutaneous lesions, isolation needs to be performed under field conditions that are susceptible to environmental contamination with bacteria or fungi, and adaptation and growth to in vitro culture failed sometimes even when organisms were present. The working assumption was that one in five attempted isolations should be successful, and a total of 50 people was the initial target for the study. It was hoped this would generate 10 or more successful isolates, then recruitment would be stopped, provided the isolates originated from at least four different villages separated by at least 10km within the Volta region. This is to ensure that adequate geographical sampling is achieved. If

these conditions are not satisfied and/or the results indicate the presence of more than one *Leishmania* species, then recruitment should be extended and further isolations performed. However, it should be noted that it is not the purpose of this project to conduct an epidemiological survey, since this cannot be properly performed until we have more information on the identity of the parasite in question, including whether more than one species is identified.

Due to logistical challenges, a total of 50 samples from 50 patients were collected and used for in-vitro culture. Patients presenting with cutaneous lesions suggestive of CL as described earlier were selected. The surfaces and about 30mm diameter around the lesions were cleaned with 70% alcohol and lesion sites anaesthetized with 1% lidocaine. For the punch biopsy sampling, gentle downward pressure was made at the raised edge of the lesion using a 0.5 -1 mm diameter punch biopsy device, to incise the skin tissue slightly deeper from that area. The tissue biopsy was immediately transferred into a prepared bijou containing 2ml Hanks Balanced Salt Solution (HBSS) supplemented with Gentamycin Sulphate and Penicillin/Streptomycin. For skin aspirate sampling, a 5ml syringe with a needle fixed to was filled with 1mL of sterile physiological saline. The tightly fitted needle to the syringe with its content was carefully advanced into the raised edge of the outer border and base of the lesion. A small amount of the saline was released into the lesion and sucked back into the syringe with the aspirating tissue fluid while rotating the needle. The content of the syringe was emptied into a sterile bijou tube containing 2ml aliquot of HBSS as above. All the samples were transported on ice at 4°C, from the field to the lab in Ghana.

**2.5 Culture of field isolates**

The collected samples (aspirates/biopsy) contained in 2ml aliquots of HBSS in sterile bijou tubes, kept on ice pack and transported from the field to the lab, were transferred into 1.5ml microcentrifuge tubes, centrifuged and washed in 2-3 changes of HBSS. The tissue biopsies were mashed in the tube with a needle to release the cells. The contents of the washed and/or mashed samples in centrifuge tube were transferred into semi-solid medium, "Sloppy Evans" and upon seeing any detectable growth, about 100µl of the content of "Sloppy Evans" were transferred into M199 medium supplemented with 20% (v/v) FBS + BME vitamins and Gentamycin sulphate in $25cm^2$ culture flasks. The contents of the culture flasks were incubated at 25ºC for 4 weeks or more and disposed when no growth is detected. On detecting any growth in any flask, the contents were carefully examined under the microscope and if *Leishmania* parasites were identified, they were then sub-passaged into fresh M199 medium and incubated at the same conditions until they reached $1.5x10^5$cells/µl. The parasites were then preserved at this growth stage by mixing culture with an equal volume of cryoprotectant (42.5ml of promastigote culture medium, +7.5ml of good quality glycerol = 15% (v/v); final concentration 7.5% (v/v)), aliquoting in cryotubes, placing these in a freezing pot and transferring to a -80ºC freezer. Some of the parasites were shipped to the Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, UK, for cryobanking.

### 2.6 Storage of samples on FTA cards

To confirm whether the parasite could be identified from field samples without culture, small amounts of the aspirates were inoculated onto FTA cards (Whatman Cat. No. WB 120204). The inoculated cards were transported to Lancaster University, UK, and diagnosis carried out.

### 2.7 In vitro culture and cryopreservation of *Leishmania* isolates

The isolates were recovered from cryobank in the Bates Lab, Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, UK. Ampoules of parasites (stabilates) were removed from liquid nitrogen and quickly thawed. The thawed contents (1ml) of cryotubes were placed into 25cm$^2$ culture flask using a sterile pipette. 4ml of the prepared M199 medium was added slowly in drops to the parasites to gradually dilute the concentration of glycerol and prevent lysing of the parasites. The culture flasks were incubated at 25$^o$C. The cultures were regularly monitored to check for growth as well as contamination by taking a drop of culture on a slide and examining under the light microscope as well as examining the whole culture flasks under an inverted microscope.

**2.8 Use of haemcytometer for cell counting**

To determine the parasite cell density, approximately 0.5ml of each culture was taken using sterile 1ml pipette and placed in a 1.5ml microcentrifuge tube. 20µl of the parasites (in the medium) was taken and added to add 20µl of buffered formalin (4%) to immobilise the parasites for easy counting. The haemocytometer with coverslip was prepared and a small volume of the parasite-formalin mixture introduced under the coverslip by touching the edge with the pipette tip and allowing the well to be filled by capillary action. The cells were allowed to settle for 2-3 minutes before they were counted, using phase contrast microscopy with x10 eyepiece and x40 objective. The cells in the four corners and centre squares (i.e. five medium-sized squares) of the middle square of the haemocytometer were counted. The cells touching the lower and left hand perimeter lines were counted whereas those touching the upper and right hand perimeter lines were ignored to prevent double counting. The cell density was calculated and the promastigotes harvested at 2.5x10$^7$ to 2.8x10$^7$/ml for making

pellets, and the remaining promastigotes cryopreserved in liquid nitrogen in 7.5% glycerol (final concentration) cryoprotectant.

# Chapter Three

## 3.0 Morphological characterisation of the parasite

### 3.1 Introduction

The demonstration of the amastigote form of the parasite through Giemsa staining of clinical sampled materials from patients, has long been used to diagnose leishmaniasis from the field and in hospitals, including in Ghana. This procedure helps in leading to appropriate treatment of the diseases in endemic communities, however, it fails to reveal the identity of the parasite responsible for the disease. For this and many other reasons the isolation of the parasite into culture is important. For instance, in a new endemic focus, there is the need to ascertain the identity of the species responsible by molecular characterisation as well as using other methods to determine if it is an emerging or re-emerging species.

The study of the cell body of the parasite can also yield useful information. Light microscopy, scanning electron microscopy and transmission electron microscopy can all be used to reveal the detailed structures of this newly isolated parasite. Also description of the different developmental stages of the parasite in vitro can be used for comparison with those of known species. For example, this can confirm the presence of stages which might contribute to infectivity, the metacyclic promastigotes. Evidence regarding motility, differential infectivity and differentiation

of various forms can help in understanding pathogenicity, such as has been revealed in some other Kinetoplastida (Broadhead *et al*., 2006; Matthews and Gull, 1997).

The different stages of the *Leishmania* parasite have their own biochemical properties (Bates, 1993), as well as structural variations that may contribute to pathogenicity and survival (Lacomble *et al*., 2009; Chanez *et al*., 2006). The development of the *Leishmania* parasite in the skin of mammalian host is simpler than the various developmental stages observed in the vectors, which can also be seen in vitro (Killick-Kendrick, 1990). There has been a clear demonstration that *L. mexicana* promastigotes in vitro progress through a range of morphologies which describe a single cell cycle (Wheeler *et al*., 2011). In the sand fly vector, different morphological forms of the *Leishmania* parasite have been revealed (Rogers *et al*., 2002; Gossage *et al*., 2003). The development of the promastigote forms also differs between *Leishmania* subgenera (*Leishmania*, *Viannia*), with a hindgut phase being found in subgenus *Viannia* (Sacks and Kamhawi, 2001). Such observations have prompted interest in the various types of promastigotes involved in the vector hosts, and whether they can be replicated by in vitro culture. For instance, procyclic and metacyclic promastigotes have been observed as two forms occurring in the exponential and stationary phases of culture, respectively (Gossage *et al*., 2003). In *L. mexicana* one morphological promastigote form in the vector, the leptomonad promastigote, was found to produce a gel-like substance, promastigote secretory gel (PSG), to facilitate parasite transmission and infection in the mammalian host by the infective form, the metacyclic promastigote (Rogers *et al*., 2002). This same study further described this and other promastigote forms that can be distinguished by morphological characterisation based on the body length, body width and the flagellum length.

It is observed that *Leishmania* promastigotes of different species can have different morphological sizes with respect to time when developing in the same vector species. This was evident in *L. mexicana* and *L. infantum* infections in *Lu. longipalpis*, although the order of the emergence of the developmental types of promastigotes (**Figure 3.1**) was the same in both *Leishmania* species (Gossage *et al*., 2003). Whether this order will hold for all the species of *Leishmania*, especially the new emerging ones with possible new natural vectors, is yet to be proven.

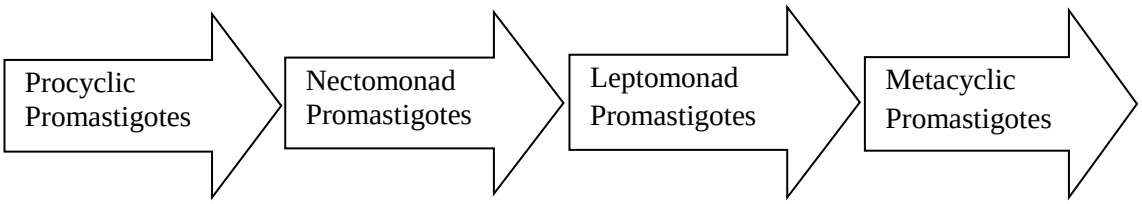



**Figure 3.1** The developmental order of the main promastigote forms.

Outside the natural vector host when their internal influence is curtailed, the pattern of the promastigote development in culture medium was similar, which indicates a programme for consistent development, although this can be somewhat affected by sub-passaging (Gossage *et al*., 2003). The role of the different morphological promastigote forms is ultimately to contribute in numbers to fully developed metacyclic forms, which can effect infection in mammalian hosts. There should be sufficient populations of the promastigote forms generated by cell dividing stages (with procyclic and leptomonad promastigotes as dividing forms), to make available enough infective forms to influence infection in the mammal host. Possibly, such developmental forms could affect conditions in the vector to help the parasite fully develop to perpetuate transmission by adaptation. However, adaptation may mean the morphological features of the promastigotes in the natural environment are different from the in vitro cultures where they are not exposed to any harsh environmental conditions, especially enzymatic reactions.

The morphologies of *Leishmania* in a growing system and the series of developmental events involving maturation of organelles, has a link with its cell cycle (Wheeler *et al*., 2011). The differences in length of the cell, its flagellum and the body width could be matched to the timings involved in the various structural and cell division. Differential position of cellular structures such as flagellum, kinetoplast and the nucleus have been used to define some life cycle stages, and the length of the flagellum can be used to infer the stage of the cell division (Casanova, *et al.*, 2009; Tyler *et al*., 2001; Wheeler *et al*., 2011).

In the case of new emerging species, the patterns of promastigote development as well as their division in the new species might be different. *Leishmania mexicana* and *L. infantum* exhibited differences in prevalence of promastigote stages even in the same host (Gossage *et al*., 2003). Adaptation to different natural hosts could be accompanied by different promastigote forms. The physiology of blood meal digestion is a potential barrier to the developmental of the promastigote in the sand fly, so in a situation where blood meal digestion is faster or delayed, there is bound to be promastigote developmental changes, which may be intrinsic to particular *Leishmania* species, and in some cases have been linked with apoptosis (Gannavaram and Debrabant, 2012; Zangger *et al*., 2002).

## 3.2 Materials and Methods

### 3.2.1 Light microscope morphometry of *Leishmania* GH5

The promastigotes used in the work described in this chapter were from the GH5 isolate, MHOM/GH/2012/GH5;LV757. GH5 promastigotes were grown for a period of 7 days in M199 supplemented with 10% FBS, BME vitamins and 25µg/ml gentamycin sulphate (M199 medium). Microscope slides were made daily, from day 1 to 7. Smears were made each day and fixed in absolute methanol (molecular grade). The slides were Giemsa stained and examined with 10x eyepiece and 100x objective under oil immersion and bright field microscopy, using a Nikon Eclipse E600 microscope, and different fields of the slides photographed. The parameters of the body length, body width and the flagellum length of 50 individual stained randomly selected cells were measured using the ImageJ software (version 1.48) programme.

### 3.2.2 Electron microscopy of GH5

#### 3.2.2.1 Electron microscopy (EM) fixative

0.1M cacodylate buffer was prepared by dissolving the required amount of sodium cacodylate trihydrate ($(CH_3)_2AsO_2Na\cdot 3H_2O$) in distilled water, and adjusting the pH to 7.4 with hydrochloric acid (HCl). Appropriate volumes of paraformaldehyde and glutaraldehyde (EM grades) were added to give final concentrations of 4% paraformaldehyde, 2.5% glutaraldehyde. This constituted the EM fixative. All the solutions were prepared in a fume hood.

#### 3.2.2.2 Promastigote processing

The promastigotes were cultured to a density of approximately $1x10^7$cells/ml using M199 medium, harvested by centrifuging at 8000rpm for 5 minutes, and the supernatant medium discarded. The cell pellets were re-suspended in Hanks Balanced Salt Solution (HBSS) and the centrifugation repeated. The pellets were then fixed in 2ml EM fixative for 2-6 hours at room temperature and then processed for either

scanning electron microscopy (SEM) or transmission electron microscopy (TEM). EM was performed with the assistance of Dr Alison Beckett, University of Liverpool.

### 3.2.2.3 Processing of promastigotes for SEM

Several 10µl samples of the fixed promastigotes were gently pipetted onto 6mm poly-L-lysine coated cover glasses and allowed to stand for 20 minutes at room temperature. The samples were washed in 2 changes for 1minute in 0.1M phosphate-buffer (pH 7.4) at room temperature. The samples on the cover glasses were stained with 2% Osmium stain (2% $OsO_4$ in $ddH_2O$) at room temperature for 1 hour, after which they were washed 5 times for 3 minutes each in distilled water. The samples were then incubated in 1% Tannic Acid in $ddH_2O$ and washed 5 times as previously. This was followed by a second osmium staining (2% OsO4 in $ddH_2O$) for 1 hour at room temperature and washed 5 times for 3 minutes each. At this stage the samples were stained in 1% Uranyl acetate (1% UA in $ddH_2O$) at 4°C overnight, after which the samples were washed 5 times for 3 minutes each in $ddH_2O$ at room temperature. The samples were then dehydrated in graded ethanol of 30%, 50%, 70%, 90% in $ddH_2O$ for 5mins each, and then 3 times in 100% ethanol. The samples were finally dried and sputter coated with 12nm Au/Pd (Gold/Palladium). The samples were then ready for examination.

### 3.2.2.4 Processing of promastigotes for TEM

The fixed samples were washed 5 times for 3 minutes each in 0.1M phosphate buffer (pH 7.4). The washed samples were embedded in 2% agarose, allowed to set and cut into $1mm^3$ pieces. These pieces were stained in 2% osmium (2% $OsO_4$ in $ddH_2O$) for an hour at room temperature, followed by 5 washes of 3 minutes each in distilled water at room temperature. The samples were stained with 1% tannic acid in distilled water at room temperature for 2 hours. A second osmium staining for an hour at room

temperature followed, after which the samples were washed 5 times for 3 minutes each in distilled water. An overnight staining with 1% Uranyl acetate (1% UA in dd$H_2O$) at 4°C was performed, and the samples washed 5 times for 3 minutes again in distilled water at room temperature. Dehydration of the samples at this stage was performed in a graded ethanol series of 30, 50, 70, 90% for 10 minutes each, then twice in 100% ethanol at room temperature, and then 2 times in 100% propylene oxide for 10 minutes. Infiltration of the samples were performed in graded medium TAAB resin 812/100% propylene oxide at a 1:2 ratio for 1 hour, then at 1:1 overnight, followed by 2:1 for 1 hour, 3 x 100% for 1 hour. The samples were finally embedded in 100% resin in a pellet mould and cured for 48hrs at 60°C. Ultra-thin sections were cut, picked up on hexagonal copper grids and observed using a Philips CM10 transmission electron microscope.

## 3.3 Results

### 3.3.1 Culture

The ability of the GH5 isolate to grow in vitro in the standard promastigote growth medium was investigated. A growth curve comparable to that shown by *L. mexicana* was obtained (**Figure 3.2**). These growth curves pattern are similar to those seen with other species of *Leishmania* (Celeste and Guimaraes, 1988).

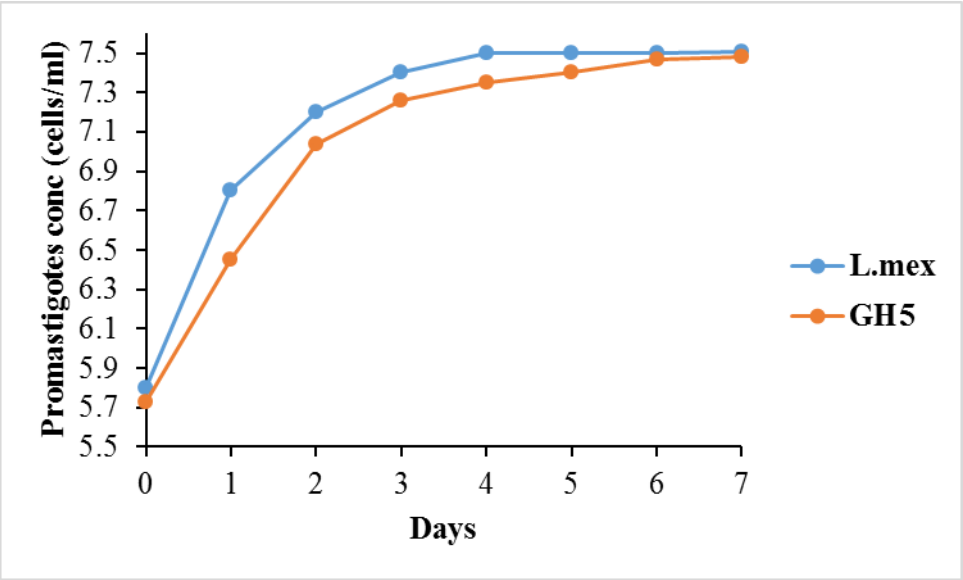



**Figure 3.2** Growth curve of the GH5 isolate (L.GH) compared to that of *L. mexicana*: (L.mex). Vertical axis displays log cell density.

There was progressive exponential growth of the GH5 isolate from day 1 until day 6 when the stationary phase was entered. Extension of this period beyond ≈10 days revealed a gradual decline in cell numbers, and although passage from this type of culture was possible, a lag phase was observed before growth was resumed. Therefore, routinely GH5 promastigotes were sub-passaged every 7 days.

### 3.3.2 Morphometry of GH5 by light microscopy

**Figure 3.3** shows the morphology of the promastigotes after Giemsa staining. Various changes were observed as the promastigotes progressed through their growth cycle. Flagella were present on days 1 to 4, after which the flagella became reduced in length, until by day 7 there were virtually no external flagella present or only a very short flagellum (**Table 3.1**). Cells dividing by binary fission were seen on day 1 and through the exponential phase, as evidenced by cells with two flagella, indicating the initiation of cell division, and the presence of doublets, cells that were still joined together at the end of cell division.

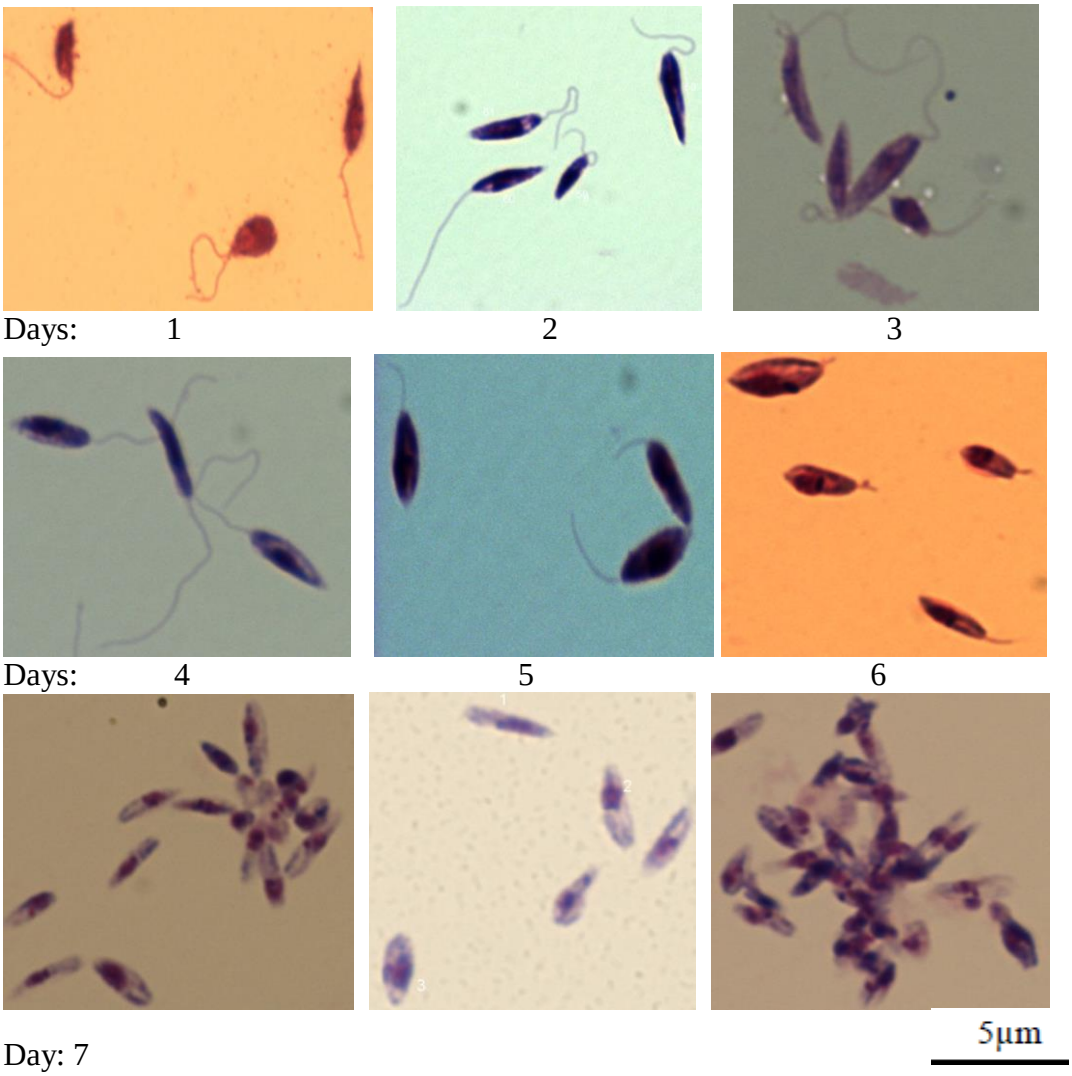



**Figure 3.3** Morphology of the Giemsa stained GH5 from days 1 – 7.

| Parameter | Average (Days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| **Body Length** | 7.54 | 8.51 | 7.37 | 11.78 | 5.56 | 6.06 | 4.72 |
| **Body Width** | 2.33 | 2.25 | 1.92 | 3.81 | 2.01 | 2.42 | 1.52 |
| **Flagellum Length** | 9.25 | 12.49 | 11.88 | 19.49 | 3.65 | 3.10 | 0.58 |

**Table 3.1** Measurements of body length, body width and flagellum length during growth in vitro. Measurements are in µm and are averages from 50 individual promastigotes per day.

These data are graphically represented in **Figure 3.4**.

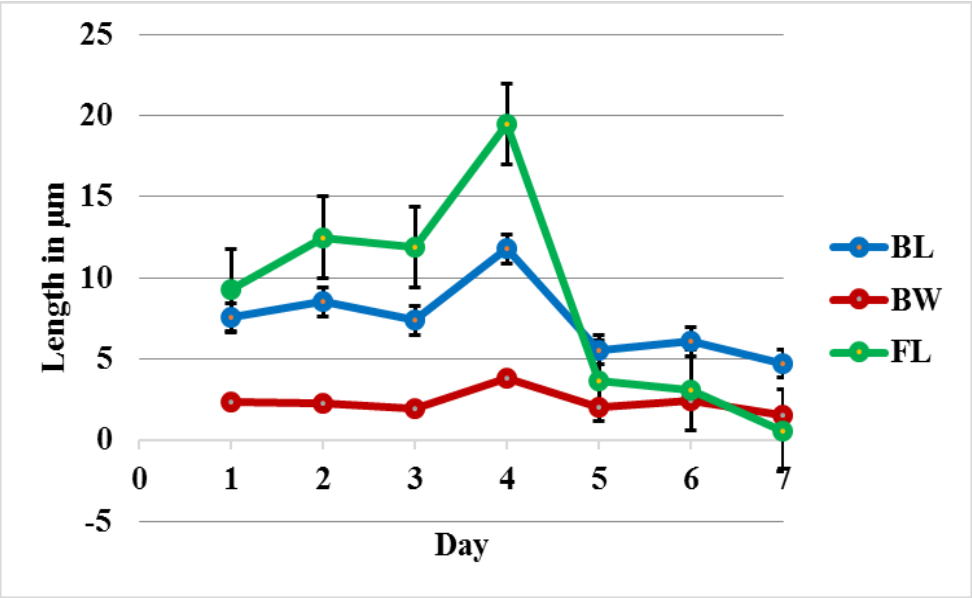



**Figure 3.4** Daily average measurements of GH5 under the light microscope.

The body width remained fairly constant throughout the days of culture, but did show an increase on day 4, at the late exponential phase. Body length in the exponential phase was greater than in the stationary phase, again peaking at day 4. Flagellum length showed the greatest variation, increasing through the exponential phase and peaking on day 4, decreasing in size until by day 7 the cells were almost aflagellate.

### 3.3.3 Electron Microscopy

#### 3.3.3.1 Scanning electron morphology (SEM)

The structures revealed by SEM are similar to those previously seen in the genus *Leishmania* (**Figure 3.5**).

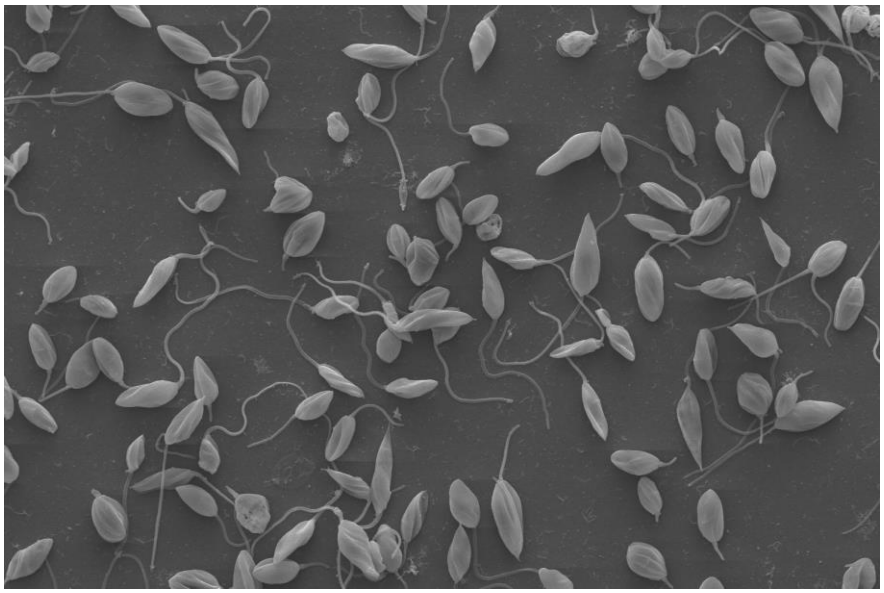



**Figure 3.5** Low power SEM view of GH5 promastigotes.

Promastigotes showing variation in body shape, width and flagellum length were observed. Some fields revealed promastigotes in a mass, similar to the rosettes seen in live cultures and by light microscopy (**Figure 3.6**).

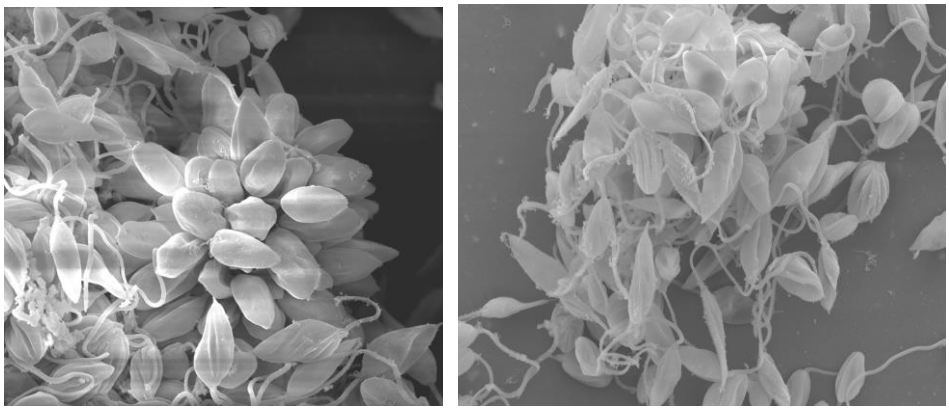


**Figure 3.6** Rosettes and aggregates of GH5 promastigotes in SEM.

Observations of single cells revealed various body shapes, including pear/tear shaped promastigote bodies of between 5-7µm (**Figure 3.7**). The body extended anteriorly to form a short or long flagellum. Some of the promastigotes were in

division with 2 flagella emerging anteriorly from the body. Some cells were blunt at their posterior end, others had a tapered posterior end.

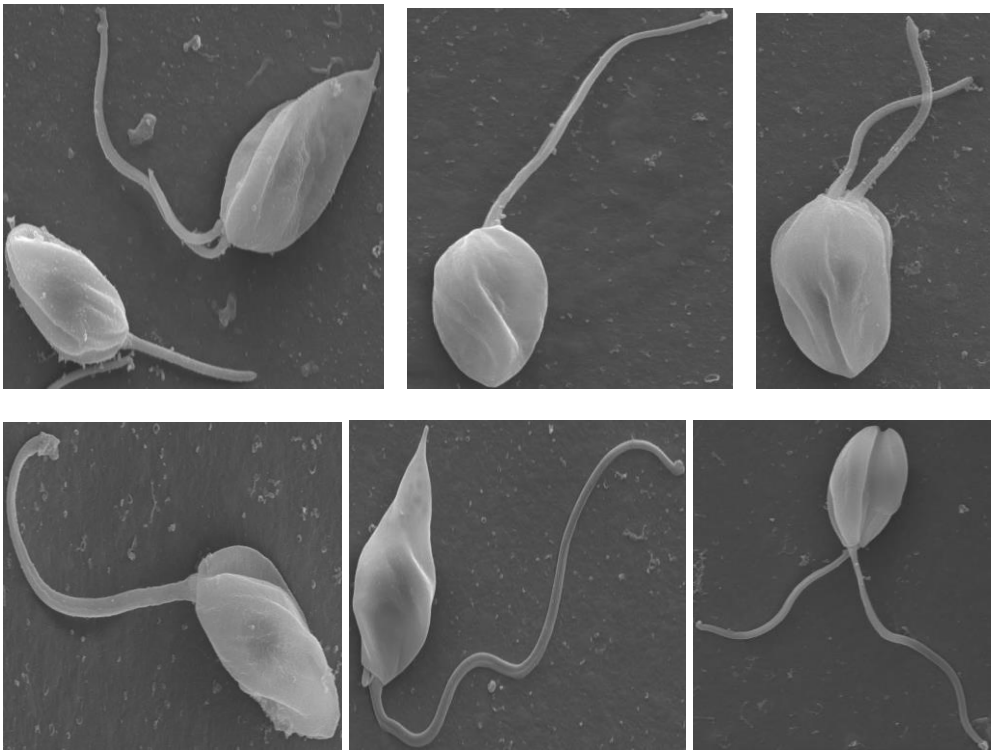



**Figure 3.7** Montage of GH5 promastigotes observed by SEM.

### 3.3.3.2 Transmission electron morphology (TEM)

The TEM revealed various internal structures of the parasite (**Figure 3.8**). The purpose was to reveal similarities or difference of GH5 in comparison to other *Leishmania* parasites characterised. Basic structures such as the kinetoplast, nucleus and flagellum in the flagellar pocket were revealed. A bilayer plasma membrane was observed, the 9+2 microtubule arrangement within the flagellum, presence of a paraxial rod, and branched cristate mitochondrion, all structures typical of *Leishmania* promastigotes (**Figure 3.8**).

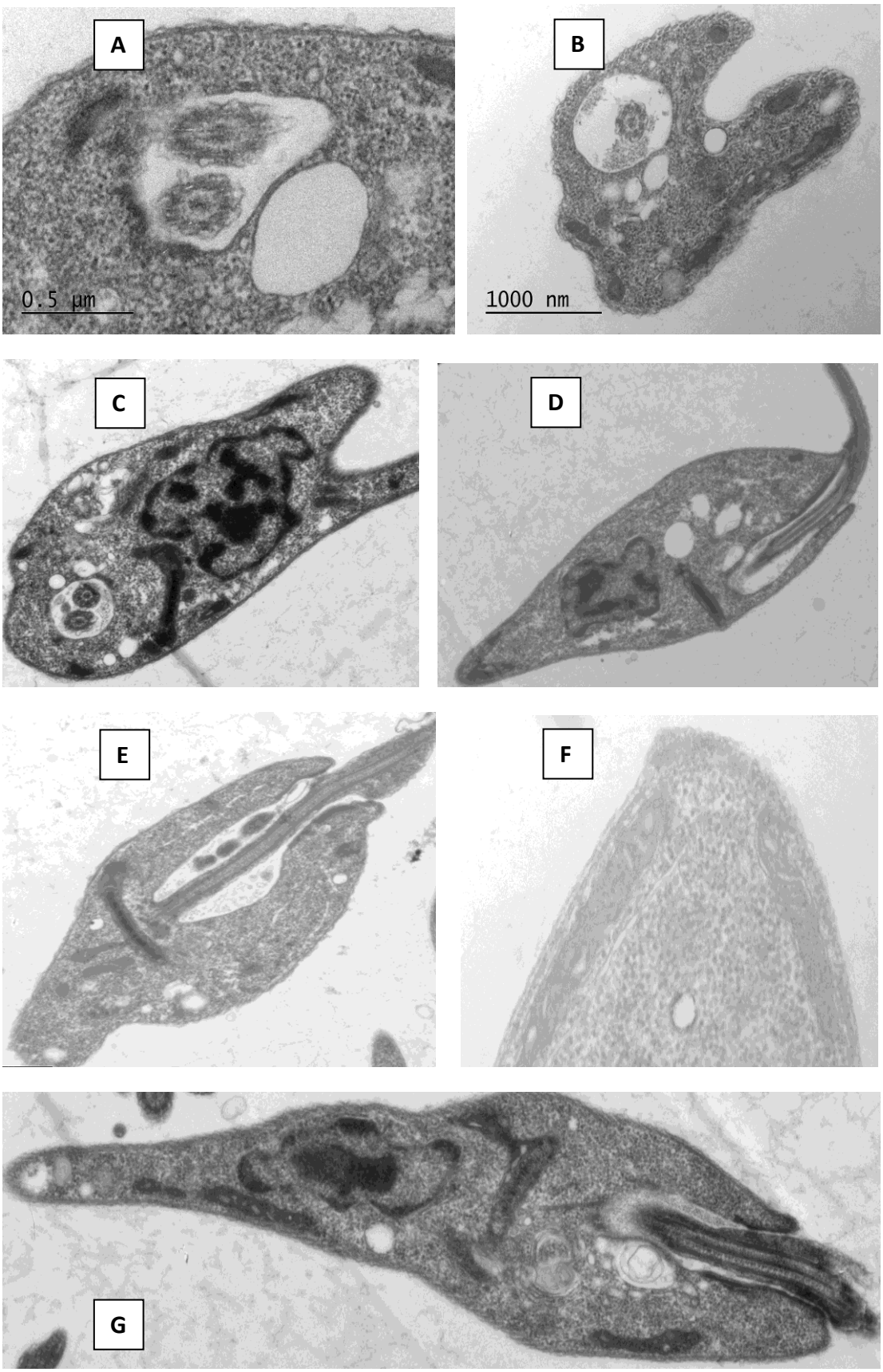



**Figure 3.8** Various TEM sections through GH5 promastigotes. A. Transverse section through a dividing promastigote showing two flagella within the pocket. B.

Transverse section showing flagellum and typical material within the flagellar pocket, probably secretory. Various cross sections through the mitochondrion can be seen at the periphery of the cell, and also transverse sections through subpellicular microtubules. C. Oblique section showing detail of the nucleus with peripheral and central chromatin, and kinetoplast between nucleus and flagellar pocket with two flagella. D. Longitudinal section showing similar features as in C, also paraxial rod can be seen in flagellum outside the cell body. E. Longitudinal section showing kinetoplast, basal body, and flagellum, amongst other features. F. Detail of anterior part of cell body showing mitochondrial sections and subpellicular microtubules. G. Longitudinal section showing multivesicular bodies in vicinity of flagellar pocket.

Various inclusion bodies were observed in the cytoplasm that are difficult to identify without immunolabelling. These could be glycosomes, lysosomes, lipid droplets or polyphosphate inclusions, all of which have been reported in *Leishmania*. Nothing was observed that was indicative of a non-*Leishmania* trypanosomatid.

### 3.4 Discussions and Conclusions

*Leishmania* promastigotes have been shown to adopt various morphological stages, which can be revealed by Giemsa staining under the light microscope. These morphological promastigote stages have been investigated in this study. Their ultrastructure could also help to understand the nature of the newly isolated parasites from Ghana and their role in the disease outcome in the endemic focus.

In the morphometric studies, the pattern of growth gives an impression of different morphological promastigote stages and forms occurring at different times during the growth cycle, such as have previously been reported in well characterised species like *L. mexicana* and *L. infantum* (Gossage *et al*., 2003, Rogers *et al*., 2002). The dividing morphological forms of *L. mexicana* are procyclic (stumpy; short flagellum) and leptomonad (small body length and body width) promastigote forms (Gossage *et al*., 2003). These may correspond to forms seen on days 1-3 and 5-6 in the

current study. The rapidly dividing promastigotes were mostly in the early phase of the growth curve which coincided with the first few days of culture. On day 4 a population of large promastigotes with long flagella were observed, which may correspond to the nectomonad forms previously described (Gossage *et al*., 2003, Rogers *et al*., 2002). These forms are non-dividing in *L. mexicana* and *L. infantum*, and here occurred at the late exponential phase when growth was slowing down. One interesting difference was in the stationary phase population. There was an absence of forms typical of metacyclic promastigotes, small highly motile promastigotes with long flagella. Instead there was a population of promastigotes with very small flagella or even aflagellate forms. It is possible that this represents a partial differentiation into amastigotes, which can sometimes occur in stationary phase of promastigote cultures. Alternatively, it could be that these do represent the mammalian-infective stages for these particular parasites, but they have a rather different morphology. More work is required to characterise these different forms and understand their significance.

Regarding the ultrastructural characterisation, both SEM and TEM analyses broadly indicated *Leishmania* promastigotes, with expected structures present and nothing unusual observed. The various structures and their relative positioning in the TEM sections and the relative sizes and the presence of the flagella in the SEM leaves no doubt these organisms are trypanosomatids, of which *Leishmania* is a member. The position of the flagellum is similar to that found in other species. It is extracellularly anterior to the kinetoplast which is intracellularly placed, which emerges from a groove, the flagella pocket (Wheeler *et al*., 2010; Dedet *et al*., 1995). The positioning of the nucleus and the kinetoplast is in similar fashion to other *Leishmania* species, as well as the proximal location of the basal body with regards to the flagellum (Wheeler *et al*., 2010). The kinetoplast and flagellum are found at the same relative position,

only varying in the distance that separates them (Kazemi *et al*., 2008). The tapering posterior end of some cells has been reported to encourage doublets in vitro culture (Wheeler *et al*., 2010). Though there have been reports of biological dissimilarities among *Leishmania* species, most of their ultrastructural features are conserved (Correa *et al*., 2007; 2005).

# Chapter Four

## 4.0 Clinical cases detection, molecular identification and analysis

**Some of the results presented in this chapter have been published in Kwakye-Nuako *et al*. (2015) *Int. J. Parasitol*.; 45(11): 679-684.**

### 4.1 Introduction

Differential diagnosis to discriminate among the agents responsible for cutaneous leishmaniasis (CL) on the basis of clinical signs alone is not reliable, but is required for proper disease management (Klaus and Frankenburg, 1999; Al-Jawabreh, 2005; Shani-Adir *et al*., 2005). Previously diagnosis has mainly relied on microscopic observation of amastigotes in stained tissue smears, in addition to culture of promastigotes from such tissue samples (Bensoussan *et al*., 2006; Magill, 2005; Vega-Lopez, 2003; Marfurt *et al*., 2003). Despite the challenges associated with these presumptive clinical diagnoses, they are commonly practiced in less resourced laboratories in highly endemic areas where improved diagnostic techniques are needed (Minodier *et al*., 1997). The inoculation and in vitro culture of the samples from patients' tissues increases the potential DNA yield through the growth of the promastigotes. Media like Schneider's *Drosophila*, M199, Novy MacNeal Nicolle

(NNN) and RPMI 1640, supplemented with foetal bovine serum (FBS) have been used in isolating the promastigote forms of the parasite from aspirates, biopsy and other clinical samples from infected human and animal hosts (Markell *et al*., 1999).

The recent improved methods for identification of the parasite to the species level have utilised molecular approaches where DNA is amplified by polymerase chain reaction (PCR), either directly from the sample source or from promastigotes generated by in vitro culture, based on a specific target gene of interest. These targets have included minicircle kinetoplast DNA, small subunit ribosomal RNA and miniexon RNA sequences, which were designed with specific primers based on the species in question (Gangneux *et al*., 2003). These techniques have been a preferred choice due to their high sensitivity and specificity, and the ability to amplify from small parasite loads in clinical samples. However, they remain relatively expensive, so cheaper equipment and reagents are needed to lower the cost of running a PCR and make the method suitable to use in facilities of limited resources (Belli *et al*., 1998).

The identification of the various species of the parasite causing different clinical forms by PCR, would be of great benefit particularly in cases where specific species of *Leishmania* require distinct therapeutic regimens (Navin *et al*., 1990). Such methods are also valuable epidemiologically in designing disease intervention control strategies (Belli *et al*., 1998; Shani-Adir *et al*., 2005). The sensitivity of the PCR has been found to be greater than conventional parasitological methods of diagnosis of the disease, though it requires some specialised equipment and technical expertise (Wilson, 1995; Bensoussan *et al*., 2006). The kDNA-PCR based technique for instance, has been extremely sensitive, by virtue of the unique properties of the target being highly abundant and containing approximately 10,000 small circular DNA minicircles (Noyes *et al*., 1998). Even in serologically negative patients, PCR has

confirmed *Leishmania* infection, achieving sensitivity as high as 98.7% (Bensoussan *et al*., 2006; Wilson, 1995).

PCR is theoretically capable of detecting one copy of a target in DNA, and in combination with restriction fragment length polymorphism (RFLP), has helped in identifying *Leishmania* species in CL foci in Sri-Lanka, Palestine and Jericho (Azmi *et al*., 2010; Schonian *et al*., 2008; Al-Jawabreh *et al*., 2004; Sawalha *et al*., 2003; Ashford 2000; Siriwardana *et al*., 2007). Thorough and accurate identification of *Leishmania* species is crucial. The technique can be applied using either genus-specific sequences or species-specific sequences for differentiation of *Leishmania* species (Azmi *et al*., 2010; Salotra *et al*., 2001; Jirku *et al*., 2006). PCR-RFLP can also give an idea of species variations involving nucleotide base alteration at sites to be digested by a restriction endonuclease, as has occurred in gp63 coding sequences (Victoir *et al*., 1998; Mauricio *et al*., 2001), kDNA amplification (Ferroglio *et al*., 2006), and miniexon sequences (Marfurt *et al*., 2003). PCR-RFLP performed on ribosomal small subunit and internal transcribed spacer 1 has been able to reveal diversity in *Leishmania* parasites, suitable for taxonomic, ecological and epidemiological studies (Rotureau *et al*. 2006).

A convenient tool for diagnosis in resource poor disease endemic settings is PCR-RFLP with clinical samples on filter paper, and could identify species like *L.* (*V.*) *braziliensis, L.* (*V.*) *peruviana, L.* (*V.*) *guyanensis,* and *L.* (*V.*) *braziliensis–peruviana* hybrids (Boggild *et al*., 2010). The method is direct and rapid, and using the amplicons of ITS1 and SLME proved to be a very sensitive method in detection of CL cases (Schonian, *et al*., 2003; Marfurt *et al*., 2003; Cupolillo *et al*., 1995). Using PCR-RFLP with 7 spliced leader gene targets it was possible to distinguish *L. tropica*, *L. aethiopica* and *L. donovani* complex parasites (Azmi *et al*., 2010). A PCR-RFLP was

also used to distinguish *L. braziliensis* and *L. amazonensis* occurring in the same endemic focus (Volpini *et al*., 2004). Furthermore, *L. infantum* variants have been discovered as agents of CL cases spreading to new areas of Tunisia by PCR-RFLP analysis (Schonian *et al*., 2008). Moreover, PCR-RFLP analysis identified the etiological agent as *L infantum* in some immunocompromised children (Cascio *et al*., 2002). These studies demonstrate that PCR-RFLP can be useful in species identification in leishmaniasis, despite some limitations. These limitations can be mitigated by sequence analysis, although this is more time consuming and expensive.

DNA sequence analysis of amplified PCR products has been applied to various targets, including kinetoplast DNA (kDNA), mini-exon DNA, ribosomal RNA, and glucose-6-phosphate dehydrogenase (Laskay *et al*., 1995; Marfurt *et al*., 2003; Castilho *et al*., 2002; Shahbazi *et al*., 2008). In a sequenced amplified conserved region common to the genus *Leishmania*, the agent responsible for CL was identified as *L. amazonensis* (Silveira *et al*., 2004). The sequenced amplicons of a short fragment of 7SL RNA gene could identify to the species and complex level, *Leishmania* isolates from the Old and New World (Zelazny *et al*., 2005). In another sequence analysis of PCR amplicons for species identification, the alignment of consensus sequences identified the following species into their assigned groups, *L. major* and *L. aethiopica*; *L. infantum, L. donovani* and *L. archibaldi*; and *L. tropica* and *L. killicki* (Gangneux *et al*., 2003). The sequencing of the 7SL RNA gene was able to discriminate reference and clinical strains including *L. major*, *L. tropica*, *L. aethiopica*, *L. braziliensis*, *L. guyanensis*, *L. mexicana* and *L. donovan*i (Stevenson *et al*., 2010). There was some genetic variability within species, with an intraspecies identity range of 99.2 to 100% (Stevenson *et al*., 2010).

6GPDH gene sequence analysis has been used to identify *L. donovani* previously typed as MON-37, in clinical isolates that cause CL in Sri Lanka, and could also distinguish this agent from the MON-2 zymodeme of India (Siriwardana *et al*., 2007). This gene can be used for identification of other *Leishmania* species due to its high degree of sequence polymorphism species and availability in Genbank (Greenblatt *et al*., 2002). In another study the order and arrangement of gene within the maxicircles of *L. major* and *L. tarentolae* were found to be the same, however, the base composition differed between the species, and the sequences showed 85-87% identity at the nucleotide and 71-94% identity at the amino acid level (Yatawara *et al*., 2008). Variations such as these and the other examples above could influence the functions of the genes in species but can only be revealed by sequencing. Moreover, sequence results also permit the phylogenetic analysis of species, providing information on the divergence of species and their relatedness.

Phylogenetic analysis of DNA sequences provides evolutionary information, placing species of *Leishmania* into phylogenetic trees. The phylogenetic relationships of the parasite were previously made based on the natural history of their vertebrate and invertebrate hosts, clinical manifestations and possibly geographical distribution (Marcili *et al*., 2014; Shaw, 1997). During evolution groups of organisms are altered over time and could undergo speciation into separate branches, hybridize together or go on extinction. For example, the taxonomy of certain species of *Leishmania* have over the years come under question (Momen and Cupolillo, 2000), which have been regrouped or reclassified including emerging species (Pothirat *et al*., 2014). Evolution in the genus *Leishmania* has occurred over long periods of time, so in the absence of a fossil record molecular approaches to generate phylogenies are useful to understand the evolutionary relationships among present-day genera (Marcili *et al*., 2014). This

has made molecular data involving protein and DNA sequences useful in the construction of phylogenetic trees (Hall, 2013). Phylogeny has made use of such data to assemble a "relationship tree" that shows the plausible relationships of various taxa. The commonest technique is the contrasting of sequences of genes via sequence alignment, and has been used to better understand the phylogenetic relationships in *Leishmania*. This has helped to reveal the distinctions among species of *Leishmania* of related identity. In one study, genetic differences between the *L. donovani* complex as opposed to *L. tropica* and *L. major* was described (Hide *et al*., 2001). Phylogenetic analysis has also provided considerable support for a Neotropical origin of the genus *Leishmania* (Noyes *et al*., 2000). There has been an argument whether *Leishmania* originated from the Old World or the New World. The origin of the New World *Leishmania* is supported by the high genetic diversity of neotropical *Leishmania* species, as well as amino acid and RNA polymerase-based trees (Lukes *et al*., 2007). Following this the lineage of *Leishmania* was scattered across Asia during the Miocene in which diversification has given rise to *L. aethiopica*, *L. major*, *L. gerbilli*, *L. turanica*, *L. tropica* and the *L. donovani* complex (Noyes *et al*., 1997; Croan *et al*., 1997).

The construction of phylogenetic trees from the sequence data generated from the PCR products is achieved in 4 steps, namely: acquisition of a set of DNA or protein sequences; alignment of the edited sequences; the tree estimation from the aligned sequences; tree presentation to depict the information gathered from the sequence data (Hall, 2013). The main aim of this part of the investigation is to unravel the identity of the *Leishmania* responsible for CL in the Volta region of Ghana and to investigate its relationship with other species, by employing the above mentioned methods.

## 4.2 Materials and Methods

### 4.2.1 Isolation/Extraction of DNA

Genomic DNA of all the *Leishmania* species (both known and unknown) were extracted using Qiagen DNeasy Tissue Kit (Cat. No. 69504). The following procedures were used according to the manufacturer's instructions. The pellets made from the *Leishmania* species were re-suspended in 200µl phosphate buffered saline (PBS). 20µl proteinase K and 4µl RNase were added, vortexed and incubated at room temperature for 2 minutes. 200µl buffer AL (without added ethanol) was added to the sample and mixed immediately and thoroughly by vortexing to yield a homogeneous solution. The contents were then incubated at 54ºC for 10 minutes. Then 200µl of ethanol (96%) was added and mixed thoroughly by vortexing to yield a homogeneous solution. The mixture was then pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube provided in the kit, and centrifuged at ≥6000xg (8000 rpm) for 1 min. The flow-through and collection tube were then discarded. The DNeasy Mini spin column was again placed in a new 2 ml collection tube provided in the kit, 500µl Buffer AW1 was added and then centrifuged for 1 min at ≥6000x*g* (8000 rpm). The flow-through and collection tube were again discarded. The DNeasy Mini spin column placed in a new 2 ml collection tube provided. 500µl buffer AW2 was added and the column centrifuged for 3 min at 20,000x*g* (14,000 rpm) to dry the DNeasy membrane. The flow-through was carefully discarded, to ensure that the column does not come into contact with the flow-through to prevent carryover of ethanol into the elution stage. The column was re-centrifuged for 1 min at 20,000x *g* (14,000 rpm) to ensure that no residual ethanol was carried over during the elution. The DNeasy Mini spin column was placed in a clean 1.5ml microfuge tube and 100 µl Buffer AE was pipetted directly onto the DNeasy membrane, incubated at room temperature for 1

min, and centrifuged for 1 min at ≥6000xg (8000 rpm) to elute the DNA. The concentration of the DNA eluted was estimated using a NanoDrop 2000c spectrophotometer (Thermo Scientific).

### 4.2.2 Molecular analysis

#### 4.2.2.1 Amplification reactions

Standard PCR reactions were carried out, unless otherwise specified. DNA amplification by PCR was performed with two different sets of primers. Amplification reactions were carried out using a G-STORM Cycler. Reactions were performed in 25 µl volumes using 2 x Reddy Mix$^{TM}$ PCR Master Mix (ABgene) (1.5 mM $MgCl_2$, 1.25units *Taq* DNA polymerase, 75mM Tris-HCl (pH 8.8 at 25ºC), 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 100 pmol of each primer, and 100 ng of target DNA. Positive and negative controls were run with each reaction.

#### 4.2.2.2 Primers used for PCR and RFLP

The primers used were as shown below:

| | |
|---|---|
| AM1 Forward | 5' CGC GTG TCG TTC GGC TTT ATG TG 3' |
| AM2 Reverse | 5' CTT ACG GAG CTT GCT GAG GTG AGG 3' |

#### 4.2.2.3 Polymerase Chain Reaction for RFLP

These primers were used to amplify an intergenic sequence between two tandemly repeated ribosomal protein S7 genes on chromosome 1 (RPS7A and RPS7B), generating a product of 1100-1400 bp. The reaction conditions were optimised to a final $MgCl_2$ concentration of 2.5 mM and a 63°C annealing temperature. PCR reaction conditions were: initial denaturation at 94ºC for 5 minutes, followed by cycles at 94ºC for 30 seconds, annealing at 63ºC for 30 seconds, and extension at 72ºC for 1 minute

30 seconds, with a final extension at 72ºC for 3 minutes. The number of cycles was 35.

#### 4.2.2.4 Primers used for PCR and sequencing

The primers used were as shown below:

BN1 Forward 5' GAA GGT CAA CAC CCT GAT CC 3'
BN2 Reverse 5' CTT CTT GGC GGT CTT CTG AG 3'

#### 4.2.2.5 Polymerase Chain Reaction for sequencing

These primers are designed to amplify an intergenic region between two tandemly repeated RPL23a (ribosomal protein L23a) genes on chromosome 6 with the expected product size of ≈595bp, depending on species. Products of these primers were used for sequence analysis for species identification and phylogenetic analysis. PCR reaction conditions were optimised to an annealing temperature of 60°C. PCR reaction conditions were; initial denaturation at 94ºC for 5 minutes, followed by cycles at 94ºC for 30 seconds, annealing at 60ºC for 30 seconds, and extension at 72ºC for 1 minute 30 seconds, with a final extension at 72ºC for 7 minutes. The number of cycles was 35. Positive and negative controls run with each reaction.

#### 4.2.2.6 Agarose gel electrophoresis

PCR products were mixed with loading buffer (20%w/v Ficoll, l00 mM EDTA, Orange G) at 4:1 ratio and loaded onto 1.5% - 2.0% agarose (Sigma) gels in 100ml Tris-acetate (TAE) buffer containing 5ng/µl ethidium bromide, along with a 1Kb DNA ladder (Invitrogen 15615-016). The samples were then subjected to electrophoresis for 45 minutes to 1 hour at 75 - 100V on gels of 8 cm length. DNA bands were examined under ultraviolet trans-illumination. By comparing the product

bands with bands from the known molecular-weight markers, the sizes of each product were determined.

#### 4.2.2.7 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

For identification of *Leishmania* species by PCR-RFLP, the PCR products were digested using *MspI* enzyme prior to electrophoresis. Ten microlitres of the PCR products were digested with 10 units of the *MspI* enzyme and appropriate buffer to bring the total reaction volume to 20µl. The reaction mixtures were thoroughly mixed and incubated at 37ºC for 2 hours. Five microliters of loading buffer (20%w/v Ficoll, l00 mM EDTA, Orange G) were added to the digested products and loaded onto 2.5% agarose gels and run at 60V for 4 hours.

#### 4.2.2.8 PCR products for sequencing

PCR products generated using the BN1/BN2 primers were subsequently cleaned using a three step procedure with a GeneJET PCR purification kit (Thermo Scientific, #K0701). In this protocol, equal volumes of the PCR reaction product were thoroughly mixed with the binding buffer. The resulting mixture was then added to the GeneJET purification column, centrifuged at 13,000g (12,000 rpm) for 60 seconds and the flow-through discard. About 700µl of the wash buffer was added to the GeneJET purification column, centrifuged for 60 seconds and the flow-through discarded. Between 20-50µl of elution buffer (sterile distilled water) was added to the centre of the GeneJET purification column membrane, incubated for 60 seconds and centrifuged for 1 minute to elute the cleaned PCR product, ready to be sent for sequencing. The DNA concentration in this cleaned PCR product was determined using NanoDrop 2000c Spectrophotometer.

The product was then sent to Source Bioscience Sequencing (Cambridge, UK) for sequencing. The resulting sequence data was BLAST searched using NCBI

Nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and GeneDB (http://www.genedb.org) to confirm identification. Multiple alignments was performed using CLUSTAL W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

#### 4.2.2.14 Phylogenetic analysis

The sequence products generated by primers designed from RPL23a genes were aligned using Clustal W2 in MEGA (version 6), and then tested with various models of sequence evolution, and the Tamura and Nei (TN93) model, which gave the best fit, used for the tree generation. The phylogenetic tress was inferred using maximum-likelihood (ML) approach. To test tree topology, bootstrap re-sampling was performed (1000 iterations). Numbers of transitions, transversions and nucleotide differences existing in *Leishmania* species were calculated.

## 4.3 Results

### 4.3.2 Field investigation

#### 4.3.2.1 Cases

In the four villages visited, 63 suspected patients were found but based on the clinical description, 50 individuals were recruited some of whose lesions are represented in **Figure 4.1.** Samples were taken from some of these and processed for in vitro culture and/or for direct PCR.

#### 4.3.2.2 Lesion observations

The lesions observed on the recruited patients during the visits appeared to be recent and ulcerated (**Figure 4.1**). Four individuals had two lesions, the remaining 46 patients had single lesion (**Table 4.1**). 44 patients had lesions on their upper extremities, and 6 patients had lesion on the lower extremities. The total number of

infected males recruited were 22 and the females were 28, with the infected adults mainly farmers.

A total of 54 lesions were seen on the 50 recruited individuals of whom 25 lesions were found on the 22 males with 3 patients having double lesions. The 28 females contributed 29 lesions with only one person having two lesions.

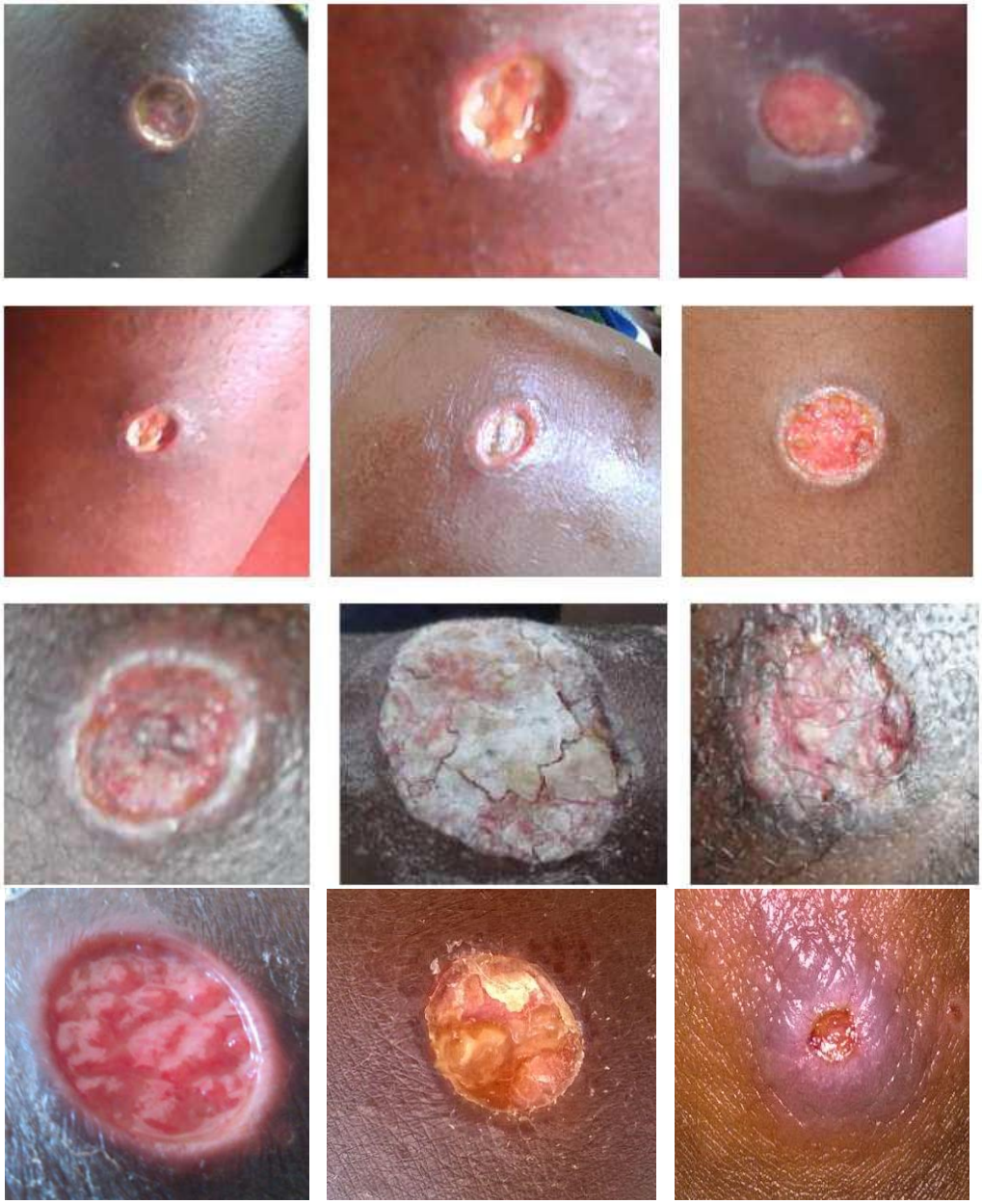


**Figure 4.1** Some examples of lesions from participants in the study.

### 4.3.2.3 Age group and gender distribution

The age and gender distribution of suspected cases recruited into the study were shown to range from 2 years to 60 years which were indicated by the lesion distribution (**Table 4.1**). Eleven lesions were found between age group 1-10 years, 15 lesions were in age group 11-20, 10 lesions were found in age group 21-30, 9 were found in age group 31-40 and 5 lesions each found in age groups 41-50 and 51-60.

| ID | Age | Sex | Lesion | | Occupation | Endemic Community |
|---|---|---|---|---|---|---|
| | | | No | Site | | |
| 001 | 60 | F | 1 | Back | Farmer | Dodome- Awiasu |
| 002 | 4 | M | 2 | Arm | NA | “ |
| 003 | 2 | M | 1 | Head | NA | “ |
| 004 | 30 | F | 1 | Arm | Farmer | “ |
| 005 | 35 | F | 1 | Arm | Farmer | Dodome-Doglome |
| 006 | 10 | M | 2 | Arm | Pupil | Dodome-Awiasu |
| 007 | 20 | M | 2 | Head | Student | Lume-Achiame |
| 008 | 25 | M | 1 | Head | Student | “ |
| 009 | 4 | M | 1 | Arm | NA | Dodome-Awiasu |
| 010 | 52 | F | 1 | Arm | Farmer | “ |
| 011 | 9 | M | 1 | Arm | Pupil | “ |
| 012 | 12 | F | 1 | Arm | Pupil | “ |
| 013 | 10 | F | 1 | Arm | Pupil | “ |
| 014 | 30 | F | 1 | Arm | Farmer | “ |
| 015 | 42 | F | 1 | Arm | Farmer | “ |
| 016 | 38 | F | 2 | Arm | Farmer | “ |
| 017 | 40 | F | 1 | Back | Farmer | “ |
| 018 | 13 | F | 1 | Head | Pupil | “ |
| 019 | 22 | F | 1 | Arm | Student | “ |
| 020 | 14 | M | 1 | Head | Pupil | “ |
| 021 | 18 | M | 1 | Head | Student | “ |
| 022 | 21 | M | 1 | Head | Student | “ |
| 023 | 22 | M | 1 | Head | Student | “ |
| 024 | 30 | M | 1 | Head | Farmer | “ |
| 025 | 48 | F | 1 | Back | Farmer | “ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 026 | 44 | F | 1 | Arm | Farmer | “ |
| 027 | 38 | F | 1 | Leg | Farmer | “ |
| 028 | 33 | F | 1 | Arm | Farmer | “ |
| 029 | 34 | F | 1 | Arm | Farmer | “ |
| 030 | 52 | F | 1 | Arm | Farmer | “ |
| 031 | 14 | F | 1 | Arm | Student | “ |
| 032 | 18 | M | 1 | Leg | Student | Hoe |
| 033 | 16 | F | 1 | Arm | Student | “ |
| 034 | 58 | F | 1 | Arm | Farmer | “ |
| 035 | 41 | M | 1 | Neck | Farmer | “ |
| 036 | 18 | M | 1 | Arm | Student | “ |
| 037 | 14 | F | 1 | Leg | Student | “ |
| 038 | 36 | F | 1 | Leg | Hair dresser | “ |
| 039 | 34 | F | 1 | Arm | Trader | “ |
| 040 | 19 | F | 1 | Arm | Student | “ |
| 041 | 10 | F | 1 | Arm | Pupil | “ |
| 042 | 8 | M | 1 | Leg | Pupil | “ |
| 043 | 17 | M | 1 | Neck | Student | Dodome-Doglome |
| 044 | 21 | F | 1 | Arm | Student | “ |
| 045 | 33 | M | 1 | Neck | Farmer | “ |
| 046 | 42 | M | 1 | Leg | Farmer | “ |
| 047 | 14 | M | 1 | Arm | Student | “ |
| 048 | 18 | F | 1 | Arm | Student | “ |
| 049 | 24 | M | 1 | Arm | NA | “ |
| 050 | 55 | M | 1 | Arm | Farmer | Dodome-Awiasu |

**Table 4.1** Summary of information gathered on the participants in the study.

In the age group 1-10, 6 of the cases were males and 2 cases were female males. In age groups 11-20 were found 7 male and 6 female cases. The 9 cases each in age groups 21-30 and 31-40 were made of 5 males; 4 females and 1 male; 8 females respectively. Within the age group 41-50 were 6 cases, 2 males and 4 females, whereas the 5 cases in age group 51-60 had a breakdown of 1 male and 4 females.

### 4.3.3 Laboratory investigations

### 4.3.3.1 In vitro culture of samples

Several attempts were made to establish in vitro cultures from patient samples, and three of these were successful. The species of the *Leishmania* parasite responsible for CL in Ghana is not certain, so these cultures were subjected to laboratory investigations by various methods, whose results are as summarised in **Table 4.2**.

| **Lab ID** | **Parasite identification/method used** | | |
|---|---|---|---|
| | **PCR (band size)** | **PCR-RFLP** | **Sequencing (% relatedness)** |
| GH005 | *Leishmania* (≈1100bp) | No match | 92.87% to *L. enriettii* |
| GH010 | *Leishmania* (≈1100bp) | No match | 92.87% to *L. enriettii* |
| GH011 | *Leishmania* (≈1100bp) | No match | 92.87% to *L. enriettii* |

**Table 4.2** Laboratory molecular identification of the *Leishmania* isolates from Ghana.

The proof of the isolation of *Leishmania* in the current study was the presence of the promastigotes in the culture flasks after 4 or more weeks of incubation. The isolates were later cryopreserved in 7.5% glycerol.

### 4.3.3.2 PCR reactions and gel electrophoresis

The DNA samples extracted from the promastigote pellets were amplified by PCR using the primers AM1/AM2 to yield a product of band sizes of ≈1,100bp, under the reaction conditions mentioned earlier. The bands generated from the PCR reaction for the samples and the controls (positive and negative controls) are shown in **Figure 4.2**.

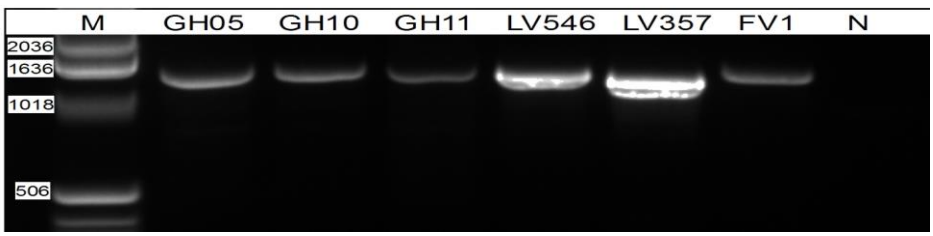


**Figure 4.2** Gel photograph samples from Ghana and positive controls, showing the bands of PCR products. {M – Molecular weight marker; 357 (LV375) – *L. tropica*; 546 (LV546) – *L. aethiopica*; FV1 – *L. major*).

#### 4.3.3.3 Restriction Fragment Length Polymorphism (RFLP)

The identities of the *Leishmania* from Ghana were explored by digested the amplicons of primers AM1/AM2 with endonuclease *Msp1*. The banding patterns generated from the new isolates did not match any of the positive controls (**Figure 4.3**) which are well characterised isolates responsible for cutaneous leishmaniasis in Africa.

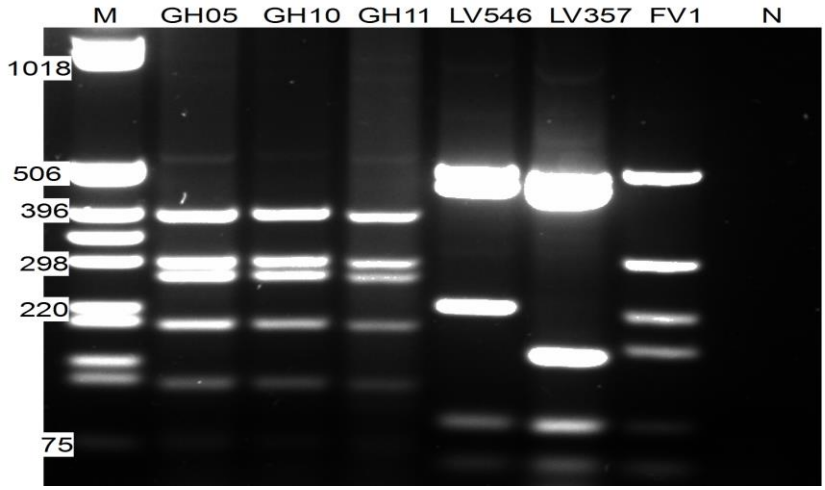



**Figure 4.3** Gel photograph of the restriction enzyme analysis on the samples from Ghana and positive controls. M – Molecular weight marker; 357 (LV375) – *L. tropica*; 546 (LV546) – *L. aethiopica*; FV1 – *L. major*.

#### 4.3.3.4 Sequencing

To further scrutinize the identity of the isolates, PCR products generated using the chromosome 6 primers BN1/BN2 were sequenced. These primers were designed to amplify an intergenic sequence between two ribosomal protein L23a genes to generate a product size of ≈500bp (Dougall *et al*., 2011).

The three sequences from the Ghana isolates aligned perfectly with each other (**Figure 4.4**), but did not match the sequences from any other known *Leishmania*

species in Genbank. The closest match to the Ghana isolates was 92.87% identity with *L. enriettii* (LV90), the remaining species were below 70% identity.

#### 4.3.3.5 Phylogeny

To discover more about the new isolate, the evolutionary history was inferred from the phylogenetic tree (**Figure 4.5**), to show the clustering of the Ghana isolates with other characterised species of the parasite. Included in this analysis were two recently described members of the *L. enriettii* complex, *L. martiniquensis* and *L. siamensis* (Pothirat *et al*., 2014). The phylogenetic trees showed the Ghana isolates to be included in the *L. enriettii* complex and closely related to *L. siamensis* from Thailand (98.3% identity)

```
GH005< GAAGGTCAACACCCTGATCCGCCCGGATGGTCTGAAGAAGGCATATATCCGCCTTTCTGC 60
GH011< GAAGGTCAACACCCTGATCCGCCCGGATGGTCTGAAGAAGGCATATATCCGCCTTTCTGC 60
GH010< GAAGGTCAACACCCTGATCCGCCCGGATGGTCTGAAGAAGGCATATATCCGCCTTTCTGC 60
       ************************************************************
GH005< CGCTCACGATGCTCTGGACACCGCCAACAAGATCGGTCTCGTCTAGCCGGAATTCGAATC 120
GH011< CGCTCACGATGCTCTGGACACCGCCAACAAGATCGGTCTCGTCTAGCCGGAATTCGAATC 120
GH010< CGCTCACGATGCTCTGGACACCGCCAACAAGATCGGTCTCGTCTAGCCGGAATTCGAATC 120
       ************************************************************
GH005< GCGTGACTAGTTTAGGTGACTTTATTTTATCAACAAATAAAGGTAAAAATGAAGTTCGGC 180
GH011< GCGTGACTAGTTTAGGTGACTTTATTTTATCAACAAATAAAGGTAAAAATGAAGTTCGGC 180
GH010< GCGTGACTAGTTTAGGTGACTTTATTTTATCAACAAATAAAGGTAAAAATGAAGTTCGGC 180
       ************************************************************
GH005< TCTTTAGAGGTCGGCAGAGGCTCTGATGGCGGCGTGGTGGCCGGTAACTCCCTATCATGA 240
GH011< TCTTTAGAGGTCGGCAGAGGCTCTGATGGCGGCGTGGTGGCCGGTAACTCCCTATCATGA 240
GH010< TCTTTAGAGGTCGGCAGAGGCTCTGATGGCGGCGTGGTGGCCGGTAACTCCCTATCATGA 240
       ************************************************************
GH005< AATGTGGTGGTGAGCTGCGGCATCCTTGTTCAAGGCAAGGACTCGGCTGTTTCTGTACCT 300
GH011< AATGTGGTGGTGAGCTGCGGCATCCTTGTTCAAGGCAAGGACTCGGCTGTTTCTGTACCT 300
GH010< AATGTGGTGGTGAGCTGCGGCATCCTTGTTCAAGGCAAGGACTCGGCTGTTTCTGTACCT 300
       ************************************************************
GH005< CGGAGCACGAGCTTCTCTTTTCTGAGGATGTATATGGAAGAGGTGGTTGTCATTGGTGCT 360
GH011< CGGAGCACGAGCTTCTCTTTTCTGAGGATGTATATGGAAGAGGTGGTTGTCATTGGTGCT 360
GH010< CGGAGCACGAGCTTCTCTTTTCTGAGGATGTATATGGAAGAGGTGGTTGTCATTGGTGCT 360
       ************************************************************
GH005< TTTTGGACTCTTGCCTGGCTGCTCCTTCAAATGAGCCGAGCTGTTCCGTTTCTTGCGGCA 420
GH011< TTTTGGACTCTTGCCTGGCTGCTCCTTCAAATGAGCCGAGCTGTTCCGTTTCTTGCGGCA 420
GH010< TTTTGGACTCTTGCCTGGCTGCTCCTTCAAATGAGCCGAGCTGTTCCGTTTCTTGCGGCA 420
       ************************************************************
GH005< ACTGACCTCTATTTTTCATCTCTCCTTTCCTACTTATTTTTGCCTTTGGTGATTATCTGC 480
GH011< ACTGACCTCTATTTTTCATCTCTCCTTTCCTACTTATTTTTGCCTTTGGTGATTATCTGC 480
GH010< ACTGACCTCTATTTTTCATCTCTCCTTTCCTACTTATTTTTGCCTTTGGTGATTATCTGC 480
       ************************************************************
GH005< CTCACATGTGTCTCCACTTGCTACAACGTTTCCCACTCGTGCTCCTCCGACAAATCCTGT 540
GH011< CTCACATGTGTCTCCACTTGCTACAACGTTTCCCACTCGTGCTCCTCCGACAAATCCTGT 540
GH010< CTCACATGTGTCTCCACTTGCTACAACGTTTCCCACTCGTGCTCCTCCGACAAATCCTGT 540
       ************************************************************
```

```
GH005< TTGATCAGGCTTAGGGTTTCATTCTCAACGAACCATGGCTGCTGCTCAGAAGACCGCCAA 600
GH011< TTGATCAGGCTTAGGGTTTCATTCTCAACGAACCATGGCTGCTGCTCAGAAGACCGCCAA 600
GH010< TTGATCAGGCTTAGGGTTTCATTCTCAACGAACCATGGCTGCTGCTCAGAAGACCGCCAA 600
       ************************************************************
GH005< GAAG 604
GH011< GAAG 604
GH010< GAAG 604
       ****
```

**Figure 4.4** Multiple sequence alignment of Ghana isolates generated from RPL23a intergenic sequence (BN1/BN2 primers).

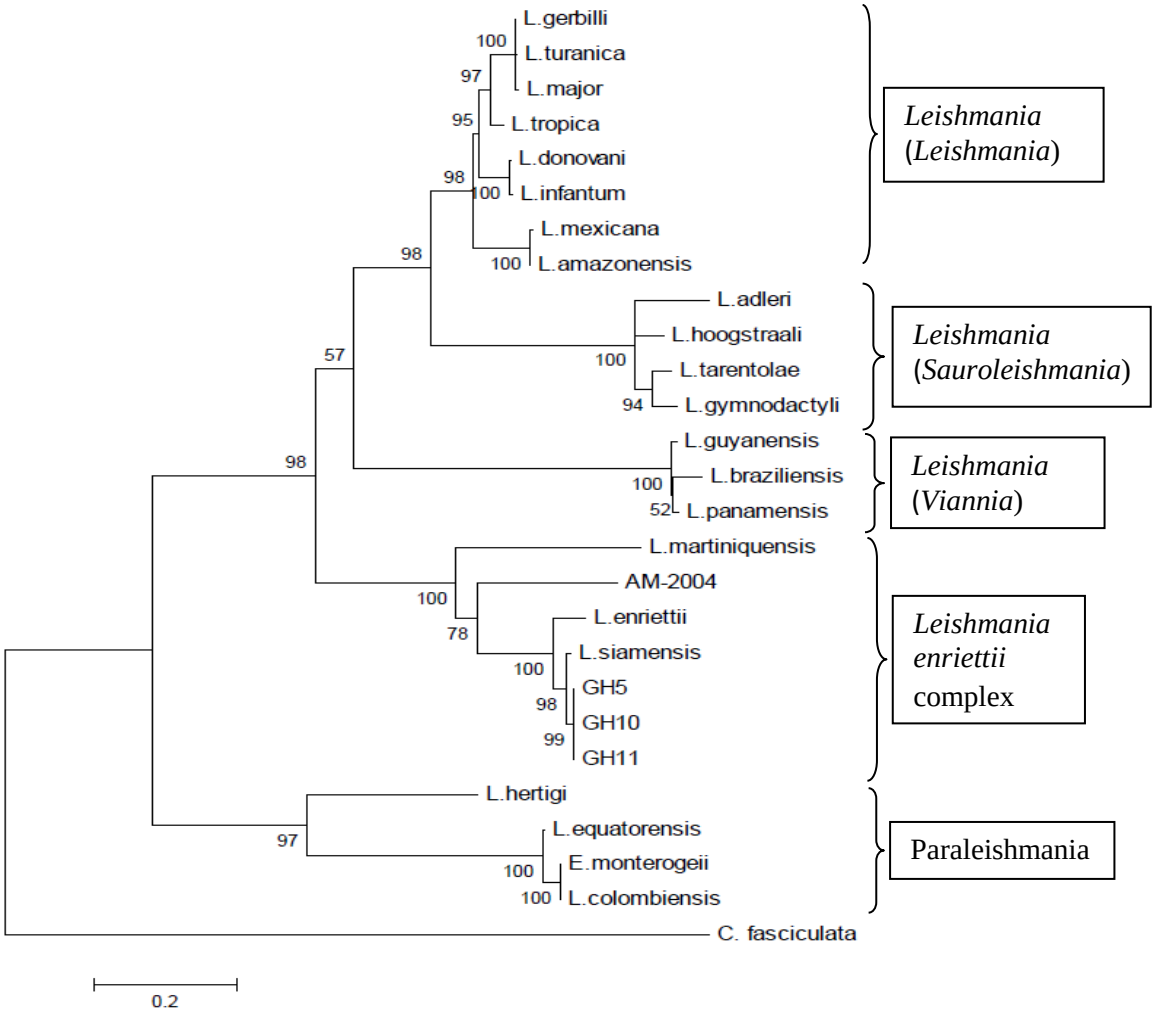



**Figure 4.5** Evolutionary relationships of *Leishmania* species. Example of a phylogenetic tree based on RPL23a intergenic sequences, involving the new species from Ghana and the reference isolates. Also included were newly available sequences for *L. siamensis* and *L. martiniquensis*.

## 4.1 Discussion

Diagnosis of CL often comes with challenges owing to the low parasite levels found in clinical samples. The use of clinical manifestations and geographical location of patients have neither been very helpful, which might also be misleading since fungal (*Paracoccidioides*) and bacterial (buruli ulcer) infections can present with similar symptoms and could interfere with the management and treatment of CL, hence the need find the identity of *Leishmania* in CL patient samples is crucial.

The emergence of cutaneous leishmaniasis (CL) in the southeastern part of Ghana in recent times needs to be addressed so that the public health implications can be fully exploited to enable planning and management in the endemic communities. It is therefore imperative to identify the etiologic agent(s) responsible for the infection.

In pursuance to contribute to the identification of the species of *Leishmania* responsible for CL in Ghana, a number of cases were detected when searches were launched and patients recruited. Although the sample size is very small in this investigation, in addition to the previous investigations (Kwakye-Nuako *et al*., 2015; Fryuff *et al*., 2006; Villiski *et al*., 2007; Kweku *et al*., 2011), a sizable number of cases have been observed over the years with some trends of case distributions in some age ranges. Without a full-scale survey conducted, these observations are consistent with increased susceptibility and/or exposure in younger and economically productive age groups.

Consistent with the current investigation, leishmaniasis is frequently found as an infection among younger people (Shoaib *et al*., 2007), which supports the findings here where majority of the cases were in the younger and productive population (1-30 years in this current study). Similar results were found in a prolonged CL study conducted, with the infections mostly associated with children ≤15 years (Al-Tawfiq and AbuKhamsin, 2004). One of the reasons for this could be that these younger age

groups were seen mostly exposing their bodies, especially where most of the lesions were located. A study has found correlation between body exposure and lesions in some other places in the world where the vector has preferred biting the exposed body parts to those covered by clothes (Talari *et al*., 2006). Hence, in any endemic focus, it is therefore advisable to wear clothes to serve as barrier to prevent the vector from biting and inoculating the agent in to the skin more especially during their active feeding periods. To further prevent the infection, sleeping in the insecticide treated bed-nets could offer some shield preventing the vector from getting in contact with humans during sleeping hours, during which period the vectors are very active (Claborn, 2010; Mondal *et al*., 2010; Courtenay *et al*., 2007).

Though too early to rule out multiple lesions, single lesions clearly characterised this investigation, consistent with some previous work (Soto *et al*., 2013), but contrary to one other study where multiple lesions were 16% greater than single lesions in the study population (AlSamarai and AlObaidi, 2009). It was observed that nearly equal numbers of CL were found in both sexes (male: female 1:1.3) as has previously found (Al-Tawfiq and AbuKhamsin, 2004), suggesting that the vector has no preference for a particular gender. On the contrary, higher number of cases in females than in males was reported from Nigeria (Okwori *et al*., 2001), even though some independent works have reported a reverse trend of CL, being more frequent in males than in females (Weigel *et al*., 1994; Shoaib *et al*., 2007). Reports of infection in females closely matching the males (81:80) as occurred in this study has been found elsewhere (Sharma *et al*., 2005).

This preliminary exploration has found the infection substantially reported in farmers, raising the suspicion that the infection could be closely associated with sylvatic rather than peri-domestic transmission, even though it is premature to make

such assumption considering the sample size, agricultural activities have been linked with CL infections in a similar climate (Yadon *et al*., 2003).

The lesions appeared mainly circular with raised edges and to some extent red margins. Their appearance was relatively uniform, perhaps indicating the presence of a single species responsible for CL, though it is quite early to speculate on this. Some authors have linked specific species of the parasite with different forms of lesions on the victims (Piscopo and Mallia, 2006). However, there is often a wide variation in lesion appearance with a single species. These lesions were found mostly on the upper part of the body compared to lower parts, in support of the vector preference of uncovered parts of the body (Sharma *et al*., 2003).

Molecular identification is the best approach when species of *Leishmania* are to be characterised to the species level. The products generated from the PCR performed on one part of samples on FTA cards yielded positive results, when microscopy had shown negative results on the other part (Belli *et al*., 1998), and FTA cards avoid loss of cultures through contamination or when not successful at all due to limited parasitic load. When the levels or density of parasites in the samples to be used are low, PCR methods have performed well as a diagnostic tool (van Eys *et al*., 1992; Weigle *et al*., 2002), whose generated product could further be analysed at sequence and/or phylogenetic level. It was therefore not surprising that PCR was the most very reliable method for the diagnosis of CL cases (Andresen *et al*., 1996; Weigle *et al*., 2002). The previous studies indicated that molecular techniques should be employed to identify the species in Ghana. The isolates from Ghana were able to be amplified by the AM1/AM2 primers previously designed (Sultan, 2008, PhD thesis). Nevertheless, characterization of *Leishmania* in any disease focus to the species level is important,

especially in situations where therapy will be needed, as well as for epidemiological investigations of newly recognised infections in communities.

The digestion of the PCR products of the AM1/AM2 primers produced an interesting pattern of bands with sizes that were different to bands of the positive controls used, which have also been found to be responsible to causing CL in Old World including Africa. In a new emerging CL focus investigated in Israel in contrast, *L. major* was quickly identified (Faiman *et al*., 2013; Gouzelou *et al*., 2012). The banding patterns of the Ghana isolates were entirely different from bands of reference isolates; *L. aethiopica, L. major* and *L. tropica* controls. The isolate has so far not show closeness to any known banding patterns in our collections after RFLP. This supports the claim that the Ghana parasites could possibly be a characterised species of *Leishmania* (Villiski *et al.,* 2007), which indicates a fascinating result, and requires confirmation. This prompted further investigations such as sequence and phylogenetic analysis.

The three isolates from Ghana aligned perfectly with each other, showing they were from the same *Leishmania* species. The isolate however could not align with characterised species of *Leishmania* parasites used in comparison, an indication that they are a new species of *Leishmania*. The closest relatedness was 92.87% to *L. enriettii*.

On the evolutionary tree using maximum likelihood, the three unknown isolates from Ghana, clustered together to form a separate and distinct clade or branch, distinct from the other characterised species. They were grouped within the *L. enriettii* complex, which includes the human pathogens *L. martiniquensis* originally isolated from Martinique Island in the Caribbean and *L. siamensis* from Thailand (Pothirat *et al*., 2014; Desbois *et al*., 2014). However, *L. enriettii* from Brazil has only been found

in domestic guinea pigs, and is non-pathogenic to humans (Machado *et al*., 1994). This makes the inference of the evolutionary history of the isolates from Ghana interesting but uncertain. This new *Leishmania* species might also have deviated from the conventional *Leishmania* vectors (investigated in Chapter 5), because a different parasite-vector combination with day-feeding midges has been suggested for another member of the *L. enriettii* complex (Dougall *et al*., 2011).

All the isolates from Ghana were from a common ecological origin. However, it is possible the agent(s) responsible for CL in Ghana might include some of the well characterised species of the parasite known in other parts of the world (Gouzelou *et al*., 2012; Siriwardana *et al*., 2007; Guerbouj *et al*., 2001). The analyses have undoubtedly implicated *Leishmania* in the ongoing CL cases in the south-eastern part of Ghana, but the species identity still remains to be named. It is therefore useful to carry out extra work to wholly understand the disease dynamics in Ghana.

# Chapter Five

# Vector Studies

## 5.1 Introduction

The most predominant sand fly vectors in Sub-Saharan West Africa are *Phlebotomus duboscqi* and *P. rodhaini*. These species have been reported in certain parts of Ghana. In Senegal and the Gambia, *P. duboscqi* has been incriminated as a vector of leishmaniasis, although *Sergentomyia* have also been suggested in some cases (Boakye *et al*., 2005; Senghor *et al*., 2011). There has therefore been speculation that *P. duboscqi* could be the vector responsible for cutaneous leishmaniasis in Ghana (Boakye *et al*., 2005), though this is not confirmed.

Many sand fly species have been collected in Ghana, for example between 1997 and 2002 in Navrongo in northern Ghana 14 different species were reported, 13 of which were of the genus *Sergentomyia* and one being *P. duboscqi* (Boakye *et al*., 2005). Additionally, 17 sand fly species, including *P. duboscqi* and *P. rodhaini*, have been identified from collections in the present endemic area of the Ho district from 2004 to 2005 (Boakye *et al*., 2005; Fryauff *et al.*, 2006). The presence of *P. rodhaini* among the identified sand fly species might explain the uncharacterised *Leishmania* found by Villinski *et al*. in 2007 in their human sample collections. Perhaps it can harbor the proposed new *Leishmania* species. It has a preference for rodents as a blood meal in Sudan (Fryauff *et al*., 2006).

Nonetheless, the low numbers of the *P. duboscqi* and *P. rodhaini* collected raises doubts about their role as vectors of *Leishmania* in Ghana (Fryauff *et al*., 2006). On the contrary, huge numbers of man-biting *Sergentomyia* species, *S. schwetzi*, *S. clydei* and *S. adleri*, have been collected in various parts of the country including the Ho district, with abundantly high numbers of *S. schwetzi*, but their role as vectors is yet to be defined (Boakye *et al*., 2005). In one recent entomological study in the endemic focus in Ghana, *Sergentomyia* showed 99.5% abundance compared to the low densities of *P. duboscqi* and *P. rodhaini* (Desewu *et al*., 2009). In one study of the vector of *Leishmania* in Ghana, *S. africana africana*, *S. squamipleuris* and *S. ghesquierei* were collected but no *Phlebotomus* species (Odoom, 2008). The evidence of *Sergentomyia* being incriminated as vectors of the Ghanaian *Leishmania* have been amplified due to the recent detection of DNA in the collected species (Nzelu *et al*., 2014), however, as has been mentioned elsewhere (Bates, 2007), there has not been any success of detecting the live parasites in the dissected vectors collected over the years. There has also been evidence incriminating *Sergentomyia* by detection of

varying blood meal sources other than lizard blood, in different forms of disease endemic foci, including Ghana (Desewu *et al*., 2009; Mutinga *et al*., 1994), as well as evidence associating canine leishmaniasis with *Sergentomyia* in Senegal (Senghor *et al*., 2011).

The reservations as to whether *Sergentomyia* species could possibly transmit any pathogenic species of *Leishmania* have been raised by several lines of evidence. The genus *Sergentomyia* is an accepted vector of the non-pathogenic *Leishmania* species responsible for the lizard form of leishmaniasis (Sadlova *et al*., 2013; Elnaiem *et al*., 1997; 1999; Senghor *et al*., 2011). However, even though implicated as vectors, examination for the presence of parasites is not always successful, for example *P. orentalis* (Gebre-Michael *et al*., 2007; 2010). Therefore, a similar situation might be happening in Ghana regarding *Sergentomyia*. In spite of its inability to satisfy one of the criteria for a vectorial role with its low man-biting activity, *P. rodhaini* is still implicated as a vector of *L. donovani* in Sudan (Elnaiem, *et al*., 2011). In an *L. major* endemic focus in Kenya with *P. duboscqi* as the confirmed vector, there was high infectivity shown in *S. ingrami,* which were regarded as possible secondary vectors of *L. major* (Mutinga *et al*., 1986).

The sand fly species *Lutzomyia longipalpis* is regarded as a permissive vector of *Leishmania* because it shows high susceptibility to various species of the parasite, supporting heavy infections for species of *Leishmania* compared to the development of parasites in *Sergentomyia* (Sadlova *et al*., 2013). The vector, *Lu. longipalpis* is regarded as an "eclectic feeder", exhibiting diversity for host blood meal preference (Macedo-Silva *et al*., 2014), which could account for their susceptibility to most of the species of the parasite. For the parasite to survive in the vector host, many factors come to play. One such factor has been found to be the regulation of the sand fly gut

enzyme levels. For instance, *L. mexicana* seems well adapted to the gut of *Lu. longipalpis* via downward regulation of trypsin, which enhances the survival of the parasite (Sant'Anna *et al*., 2009). Though they are natural vectors of *L. infantum* in the New World, this particular characteristic has made *Lu. longipalpis* very useful in the investigation into the potential vectors of the different species of the parasite, aside from the *L. infantum* they naturally transmit. Mechanisms where the promastigote forms of the parasite ensure they are not excluded from the gut of the vector in the course of the defaecation are vital in infection establishment and in subsequent transmission of the parasite (Vaidyanathan, 2004). For an infection to be sustained, there has to be vector-*Leishmania* species compatibility, in other words the vector should be a natural host of that particular species (Santos *et al*., 2014). Some species of *Leishmania* have been found to reduce the proteolytic activities of their natural vectors, which might have enhanced their infectivity in the vector host, whereas the same proteolytic activities have been found to increase in certain non-natural-species interactions, hence infectivity could not be sustained (Schlein and Romano, 1986)

### 5.2 Exceptional vectors of emerging *Leishmania* species

Insect vectors are a fundamental component in many diseases and their outcome. They play an important role as one of the hosts to perpetuate vector-borne diseases by acting as a bridge between the causal agent and the reservoir or definitive hosts. *Simulium* spp. and *Culicoides* spp. have both been suspected to be transmitting the same parasite, *Mansonella* sp. in the same disease endemic focus (Shelley and Coscaron, 2001; Lowrie and Raccurt, 1981), indicating that there can be varying vectors transmitting similar agents. It will not be surprising if this is the case for other parasites. For instance, *Leishmania* parasites may not be exclusively transmitted by

sand flies, and there is room for exceptions (Killick-Kendrick, 1999). There are no exclusive rights to "one vector, one agent".

There currently is no evidence of midges as vectors in Ghana. There is only speculation, since they have been implicated as vectors of the closely related species from Australia ("*L. australiensis*"), they could possibly be the vectors in Ghana as well. There is also no information on the identity of species of biting midges in Ghana, which an ongoing study seeks to unravel. The only evidence is the abundance of midges in the endemic community, which includes man-biting species whose presence is felt in early evenings and mornings.

The male and the female *Culicoides* species feed on nectar, but female biting midges include haematophagous, as well as nectarphagous and entomophagous, insects (Filimonova, 2005). In addition to the nectar, the female flies require blood for their egg development (Mellor *et al*., 2000). They have been implicated as vectors of viruses to various mammals and animals and have recently received a lot of attention in veterinary medicine as a vector for bluetongue virus in cattle in Europe (Lassen *et al*., 2012; Filimonova, 2005; Megahed, 1956). Though they have been found to transmit parasites to various animals and mammals, not much is known about their parasitic vectorial competence. Some evidence has shown *Culicoides* species to be involved in transmission of parasites to lizard, birds, horses, and other mammals, transmitting parasites like *Onchocerca*, *Mansonella*, and other filarial worms among others. But their incrimination to be the vector of other parasitic agents including *Leishmania* is not proven (Dougall *et al*., 2011). However, some good reasons to consider vectorial roles for *Culicoides* species are their abundance and wide dispersal (Lassen and Nielsen, 2011). Though these factors can help in a vectorial role, other factors also need to be satisfied to incriminate a vector for *Leishmania*. These include,

amongst others: the availability of the vector and the reservoir host in the endemic focus (Sadlova *et al*., 2013); ability of the vector to feed on the hosts, i.e. the display of host-biting behaviour (Sadlova, *et al*., 2013); ability of the parasite to fully develop to the infective stage in the vector beyond blood meal digestion; the identity of the parasite in the vector collected from the wild and that in the hosts; and the vector is able to transmit the parasite through feeding on the hosts (Lawyer and Perkins, 2000; Killick-Kendrick, 1999).

*Culicoides* species have characteristics like pool-feeding that are required in a *Leishmania* vector. Their cosmopolitan nature could also contribute to their emergence as a vector to many infectious agents. They are found everywhere on the globe, from the tropics to the tundra, and from sea level up to a height of 4000m, with the exception of environmental conditions at the extreme polar regions (Lessen *et al*., 2012; Mellor *et al*., 2000). They also exhibit longevity, which is a benefit as a competent vector for parasitic agent(s). A few species have been found to survive for 92 days after their blood meal, others have been found to survive for a few weeks to several months (Mehlhorn *et al*., 2008; Goffredo *et al*., 2004). *C. sonorensis* has been speculated to survive for three months after their blood meal (Lysyk and Danyk 2007). They are also able to survive varying temperatures (Lassen and Nielsen, 2011). For instance, different species such as *C. sonorensis* and the *C. obsoletus* complex have been found to survive temperatures ranging from 10°C to 25°C (Lysyk and Danyk 2007; Goffredo *et al*., 2004). Another feature which might contribute to their robustness as carriers of pathogens is their diverse host preference (amphibians, reptiles, other insects, birds, mammals), and their quick blood meal digestion (Lassen *et al*., 2012; Lassen and Nielsen, 2011). As hematophagous insects, they are reported to digest their blood meal within 48 hours after feeding (Megahed, 1956), which

probably explains the reason for their recurrent feeding behaviour, as opposed to the 3.5-4 days which is more typical in other species (Filimonova, 2005). *Culiciodes* species have the unique behaviour of expending nearly 90% of their time after blood feeding in digesting its feed for egg development, hence are not frequently seen except in flight (Mullens *et al*., 2004). Aside from their ability to transmit pathogens of public health and veterinary importance, they can also be present as a biting nuisance. For instance, their painful bites can produce dermatitis which affects the skin and the general health of their hosts (la Puente *et al*., 2015).

## 5.3 Material and Methods

### 5.3.1 Vector infection study

Cultures of GH5 were grown from infected macrophage THP1 cell lines in M199 medium, to the promastigote concentration of $1.24x10^6$cells/ml. This promastigote concentration was cryo-preserved to be used to infect the permissive vector *Lutzomyia longipalpis* and the midges.

### 5.3.2 Blood meal preparation

1ml of sheep blood was put into three 1.5ml microfuge tubes. The tube and the contained blood were centrifuged at 7,000 rpm for 3 minutes at 4°C. The supernatants (serum) were transferred into new microfuge tubes and placed in a heating block to heat inactivate at 56°C for 1 hour, while retaining the residual pellets under sterile conditions in the culture hood. The pellets were washed in 2-3 changes by re-suspending them in 0.5ml M199 (without additives) and centrifuged, the supernatants were discarded after each wash. The washed pellets and the heat-inactivated serum were reconstituted by combining them and kept at room temperature, ready for use.

### 5.3.3 Parasite preparation

The *Leishmania* isolate GH5 was prepared to a final density of $2.0x10^6$cells/ml. Approximately 5ml of culture was centrifuged at 13,000 rpm for 3 minutes. The pellets of the parasites were re-suspended in 1ml M199 (without additives) for a second wash by centrifuging at the same speed. To make the blood meal for the sand fly infections, the pellets were gently re-suspended into a final volume of 2 ml by adding the prepared heat-inactivated blood, this was then used to feed the sand fly. Thin blood film slides were prepared from the blood meal, fixed in absolute methanol and stained with Giemsa, to know the stage of the promastigote used for the infection. A drop was used to prepare thin blood smears for air drying, followed by staining.

### 5.3.4 Feeding to establish infection

The prepared chicken membrane was filled with a 2ml blood meal and placed in the membrane feeding apparatus (HEMOTEK) connected to the feeding block which provides temperature of 37°C to the feeding membrane, which is ideal for flies to feed. The feeder was placed onto a cage of 5 day old sand flies for 2 hours, for the flies to feed. The blood fed females were then selected and placed into a new cage and kept in 24°C incubator for 7 days.

### 5.3.5 Dissection and examination

Ten flies were dissected each day from day 1 – 7, and the gut (foregut, midgut and hindgut) contents were examined with x40 lens under the microscope for colonisation, attachment and development into the stages of *Leishmania* parasites. The flies were mounted in cold PBS with 0.05% Tween.

### 5.3.6 *Leishmania* GH5 and biting midges

Five day old midges, *Culicoides sonorensis,* were obtained from The Pirbright Institute, UK, and were infected with $2x10^6$/ml cell density of GH5 promastigotes for

1 hour, prepared as described above. The fed midges were separated from the unfed midges and kept in a separate cage for onward dissection as previously mentioned.

## 5.4 Results

The potential vector of *Leishmania* species responsible for CL in Ghana is still unknown which sparked the investigation. To further probe this, the post blood meal infection establishment in two insects, *Lu. longipalpis* and *C. sonorensis*, were studied.

### 5.4.1 Infection and development of *Leishmania* GH5 in the vectors

The infectivity and parasite development in both vectors were followed from day 1 to 10 post blood meal, by dissecting these vectors to examine the gut contents. The various levels of infection were classified as heavy (>1000 promastigotes), moderate (100-1000 promastigotes) and light (<100 promastigotes) infections.

In **Figure 5.1** it can be seen there was gradual decreasing level of infection from day 1 to day 3, after an overall average of 65% infection was established in *Lu. longiplapis* on day 1, which was maintained in the first 2 days post blood meal with various levels of infection. At day 1, the infections were classified as 50% heavy and 15% light with no moderate infections recorded. The same 65% overall infection rate was retained in day 2 with 10% of day 1 heavy infections changing to moderate infections, 40% heavy and 15% light infections remained.

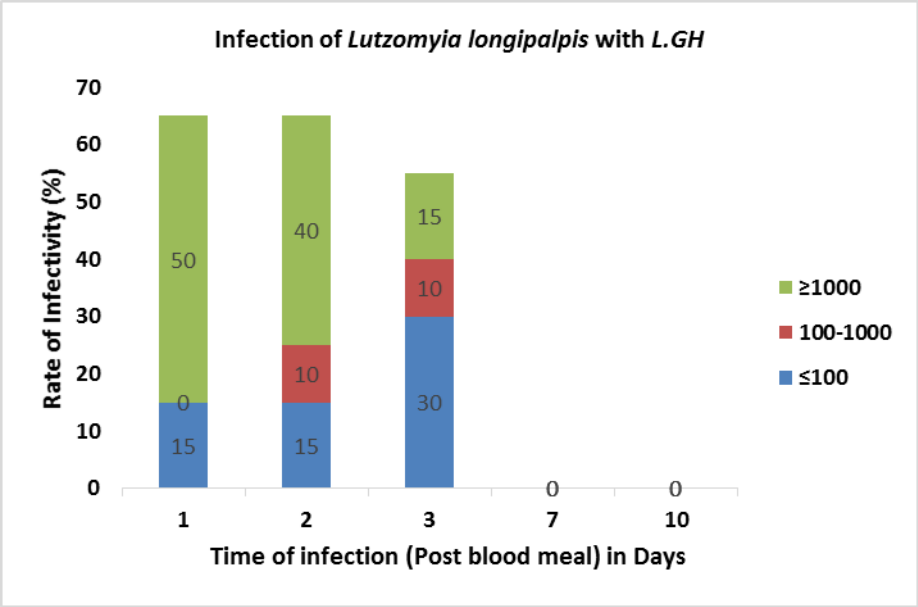



**Figure 5.1** *Lutzomyia longipalpis* infection with *Leishmania* GH5. Combined results from two independent experiments.

At day 3, the infectivity had reduced to 55% with heavy, moderate and light infections recorded as 15%, 10% and 30%, respectively. The reduction in heavy infections was accompanied by an increase in light infections.

Blood meal remnants were observed in the midgut from day 1 to 3 in this vector, an indication that the blood meal was not fully digested at day 3. Parasites could be seen in the blood meal in the peritrophic matrix, which might have offered the parasites protection from the gut content of the vector. The enclosed parasites in the peritrophic matrix were localised in the midgut of the vector. Even though there were fast moving slender nectomonad-like promastigotes on day 3, they were not sustained after day 7 to 10, there were no infections established. There was also complete digestion of the blood meal by day 7, with development of eggs in all the dissected *Lu. longipalpis*. Since there were no infections beyond day 3 there could not be colonisation of the stomodeal valve, a scenario which plays significant role in the mechanism of infection by the vector. It could therefore be inferred that the parasites

in the *Lu. longipalpis* were excreted with faeces after the digested. In other words, the promastigotes could not develop any attachment mechanism to withstand the current in the gut of this particular vector after digestion, and relied solely on their enclosure in the peritrophic matrix to live for a while in the vector.

However, the other vector, *Culicoides sonorensis*, exhibited entirely different characteristics with overall average infection rate of ≈ 80% (**Figure 5.2**).

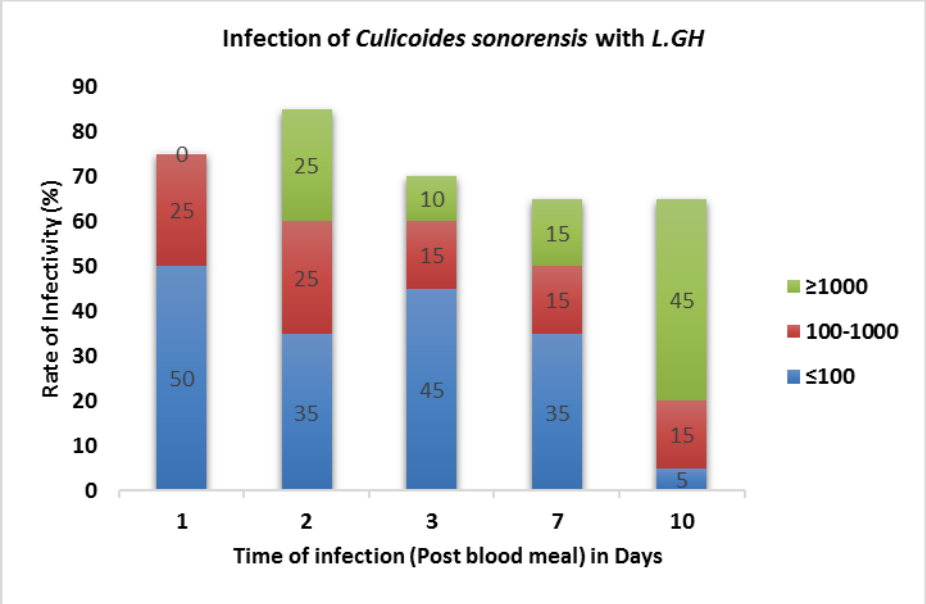



**Figure 5.2** *Culicoides sonorensis* infection with *Leishmania* GH5. Combined results from two independent experiments.

On day 1 75% of the midges displayed infections, there were no heavy infections seen (more than 1000 promastigotes/midge), but 50% light and 25% moderate infections. Infection rate increased to 85% in day 2, which may be simply due to experimental variation or perhaps growth of parasites so they became more obvious. Supporting the latter interpretation, light infections were reduced to 35%, 25% moderate infections remained, but interestingly 25% of the midges displayed heavy infections. Also, at this time there was complete digestion of the blood meal, being completed by 44 hours in the *C. sonorensis* in this experiment. During this time

the parasites transformed to long slender nectomonad-like promastigotes. The fast transformation could be due to the quick digestion of the blood meal, which will be followed by defaecation, with the possibility of the parasites getting excreted from the midgut of the vector. The parasites would then have to develop mechanisms to survive, if midges were indeed the vectors of this *Leishmania* species. Egg development had started at day 2 and continued to day 7. On day 3 a reduction in infections was seen down to 70%, but still 15% moderate infections and 10% heavy infections were recorded, and there was massive egg development in all the dissected insects. Beyond day 3 to 10, there were still infections maintained to a level of 65% at various infection intensities. By day 7, 35% light infections and 15% each of moderate and heavy infections were sustained. However, the promastigotes were now often in the region of the stomodeal valve at the midgut/foregut junction, and occurred in large masses (**Figure 5.3**). It was not clear what was holding these masses together, but it could possibly be PSG-related material.

In addition, promastigotes were observed in other parts of the midgut (**Figure 5.4**). At the same time there were some promastigotes transformed from leptomonad-like to stumpy haptomonad-like forms. These are clear indications that the parasites had developed some mechanisms to survive beyond staying in the peritrophic matrix, but the nature of these is unknown. No obvious gel-like PSG substance was observed by microscopy despite colonisation of the anterior midgut by the parasites, although it could be present but at a low concentration or lacking in the colouration seen in sand fly infections. One idea was that, following the escape from the peritrophic matrix the promastogotes were exposed to midgut content, PSG might help to protect the parasites from the midgut digestive enzymes. For the parasites not to have been excreted after digestion and defaecation, implies that the parasite developed some

means of attachment to the epithelia walls of the midgut, to withstand the peristaltic current in the gut. In some of the midges infections were maintained up to day 16 post blood meal.

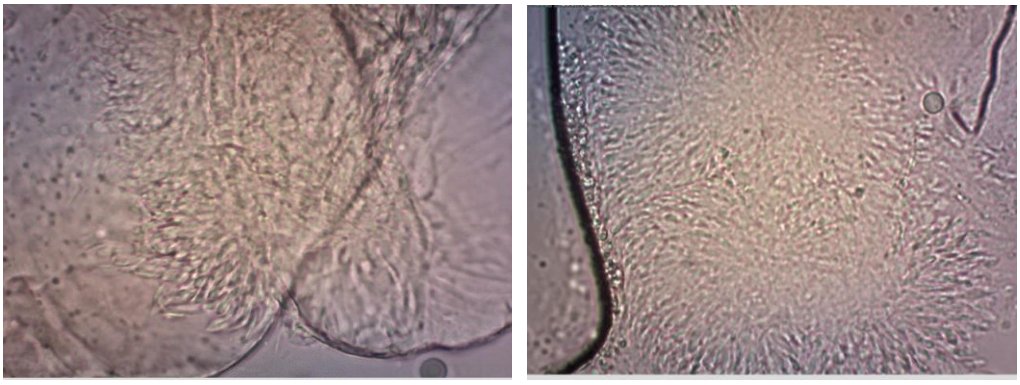


**Figure 5.3** Aggregates of *Leishmania* GH5 promastigotes seen in the stomodeal valve of *C. sonorensis*. Phase contrast microscopy at x400 magnification.

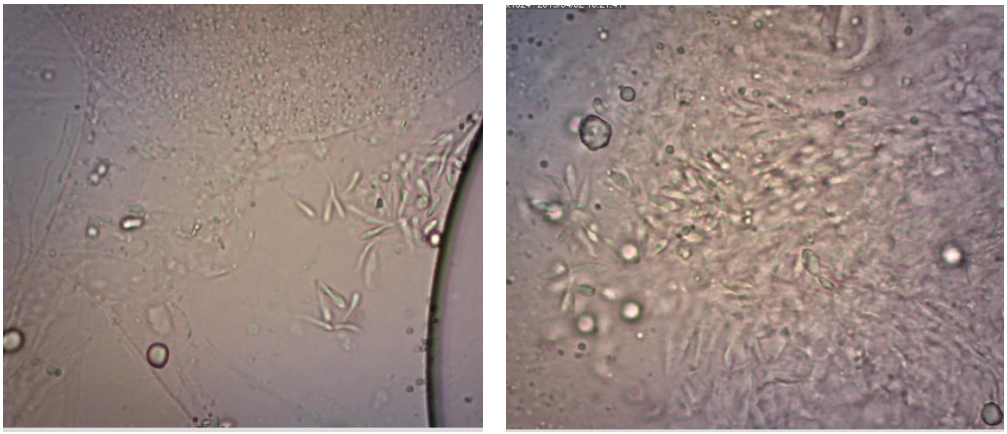


**Figure 5.4** Promastigotes observed in gut locations other than the stomodeal valve of *C. sonorensis*. Phase contrast microscopy at x400 magnification.

At day 10 there were heavy infections in 45% of the midges, 15 % with moderate and 5% with light infections, with a total 65% rate of infections as at day 7. In midges with light infections very few had the parasites localised in the stomodeal valve. However, in the case of moderate and heavy infections almost all the parasites colonised the stomodeal valve from day 7 and beyond. The stomodeal colonisation is very significant as it is an important mechanism playing a role in the regurgitation in sand fly vectors. It is also intriguing how fast (44 hours) the parasites became established into gut of the midges, knowing that it takes 3 days or more for

*Leishmania* to become established in their sand fly hosts, which is the period until blood meal digestion is completed.

### 5.5 Discussion

Since the vectors are instrumental in *Leishmania* transmission in the CL endemic focus of Ghana, it is important to investigate the potential vectors. Here two available colonies of insects were used, but it is important to note that neither of these model vectors can be the actual vector species of Ghanaian *Leishmania*. However, they may give some indication as to the identity of the real vector. There were significant differences in the intensities and the rate of parasite infections in the two vectors. Potential causes of such differences seen in previous studies that relate to digestive physiology include peritrophic matrix (PM) kinetics and timing of defaecation in the vectors after blood meal digestion.

The PM has both a protective role and represents a barrier that must be negotiated (Pimenta *et al*., 1997). The PM has a role to play in helping the parasite to adapt to the transitional environment where it finds itself. The timing of the PM breaking significantly affects the parasites' survival or elimination from the gut of both permissive/natural and resistant vectors. The normal trend of the PM degradation at day 3 or 4 in the sand fly vector was observed, but infectivity beyond PM degradation was nil. The relatively quick degradation of the PM has influence on the infection establishment, as was observed in post blood meal infectivity in *Lu. longipalpis* (Sadlova *et al*., 2013).

Colonisation of the anterior mid-gut of the vectors of *Leishmania* parasites is a requirement for onward transmission of the parasite by the vector during biting (Sadlova *et al*., 2013). However, that was not the case in *Lu. longipalpis* in this study. The reason(s) as to why there were no infections establishment in this vector could not

be clarified by the current investigation. For infections to be established in the vector, the parasite would have to resist certain challenges in the gut of the vector. Among such challenges are proteolytic enzymes in the gut of the vectors which is produced to expedite blood meal digestion. The presence of some species of the parasite can either up- or down-regulate the proteolytic enzymes, which can influence their survival in the gut of their vectors (Santos *et al*., 2014). This may have had effects on the parasite in establishing infection. Many existing data available have demonstrated that in a case where the *Leishmania* promastigotes are able to induce digestive proteases reduction in the gut of the vectors, it has been to the advantage of the parasite in the infection establishment (Santos *et al*., 2014) and possibly the parasites selective fitness. In a study on a diet which reduces the production of the proteases, the survival of *L*. *donovani* increased in an unnatural vector *P*. *papatasi* (Schlein and Jaccobson, 1998). Fast digestion was proposed to play a role in non-establishment of infection in *Sergentomyia* vectors (Strelkova, 1974; Lawyer *et al*., 1990), but where blood meal digestion was delayed for 3 days, there was still no infection as in the case of prolonged digestion in *S. schwetzi* even though it was influenced by decreased temperature (Sadlova *et al*., 2013). This demonstrates that prolonged or delayed digestion does not wholly affect infection development in the sand fly vectors. It seems to be more to do with the gut movement (defaecation) than the digestion, which affects the survival of the parasite in the gut of the vector. According to Sadlova *et al*., (2013), the relative movement of the parasite from the midgut of the vector *Sergentomyia* through defaecation was more impactful than quick digestion with regards to parasite retention to constitute infection in the vector. This was mediated by the degradation of the PM.

Interference with the PM played a significant role on the infection development in *L. major-P. papatasi* interaction, when chitinase, to reduce PM formation, was added to the blood meal (Pimenta, *et al*., 1997), suggesting that at the first 3 days of the infection of the GH5 with *Lu. longipalpis*, the PM would have ostensibly been formed to protect and retain the parasites in the midgut of the *Lu. longipalpis*. If the parasite at the time of dissolution of the PM could not transform to the forms (highly motile leptomonad and nectomonad promastigotes) which could attach to the epithelia of the midgut of the vector, the parasites could be swept away by the gut content movement (Sadlova and Volf, 2009). Though important as a barrier, the degraded PM at the suitable time exposes the trapped parasite to salivary-derived signal molecules. This aids their rapid transformation to flagellated highly motile forms (leptomonad and nectomonad promastigotes) to survive the next period in the gut of the vector, by attaching to the mid-gut epithelium (Wilson *et al*., 2010; Bates and Rogers, 2004), such forms were seemingly present in the *L. longipalpis*-GH5 interaction. The parasites suspected to be enclosed in the PM in day 3 were much different from the promastigotes that initiated the infection. The former were highly motile and slender forms. This is in conformity with the fact that an intact PM though delays transformation of the promastigote from procyclic to other forms for attachment to the mid-gut epithelia (Sadlova *et al*., 2013), there were still some form of transformation in the parasites since there were still blood in the vector, indicating no defaecation. It would be clear if these parasites had transformed to nectomonad-like promastigotes and attached to the epithelium of the midgut to perpetuate infections beyond day 3 in this particular *Lu. longipalpis*-GH5 interaction, but the opposite happened, in which case they were unable to survive, due possibly to the defaecation by the vector, as previously reported (Sadlova *et al*., 2013). Enough time

was provided for these parasites to attach when they were freely swimming the midgut at the delay of the defaecation. This implies that there are additional factors to the attachment other than to delay defecation in the permissive vector.

*Lutzomyia longipalpis* is susceptible to most well characterised species of *Leishmania* (Volf and Peckova, 2007). However, it has displayed resistance to this new species of *Leishmania* isolated from Ghana. Parasite susceptibility has been attributed to several factors. In mechanical obstruction of cardiac or stomodeal valve for instance, the parasites could be retained, develop to infective forms and facilitate their transmission through the "blocked fly" phenomenon and regurgitation (Volf *et al*., 2004). During the obstruction process, the stomodeal valve is compromised through the colonisation by the parasites, which occlude uni-directional flow of the blood meal into the midgut (Jefferies *et al*., 1986). This enhances regurgitation on second feeding releasing caught up parasites for transmission to hosts, hence the colonisation of the stomodeal valve is very important in parasite transmission. The enhancement of the blockage and colonisation requires a gel-like plug (Rogers *et al*., 2002; 2004) which was absent in the *Lu. longipalpis* infections.

Pathological obstructions have also been detected during parasite localisation in the mid-gut of the vectors, which has been boosted by chitin-lytic enzymes which destroy the chitin lining of the stomodeal valve (Schlein, 1991; 1992). The enzyme production is inhibited by the presence of a blood meal, and after digestion compromises the structural integrity of the stomodeal valve, but this could only affect transmission when there were retained parasites which was lacking in *Lu. longipalpis*. There is also a record of detachment of the stomodeal valve lining from its epithelial walls, a clear demonstration of structural destruction during infection in *Lu. longipalpis-Leishmania* interaction (Walters, 1993). The filamentous structure at the

apical end of the stomodeal valve connects the cell membrane to the inner lining of the valve, which together controls the functions of valve. Such arrangements are destroyed and degraded during infection in the vector (Volf *et al*., 2004), apparently by chitinolytic enzymes. This enzyme and others have been found to degrade the chitin lining detaching the lining from cells to expose tissues to the effects of promastigote secretary gel (PSG) (Rogers *et al*., 2002; Volf *et al*., 2004). In uninfected vectors, the integrity of the rich filamentous chitin apical layer of the stomodeal valve is maintained, contrary to the detached chitin layer at the apical end with degraded filamentous structures, in infected vectors (Volf *et al*., 2004). It is important that the localisation of the stomodeal valve plays significant role in the lives of the parasites in their transition to their next vertebrate hosts destination. It is therefore becoming clear that the permissive vector *Lu. longipalpis* is refractory to the *Leishmania* parasite isolated from Ghana.

The development of the *Leishmania* parasite in blood sucking insects is of paramount interest due to dynamics of development in the vectors. *Leishmania* parasite have been found to develop in *Culicoides nubeculosus* until 2 days post blood meal but reported not to have survive beyond this period (Seblova *et al*., 2012). In this current study, *Culicoides sonorensis* was infected to investigate a possible role for midges, since there are similar family of midges in the endemic area in Ghana, though the identity of the *Culicoides* is not known. In the *Culicoides sonorensis* the digestion was within 44 hours, faster than anticipated, which one would have thought could downplay the infectivity and its rates in this vector. The swift digestion of blood meal in this experimental vector is in line with pervious work done where *Culicoides* species have been found to have digested their blood meal within 48 hours (Megahed, 1956). Fast digestion in some *Sergentomyia* has been blamed to have negatively

affected infection establishment in this vector (Strelkova, 1974; Sadlova *et al*., 2013). On the contrary, the fast digestion appears to have had no negative impact on infectivity in the *Culicoides*. For these new species to have adapted to the mid-gut of the *Culicoides sonorensis* within such a short period of digestion (44 hours), would denote that, the parasites would have evolved such adaptive features as some point in their natural environment. It is recorded that, after 72 hours of blood meal, the stomach become empty and the PM membrane is discharged with the faeces in well studied *Culicoides* species (Megahed, 1956). To this end, if infections is not established in 3 days in *Culicoides*, the parasites could perhaps be excreted, which was not in the case of these *Culicoides*, rather the infectivity was retained after day 3 and onwards. The most intriguing is the adaptation of the *Culicoides* to these new species of *Leishmania*, to which the permissive sand fly vector was resistant. The PM thus functions importantly by limiting the number of promastigotes that survive to infective stage to colonise the stomodeal valve in *C. sonorensis*. This might have been heightened to support the fast development of the parasite in the *Culicoides sonorensis*, since parasites survival in any host is dependent on how fast the parasite can develop (Hurd, 2003). A chitinous PM is present in several haematophagous Diptera, which is formed by the epithelium each time the midgut becomes filled with blood meal but it is evacuated with the excreta when digestion is completed (Megahed, 1956). The PM could influence the penetration of parasites into the mid-gut walls, an assumption that the disease causing agent transmitted by these haematophagous insects, is dependent on the characteristics of the PM (Megahed, 1956; Yaguzhinskaya 1940).

Even though less differentiated mid-gut, as it had been noticed in some species of *Culicoides* (Megahed, 1956), it could still augment colonisation by this new species

of the *Leishmania* parasite. The blood meal seemed concentrated in the mid-gut at the early stages, which could have facilitated the colonisation of the parasite in the stomodeal valve, as a way to manage localisation of the meal. This apparently might help the contraction of the mid-gut centrally, upon encounter with blood meal, with characteristic hourglass shape as in the case of *Culicoides nubeculosus* (Megahed, 1956). Blood meal movements are well regulated by a dual purpose sphincter, which holds back the blood meal allowing only waste products out as well as regulating the opening of the Malpighain tubules preventing the faecal matter entering back to the blood meal in the gut (Megahed, 1956).

The gut structural differences in the vectors and the relative interfaces with this parasite in no doubt would contribute to their susceptibility or resistance to infection. As to whether the morphological differences between the mid-gut in *Culicoides* and *Lutzomyia* species have played a role in the differential infectivity could not be fully examined. In certain *Culicoides* species, there was found to be striations in the stomach wall, which is lacking in certain species of some sand fly species such as *Phlebotomus papatasi* (Megahed, 1956). There could have been certain (physiological, mechanical, anatomical) adaptations evolved due to the vector-parasite interactions, to the parasites' advantage. For the parasite to have survived various activities in the midgut of the *Culicoides* suggests that they were able to induce decreased digestive enzymes production to their advantage. Similar reduced production of proteases have occurred to prolong digestion for the promastigotes to have access to nutrients for their development (Dillon and Lane, 1993). The time of the digestion of the blood meal in the *C. sonorensis* however, appeared faster in this current experiment.

Experimental infection of the species of *Leishmania* with their natural and unnatural vectors have produced varying levels of proteases to either increase or decrease the parasites development in the vectors (Sant'Anna *et al*., 2009). In the current work, there were no assessment of these, but there could have been production of proteophosphoglycans which have been found to protect the parasites from harsh mid-gut conditions (Secundino *et al*., 2010). Differential pH conditions of different vectors in the presence of blood meal could have also contributed to the survival of the promastigotes (Bates and Tetley, 1993). Conducive pH in the *Culicoides sonorensis* could have supported the survival of this new species of *Leishmania*. In their natural vector *Lu. longiplapis*, *L. infantum* promastigotes could reduce the pH in the gut of the vector to invariably decrease the activity of the proteolytic enzymes to enhance their development (Santos *et al*., 2014), as has similarly been found in *P. papatas*i - *L. major* interactions (Schlein and Romano, 1986). The lower pH levels in infected vectors have been observed as early as between 48-55 hours after infection when there is still blood meal, and re-acidification when the blood meal is completely digested (Santos *et al*., 2014; Santos *et al*., 2008), after which the parasites would have been established and developed in the mid-gut and would not be affected by increased pH (Bates, 1994). Elsewhere in other haematophagous insects, the reduced pH levels and their interplay of reduced proteolytic activity have slowed defaecation (Caroci and Noriega, 2003), which could hinder the elimination of the parasites from the gut until infection is established. It is not clear if this new *Leishmania* species have had *Culicoides* species as a natural vector in the wild, to have manipulated their survival in this experimental study. There could only be a prediction that the parasite competed favourably with the vector for nutrients for their development in the midgut of this vector.

### 5.6 Conclusion

To conclude, the human pathogenic Ghanaian *Leishmania* parasite heavily infected *Culicoides sonorensis* (without metacyclic promastigotes), colonizing the midgut and stomodeal valve up to and even beyond day 10. This supports the idea that midges could be vectors of this new species of the parasite in Ghana, and successfully transmit the parasite. Since midges are repetitive feeders, a second blood meal could help the parasite to probably survive longer and trigger molecules might help them develop to the metacyclic promastigotes. The different morphological structure of the *Culicoides* and sand fly needs to be studied in detail. Moreover, the mesh-gelatinous-like appearance somewhat around the parasite in the midgut of the *Culicoides* requires further investigation.

## Chapter Six

## 6.0 Rapid test diagnosis (CL Detect™)

### 6.1 Introduction

The reliance on clinical symptoms and outcomes alone is not very reliable in the diagnosis and the management of leishmaniasis in endemic foci. The over dependence on these clinical signs for diagnosis was due largely to necessity, rather than choice (Reed, 1996). Therefore, various additional tools including parasitological methods, antigen detection platforms and molecular techniques have come into use to help in the diagnosis of the disease, nonetheless, there are various limitations with the use of some of these in resource poor settings where the disease is highly endemic.

The direct detection of the amastigote form of the parasite in Giemsa stained patients' samples has been a useful approach, but requires examination by an

experienced microscopist, and can lack sensitivity and specificity (Sunder and Rai, 2002). Also invasive procedures of sample collection are sometimes needed, for example splenic aspiration, which carries an amount of risk (Zijlstra *et al*., 1992; Kager and Rees, 1983). The method is not very suitable for younger patients, difficult to perform on patients with mild splenomegaly, and is contraindicated in thrombocytopenic, severely anaemic, pregnant and moribund patients who could experience active bleeding (Mbui *et al*., 2013).

Even though microscopy remains widely practised, the detection of antibodies against *Leishmania* antigens has become useful because of such constraints associated with parasitological diagnostic procedures. Some of these procedures have been developed into so-called Rapid Diagnostic Tests (RDTs), meaning they can be quickly and easily performed at point of care. These complement other existing diagnostic procedures such as parasitological procedures and molecular methods like PCR. Direct agglutination tests (DAT) and immunochromatographic rapid tests have been improved to be more specific and sensitive, although molecular methods are still required to identify to the species level, which can be important in optimising treatment.

In a given endemic region of American cutaneous leishmaniasis involving LCL, DCL and MCL caused by probable co-existing species like *L. mexicana*, *L. amazonensis*, *L. venezuelensis*, *L. pifanoi*, and *L. braziliensis* complex, for instance, diverse responses to therapy may ensue (Monroy-Ostria *et al*., 2014; Hernandez-Montes *et al*., 1998; Romero *et al*., 2001). Differentiating the clinical manifestation with its causal agent(s) would require diagnostic procedures to distinguish the different species. Since the inception of PCR, several molecular targets including minicircle kinetoplast DNA (kDNA), the miniexon (spliced leader RNA) gene, the

gp63 PCR-RFLP, and the rRNA internal transcribed spacer (ITS) have successfully been employed in the diagnosis of the diseases in many foci with co-existing species (Hernandez-Montes *et al*., 1998; Fernandes *et al*., 1994; Victoir *et al*., 2003; Davila and Momen, 2000; Reithinger and Dujardin, 2007; Bensoussan *et al*., 2006).

Although accurate, these molecular methods demand huge resources and competence for execution, a problem in the resource constrained areas where the disease is prevalent. Alternatively, recombinant antigens to detect the antibodies against the parasite have been produced over the years for both serological and cellular assays. These are geared towards the development of standardised diagnosis to ensure rapid and easy to perform procedures with increased sensitivity and specificity, and to positively influence the management of leishmaniasis especially in the neglected endemic areas. Elevated antibody titres are correlated with disease severity (Herwaldt, 1999). Evidence for leishmaniasis in Australia was supported by the detection of antibodies in the sera of kangaroos, showing previous exposure to the *Leishmania* isolated from one animal (Rose *et al*., 2004).

Methods like the indirect immunofluorescence antibody tests (IFA) and direct agglutination test (DAT), both of which make used of whole promastigotes as antigen source, have been successfully used in leishmaniasis diagnosis and management for several decades. Enzyme-linked immunosorbent assay (ELISA) methods, which use parasite lysates, have also been used and show increased specificity and sensitivity (Laty *et al*., 1979; Edissian and Darabian, 1979; Dye *et al*., 1993; Reed, 1996). Some of these procedures have been adapted to the field setting, particularly DAT and ELISA, with improved instrumentation and reagents. In some instances where there is an asymptomatic infection but with an anticipation of following acute infection, intradermal skin tests that reveal delayed-type hypersensitivity responses have proved

useful. Sometimes a positive response is indicative of self-healing following exposure (Reed, 1996).

Nonetheless, most of these procedures suffer disadvantages in one form or another. For example, although the DAT gives an easily recognizable result due to agglutination formation, it requires micropipettes and microtitration plates, and whose results require 18 hours before they can be read even when performed on-site, and so has undergone restricted laboratory use (Chappuis *et al*., 2006). This has therefore made DAT unattractive and inconvenient for diagnosis in the field, in spite of its robustness (Abdallah *et al*., 2004; Chappuis *et al*., 2006). The formol gel test (FGT) previously used in VL diagnosis in Indian and East Africa was convenient, reasonably cheap and easy to perform but was suspended based on poor sensitivity (Chowdhury *et al*., 1992). Another quick urine antigen-based test, the latex agglutination test (KAtex), has also been developed with excellent specificity but has a varying sensitivity (Attar *et al*., 2001; Rijal *et al*., 2004). Other recombinant membrane glycoproteins (gp) such as gp63, gp70, and gp72, and A2, with different molecular weights have been used to enhance ELISA sensitivity, and have proved useful for diagnosis of canine VL (de Paiva-Cavalcanti *et al*., 2015; Gomes *et al*., 2008; Carvalho *et al*., 2002). The DAT, rK39 dipstick, FGT and KAtex test kits have been individually used and evaluated in Nepal. The following sensitivities and specificities of the tests were recorded: FGT had a 52% and 97%; rK39 had 89% and 90%; KAtex had 57% and 98%, respectively. The results were produced in under 10 minutes for FGT and rK39 but 20 minutes for KAtex. Regarding overall performance, rK39 was excellently reproducible and of higher sensitivity in a faster turnaround time, simplicity of procedure and easier results interpretation than FGT and KAtex (Chappuis *et al*., 2006). On the other hand, some brands of rK39 antigen test have

performed very poorly in certain highly VL endemic areas in Africa and are regarded as inaccurate in diagnosis of VL relapse, compared to DAT (Hailu 1990; Zijlstra *et al*., 2001; Veeken *et al*., 2003). The cost of the rK39 test is less than most of the rapid diagnostic tests (RDTs) according to the meta-analysis done by Chappuis *et al*., (2006).

In an improved procedure for VL, *L*. *chagasi* K39 antigen copiously distributed on the amastigote cell and serologically shared by *L*. *donovani* complex (*L*. *donovani*, *L*. *infantum*) have enhanced VL immunodiagnosis, with 98% and 100% degree of sensitivity and specificity, respectively (Burns *et al*., 1993). The rK39 is considered the best first-line single diagnostic test for VL diagnosis in Bihar, India, becoming useful in VL diagnosis in this resource poor area, regardless of its poor performance in Sudanese VL patients (Chappuis *et al*., 2006; Mabey *et al*., 2004). The rK39 reagent has thus, demonstrated higher antibody titre levels which correlates with parasite numbers. The characteristic high epitope density on rK39, a recombinant polypeptide of 39 amino acid repeat as a portion of a larger protein in the kinesin family, have been adapted in rapid tests for quick specific antibody binding in seconds with fast test completion. The rK39 reagents have also been found to distinguish among species, for instance *L*. *donovani* from CL causing agents (Reed, 1996). Additionally, one validated meta-analysis on rK39, have largely looked at the rate of performance of sensitivity and specificity which respectively recorded higher percentages, in South Asia and Sudan, excluding other endemic areas like East and central Africa (Mbui *et al*., 2013). In different endemic areas, investigation on rapid test platforms (rK39) from DiaMed AG, Switzerland and Inbios, USA, recorded higher sensitivities and specificities with marked difference (Chappuis *et al*., 2005; Boelaert *et al*., 2008). More studies undertaken using rK39 recombinant antigen rapid

immunochromatographic test platform, have had high sensitivity and specificity in VL patients (Brandonisio *et al*., 2002; Rouf *et al*., 2009). Some previous validations of rK39 have yielded sensitivity and specificity of 100% upper limits (Schallig *et al*., 2002; Carvalho *et al*., 2003). Similarly, in a meta-analysis involving several research centres, rK39 rapid test for the diagnosis of VL produced sensitivity and specificity values of 93.9% and 90.6%, respectively on an average (Chappuis *et al*., 2006). In another work based on the same recombinant protein rK39, 100% specificity and 96 % sensitivity was achieved for the diagnosis of VL in one endemic area (de Paiva-Cavalcanti *et al*., 2015).

A new improved Signal-KA rapid test kit derived from rKE16, a recombinant antigen from an Indian *L. donovani* strain, has also been used in the diagnosis of immunocompetent VL cases in Spain with recorded sensitivity and specificity of 92% and 99%, respectively (Canavate *et al*., 2009; Sivakumar *et al*., 2006). In a revised Kenyan national VL diagnosis protocol, rKE16 Signal-KA and rK39 DiaMed IT-Leish were implemented as a clinical diagnosis regimen (Mbui *et al*., 2013). The ELISA-rK39 has been excellent in diagnosis, but requires use of serum for visceral leishmaniasis. In 272 culture and/or PCR confirmed LCL human cases resulting from *L. braziliensis*, a 100% serum positive rate was obtained for Kalazar Detect® (Inbios, Seattle, WA) a rK39-based immunochromatographic recombinant antigen test platform (Molinet *et al*., 2013). Both recombinant proteins rK39 and K26 fixed on nitrocellulose paper constitute another rapid test platform, TRALd (Rapid Antibody Test *Leishmania donovani*) have achieved 100% sensitivity and 98% specificity (Gomes *et al*., 2008; Srivastava *et al*., 2013; de Paiva-Cavalcanti *et al*., 2015). The development of rK39 antigen in immunochromatographic platforms seems helpful. Its diagnosis in VL comes with quick result, easy to perform and interpret results

(Boelaert *et al*., 2007). However, the use of rK39 is questionable in clinical cases where cutaneous or sub-cutaneous disease is only involved. In a study involving rK39 antigen where sera of CL patients were used, they produced a 20% positive rate (Hartzell *et al*., 2008), well below that produced by sera from VL. This requires an antigen that will be able to detect the agents responsible for CL and possibly VL.

This then indicates that, irrespective of their wide usage, such methods are confronted with limitations, for instance, some of these methods have had to contend with turn-around time, species specificity, expertise in results interpretation for titre and cut-off values, sample storage, cost, large-scale result production, specificity and sensitivity, and all have been limited to VL endemic areas detecting *L. donovani* antigens. An antigen in immunochromatographic designed for diagnosis of CL will become convenient. In addition, most of the RDTs methods mentioned above have and still require the use of bone marrow, skin biopsy, spleen aspirate, which are not only invasive but coupled with high risk to the hosts. These antibody detection tests are rapid, cheap, and easy to perform. More importantly, the ability to develop such antibody detection procedures into less invasive sampling methods, user and field-friendly, with easy to interpret results, no need for elaborate skills, would be more ideal and highly useful in poor endemic disease foci, especially in Ghana. These are among the reasons to develop a rapid test procedure in the lab using InBios rapid test kit, to diagnose the CL occurring in Ho, in the Volta region of Ghana, as well as to evaluate the performance of the test platform.

## 6.2 Materials and methods

### 6.2.1 Rapid test assay in the lab

Rapid, none/less invasive, more sensitive and reliable test in any endemic focus of the leishmaniasis is very helpful, in diagnosis and in the epidemiological survey. In trying

to do this in the lab with the notion to extend to the field CL *Detect*$^{TM}$ (InBios, Seatle, USA), qualitative, membrane based immunoassay for the detection of *Leishmania* antigens present was used for the design. The test kit platform has a high affinity polyclonal antibody coated on the membrane on the test line (**T**), to detect peroxidoxin antigen of the CL causing species.

### 6.2.2 Preparation of test samples using promastigote pellets

The late log phase promastigotes of *Leishmania* (from Ghana and other known species) were grown to 2.5x$10^7$cells/ml in M199 medium supplemented with 10% FBS with BME vitamins and gentamycin sulphate, incubated at 26°C. 1ml (1000µl) of the suspended parasites were aliquoted into 6 microfuge tubes. The tubes were spun at 13000 rpm for 5 minutes at 4°C to obtain a tight pellet. The supernatants were carefully removed without disturbing the pellets and transferred to -80°C before use.

### 6.2.3 Test of the sample

Each of the pellets removed from -80°C allowed to assume room temperature, re-suspended and were five-fold serially diluted in the lysis buffer supplied with the kit. 20µl of the lysed pellet was added to the inoculation area of the test strip, 3 drop of the chase buffer was then added and allowed to stand for a while. The sample chromatographically migrated up the strip with the help of the chase buffer, reacted with conjugated antibody in the membrane to produce red line, when the test is positive. No red line is produced when the test is negative, meaning there is no or undetected antigens in the samples. The experiments were performed in triplicate in all cases.

## 6.3 Results

Varying degrees of red bands were developed in all the species in addition to the *Leishmania* GH5, used according to the field-friendly protocol (**Figure 6.1**). The appearance of red bands showed reactivity of the *Leishmania* antibody in the test line reacting with the antigen in the lysate. At the top three concentrations equivalent to $5x10^5$, $1x10^5$ and $2x10^4$ cells/ml, antigens were detectable by pre-coated antibodies in all the species at varying intensities at a decreasing order. In isolates LV108, LEM2494 and LV546, there were no further antigen detection beyond the third concentration ($2x10^4$ cells/ml). The antigens in the isolates *Leishmania* Ghana, M379, and *L. siamensis*, were detectable at the fourth concentration $4x10^3$cells/ml, after which they were not detectable. For isolate FV1, the antigens were detectable up to the 5$^{th}$ concentration ($8x10^2$cells/ml), but not in the last concentration (160 cells/ml).

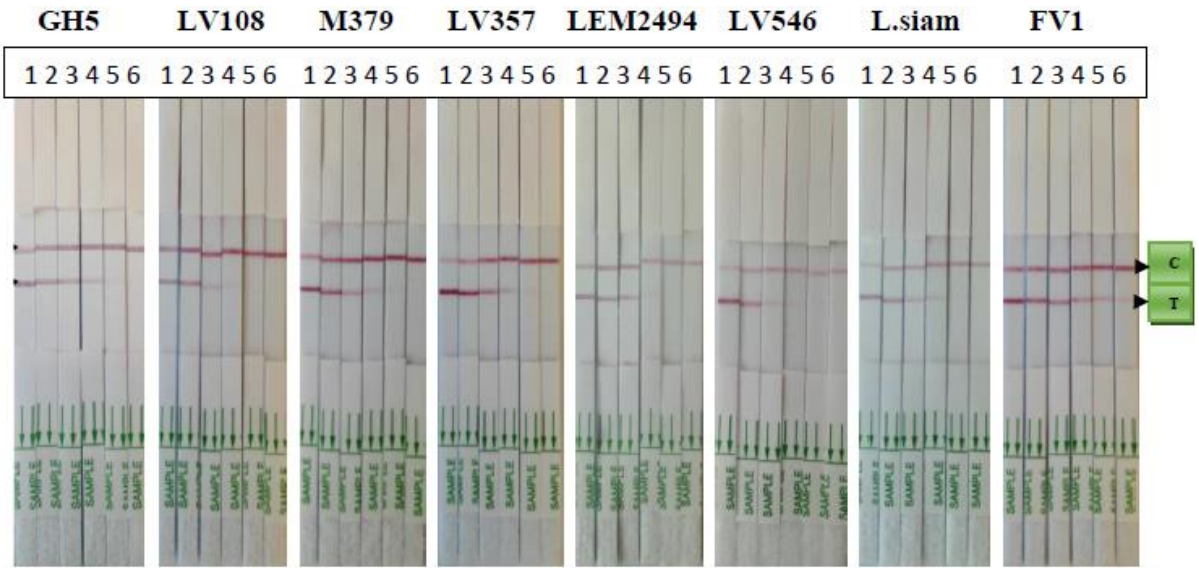



**Figure 6.1** Antigen-antibody reactions in the various *Leishmania* isolates. Cell densities: 1: $5x10^5$; 2: $1x10^5$; 3: $2x10^4$; 4: $4x10^3$; 5: $8x10^2$; 6: 160 cells/ml. C= control band; T= test band. Isolates: GH5-Ghana; LV108-*L. tarentolae*; M379-*L. mexicana*; LV357-*L. tropica*; LEM2494-*L. martiniquensis*; LV546-*L. aethiopica*; L.siam-*L. siamensis*; FV1-*L. major*.

## 6.4 Discussion

The symptoms associated with leishmaniasis are not pathognomonic since the signs have closely been linked with other protozoa infections notably malaria. This additionally has compromised the use of parasitological diagnosis. There has been several ways of diagnosis for appropriate intervention of leishmaniasis especially in a community where such interventions are lacking. Today's disease intervention specially regarding leishmaniasis is becoming complex in the instance of new emerging species and resistant strains. This therefore calls for meaningful diagnosis and the identification of the *Leishmania* responsible for particular symptoms, especially point-of-care (POC) diagnosis.

Quick detection of the antigen of *Leishmania* parasites as immune-diagnostic methods have usually based on immunofluorescence tests, ELISA, and direct agglutination assays, where the total extracts of the parasite as antigen have not always yielded conclusive results. The CL *Detect*™ is a qualitative CL Rapid Test kit from InBios (Seattle, USA) utilising an immunochromatographic *in vitro* assay procedure for quick clinical detection of *Leishmania* species in patient skin lesion samples resulting from the species responsible for cutaneous leishmaniasis. These CL causing species of the genus are mostly *L. major*, *L. tropica*, *L. aethiopica, L. mexicana, L. braziliensis* among others from both the Old and New World. There are also new emerging species of the genus which are responsible for CL, some of whose identities are still uncertain, namely *L. siamensis*, *L. martiniquensis*, isolates from Ghana (GH5) and isolate from Australia (AM-2004). It is noteworthy to investigate antigen detectability across the CL causing *Leishmania* as well as any of these emerging isolates, to ascertain the broad antigen detection spectrum of this CL *Detect*™ kit. This test device is coated with purified polyclonal antibody which has affinity to the parasite antigen peroxidoxin found in *Leishmania*.

There have been attempts to screen antibodies to a number of conserved recombinant proteins among the species of *Leishmania*. Among these proteins were two peroxidoxin family proteins homologous, *Li*mTXNPx and *Lic*TXNPx, characteristically expressed in all stages of *Leishmania* (Santarem *et al*., 2005). The protein peroxidoxin incorporated into this test device (CL *Detect*™) is ubiquitous in *Leishmania* parasites with unique function of defence against hosts' reactive oxygen species (ROS) and reactive nitrogen species (RNS) in addition to its drug target, vaccine (Gretes *et al*., 2012) and possibly diagnostic potentials. Though pervasive in parasites its unique presence and sampling method in the *Leishmania* parasite is not likely to interfere with its diagnostic potential in the CL patients, to have any worries on cross-reactivity with other protozoa and dermatological agents. Once it is associated with virulence and protection (Gretes *et al*., 2012) in the interest of the parasite, it would undoubtedly be expressed in the mammalian hosts, with the expectation that the amount would be enough to escape detection. As a transient protein, which is copiously induced at pathology and infection establishment (Santarem *et al*., 2005) will be ideal for diagnostic as well.

All the isolates used except LV108 were CL causing species but there were no inconsistencies in the red band formation to compromise the antigen's detectability. The polyclonal property of the coated antibody on the test line could have possibly made the detection of the antigen in all the isolates possible, since the antigen, peroxodoxin is shared among the species of *Leishmania* amastigotes in addition to promastigotes (Harder *et al*., 2006; Jirata *et al*., 2006). Purposefully designed and coated with polyclonal antibody to detect antigen peroxidoxin on amastigotes of the *Leishmania*, the test platform have been able to detect at varying concentrations, the

antigen on the promastigote as well. This makes the test kit very convincing in field detection of the parasite in both the human and possibly the vector hosts.

The use of the lysate from the culture samples is very encouraging since, lysate from the infected part of the CL is reachable with little or no risks. It will be ideal if the swabbing of the open lesions of CL would yield excellent results. This is in no doubt possible since swabbed samples were detectable by PCR-ELISA technique in CL samples (Adams *et al*., 2014), through a slight modification from the anticipated field procedure. Moreover, lower concentrations detectable by the use of swabs to sample from the aspirate, which have proven to be highly sensitive (Adams *et al*., 2014), would be ideal to use in this method to help reduce the invasive sampling techniques associated with CL.

While other diagnostic techniques and protocols have used antigens in the test kits to detect antibodies from the patients' samples (Chappuis *et al*., 2006), this method conversely, used the antibody in the test kit, specific to amastigote antigen, the form of the parasite expected to be found in the patients' sample (Reed, 1996), which apparently would be in the lysate. This is ideal since it will not look at the exposure of the victim to the parasite or active infection, but it would predict the presence or the absence of the parasite in the patients, depending on the results outcome.

The recombinant peroxidoxin protein *Lic*TXNPx in a study by Santarem *et al*., (2005), had emerged as a suitable antigen in leishmaniasis diagnosis for patients, where the optical density (OD) values using the antigen were higher than the OD values obtained when the total extracts of the parasite were used. Moreover, the protein peroxidoxin is found to be specific in its detection regardless of presence or absence of the antibodies to the parasite as against the crude extracts of the parasite. This is demonstrated in the fact that while other kits would detect the parasite

antibodies in the treated individual within a short period, the platform containing peroxidoxin could detect declining parasites in the treated individuals, but the antibodies levels in patients detectable by the conventional crude extract look the same as before treatment (Santarem *et al*., 2005). In the CL individuals where parasite load is reduced but not wholly absent in the samples, there is the likelihood of this platform being able to detect *Leishmania* in the patients. There is also the likelihood to reduce cross reactivity since it is highly specific and precise to the *Leishmania* antigens. Furthermore, in instances where *Leishmania* infections could induce the production of immunoglobulins with large variety of antibodies (Bohme *et al*., 1986), this would require precision in detection. In an endemic community such as the area of this current study, mild or asymptomatic exposure to *Leishmania* would still have circulating antigens. The peroxidoxin in the test kit has the potential to make the distinctions.

These proteins are highly immunogenic and non-secretory intracellular highly conserve proteins which elicit strong immune response (Soto *et al*., 1993, 1999; MacFarlane *et al*., 1990; Requena *et al*., 2000). Perioxidoxin is therefore capable of immunomodulation devoid of immune pressures (Santarem *et al*., 2007) and hence could be detected when there is an infection. This will be very important in a situation where the victims have become used to the infection to the point that, their system does not succumb to the presence of the parasite or stressed, to enhance antigen proliferation and detection.

The recombinant protein peroxidoxin is commonly associated with many parasites and other infectious agents (Gretes *et al*., 2012), which is expected to share some cross reactivity with *Leishmania* species. This rapid test kit would have to contend with cross reactivity and in a community where *Plasmodium* is common, and

having similar peroxidoxin with *Leishmania* (kinetoplastida). The protein's cross-reactivity in CL diagnosis, would be managed from their unique expressions at the infection periods (onset of leishmaniasis, expression at pathology).

### 6.5 Conclusion

In looking for point of care, field friendly and less invasive diagnosis for CL in the endemic are in Ghana, there looks to be a promise from the CL *Detect*™, InBios, Washington DC, USA, though a bit early to pre-empt, since the field study is yet to start. However, with the lower quantities of the antigens detected across a range of *Leishmania* species, it is tempting to believe that the anticipated aims would be fulfilled.

## Chapter Seven

## 7.0 *Leishmania* promastigotes susceptibility to cryptolepine

### 7.1 Introduction

Many protozoan chemotherapeutic drugs have long been used and have suffered drug resistance, which require urgent review (Polonio and Efferth, 2008). Among such diseases is leishmaniasis which have long been linked with poverty stricken habitation hence little or no interest of the pharmaceutical companies to develop current and better drugs for intervention due mainly to lack of economic value (Hazra *et al*., 2012). In spite of this, the affected victims, have for over the years resorted to the use of medicinal plants as treatment of various forms of infections including parasites, to mitigate the symptoms associated with these infections (Chan-Bacab and Pena-Rodriguez, 2001; Fournet and Munoz, 2002), probably due to indigenised properties of the natural products, readily availability and close to the victims of these diseases.

The Tropical Diseases Program of the World Health Organization (TDR/WHO) with the Drug Discovery Research Program on the above remit has deemed it a priority that pharmacological investigation of plants be made to ascertain their potency (Chan-Bacab and Pena-Rodriguez, 2001). The pursuit of specific inhibitors, in parasite control intervention, without adverse effect to the host has thus become vital (Santos *et al*., 2008).

The unique structures and the varied chemical properties have augmented the phenomenal role played by natural products in the drug discovery process, making them good candidates to lead drug development (Laurella *et al*., 2012; Harvey, 2008). They contain active compounds effective against the treatment of leishmaniasis and present a cheaper alternative to the existing commercially available drugs, which are within the reach of those in need. The application of some of these natural products has yielded between 72.9 - 99.9% parasite suppression in both *in vivo* and *in vitro* investigation (Mishra *et al*., 2011). Such products have demonstrated varying mechanisms of activity such as apoptosis and respiratory inhibition, against most pathogenic species of *Leishmania* from both Old and New World, with highly potent activity to species like *L. donovani*, for instance in the case of compounds cephaeline and emetine ($IC_{50}$ at 0.03µg/ml), but showed toxicity topically against *L. major* treatment (Muhammad *et al*., 2003). Some alkaloids of marine sponges have demonstrated a great deal of antileishmanial activities against a selected number of the parasite species at promastigote and amastigote stages (Mishra *et al*., 2011). Many of these natural products have demonstrated excellent therapeutic index in advanced stages of clinical trials (http://www.cdriindia.org/Picroliv.htm). At certain lower concentrations, there have been records of impaired structural changes in the developing promastigotes by the presence of some of the natural products, through

protein kinase, an enzyme pathway required in cellular functions (Chan-Bacab, and Pena-Rodriguez, 2001). Other inhibition activities such as acting on the membrane of the *Leishmania* parasite to alter its membrane potential have been associated with some natural products (Delmas *et al*., 2000). Moreover, modulation of phagocytic action by the macrophages has been observed by the administration of some natural product derivatives (Monzote *et al*., 2007; Delorenzi *et al*., 2001). Presented below (**Table 7.1**) are natural products which have demonstrated some level of leishmanicidal/static activities to various forms of the parasite.

| Natural Product | Susceptible species | Stage of species | Plant |
|---|---|---|---|
| **Alkaloids**<br><br>Quinoline | *L. braziliensis*<br>*L. amazoniensis* | Amastgotes (*in vivo*)<br>Promastigotes | *Galipea longiflor* (Rutaceae)<br>*Dictyoloma peruviana* (Rutaceae) |
| Indole | *L. major*<br>*L. donovani*<br>*L. amazoniensis* | Promastigotes<br><br>Amastigotes (in vitro) | *Corynanthe pachyceras* (Rubiaceae)<br>*Kopsia griffithii* (Apocynaceae)<br>*Peschiera van heurkii* (Apocynaceae) |
| Isoquinoline | *L. guyanensis*<br>*L. braziliensis*<br>*L. major*<br>*L. amazoniensis*<br>*L. donovani*<br>*L. mexicana*<br><br><br><br><br>*L. major* | Promastigotes<br><br><br><br><br><br><br>Amastgotes (*in vivo*) | *Annona foetida* (Annonaceae)<br>*Guatteria foliosa* (Annonaceae)<br>*Annona spinescens* (Annonaceae)<br>*Guatteria dumetorum* (Annonaceae)<br>*Guatteria amplifolia* (Annonaceae)<br>*Unonopsis buchtienii* (Annonaceae)<br><br>- (Annonaceae)<br>- (Menispermaceae)<br>- (Berberifaceae) |
| Naphthylisoquinoline | *L. major*<br>*L. donovani* | Promastigotes | *Ancistrocladus ealaensis* (Ancistrocladaceae)<br>*Ancistrocladus tanzaniensis* (Ancistrocladaceae) |
| Bisbenzylisoquinolinic | *L. braziliensis*<br>*L. amazoniensis*<br>*L. donovani* | | *Albertisia papuana* (Menispermaceae)<br>*Pseudoxandra sclerocarpa* (Annonaceae)<br>*Gyrocarpus americanus* (Hernandiaceae)<br>*Caryomene olivasans* (Menispermaceae) |
| Steroidal | *L. donovani* | | *Holarrhena curtisii* (Apocynaceae) |
| Benzoquinolizidine | *L. major*<br>*L. donovani* | | *Psychotria klugii* (Rubiaceae) |
| Diterpene | *L. infantum* | promastigotes | *Aconitum, Delphinium* and *Consolida* species |
| Pyrrolidinium | *L. donovani* | Axenic amastigotes | *Phlomis brunneogaleata* (Lamiaceae) |
| Acridone | *L. major* | Amastigotes<br>Promastigotes | *Thamnosma rhodesica* (Rutaceae) |

| | | | |
|---|---|---|---|
| β-Carboline | *L. amazonensis* | Amastigotes | *Peganum harmala* (Nitrariaceae) |
| **Quinones**<br>Primin | *L. donovani* | Promastigotes | *Primula obconica* (Primulaceae) |
| bis-naphthoquinone | *L. donovani* | Promastigotes | *Diospyros montana* (Ebenaceae) |
| Plumbagin | *L. donovani*<br>*L. amazonensis*<br>*L. venezuelensis* | Amastigotes | *Plumbago zylenica* (Euphorbiaceae) |
| Biplumbagin | *L. braziliensis*<br>*L. amazonensis*<br>*L. donovani* | Promastigotes | *Pera benensis* (Euphorbiaceae) |
| Lapachol | *L. donovani* | Amastigotes | *Tecoma* species (*Bignoniaceae*) |
| Jacaranone | *L. amazonensis* | Promastigotes | *Jacaranda copaia* (Bignoniaceae) |
| Hydropiperone | *L. braziliensis*<br>*L. donovani*<br>*L. amazonensis* | Promastigotes | *Peperomia galioides* (Piperaceae) |
| Aloe-emodin | *L. donovani* | Promastigotes<br>Amastigotes | *Stephania dinklagei* (Menispermaceae) |
| **Terpenes**<br>Iridoids | *L. donovani* | Amastigotes<br>Promastigotes | *Nyctanthes arbortristis* (Oleaceae) |
| Monoterpenes | *L. amazonenesis*<br>*L. braziliensis*<br>*L. donovani* | Promastigotes | *Oxandra espintana* (Annonaceae) |
| Sesquiterpenes | *L. amazonensis*<br>*L.major*<br>*L. donovani* | Promastigotes | *Munnozia maronii* (Asteraceae)<br>*Vernonia brachycalyx* (Asteraceae)<br>*Jasonia glutinosa* (Asteraceae) |
| Diterpenes | *L. amazonenesis*<br>*L. braziliensis*<br>*L. donovani* | Promastigotes<br>Amastigotes | Croton plant (Euphorbiaceae)<br>*Polyalthia macropoda* (Annonaceae)<br>*Sideritis varoi* (Lamiaceae)<br>*Holarrhena floribunda* (Apocynaceae) |
| Triterpenes | *L. amazonenesis*<br>*L.donovani* | Amastigotes<br>Promastigotes | *Jacaranda copaia* (Dilleniaceae)<br>*Doliocarpus dentatus* (Dilleniaceae)<br>*Celaenododendron mexicanum* (Euphorbiaceae) |
| **Saponins** | *L. infantum*<br>*L. tropica*<br>*L. major* | Amastigote<br>Promastigotes | *Hedera helix* (Araliaceae)<br>*Buddleja madagascariensis* (Loganiaceae)<br>*Asparagus africanus* (Liliaceae) |

| | | | |
|---|---|---|---|
| **Phenolic derivatives** Chalcones | *L. amazonenesis*<br><br>*L.major*<br>*L. donovani* | Amastigote Promastigotes | *Piper aduncum* (Piperaceae)<br><br>*Glycyrrhiza species* (Fabaceae) |
| Flavonoids | *L. amazonenesis* | Amastigotes Promastigotes | *Celanodendron mexicanum*<br>*Acacia greggii, A. berlandieri* (Fabaceae)<br>*Salvia tomentosa* (Labiatea) |
| Lignans | *L. infantum* | Amastigotes | *Haplophyllum bucharicum* (Rutaceae) |
| Coumarins | *L. major* | Promastigotes | *Vernonia brachycalyx* (Asteraceae) |
| Curcumins | *L. major* | Promastigotes | *Curcuma longa* |

**Table 7.1** Natural products investigated for their antileishmanial activity (Chan-Bacab and Pena-Rodriguez, 2001; Mishra *et al*., 2011).

It is evident that natural products and many others have shown substantial efficacy against the different forms of the *Leishmania* parasite, and further exploration on many others for their usefulness in treating the parasite, especially among the indigenes who considerably suffer the scourge of the disease will be desirable. One of such indigenous West African flowering shrub plant *Cryptolepis sanguinolenta*, Periplocaceae, from which cryptolepine has been derived.

Most importantly, the aqueous root extract of *Cryptolepis saguinolenta* has been found efficacious against *Plasmodium* sp., and has traditionally been used in Ghana extensively as antimalarial (Ansah and Gooderham, 2002). In an investigation to ascertain the medicinal plants used in malarial treatment in Ghana, *Cryptolepis sanguinolenta* was found to be the highest among the plant preparations for such treatment (Osei-Djarbeng *et al*., 2015). Cryptolepine has also been used in the plasmodium treatment across West Africa (Wright *et al*., 2001; Onyeibor *et al*., 2005). This parasitic treatment potency has been attributable to its active component and naturally occurring indoloquinoline alkaloid, cryptolepine, which have equally been deemed anticancer due to its ability and uniqueness as non-alternating DNA

intercalating (Lisgarten, *et al*., 2002; Ansah and Gooderham, 2002). The alkaloid has also been found to induce apoptosis in some cells (Dassonneville *et al*., 2000; Bierer *et al*., 1998). Other occurrences such as mitochondrial membrane depolarization and chromosomal DNA degradation into oligonucleosomal fragments, an indication of apoptosis were associated with the activities of cryptolepine against the promastigote of *L. donovani* (Hazra *et al*., 2012). The cell tolerable level in terms of toxicity of both the extracts and its major alkaloid of *Cryptolepis sanguinolenta* is found to be 32.9µg/ml and 4.3µM respectively and both have equally been found to induce apoptosis, with non-existing mutagenesis at higher concentrations of 100µg/ml for the aqueous extract and 5µM for cryptolepine (Ansah and Gooderham, 2002; Dassonneville *et al*., 2000). The various synthetic derivatives of cryptolepine have proved efficacious against *L. donovani* in promastigotes and its Sodium stibogluconate (SbV) resistant intracellular amastigotes strain from clinical isolates (Hazra *et al*., 2012). In an African sleeping sickness investigation, *Trypanosoma brucei brucei*, were found susceptible to some cryptolepine analogues (Oluwafemi *et al*., 2009).

Here, the efficacy of the pure active alkaloid, cryptolepine is investigated on the promastigotes of *L. mexicana* and Ghana *Leishmania* isolate. The molecular structure (**Figure 7.1**) and the plant are presented below (**Figure 7.2**). The cryptolepine is a 95% purified active alkaloid obtained from the plant *Cryptolepis sanguinolenta*, the common name in Ghana is nibima, donated by Dr Elvis Ofori Ameyaw, University of Cape Coast, Ghana.

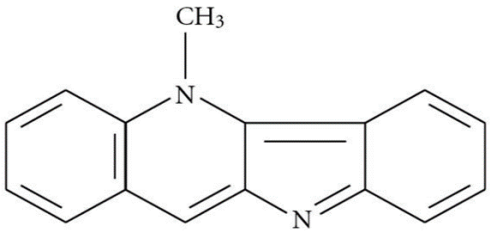



**Figure 7.1** Structure of cryptolepine (Molecular weight: 258.069g/mol; Molecular formula: $C_9H_8BrNO_3$).

**Figure 7.2** Picture of *Cryptolepis saguinolenta*.

## 7.2 Materials and Methods

Drug susceptibility to *Leishmania* species is an urgent pursuit and among the top priority on WHO drug discovery agenda. This was necessitated in the investigation of compounds and extracts of microbial susceptibility in some part of tropical Africa, in the current study. Even though susceptibility of the *Leishmania* promastigotes is not a true reflection of the compound sensitivity, it does give a quick insight into the antileishmanial activity the compound under investigation and a precursor for axenic and intracellular amastigotes investigation.

### 7.2.1 Growth curves of isolates

The isolates *L. mexicana* (M379) and GH5 (new species of *Leishmania* isolated from Ghana) were culture *in vitro*. The culture medium used in all cases was M199 supplemented with 10% FBS, BME vitamins, gentamycin sulphate as previously mentioned. This preliminary in vitro culture was to ensure the cells viability among other things.

### 7.2.2 Amphotericin B susceptibility test

Initially, different concentrations (0.04µg/ml, 0.1µg/ml, 0.25µg/ml, 0.625µg/m and 1.56µg/ml) of Amphotericin B (Sigma-Aldrich Co Ltd, UK), were prepared in DMSO (dimethyl sulfoxide) and tested on *L. mexicana* (M379) promastigotes. This was to establish at which concentration of the preparation would be efficacious and used as positive control in the anti-promastigotes assay in the test compound. The promastigotes of *L. mexicana* (M379) was used as the reference *Leishmania* isolates.

### 7.2.3 The alkaloid and its action on *L. mexicana* and *Leishmania* GH5

The promastigote of *L. mexicana* (M379) and *Leishmania* GH5 (new species of *Leishmania* isolated from Ghana) was investigated for its susceptibility to different concentrations (0.04µg/ml, 0.1µg/ml, 0.25µg/ml, 0.625µg/m and 1.56µg/ml) of cryptolepine. Amphotericin B (Sigma-Aldrich Co Ltd, UK) was used as reference positive control drug.

The promastigote at the early log phase of cell concentration $5x10^5$cell/ml in volumes of 10 ml were seeded into the culture flasks. A volume of 50µl of the concentrations of the compounds (0.04µg/ml, 0.1µg/ml, 0.25µg/ml, 0.625µg/m and 1.56µg/ml), prepared from DMSO were added to the seeded promastigotes in the flasks in addition to two set of controls, positive controls using 50µl Amphotericin B

at 1.56µg/ml and 50µl DMSO as negative control. The experimental flasks were incubated at 26°C for 7 days.

To determine daily promastigotes inhibition or otherwise for the incubation period, the cells in each flask were counted daily using haemocytometer and recorded. The experiments were performed in triplicate and the results expressed as the mean of three individual experiments. The evidence of growth as well as inhibition of the promastigotes were observed which is an indicative of colour changed in the flasks. The row score of cell densities entered on the excel spreadsheet and graphically expressed. The dose response of both the test compound and the control drug were determined using GraphPad Prism for Windows 5 (GraphPad Software, San Diego, CA, USA). Doses for 50% of the maximal effect ($ED_{50}$) for each drug were determined by using an iterative computer least squares method, following non-linear regression (3-parameter logistic) equation.

### 7.2.4 Other factors influencing the effect of cryptolepine

Regular addition of cryptolepine at 24 hours interval was investigated to find out its effect on the inhibition of the promastigotes of the isolates. The promastigotes of *L. mexicana* (control isolate) at density of $5x10^5$ cell/ml were seeded with the different concentrations (0.04µg/ml, 0.1µg/ml, 0.25µg/ml, 0.625µg/m and 1.56µg/ml) of cryptolepine and the controls. Same cryptolepine concentrations were added to the each respective flasks from day 1-7. The numbers of promastigotes were determined by the haemcytometer before each cryptolepine additions. The inhibitory effects of the cryptolepine on the promastigotes were determined by counting live parasites.

Additionally, cryptolepine concentration at 1.56µg/ml was considered to infer the stability of the compound at the experimental conditions. Of the two flasks of similar cryptolepine concentration (1.56µg/ml), one flask was incubated at 26°C for 7

days in the M199 and the other flask freshly prepared. Promastigotes of the isolates at $5x10^5$ cell/ml were added to each flask and incubated at 26°C for 7 days. The promastigotes of *L. mexicana* (control isolate) densities in each flask were determined daily for the incubation period, changes in the flasks were observed and the promastigote densities determined.

### 7.3 Results

Normal growth curves were exhibited by the two isolates. This was done to help determine when to harvest the early log phase ($5x10^5$cell/ml) promastigotes for the compound susceptibility tests. The standardisation of the chemotherapeutic assay of the species of *Leishmania*, inferred from the normal growth curves of both species was also to ascertain the viability of the isolate in storage (**Figure 7.3**). This would help to confidently conjecture the response of the compound on administration. The growth of the parasites entered the log phase on day 1 and plateaued on day 4. The assays were purely to explore the inhibition effects of the cryptolepine vis-à-vis Amphotericin B positive control.

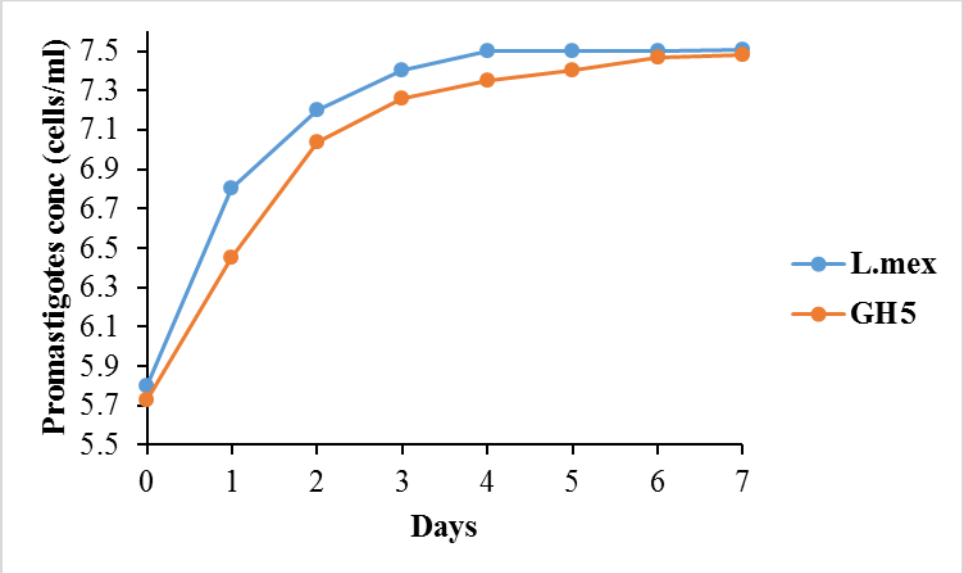
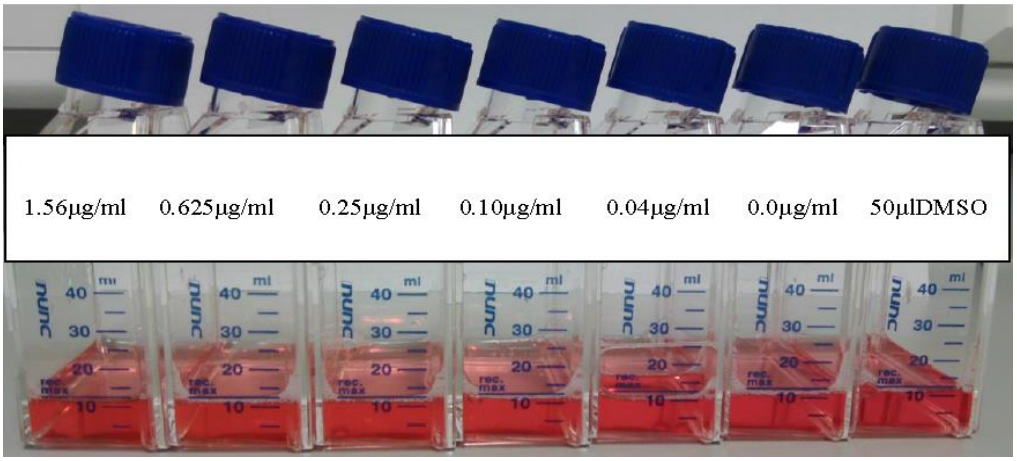



**Figure 7.3.** The normal growth curve of *L. mexicana* and GH5 promastigotes in medium M199.

### 7.3.1 Susceptibility of *L. mexicana* promastigotes to Amphotericin B

There were clear demonstration of growth in the culture flasks at day 7 in the concentrations of 0.04µg/ml, 0.1µg/ml, 0.25µg/ml, M199 and DMSO. Growth were somewhat inhibited in the flasks of concentrations 0.625µg/m and 1.56µg/ml, which were finally determined by the plots of promastigotes concentration (cells/ml) with growth periods (days).

**a).**




**b).**

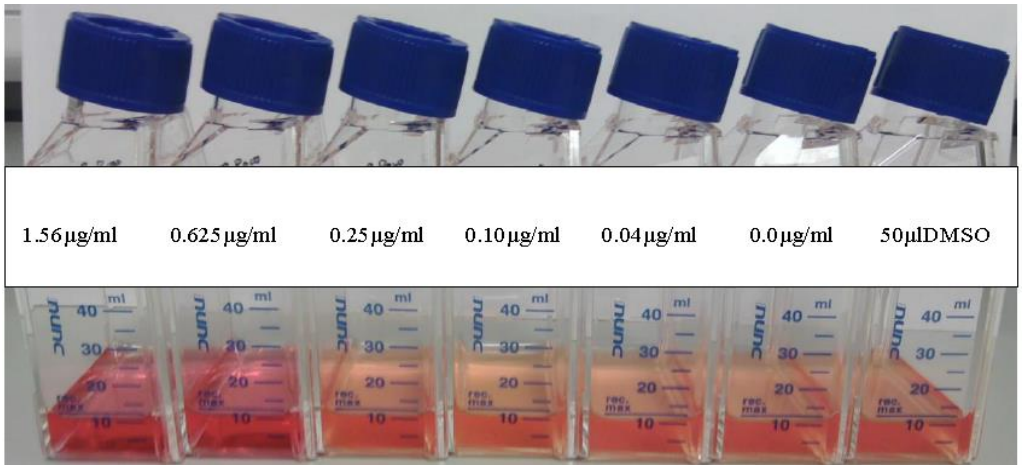



**Figure 7.4** *L. mexicana* flasks seeded with Amphotericin B at concentrations of 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml; Negative controls: M199 (0.0µg/ml) and DMSO (50µl). **a).** Growth at day 1; **b).** Growth at day 7.

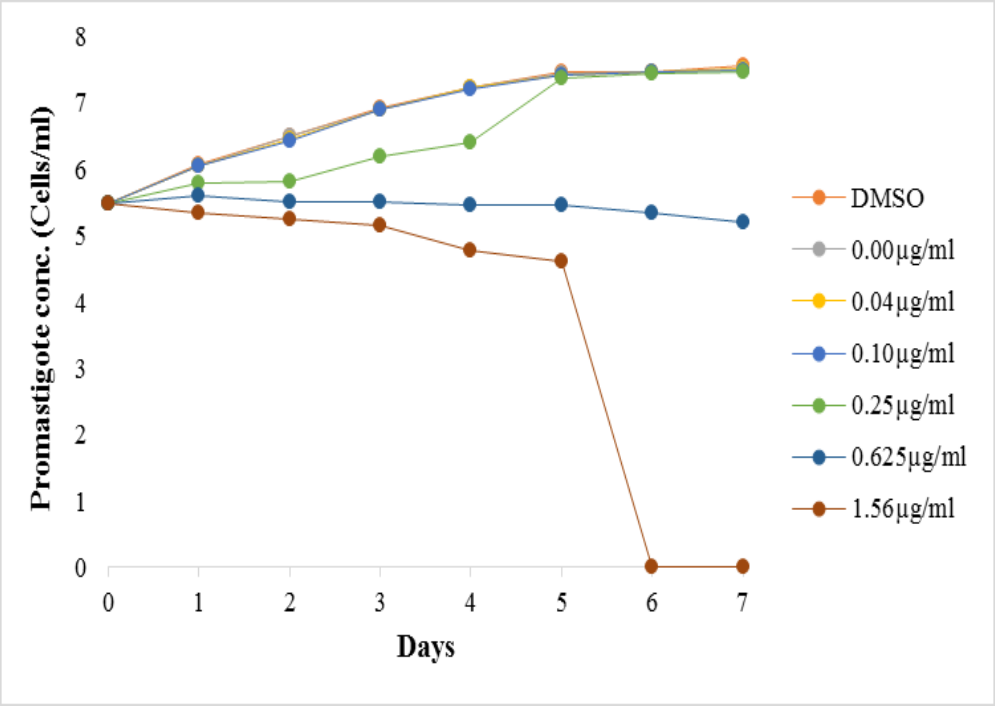


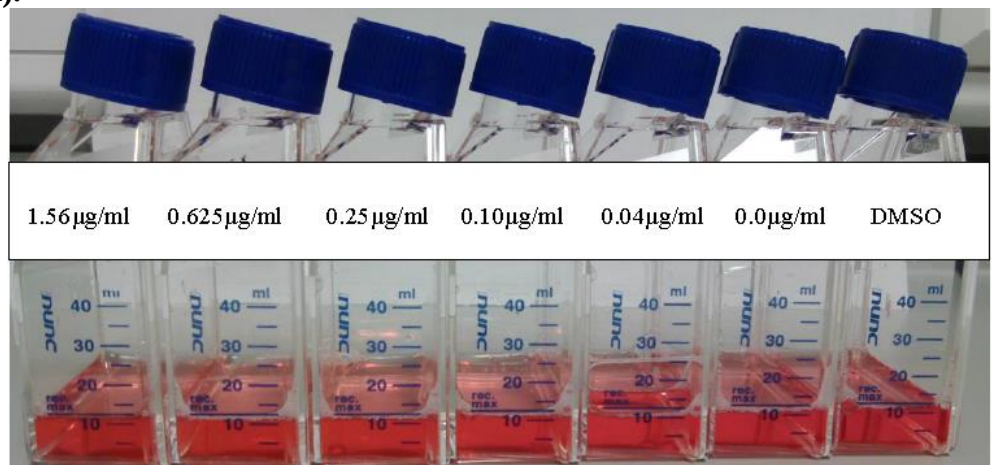

**Figure 7.5** The inhibitory effect of the Amphotericin B (Powder) on the promastigotes of *L. mexicana*. Negative controls: DMSO (50µl); M199 (0.0µg/ml).

There was marked inhibition of growth activities in flasks of concentrations 1.56µg/ml and 0.625µg/ml Amphotericin B compared to concentrations 0.25µg/ml, 0.10µg/ml and 0.04µg/ml and the controls (**Figure 7.5**), promastigotes were detectable by the haemcytometer throughout the incubation period, except in days 6-7 in flasks of 1.56µg/ml Amphotericin B concentration, which had shown consistent promastigote drop from day 1 till the final drop in days 6-7, according to the graphical representation (**Figure 7.5**). There was marginal increase in promastigote densities in flask 0.25µg/ml from days 1-4 but became equally as high as the controls from day 5-7. This demonstrated initial decline of the promastigote densities but to some point where the decline was given up to the appreciable numbers of promastigotes density (apoptosis-autophagy concept). In the flask of 0.625µg/ml Amphotericin B concentration, there were consistent density of promastigotes.

### 7.3.2 Susceptibility of *L. mexicana* promastigotes to cryptolepine

In the seeded *L. mexicana* flasks with cryptolepine, the activities as occured in the culture flasks with respective concentrations were observed and recorded from day 1-7 of incubation (**Figure 7.6**). There less significant antiparasitic activity in all the flasks seeded with various concentrations of cryptolepine (0.625µg/ml, 0.25µg/ml, 0.10µg/ml, 0.04µg/ml, 0.0µg/ml, 50µlDMSO), except in the flask with 1.56µg/ml cryptolepine, where there was significant level of inhibition.

**a).**




**b).**

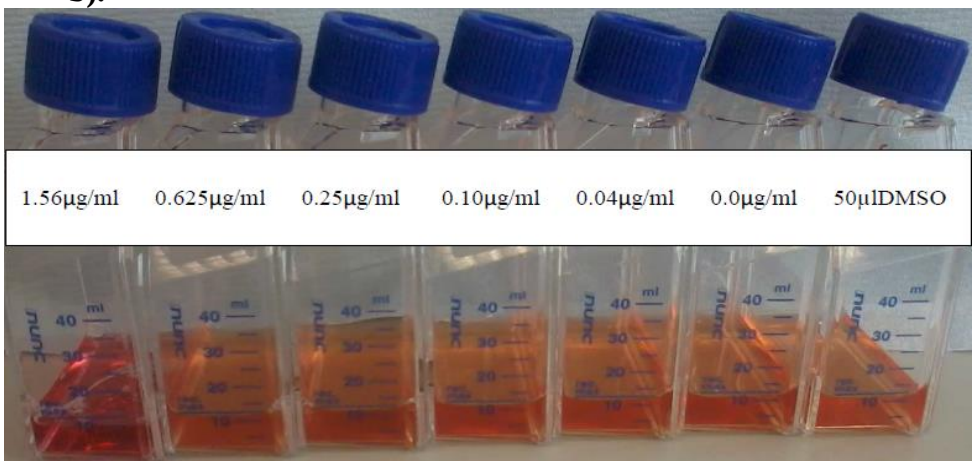



**Figure7.6** *L. mexicana* flasks seeded with cryptolepine at concentrations of 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml; Negative controls: M199 (0.0µg/ml) and DMSO (50µl). **a).** Growth at day 1; **b).** Growth at day 7.

This was evidenced in the fact that the initial colour remained fairly the same from start to the completion of the experiment on day 7. This was due to the fact that the, phenol red turned yellow from brick-red, an indication of growth of the promastigotes to produce acid. When there is no effect of the compound, the intensities of the colour changes increases with time till on the final day of the experiment where the intensities were the same. In the concentration where the effects of the compound was not provoked, the intensity of the colour of the medium remained virtually the same as from day 1 as it occurred in flask 1.56 µg/ml cryptolepine.

The information from the colour changes were transformed into the growth inhibition/promotion curves (**Figure7.7**), where the promastigote cell densities were determined by the haemocytometer.

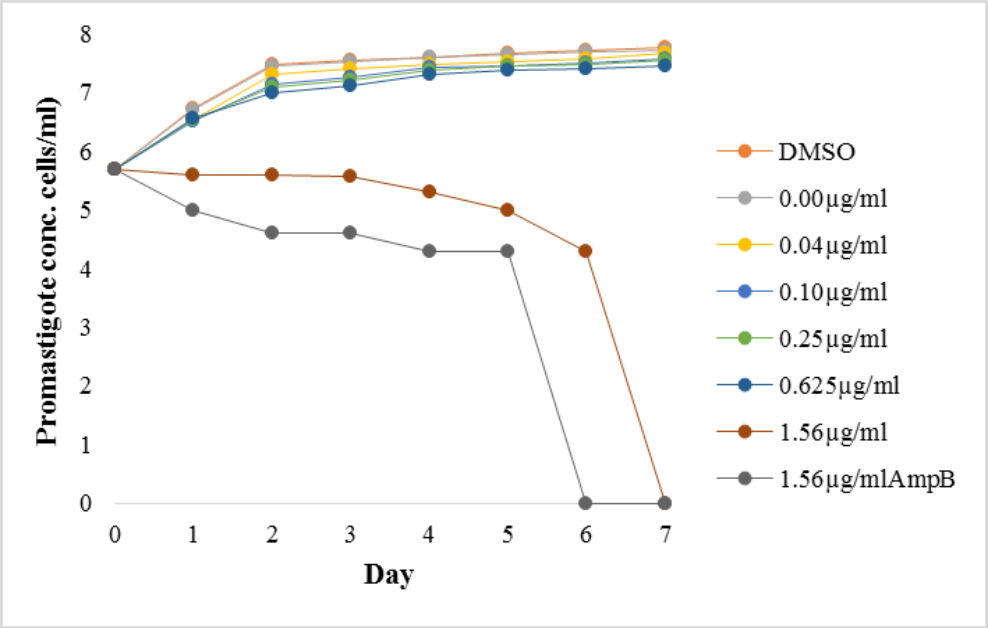



**Figure 7.7** The inhibitory effect of the cryptolepine on the promastigotes of *L. mexicana*. Negative controls: DMSO (50µl); M199 (0.0µg/ml). Positive control: 1.56µg/ml Amphotericin B.

Apart from the flask with 1.56µg/ml cryptolepine concentration, which showed clearly distinct inhibition effect (equivalent to the positive control) of the

compound, the remaining concentrations, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml (and the negative controls) showed pronounced promastigotes growth. There was gradual decrease of the promastigote density with time in the 1.56µg/ml cryptolepine concentration flask from day 1-7, compare to the two control flasks, 0.0µg/ml and 50µl DMSO. There were virtually no detectable parasites in this (1.56µg/ml seeded concentration flask), at day 7 and beyond the experiment period. Thus, *L. mexicana* have demonstrated some level of susceptibility to cyrptolepine at 1.56 µg/ml.

On the other hand, the concentrations 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml, produced intense decrease in promastigotes densities in increasing order of cryptolepine concentration, through the incubation period, in equal measure comparable to the negative controls (DMSO and M199). The inhibitory effect of the cryptolepine on *L. mexicana* increases with decreasing concentration which implies decreasing promastigote densities, and vice versa. This indicates that at higher concentration the *L. mexicana* of the compound, promastigote growth could be inhibited.

### 7.2.3 Other factors influencing the effect of cryptolepine

There were additional inhibition effects on the *L. mexicana* promastigotes particularly in flasks of cryptolepine concentrations 1.56µg/ml and 0.625µg/ml demonstrated by colour change observation (**Figure 7.8**).

The inhibitory effects of the remaining concentrations were similar to the previous experiment where there were little or no effects on the promastigote at cryptolepine concentrations 0.25µg/ml, 0.10µg/ml and 0.04µg/ml which compared well to the negative controls (M199 and DMSO).

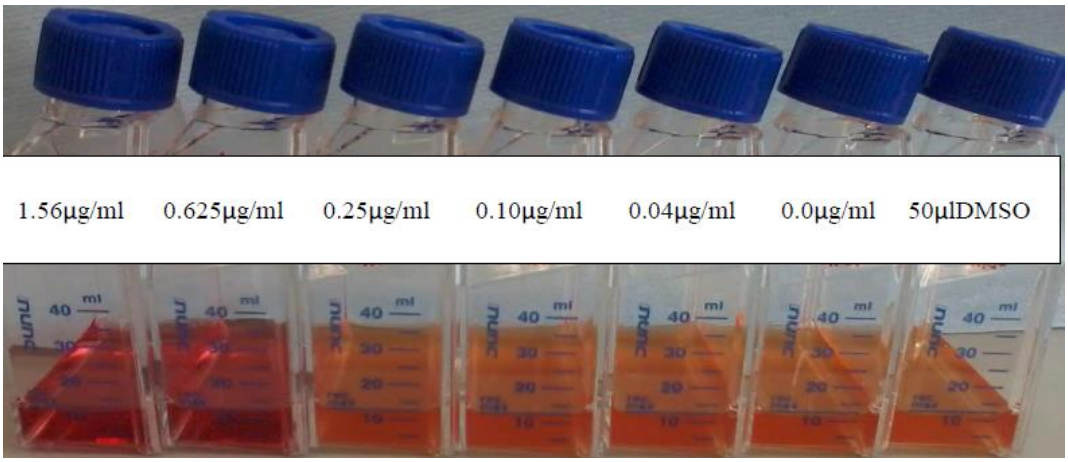



**Figure 7.8** Continuous cryptolepine addition to *L. mexicana* promastigotes at 24 hours interval, 7th day observation.

The promastigote densities detectable by haemocytometer showed inhibition effect of the compound at both 1.56µg/ml and 0.625µg/ml. The concentration 1.56µg/ml in this set up was comparable to 1.56µg/ml Amphotericin B positive control (**Figure 7.9**). There were no cryptolepine effects at concentration 0.25µg/ml, 0.10 µg/ml and 0.04µg/ml, which similarly match the negative controls (M199 and DMSO).

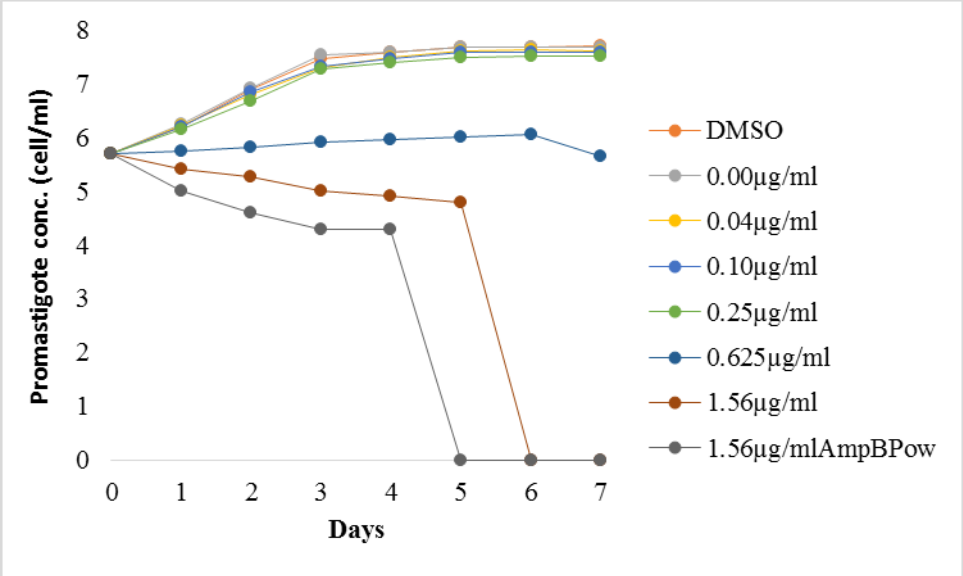
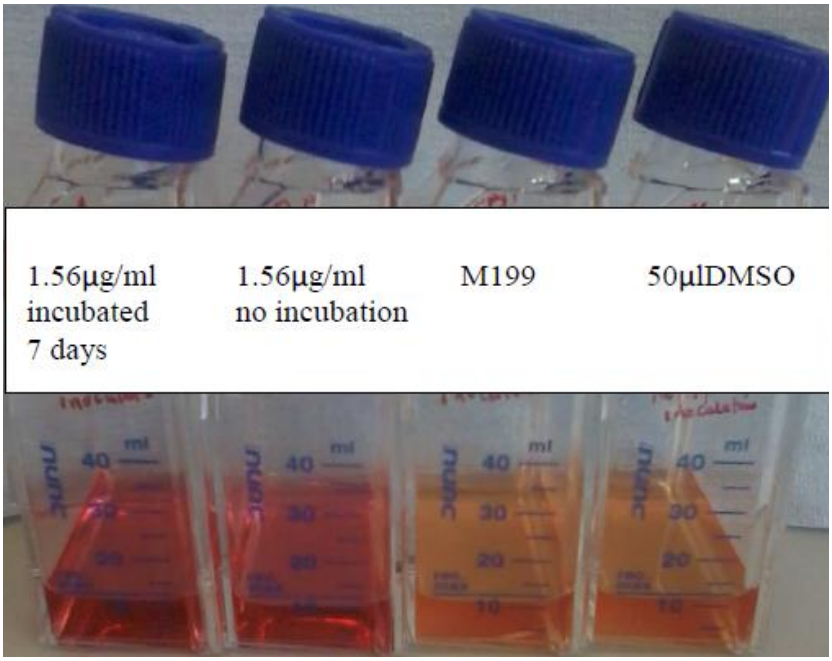



**Figure 7.9** Continuous cryptolepine addition to *L. mexicana* promastigotes at 24 hours interval.

On the cryptolepine stability, there were no pronounced difference between the 7 days incubated and freshly inoculated cryptolepine at 1.56µg/ml concentration as demonstrated in the observed flasks and the graph. The promastigotes densities in both flasks were virtually equal (**Figure 7.10).**

**a)**




**b)**

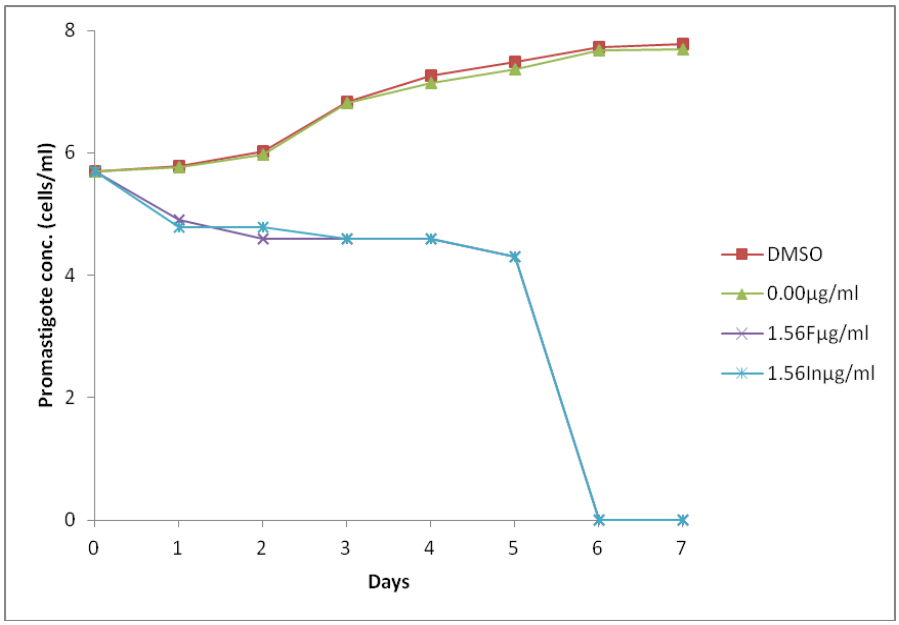
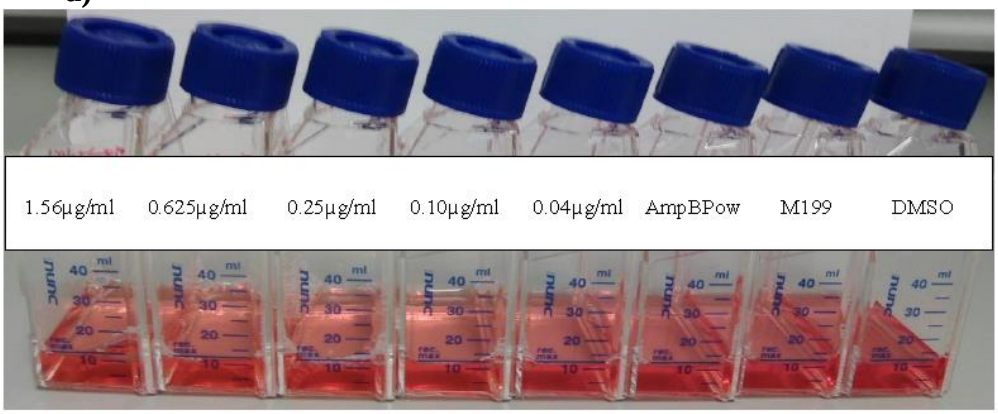



**Figure 7.10** The stability of cryptolepine in M199 at the experimental incubation conditions. Fresh and incubated cryptolepine in M199 before the promastigotes were added. **a).** as observed in the flasks; **b).** Promastigote densities.

### 7.3.4 Susceptibility of L. GH to cryptolepine

On the GH5, the promastigote growth inhibitory effects of the cryptolepine on the contrary were quite astonishing, paralleled to the inhibition effect on *L. mexicana* promastigote at those same concentrations. There was no pronounced growth inhibition observed in the croptolepine seeded flasks at all concentrations including the controls (1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml, 0.0µg/ml and 50µlDMSO). The promastigotes growth promotion activities were clearly observed in the flasks which were strongly depicted in the colour changes from days 1-7, except in the positive control flask (**Figure 7.11**).

**a)**




**b)**

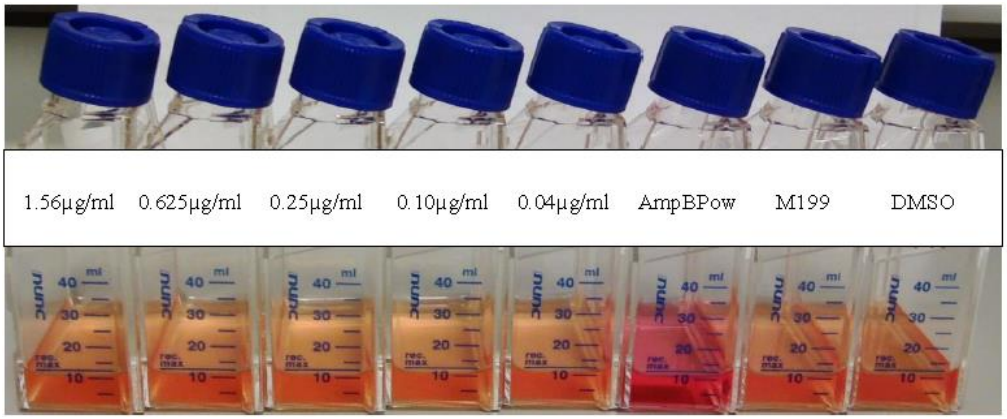



**Figure 7.11** GH5 flasks seeded with cryptolepine at concentrations of 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml; Amphotericin B (1.56µg/ml) positive control; Negative controls: M199 (0.0µg/ml) and DMSO (50µl). **a).** Growth at day 1; **b).** Growth at day 7.

The GH5 promastigotes densities were much pronounced in all the concentrations as detectable by the haemocytometer (**Figure 7.12**). None of the administered concentrations; 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml of the cryptolepine could demonstrate any promastigote inhibitory effect on the GH5. At all the concentrations, their effects were in step with the negative controls (M199 and DMSO). The promastigotes densities compared with the negative controls were basically the same.

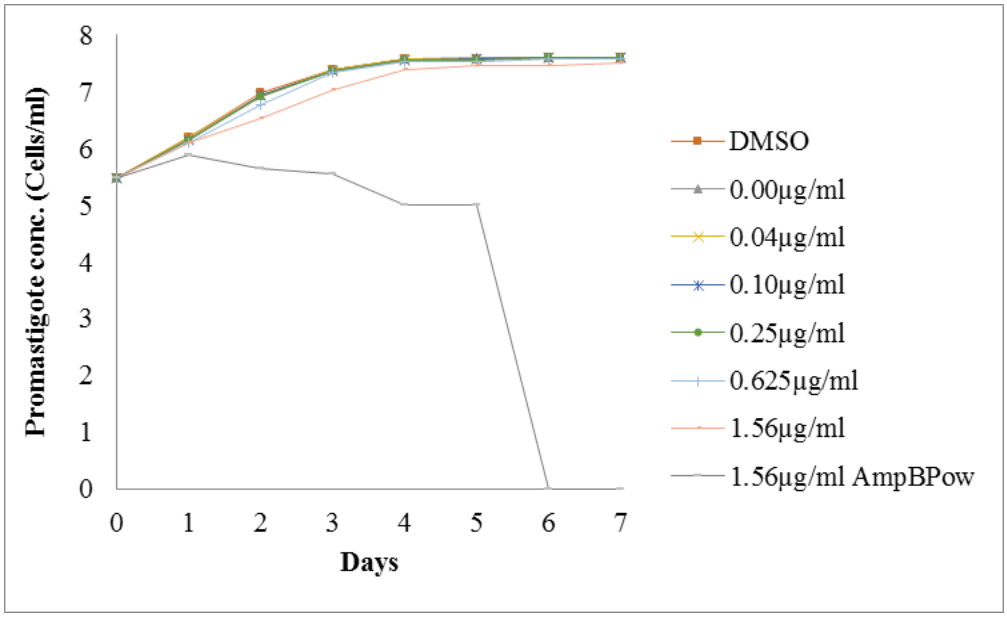
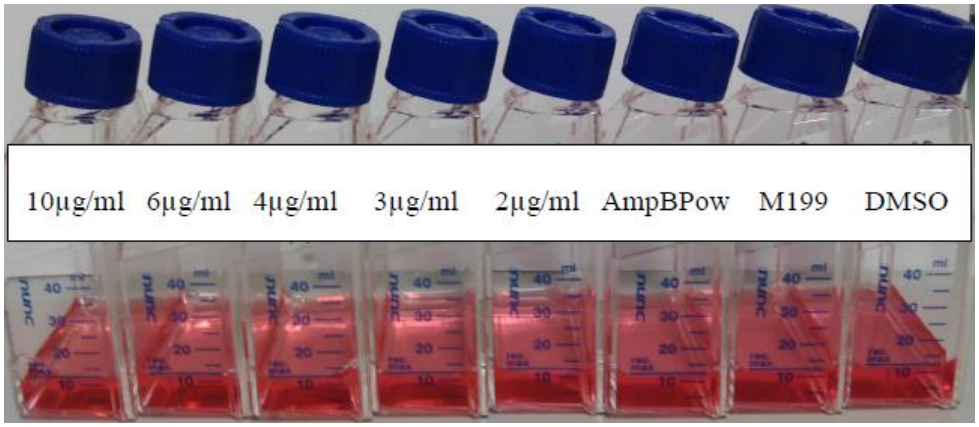


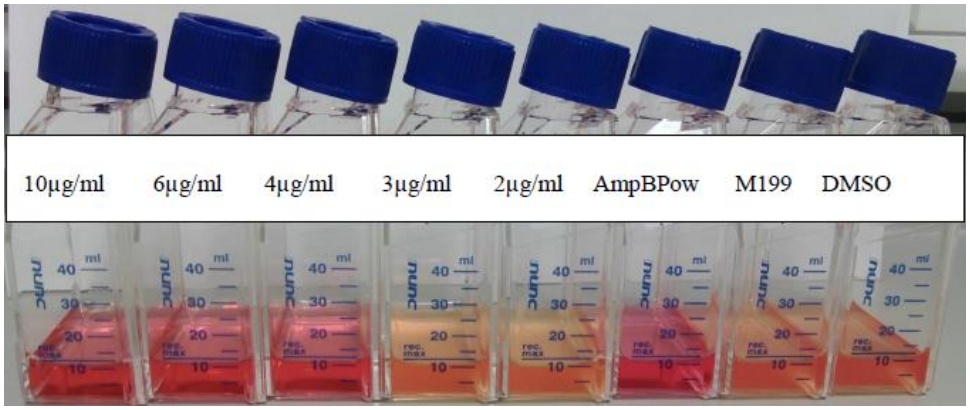

**Figure 7.12** The inhibitory effect of the cryptolepine on the promastigotes of GH5. Negative controls: DMSO (50µl); M199 (0.0µg/ml). Positive control: 1.56µg/ml Amphotericin B.

The slight increase of the cryptolepine concentrations (10µg/ml, 6µg/ml, 4µg/ml 3µg/ml, 2µg/ml) nonetheless, produced some level of promastigote inhibition in at least top three concentrations, 10µg/ml, 6µg/ml, 4µg/ml (**Figure 7.13**). However, none of the inhibitory effects could matched the inhibition produced by the reference drug at 1.56µg/ml concentration, eventhough there was no promastigote detected beyond the 7th day verified by sub-passaging.

**a) (i)**




**a) (ii)**




**b)**

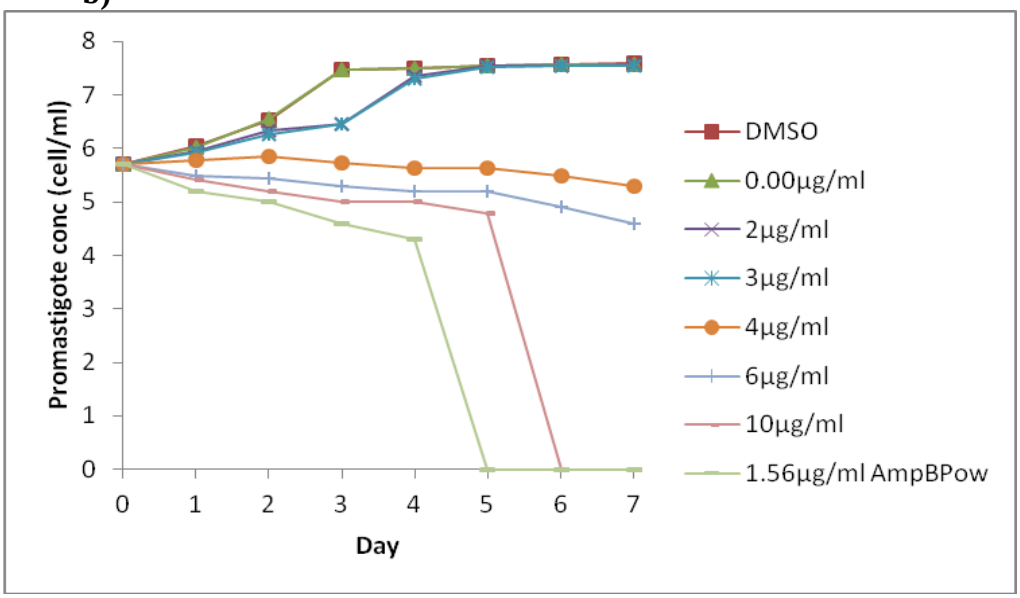



**Figure 7.13** The inhibition effect of cryptolepine on GH5 promastigotes at higher concentrations. Positive control: 1.56µg/ml Amphotericin B; Negative controls: M199 (0.0µg/ml) and DMSO (50µl). **a) (i)** and **(ii)** observed inhibition activities from days 1-7; **b)** promastigote densities detectable by the haemocytometer.

Intruigingly the GH5 promastigotes cluster under nomal growth conditions (M199 supplemented with 10% FBS, BME vitamins). At higher concentrations of the cryptolepine (10µg/ml and 6µg/ml) and in 1.56µg/ml Amphotericin B, there was no clustering of the promastigotes, the cells of the promastigotes seem disentigrated. Even though the scope of this study does not cover investigation into why such clustering, it is still worth mentioning.

In the case of GH5 susceptibility to Amphotericin B at the concentrations 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml, there was pronounced growth inhibition of the GH5 promastigotes in the top two concentration (1.56µg/ml, 0.625µg/ml), similar to that observed for Amphotericin B against *L. mexicana* (**Figure 7.14**).

In the promastiogtes densities determination by the haemcytometer, the two top concentrations (1.56µg/ml and 0.625µg/ml) of Amphotericin B produced marked inhibition of growth. There were no detecteable GH5 promastigotes by day 6 at 1.56µg/ml Amphotericin B, whereas in the case of concentration 0.625µg/ml, promastigotes were non-detectable at day 7 (**Figure 7.15)**. The Amphotericin B concentration 0.25µg/ml inhibited the promastigotes growth from days 1-3, very close to the growth inhibition pattern demonstrated by concentration 0.625µg/ml. The growth inhibition was surprisingly reversed from day 4 and progressively promoted through to day 7 in 0.25µg/ml (**Figure 7.15**), becoming quite close to the negative controls (M199 and DMSO). Contamination of the media was checked to rule out the possibility of other parasitic and microbial growth.

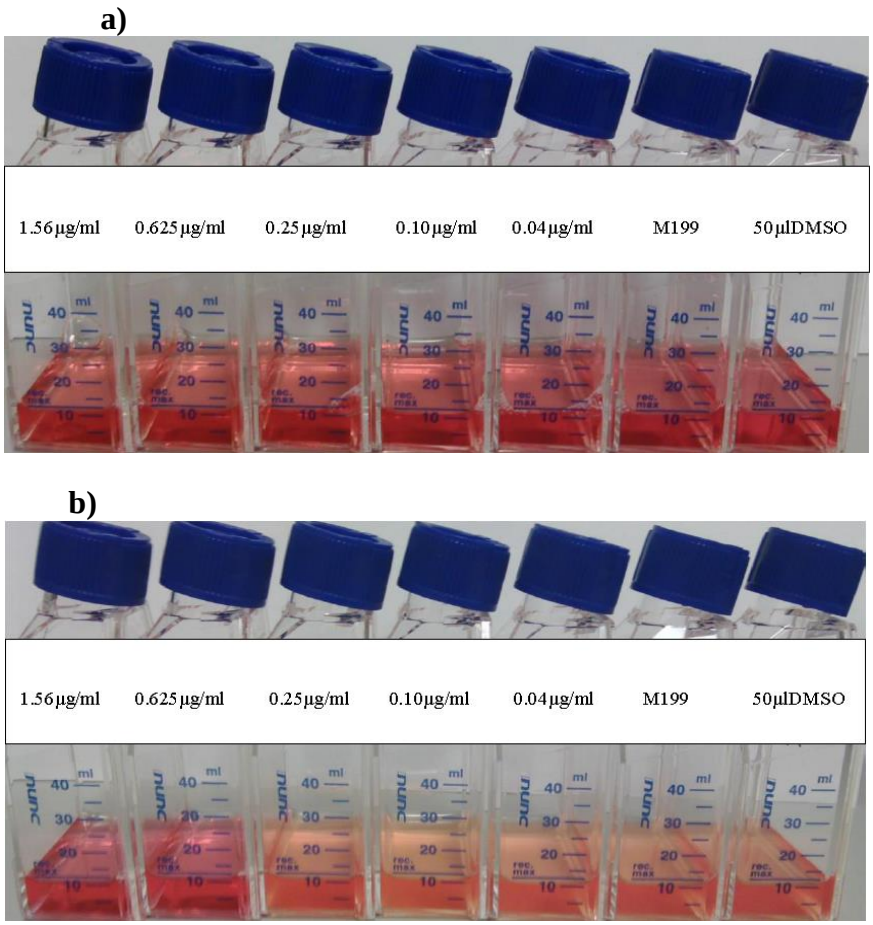



**Figure 7.14** GH5 flasks seeded with Amphotericin B at concentrations of 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml. Negative controls: M199 (0.0µg/ml) and DMSO (50µl). **a).** Growth at day 1; **b).** Growth at day 7.

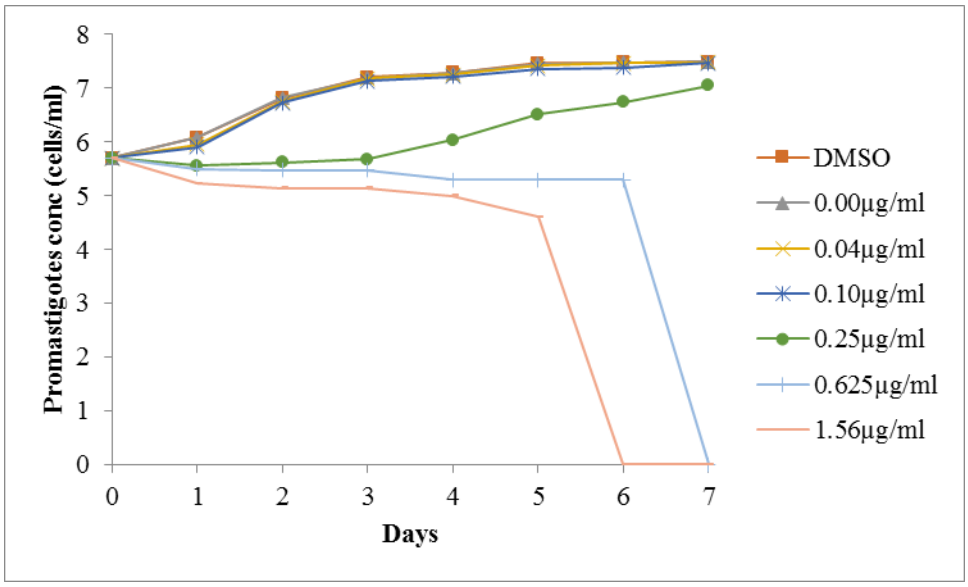



**Figure 7.15.** The inhibitory effect of the Amphotericin B on the promastigotes of GH5. Negative controls: DMSO (50µl); M199 (0.0µg/ml).

### 7.4 Dose responses

The effects of the different concentrations of the cryptolepine and control drug Amphotericin B, at day 2 time points, were used to estimate the effects of cryptolepine and Amphotericin B on the *Leishmania* isolates used in the study. The antilog of $EC_{50}$ values inferred from the inhibition concentrations of the dose response curves were equally computed by the software. At one set of concentrations (lower concentrations: 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml), the effects were clearly demonstrated in the **Figure 7.16a**.

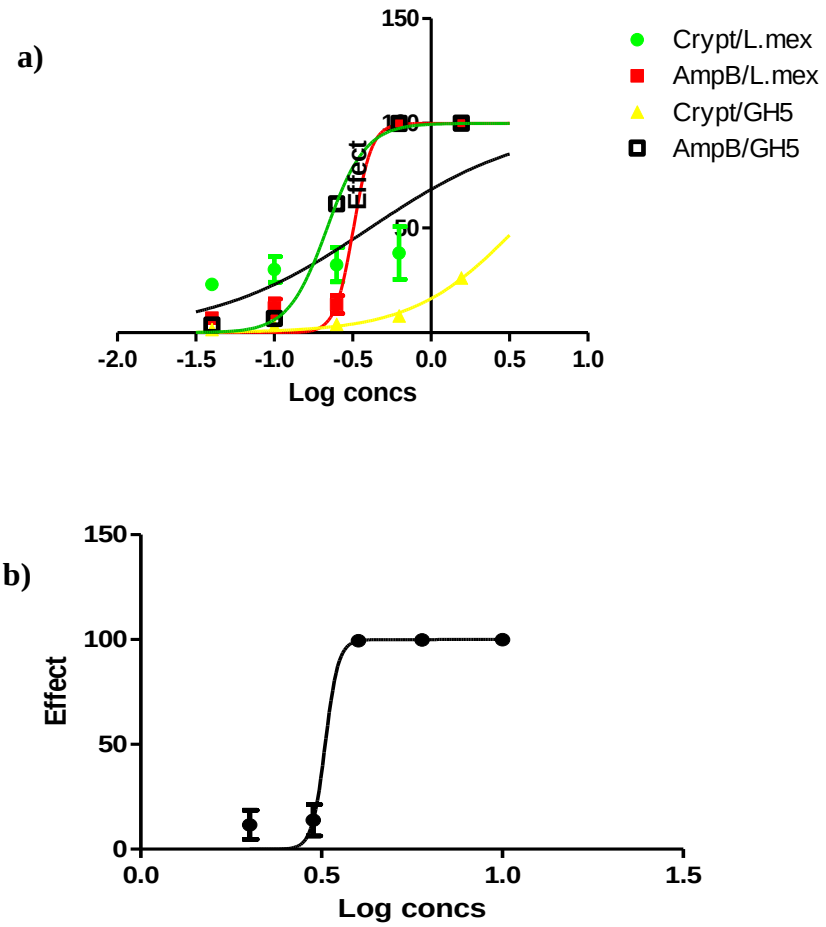



**Figure 7.16** Dose response curves at day 2 time points. **a).** Cryptolepine and Amphotericin B on both *L. mexicana* and GH5 at lower concentrations (1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml and 0.04µg/ml); **b).** Cryptolepine on GH5 at higher concentrations.

The cryptolepine was more effective on the *L. mexicana* than on the GH5 isolates, with the *L. mexicana*-cryptolepine effect producing a gently sloped curve with $EC_{50}$ value of 0.4078 more towards agonist than antagonist (**Table 7.2**). Steeply sloped curves were generated for the effect of Amphotericin B on both isolates whose $EC_{50}$ values were 0.3202 (AmpB/L. mex) and 0.2174 (AmpB/GH5) (**Figure 7.16** and **Table 7.2**).

| | **Concentrations** | | | | |
|---|---|---|---|---|---|
| | **[a]Lower** | | | | **[b]Higher** |
| **Treatment** | **Crypt/L.mex** | **AmpB/L.mex** | **Crypt/GH5** | **AmpB/GH5** | **Crypt/GH5** |
| **$EC_{50}$** | 0.4078 | 0.3202 | 3.514 | 0.2174 | 3.237 |

**Table 7.2** $EC_{50}$ values obtained from the effects of cryptolepine and Amphotericin B. [a]Lower concentrations: 1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml, 0.04µg/ml; [b]Higher concentrations: 10µg/ml, 6µg/ml, 4µg/ml 3µg/ml, 2µg/ml.

The dose response of cryptolepine on GH5 was not highly positive as portrayed by the virtually flattened dose response curve whose $EC_{50}$ value 3.514µg/ml, was very high, far above the effective drug value range (1). On increasing the concentration to check the cryptolepine effectiveness on the GH5, the $EC_{50}$ value could only come to 3.237 µg/ml (**Table 7.2**) with a very steeply sloped dose response curve, which is shifted more towards the right, this indicates reduced potency (**Figure 7.16b**).

### 7.5 Discussion

The recommended drugs for the treatment of leishmaniasis are fast becoming ineffective and noncompliant, characterized by long course of treatment, toxic side effects, chemo-resistance and ridiculous costs (Iwu *et al.*, 1994). These has necessitated urgent quest for new drugs, molecular targets, and novel therapeutic

strategies to treat the infection, of which plant alkaloids have been appraised and promising. One of such plant alkaloids is cryptolepine, purified from *Cryptolepis sanguinolenta*, common plant in West Africa, which has traditionally been used to malaria among other protozoa diseases (Ansah and Gooderham, 2002).

Cryptolepine have generally been found efficacious against some species of *Leishmania* including its different synthetic derivative forms, aside its effect on protozoa (Hazra *et al*., 2012; Wright *et al*., 2001; Onyeibor *et al*., 2005; Ansah and Gooderham, 2002; Oluwafemi *et al*., 2009). The compound seems to have inhibited promastigotes of visceral leishmaniasis causing *L. donovani*, however the derivative form could inhibit intracellular amastigotes, in addition to the commercial drug resistant *Leishmania* (Hazra *et al*., 2012). In one recent investigation, cryptolepine and its derivatives have been accessed to work within 50µM, as the highest tested dose ($IC_{50}$ = 50µM) at which some of the derivatives influenced by the positioning of a halogen on the canonical ring of crytolepine, were found non-efficacious (Hazra *et al*., 2012), which on the contrary, is higher than the 1.56µg/ml concentration in the current investigation.

In other to predict the effect of the cryptolepine inhibition on the promastigotes of the *Leishmania* species, the normal growth density curve and the current susceptibility curve, were compared. When the cryptolepine was applied, there were generally some level of initial promastigote density appreciation but could be overwhelmed after few days, at the concentrations where the promastigotes inhibition were effective. Many characteristic influence of cryptolepine have been mentioned including autophagy-apoptotic phenomenon on *Leishmania* at the treatment of cryptolepine (Hernandez-Chinea *et al*., 2015; Hazra *et al*., 2012; Sengupta *et al*., 2011), which could have also been experienced in this investigation. This is due to the

morphological changes which occurred in the promastigote cell (Hazra *et al*., 2012), though apoptotic characteristics were not investigated.

At the initial application of the compound at effective concentrations (1.56 µg/ml), there were substantial escalation in promastigotes densities, few hours to days. This could be due to the recognition of the ability of the cells to resist the effects of cryptolepine, which has been reported to exert dysfunctional characteristics in certain species of *Leishmania* promastigote (Sengupta *et al*., 2011). The defensive mechanisms exhibited by the promastigotes of *L*. *mexicana* on which cryptolepine were effective, were later overwhelmed (Thorburn, 2008; Scherz-Shouval and Elazar, 2007), which led to the decline in the promastigote densities over time, necessitated by the harsh environmental stress induced by the addition of the effective concentration of the cryptolepine (Levine and Klionsky, 2004). This overwhelming gradual decrease in densities of *L*. *mexicana* promastigotes have been attributable to the cryptolepine killing the promastigotes by apoptosis in certain studies (Sengupta *et al*., 2011), contrary to cryptolepine proposed mechanism in other protozoa (Wright, 2005) as well as the old assertion that apoptosis does not occur in unicellular organisms (Khademvatan *et al*., 2009).

The most effectiveness of the cryptolepine on the density of *L*. *mexicana* marked by the reduced number of promastigotes, was felt at a concentration 1.56µg/ml, same as that of the amphotericin B concentration howbeit less effective than the Amphotericin B (which has deoxycholate component). It is not surprising though since some derivatives of cryptolepine have performed creditably well than the pure compound, even though cryptolepine performed at an $IC_{50}$ of 1.6 µM (Hazra *et al*., 2012). Certain derivatives of cryptolepine have been involved in the interference of apoptosis (Hernandez-Chinea *et al*., 2015), in which case there are alterations in the

cell structure. Similar cellular malfunctions were exhibited at the incubation days when the effects of the cryptolepine were graphically pronounced. It is therefore speculated that, analogous topoisomerases inhibition which are induced in several cryptolepine derivative investigations were experienced in this study. Additionally, the promastigote cells of the *L. mexicana* where the cryptolepine was effective underwent shrinkage and assuming amastigote-like structures. In some other cells elsewhere, cytoplasmic and nuclear shrinkages together with chromatin condensation, DNA fragmentation and multiple membrane-bound apoptotic bodies have been characterised with apoptosis and reported (Hernandez-Chinea *et al*., 2015). Though the pattern of apoptosis in kinetoplastida is not fully understood (Lee *et al*., 2002; Arnoult *et al*., 2002; Mukherjee *et al*., 2002), parallel cellular interference could hazardously have been guessed in this study, despite the asserting that apoptosis seemingly becoming the preferred parasiticidal mechanism (Lee *et al*., 2002). The dysfunctional promastigotes cells at the effective cryptolepine concentrations in *L. mexicana*, could be attributable to ROS (reactive oxygen species) induced in the promastigotes cells, during cryptolepine addition, as reported previously (Sengupta *et al*., 2011).

The inhibition effect of the compound in the current study on *L. mexicana* promastigotes was independent on the stability of the cryptolepine, in other words, the stability of the compound was not affected by the experimental conditions, predicting a sealing effect of the cryptolepine which needs further investigation. The cryptolepine on application from the start of the experimental set up did not change. This implies that the compound was quite stable in vitro and effective under the experimental conditions against *L. mexicana* promastigote. The daily application of

the cryptolepine could not influence the inhibition of the promastigote either compare to one-time application from day zero.

The dose response curve was perfect for the *L. mexicana*-cryptolepine, whose $EC_{50}$ value of 0.4078µg/ml was within a reasonable and tolerable limit, which was even higher than that of the *L. mexicana*-Amphotericin B of 0.3202µg/ml. Assuming the highest limit of the dose concentration of 50µM in other investigations have been the performance of the cryptolepine (molecular weight ≈ 258g/mol) in Hadza *et al*., (2012) on *L. donovani*, an approximated $IC_{50}$ of 12.9µg/ml (50µM) could have been obtained, roughly thirty-two times better in the current study than the previous one. This would have been on the higher side and possibly intolerable to the host cells, compare to the $EC_{50}$ of 0.4078µg/ml of cryptolepine on *L. mexicana,* in in vivo study. In a very recent study of synthetic compound related to cryptolepine on similar species, *L. mexicana* (MHOM/BZ/62/BEL21 strain), the $IC_{50}$ values of more effective to least effective of the synthetics ranged between 1.01 to 4.83µg/ml (Hernandez-Chinea *et al*., 2015). This is higher than the amount of cryptolepine require to inhibit 50% promastigotes of *L. mexicana* in the current investigation. Hence the cryptolepine concentration (1.56µg/ml), has performed better in this study. A further increase in the concentration or modification of the compound in the current study would perform better to give an $EC_{50}$ value within approximated agonist range, to make the compound more agonist than partial agonist as it is depicted in the recent study, taking into account the toxicity at a slight increase. However, in *L. major* in vitro susceptibility study liposomal and non- liposomal forms of clarithromycin, a dose response with $EC_{50}$ concentration of 169µg/ml and 253.6µg/ml respectively where reported (Sazgarnia *et al*., 2011), which is seems ridiculously high on an in vitro promastigotes, bearing in mind an anticipated in vivo study.

At the concentration 1.56µg/ml of the cryptolepine, the promastigotes of GH5 were inadequately inhibited even beyond the 7 days incubation periods. Cryptolepine could barely influenced its inhibition effects on the promastigotes of GH5. A small amount of effect was experienced only when the concentrations compound was increased approximately ten times (10x). The increased in concentration produced unfavourable dose response curve which is prospective to be toxic, reinforcing the property of sealing effect of cryptolepine. The $EC_{50}$ (3.237) at higher concentrations (10µg/ml, 6µg/ml, 4µg/ml 3µg/ml, 2µg/ml) did not differ much from when the lower concentrations (1.56µg/ml, 0.625µg/ml, 0.25µg/ml, 0.10µg/ml, 0.04µg/ml) of $EC_{50}$ value (3.514). This has made cryptolepine antagonist towards GH5. The only explanation regarding the resistance of GH5 to this concentration of cryptolepine, compare to its inhibition on the *L. mexicana* in the current study is the fact that, the species of the parasite used in this investigation are of two different clades on the phylogenetic tree (Kwakye-Nuako *et al*., 2015), despite both have been responsible for CL, in a similar or different geographical settings (Desbois *et al*., 2014; Machado *et al*., 1994; Noyes *et al*., 2002), which would therefore expected to respond differently to the compound. Moreover, *Cryptolepis sanguinolenta* (nibima) is a common plant whose concoction is available on the market (herbal products) enjoy wide unregulated usage in Ghana for the treatment of ailment including malaria (Osei-Djarbeng *et al*., 2015; Tempesta, 2010; Lisgarten *et al*., 2002; Ansah and Gooderham, 2002; Laryea *et al*., 2009; Wright, 2007) as well as other microbes (Mills-Robertson *et al*., 2012). The species GH5 might have thus been pre-exposed to the active compound through the whole plant usage (concoction) across the country. This therefore might have induced certain level of resistance in the GH5, beyond the threshold at which the cryptolepine concentrations currently used in this investigation

would be able to avert. Furthermore, the concentrations at which this compound is studied in relation to its effects on *Leishmania* have been above the current concentrations used in this study (Hazra *et al*., 2012), which would have provoked their autophagic properties to sustain the growth of promastigotes of GH5, beyond the threshold of at which apoptosis could not counteract, since there is a report of autophagic response to cryptolepine treatment (Sengupta *et al*., 2011) to enhance the promastigote growth. An increased concentration of the cryptolepine could not be helpful either, since it could make the compound toxic to the host cells as indicated by the dose response curve. This would therefore require a substitute on or modification of the compound to make it more effective to the GH5 at a tolerable concentration.

### 7.6 Conclusion

Various ailments treated with cryptolepine have been reported including protozoa. Recent investigations on *Leishmania* species have come up for studies including *L. donovani* (Hazra *et al*., 2012; Sengupta *et al*., 2011). The inhibition effects of the cryptolepine on *L. mecicana* and newly isolated species from Ghana GH5 promastigotes, both responsible for CL, is studied in this current investigation and under continuous investigation. The compound, cryptolepine, have shown inhibition on the promastigotes at varying effectiveness on some species at diverse concentrations. It has demonstrated some level of activity against well characterised *L. mexicana* but not the newly emerged GH5 in this current study. A derivative of the compound through modification would make it useful candidate for some species of *Leishmania* especially GH5 for indigenous use, based on its wide usage and availability in Ghana. This particular study focused on the inhibition of the promastigotes of *Leishmania* species. Further study to focus on the clinically relevant stage of the *Leishmania*, axenic and intracellular amastigotes would fully divulge the

drug candidature of the pure compound cryptolepine against GH5 and other closely related species. It is ideal to present drug screening procedures that reflects the in vivo, though quite arduous. The design of axenic and in vitro intracellular macrophages of the parasite would closely represent such in vivo procedures (Callahan *et al*., 1997), where important properties among others, like ROS (reactive oxygen species) displayed by *Leishmania*-infected animal cells (macrophage cell lines) when cryptolepine is applied, is hoped to enhance the inhibition of the *Leishmania* promastigotes, where apoptosis and ROS have been linked (Sengupta *et al*., 2011). Other natural products and/or their derivatives have extensively been studied on *L. mexicana* (Abdala *et al*., 2002), but not with cryptolepine. An extensive effort to confirm or otherwise, the effect of the compound on both *L. mexicana* and GH5 promastigotes would be incentive since it is commonly used in Ghana. The effect of the pure compound cryptolepine from this common indigenous plant *Cryptolepis saguinolenta* (nibima) in Ghana is barely unknown. This seems to be the first report of the susceptibility of 95% purity of isolated cryptolepine from its parent plant on promastigotes of both species of *Leishmania* responsible for cutaneous leishmaniasis.

With its deoxycholate constituent, the reference drug Amphotericin B, with its anti*Leishmania* (Manandhar *et al*., 2008) properties, could marginally perform roughly 1x ($EC_{50}$ = 0.3202µg/ml) better than this purified and indigenously isolated cryptolepine ($EC_{50}$ = 0.4078µg/ml) at the minimal concentrations used in this study on *L. mexicana* promastigotes.

## Chapter Eight

# 8.0 General Discussion

## 8.1 Introduction

Leishmaniasis is now a public health problem worldwide. It was previously associated with the tropics and sub-tropics in a disproportionate manner, but now it is of global importance due to increasing international travel, military intrusions, global integration, mass exodus of refugees, tourism and intrusion into the disease environment through urbanisation. This has therefore triggered new foci of the disease with interesting dynamics in relation to the parasite, vectors and transmission, management interventions and the search for therapeutic routines. Emergence of autochthonous leishmaniasis has occurred in new foci in Africa, Australia, South-east Asia, and the Caribbean. Such emerging foci are of much interest and concern, and require investigation into the disease and its outcomes.

Various species related to *Leishmania enriettii* have recently been discovered in a somewhat complex and new clade in the *Leishmania* taxa. A previously presumed monoxenous trypanosomatid confirmed as the cause of skin lesions in the Caribbean was documented (Dedet *et al*., 1995). It was later recognised that it could possibly be a new species requiring classification among *Leishmania*. The advancement in diagnostic molecular tools confirmed the species as *Leishmania* (Desbois *et al*., 2014), and was subsequently grouped into the *L. enriettii* complex (Dougall *et al*., 2011). Additionally, other similar species of *Leishmania* have emerged from Thailand and Australia whose vectors are still debateable (Pothirat *et al*., 2014; Dougall *et al*., 2011). In Sri-Lanka CL was found to have been caused by *L. donovani* (Siriwardana *et al*., 2007), which is unusual for this agent. Around the same time Ghana was first experiencing CL cases whose identity, vectors and reservoirs were unknown.

## 8.2 Conclusions

Emerging species of *Leishmania* has been the focus of this study, where a new species believed to be in the *L. enriettii* complex, is implicated for the first time in Ghana and for the first time over several decades in Africa. The CL in that south-eastern part of Ghana has being trending, gaining prominence among the indigenes where it is referred to as "agbamekanu". This has led to the identification and diagnoses of new species of *Leishmania*, from CL patients in a focus at that south-eastern part of Ghana (Kweku *et al*., 2011) after there has been speculations of suspected human cases since 1999. The simple PCR bands confirmed the disease was caused by *Leishmania* whose identity could not be determined from this method, when the samples from patients were compared with the band sizes of positive controls of *L. tropica*, *L. aethiopica* and *L. major*. The identity of this new *Leishmania* species could neither be inferred from the PCR-RFLP analysis even though clear multiple bands were established, after their PCR products were digested by *Msp1* endonuclease. The identity as a probable new *Leishmania* species, which is yet to be officially named, was revealed by sequence and phylogenetic analysis as was the case of new species emerged from Australia and Thailand (Dougall *et al*., 2011; Pothirat *et al*., 2014). There has not been the case of imported influx of infected patients from other known leishmaniasis endemic foci as all the patients were indigenes, contrary to the first few CL cases earlier reported in Namibia in the south-western part of Africa, which were purely imported despite the identification of sand flies, though some few indigenes have lately been reported to have had the disease (Grove, 1970; 1989).

Thought to be *L. major* according to ITS-1 gene analysis (Fryauff *et al*., 2006), a subsequent report could not confirm or otherwise of the identity of the Ghanaian *Leishmania*, but asserted an uncharacterised *Leishmania* species (Villinski *et al*.,

2007). These controversies necessitated the investigation into the identity of the *Leishmania* species responsible for the CL. The current identification and confirmation was made possible through a published molecular tool based on the ribosomal protein L23a intergenic spacer (Dougall *et al*., 2011) in addition to ITS and RNA Pol II gene analysis (Kwakye-Nuako *et al*., 2015). These sequences undoubtedly have confirmed the identity of the parasite responsible for CL in Ghana to be a newly isolated species of *Leishmania* belonging to the new clade *L. enriettii* complex. The electron micrographs have as well confirmed the identity to be *Leishmania* with morphological semblance, showing both short and long flagella, especially in the SEM evidence. The TEM confirmed well demarcated flagellar pockets within which the flagella insert, similarly found in the species which was responsible for CL in Martinique (Noyes *et al*., 2002), in addition to clearly demonstrated nucleus and kinetoplast adjacent to each other, exactly as in amastigote-like forms previously reported. This current Ghana and the previously reported species have shown dissimilarity from *Trypanosoma* and other closely related species (Dedet *et al*., 1995; Desbois, *et al*., 2014). Both species have thus demonstrated different morphological forms in culture, which seem to be the characteristics of these new emerging parasites, clustering in growth medium. In the case of Martinique's isolates, they were previously described as paramastigotes and opisthomastigotes (Dedet *et al*., 1995). Nonetheless, no such similarities have been found for species isolated from Ghana despite the different forms they exhibit in culture.

The isolates from Ghana have still not been infectious to any lab strain mice, in contrast to *L. martiniquensis* which has recently been infectious to Balb/C mice (Noyes *et al*., 2002; Garin *et al*., 2001), in addition to a possible show of opposing intracellular form (amastigotes) characteristics from other *Leishmania* species with

varied behaviour in cultures. The isolates from Ghana caused localised CL, contrary to that of *L. martiniquensis*, probably due to the immunocompromised state of the victims (HIV/AIDS) on the island. This was not a deviation as *Leishmania* infections are dependent on the immune status of the hosts. The species of *Leishmania* responsible for diffused cutaneous leishmaniasis in the Dominican Republic (Caribbean), was found to be entirely different from any New World species though it was presumed to be close to *L. mexicana* complex (Kreutzer, 1990; Schnur *et al*., 1983). This reinforces the previous contentious identity of *Leishmania* species from the Caribbean in past investigations, which until recently emerged as belonging the *L. enriettii* complex (Desbois, *et al*., 2014), a relation to Ghana isolate.

Since it has emerged as a new *Leishmania* species, it is ideal to classify and name it appropriately after adequate evidence have been adduced from very good gene sequence analysis, which is robust in terms of phylogeny over a long evolutionary distance with a strong bootstrap support. This is in agreement with similar argument which had been suggested previously from the use of 18S genes where genetic diversity is found among the *Leishmania* species (Noyes *et al*., 2002).

Surprisingly, the identity of the vector(s) of the current *Leishmania* isolated from Ghana are still not fully revealed just like Caribbean *Leishmania* species. In the Caribbean, *Lutzomyia* species (*Lutzomyia christophei*) is the implicated vector in Dominican Republic, which was the least collected species in a field investigation. However, the species of the *Leishmania* (*Leishmania*-Isabel; MHOM/DO/79/Isabel, WR338c) from Dominican Republic was experimentally found to have suprapylarian midgut development in *Lutzomyia longipalpis* (Johnson *et al*., 1992; Lainson, 1983). Even though not prevalent, the distribution of *Lutzomyia christophei* in the wild were found to coincided with the disease and were the only species which fed on humans

(Johnson *et al*., 1992). This might be shifting the course of the new emerging *Leishmania* species of the *L. enriettii* complex and their interactions with vector species. With recent tools for studying the insect-*Leishmania* interaction, such hidden knowledge will be unravelled, where the developmental stages of the *Leishmania* in their vectors are now known (Bates and Rogers, 2004).

The vector(s) of this current isolated *Leishmania* species though not fully known, the speculation on biting midges as a likely vector is supported by the results presented here. This vector, though outside the known sand fly vectors, will not be too much of a surprise since a similar vector has already been implicated (Dougall *et al*., 2011). These information coupled with the new *Leishmania* species emerging elsewhere, have renewed the interest of the disease epidemiology and management across the world.

The recent outbreak of leishmaniasis in the south-eastern part of Ghana has necessitated the need for diagnosis in the endemic area. The victims do not report to any facility where they could properly be managed and the records on the disease taken for any intervention plans. Since it was first reported, the disease has not been clinically diagnosed anywhere especially with point of care diagnostic tool. The victims are basically very poor, located in the very remote part of the district with no access to health facility or unable to afford one. As part of the current study therefore, a point of care diagnostic procedure is being sought to initiate the diagnosis of the diseases in the endemic community, hence the use of CL Detect$^{TM}$ developed based on enzyme peroxidoxin. The perioxidoxin which is found abundant in amastigotes could be detected in lower quantity by CL Detect$^{TM}$ (InBios, Seatle, USA), when the promastigotes were used. Designed as a rapid test kit, it would come as handy qualitative diagnostic tool to quickly detect the presence or otherwise of the parasite in

patients on the field. This is yet to be rolled out after it had successfully detected the antigens in promastigotes in the lab. No such diagnostic procedures have been carried out on the field. It is presumed to be a quick diagnostic tool for trial and possibly implemented on the field.

The antigen perioxidoxin has not been developed to be used as diagnosis for rapid detection of the parasite in clinical cases. If successful, this will be a useful diagnostic platform on the field. Many similar rapid diagnostic kit have been designed to detect the parasite in urine, blood, but are targeted against the VL form of the disease. For instance, rK39 has extensively been used but have performed better in the VL endemic areas, than CL endemic areas (Hartzell *et al*., 2008; Boelaert *et al*., 2007). The small amount of promastigotes detectable by this kit is look promising and might perform better in this CL endemic area, just as other diagnostic kit have achieved greater diagnostic results in other clinically manifested forms (Molinet *et al*., 2013). Not many rapid test diagnostic kits have been design for CL diagnosis as in the case of rapid test kits for VL diagnosis. Therefore the kit from InBios would become ideal in an anticipation of the small quantity of peroxidoxin it could detect in real-time regarding abundance in *Leishmania* (Daifalla *et al*., 2015), if it could detect small quantities in promastigotes.

Due to the self-limiting nature of the disease, the treatment of CL is dependent on the *Leishmania* species involved and the potential to disseminate into other parts of the body to cause complex forms of the disease. This implies the treatment of CL is not urgent. However, simple diagnostic test will greatly help to enhance treatment, mostly dependent on the commercially available drugs. These drug have posed problems with regards to toxicity, adverse side effect (Croft *et al*., 2006) and more importantly inaccessible to the poverty stricken victim in terms of affordability (Iwu *et*

*al*., 1994). The treatment of CL at any foci would aim importantly at preventing complex disease situations such as mucosal involvement and relapse, as well as accelerating wound healing lesion, decrease parasitic load in victims from acting as reservoir in a given community (Bukar *et al*., 2015). By these, any successful treatment devoid of trial and error is crucial.

The commercial availability of the leishmaniasis drug on market does not help the victims of this particular endemic focus i.e. Ho district in the Volta region of Ghana, hence the need for indigenous treatment would be of immense important. Some of the victims have resorted to the use of local herbs to treat the disease which they anecdotally claim to be useful. Scientific investigation to ascertain the genuineness of this claim was therefore essential. One active compound is cryptolepine, in one the plants has been in used across the country for malaria and other protozoa treatment as well as various aliment (Ansah and Gooderham, 2002; Wright *et al*., 2001; Onyeibor *et al*., 2005). This compound was however inactive against the *Leishmania* Ghana. Nevertheless, cryptolepine applied to the control isolates, *L. mexicana*, which is responsible for CL in the New World, was found to exhibit the activity of the promastigotes of the species. This is not surprising though since other derived forms of the compound have been found active against other species of *Leishmania* (Hazra *et al*., 2012). The explanation that could be ascribed to the inactivity of cryptolepine on *Leishmania* Ghana has to be a possible pre-exposure to the compound, from the abundant use of the whole plant in treating malaria among other ailments in Ghana. This would have induced some level of resistant mechanism against the compound. Other inherent parasitic factors could have contributed to the resistance posed to the application of cryptolepine but they remain uncertain as far as this current investigation is concerned. The focus was primarily on the in vitro

inhibition activity of the compound on *Leishmania* Ghana and *L. mexicana* promastigotes growth. The concentration of the cryptolepine needed to inhibit the promastigote growth of the control isolates *L. mexicana*, was found to be within the tolerable range to the animal cells (Ansah and Gooderham, 2002; Dassonneville *et al*., 2000), contrary to the concentration that could inhibit *Leishmania* Ghana.

### 8.3 Recommendations

- On confirming the disease in that particular focus, further molecular methods should be carried out to confirm or otherwise any mix infections in the community and its catchment area.
- Once the disease is confirmed measure of routine diagnosis should be established to encourage at least indigenous treatment regimen for the victims.
- More leishmaniasis investigations need to be carried out across the country to know which other part of the country is endemic with leishmaniasis.
- Vector incrimination studies on both the sand flies found in the community as well as biting midges should urgently be undertaken.
- The mechanism of resistance demonstrated by the *Leishmania* Ghana against the action of cryptolepine should be fully investigated.
- The activity of the cryptolepine on other Old World agents responsible for CL should also be investigated alongside *Leishmania* Ghana and *L. mexicana*.
- More investigation into many indigenous plants should be considered to offer alternative to cryptolepine when resistance ensue.

### 8.4 Ongoing research priorities

- Rapid test diagnostic procedure is being established using CL Detect®, InBios, Washington, USA, for routine diagnosis of the disease in endemic community in Ghana.
- Further investigation in both lab and on the field is ongoing to confirm or deny biting midges as vector of *Leishmania* Ghana.
- The identity of the midges in Ghana which have never been studied is on course.
- More field investigation further away from the present community is ongoing to confirm or deny differential identity of the *Leishmania* in that part of the country.
- The search for the reservoirs, though yet to vigorously be pursued, is anticipated.
- A derived form of the crytolepine to be active against *Leishmania* Ghana is currently being pursued to ensure that small amount absorbable by the promastigotes is found in vitro.
- The activity of cryptolepine on the *Leishmania* species closely related to *Leishmania* Ghana are being considered.

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# Appendix

## Aligned Sequence

```
C.fasciculata         -----CCTCGTTTAAGTGACGTTTGA--------------ATCTTCT---------CGTT
L.hertigi             -----TCTTGTCTAAGCAGAATTTGA--------------ATCATGC------------A
E.monterogeii         -----TCTCGTTTAAGCAGAATTTGA--------------ATCATGT------------G
L.colombiensis        -----TCTCGTTTAAGCAGAATTTGA--------------ATCATGT------------G
L.equatorensis        -----TCTCGTTTAAGCAGAATTTGA--------------ATCATGT------------G
L.braziliensis        -----TCTTGTCTAAGCAGAATTTGGCGGTGGTGCGGCTACTTTTGGGATTATTTTTTTA
L.panamensis          TCTTGTCTAGTAAACGAGAACTTGACTGGGGTGCACCATACTTTTGGGAATATTTTCTTA
L.guyanensis          TCTTGTCTAGTAAACGCAGAACTTGACTGGGGTGCAGCTACTTTTGGGATTATTTTCTTA
L.adleri              ----CCTCGTCTAACACGGTATTCGC--------------AGGACGTAATGTTGTTTGTT
L.hoogstraali         ---GCTTGTTTAAAAACCGGATTTGC--------------CAGACGTG-------ATTTT
L.tarentolae          -----TCTTGTCTAAGCAGTATTTGC---------------TGACGT--------GATTT
L.gymnodactyli        ---GCTTGTCTAAAAGCAGTATTCGC--------------ATGACGTG-------ATTTT
L.mexicana            -----TCTCGTCTAAGCAGAACTTGG--------------ATGATGCG-----------A
L.amazonensis         -----TCTTGTCTAAGCAGAACTTGG--------------ATGATGCG-----------A
L.donovani            -----TCTTGTCTAAGCAGAATTTGG--------------ATGATGCG-----------A
L.infantum            -----TCTCGTCTAAGCAGAATTTGG--------------ATGATGCG-----------A
L.tropica             -----TCTTGTCTAAGCAGAATTTGG--------------ATGATGCG-----------A
L.major               -----TCTTGTCTAAGCAGAATTTGG--------------ATGATGCG-----------A
L.gerbilli            -----TCTTGTCTAAGCAGAATTTGG--------------ATGATGCG-----------A
L.turanica            -----TCTTGTCTAAGCAGAATTTGG--------------ATGATGCG-----------A
AM-2004               ----ACTCGTCTAAAGCGGAATGCGA--------------ATCGCGCG-----------A
L.martiniquensis      -----CCTCGTCTAGGTGGAATCGGA--------------ATCGAGCG-----------A
L.enriettii           -----TCTCGTCTAGCCGGAATTCGA--------------ATCGCGTG-----------A
GH5                   -----TCTCGTCTAGCCGGAATTCGA--------------ATCGCGTG-----------A
GH10                  -----TCTCGTCTAGCCGGAATTCGA--------------ATCGCGTG-----------A
GH11                  -----TCTCGTCTAGCCGGAATTCGA--------------ATCGCGTG-----------A
                                *

C.fasciculata         TTACTTTCATCATTTTGCATTGAATTCCAAAACAAATAAAAATATTGATGACAGCTCCTT
L.hertigi             ACTGGTTTGGGTTTTTTTTCTTCATT-----TCATAAAGAACGAAACGTTAGATTTTTAT
E.monterogeii         GTTGTTTTGAGTTATTTCATTAAAAA-----AA-GAAAGAAAAAACCATAGTCGCCAGTT
L.colombiensis        GTTGTTTTGAGTTATTTCATTAAAAA-----AA-GAAAGAAAAAACCATAGTCGCCAGTT
L.equatorensis        GTTGTTTTGAGTTATTTCATTAAAAA-----AA-GAAAGACAAAACCAGAGTCGCCAGTT
L.braziliensis        TTCCCTTTTTTTTTTTTCACTTCAAC--A--ACAAAAAAAAAATGAGGGGCAAGGCGCGCT
L.panamensis          TTCCTTTT------TTTTTTCAGTTC--A--ACAACAAAAAGTGAGGGGCAAGGCGCGCT
L.guyanensis          T-TCCCTT------TTTTTTCAGTTC--A--ACAACAAAAAGTGAGGGGCAAGGCGCGCT
L.adleri              TGTTCTTTTCATGACTTCATTTTCTGGGA--AAAAAGGGAGAGTAGACGCAACG----GT
L.hoogstraali         TTTTTTTTCATGA-CTTCATTTTATGAAA--AAAAAACGAGAATAGAAACGAAT----GT
L.tarentolae          TTTTTTTCCATGACTTCATTTTATGAAAA--AAAAAAAGAGAATAGAAACGAAT----GT
L.gymnodactyli        TTTTTTTCCCAAGACTTCATTTATGAAAA--AAAAAACGAGAATAGAAACGAAT----GT
L.mexicana            TTACTTG-CGATGATTTCATTTTATCA----ACAAAA--AAT-AAGTGCAAACGGTATCT
L.amazonensis         TTACTTG-CGATGATTTCATTTTATCA----ACAAAA--AAT-AAGTGCAAACGGTATCT
L.donovani            TTACTTG-AGATAATTTCATTTTCAT------CAGCAAAAAC-GAAGTGCAACGGTATCT
L.infantum            TTACTTG-AGATAATTTCATTTTCAT------CAGCAAAAAC-GAAGTGCAACGGTATCT
L.tropica             TTACTTG-AGATAATTTCATTTTATCA----ACAACAAAAAA-GAAGTGCAACGGTATCT
L.major               TTACTTG-AGATAATTTCATTTTATCA----ACAACGAAAAA-GAGGTGCTACGGTAACT
L.gerbilli            TTACTTG-AGATAATTTCATTTTATCA----ACAACGAAAAA-GAAGTGCTACGGTAACT
L.turanica            TTACTTG-AGATAATTTCATTTTATCA----ACAACGAAAAA-GAAGTGCTACGGTAACT
AM-2004               CTCCTTTTAGCTGACTTTATTTTGGCCCA--CGAAAGAAAAGTAAAAGCGCACTGTTGCC
L.martiniquensis      CTTGTTT-AGATGATTTTATTTTATC--A--GCAAAGAAAAGAAAAAGTGAGCTCTGGCC
L.enriettii           CTAGTTT-AGGTGACTTTATTTTATC--A--ACAAATAAAAGTAAAAATGCACGTTGGCT
GH5                   CTAGTTT-AGGTGACTTTATTTTATC--A--ACAAATAAAGGTAAAAATGAAGTTCGGCT
GH10                  CTAGTTT-AGGTGACTTTATTTTATC--A--ACAAATAAAGGTAAAAATGAAGTTCGGCT
GH11                  CTAGTTT-AGGTGACTTTATTTTATC--A--ACAAATAAAGGTAAAAATGAAGTTCGGCT
                                *                   *

C.fasciculata         GTATGTGTAACTGTGTGTGTGTGCCGTAGCGCTCCTCACAGTAGAGAG-----ATGGCGG
L.hertigi             TCAGATCC--GTA-G-ATCTTGACCGGAGCCACAAGGGCGTCATAGCG------------
E.monterogeii         CCGTGTCA--GTAGG-AGAGGGACAGTAGTTGGAAAGGAGCCATATCA------------
L.colombiensis        CCGTGTCA--GTAGG-AGAGGGACAGTAGTTGGAAAGGAGCCATATCA------------
L.equatorensis        CCGTGTCA--GTAGG-AGAGGGACAGTAGTTGGAAAGGAGCCATATCA------------
L.braziliensis        CTGTGGCT----C---------TCCAGAGGCGGACAACAGCTCTGTGAGAGGGGGCGCCCT
L.panamensis          CTGTGGCT----C---------TCCAGAGGCGGACAAGGGCTCTGTGAGAGGGGGCGCCCT
L.guyanensis          CTGTGGCT----C---------TCCAGAGGCGGACAAGGGCTCTGTGAGAGGGGGCGCCCT
L.adleri              GTGTTGTTGGCTT---------TCCAGCGGCCAGCGAAGTAACGGAGGG------TGGCGC
L.hoogstraali         GTGTTGCTGGCTT---------TCCAGCGGCCAGCGAAGTAACGGAGGG------TGACGC
L.tarentolae          GTGTTGTTGGCGT---------TCCAGCGGCCAGCGAAGTAATGGAGGG------TGGCGC
L.gymnodactyli        GTGCTGTGGGCGT---------TCCAGCGGCCAGCGTAGTAACGGAGGG------TGGCGC
L.mexicana            TTGGTTCT--------------CCAGAGGCCCGCGAAGGCTCTTAGAG------TGGTGT
L.amazonensis         TTGGTTCT--------------CCAGAGGCCCGCGAAGGCTCTTAGAG------TGGTGT
L.donovani            TTGGTTCA--------------CCAGAGGCCGG-CAAAGCTCTTAGGG------TGGTGT
L.infantum            TTGGTTCA--------------CCAGAGGCCGG-CAAAGCTCTTAGGG------TGGTGT
L.tropica             TTGGTTCA--------------CCAGAGGCTGGCGAAGGCTCTTAGGG------TGGTTT
L.major               TTGGTGCA--------------CCAGAGGCTGGCGACGGCTCTTAGGG------TGGTTT
L.gerbilli            TTGGTGCA--------------CCAGAGGCTGGCGACGGCTCTTAGGG------TGGTTT
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L.turanica            TTGGTGCA---------------CCAGAGGCTGGCGACGGCTCTTAGGG------TGGTTT
AM-2004               TCTTTTCGGGGGAGGCAGGGGAGGGGGACGCGGCAGAGGCTGTAAGGT------TCGTGG
L.martiniquensis      TT--------------------TTAGAGGGCATCAGACGCTCTGAGGG------TGATGC
L.enriettii           CT--------------------TTAGAGGTCGGCAGAGGCTCTGATGG------CGACGT
GH5                   CT--------------------TTAGAGGTCGGCAGAGGCTCTGATGG------CGGCGT
GH10                  CT--------------------TTAGAGGTCGGCAGAGGCTCTGATGG------CGGCGT
GH11                  CT--------------------TTAGAGGTCGGCAGAGGCTCTGATGG------CGGCGT
                                              *

C.fasciculata         ACTTTTTTTCCCTGCCCTCTCAAATGAGAGCTGATCTGCGC-GCTGGTAGGTGCATTTTG
L.hertigi             ----AATCGTGGCTCCTGCA---------TGGCACTGAGGCGACCCCGCTGCACTGTGTGG
E.monterogeii         ----AAATGCTGCCTTCTCC---------AGAATATGCAATGATCCAGGGAGACTGTGCTG
L.colombiensis        ----AAATGCTGCCTTCTCC---------AGAATATGCAATGATCCAGGGAGACTGTGCTG
L.equatorensis        ----AAATGCTGCCTTCTCC---------AGAATATGCAATGATCCAGGGAAACTGTGCTG
L.braziliensis        GGCTCGGGTGCAACCCTTAGGGT-TCACGGAGGTGTGACCAGCTGCAGGCATCGCGGTTT
L.panamensis          GGCTGGTGTGCAACCCTTAGGGT-TCACGGAGGTGTGACCAGCTGCAGGCATCGCGGTTT
L.guyanensis          GGCTCGTGTGCAACCCTTAGGGT-TCACGGAGGTGTGACCAGCTGCAGGCATCGCGGTTT
L.adleri              GATGAGTTGTTACGCCTTATGG--GTACACCGAGGACGGCCATCGTTGGTGTCTATGCTG
L.hoogstraali         GATGAGTTGTTACTCCTTATG---GCACACCGAGGGCGGCCATCGTTGGTGTCTATGCTC
L.tarentolae          GATGAGTTGTTACTCTGTATG---GTACGCCGAGTGCGGCCATCGTTGGTGCCTCTGCGC
L.gymnodactyli        GATGAGTTGTTACTCCGTATG---GTACACCGAGGGCGGACATCGTTGGTGTCTATGCTC
L.mexicana            AATGA-GCTGTTACCTCTGT----GGTACACTGTGGCGATCAGCTGCGGTGTCTGTGTGC
L.amazonensis         AATGA-GCTGTTACCTCTGT----GGTACACTGTGGCGATCAGCTGCGGTATCTGTGTGC
L.donovani            AATGG-GCTGTTAGCGCTAC----GGTACACTGAAGCGATCAGCTGCGGTATCTGTGTGA
L.infantum            AATGG-GCTGTTAGCGCTAC----GGTACACTGAAGCGATCAGCTGCGGTATCTGTGTGA
L.tropica             AATGA-GCTGTTACCTCTAT----GGTACACTGAAGCGATCAGCTGCGGTATCTGTGTGC
L.major               AATGA-GGTGTTACCTCTAT----GGTAAACTGAAGCGATCAGCTGCGGTATCTTTGTGC
L.gerbilli            AATGA-GGTGTTACCTCTAT----GGTAAACTGAAGCGATCAGCTGCGGTATCTTTGTGC
L.turanica            AATGA-GGTGTTACCTCTAT----GGTAAACTGAAGCGATCAGCTGCGGTATCTTTGTGC
AM-2004               TGTGGCCAGCGACTCCCTCC----CATGGTCTGCGGTCATGAGCCGCGGCGTCCTTGCTT
L.martiniquensis      GCTGAACAAGGACTCGCGAT----TGTGCATTGCGGTGATGAGTTGTGGTGTCTCTGTTT
L.enriettii           GGCGGCCGGTAACTCCCTAT----CATGAAATGTGGCGGTGAGCTGCGGCGTCCTTGTTC
GH5                   GGTGGCCGGTAACTCCCTAT----CATGAAATGTGGTGGTGAGCTGCGGCATCCTTGTTC
GH10                  GGTGGCCGGTAACTCCCTAT----CATGAAATGTGGTGGTGAGCTGCGGCATCCTTGTTC
GH11                  GGTGGCCGGTAACTCCCTAT----CATGAAATGTGGTGGTGAGCTGCGGCATCCTTGTTC

C.fasciculata         TGTACCAGACTTTTGTACTAGCTGCGTGCAGAGA-ACAAGACGTGCACGAAGCCGGAACT
L.hertigi             GCGTCAAAATTGTGCTTCTTCGCGCATCCGTGAT---TCTGTAGCTTCCA---AGAGTCT
E.monterogeii         GCGTTAAACGTATGCTCGTTGGCACACCAACTATTATTCTGTAGTTTCTC---CTGACAC
L.colombiensis        GCGTTAAACGTATGCTCGTTGGCACACCAACTATTATTCTGTAGTTTCTC---CTGACAC
L.equatorensis        ACGTTAAACGTATGCTCGTTGGCACATCAATTATTATTCTGTAGTTTCTC---CTGACAC
L.braziliensis        GCGTCGAGGGTGCACTGCTGTCTGCGTCTCGGAGCGTTGGGTTTTGCTTC---TGTGAGG
L.panamensis          GCGTCGAGGGTGCACTGCTGTCTGCGTCTCGGAGCGCTGGGTTTTGTTTC---TGTGAGG
L.guyanensis          GCGTCGAGGGTGCACTACTGTCTGCGTCTCGGAGCGCTGGGTTTTGTTTC---TGTGAGG
L.adleri              ACGTCTAGGATGCGCTGGGCTCTCGACCGTGGAGTTGGGGACTGGCGCTG---AAGGTGT
L.hoogstraali         ACGTCTAGGATGCTCTGGTTTCTGCACCTTGGAGTTTGCGATTGGCACTG---AAGGTGT
L.tarentolae          ACGTCTAGGATGCGCTGGTTTCTGCACCTTGGAGTTGGAGATGGGCGCAG---AAGGTGT
L.gymnodactyli        ACGTCTAGGATGCGCTGGTTTCTGCACCTTGGAGTTGGAGATGGGGGCAG---AAGGTGT
L.mexicana            ACGCCAAGGGTGCGCTGGTCTCTGCATCTCCGAGTTTGTGCTTTGCTCTG---AGGATGT
L.amazonensis         ACGCCAAGGGTGCGCTGGTCTCTGCATCTCCGAGTTTGTGCTTTGCTCTG---AGGATGT
L.donovani            GCGTCAAGGGTGCGCTGGTTTCTACATCTCCGAGTTTTTGATTCGCTCTG---AGGATGT
L.infantum            GCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTTTGATTCGCTCTG---AGGATGT
L.tropica             GCGTCAAGGGTGCGCTGGTTTCTGCATCTCCGAGTGTGGGATTCTCTCTG---AAGATGT
L.major               GCGTCGAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTGTGATTCTCTCTG---AAGATGT
L.gerbilli            GCGTCGAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTGTGATTCTCTCTG---AAGATGT
L.turanica            GCGTCGAGGGTGCGCTGGTTTCTGCATCTCCGAGTTTGTGATTCTCTCTG---AAGATGT
AM-2004               GCGCCGAGGGATCGGTCGACTCTGTATGTTGGAGCTCAAGCCTTCCTGTG---CTGAAGA
L.martiniquensis      ACGCACAGGGCTCCGCCGCCTCTGTATCTTGGAGCTCGGGATTTTTTTTC---TCTGAGA
L.enriettii           GAGGCAAGGACTCGGCTGTTTCTGTACCTCGAAGCACGAGCTTCTCTTTT---CTGTGGA
GH5                   AAGGCAAGGACTCGGCTGTTTCTGTACCTCGGAGCACGAGCTTCTCTTTT---CTGAGGA
GH10                  AAGGCAAGGACTCGGCTGTTTCTGTACCTCGGAGCACGAGCTTCTCTTTT---CTGAGGA
GH11                  AAGGCAAGGACTCGGCTGTTTCTGTACCTCGGAGCACGAGCTTCTCTTTT---CTGAGGA
                            *

C.fasciculata         GTGTTCTCTAGACGTTGAGCTGGTTATTATAGGTTAATTGAGCTGCTTTGGTTGCAGTTC
L.hertigi             G-CAAGC-----GAAAGGAGCTCGAAGGTAATGATTTTTGA---------CTCCAACACT
E.monterogeii         AAGAAAGGC--AAGTATGTTCCCCAAGGAAATGATCTTTTG------CCTCTTCTATACT
L.colombiensis        AAGAAAGGC--AAGTATGTTCCCCAAGGAAATGATCTTTTG------CCTCTTCTATACT
L.equatorensis        AAGAAAGGC--AAGTATGTTCCCCAAGGAAGTGATCTTTTG------CCTCTTCTATACT
L.braziliensis        ATGTAGG----AGGAAAGACGGTGCTTTGCAGTGCTGCTTT------TCTTTTTCCGCGC
L.panamensis          GCGTAGG----AGGAAAGACGGTGCTTTGCAGTGCTGCTTT------TCTTTTTCCGCGC
L.guyanensis          GCGTAGG----AGGAAAGACGGTGCTTTGCAGTGCTGCTTT------TCTTTTTCCGCGC
L.adleri              ACCGCAGGGAGAAAACGAAGAAAGCGTTAAAGGGCCGCTGT------TGCACCCTCGCGC
L.hoogstraali         ACCGGAGGGAGAAAA---AGAAGGCGTTAAAGGGCCGCTGT------TGCACCCTCGCGC
L.tarentolae          ACCGGACGGAGAAAACGAAGAAAGCGTTGAAGGGCCGCTGT------TGCACCTTCGCGC
L.gymnodactyli        ACCGGACAGAGAAAACGAAGAAAGCGTTAAAGGGCCGCTGT------CGCACCCTCGCGC
L.mexicana            ATCG--------CAA---AAAGCGCGTTGCGGTGCTGTTTT------CCTGCTCCTGCGC
L.amazonensis         ATCG--------CAA---AAAGCGCGTTGCGGTGCTGTTTT------CCTGCTCCTGCGC
L.donovani            ATCG--------CAA---AAAGCACGCTGCGGTGCTGCTTT------ACTACTCCTGCGC
L.infantum            ATCG--------CAA---AAAGCACGCTGCGGTGCTGCTTT------ACTACTCCTGCGC
L.tropica             ATCG--------CAA---AAAGTGCGTTATGGTGCTGCTTT------TCTACTCCTGCGC
L.major               ATTG--------CAA---AAAGCGCGCTGCGGCGCTGCTTT------TCTACTCCTGCGC
L.gerbilli            ATTG--------CAA---AAAGCGCGCTGCGGCGCTGCTTT------TCTACTCCTGCGC
L.turanica            ATTG--------CAA---AAAGCGCGCTGCGGCGCTGCTTT------TCTACTCCTGCGC
AM-2004               CAAA-CG----AGCGGGAGTCACTTGACC---GGCGCTTTT------CAGACTCTCGCAC
L.martiniquensis      GCGTATG----ATCAGAAGACACTTGAAAG---T-GCCTCT------TCGACCCTTGCCC
L.enriettii           -TGTATA----TGGAGGAGGTGGTTGGCATTGGTGCTTTTT------TGGACTATTGCCT
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GH5                 -TGTATA----TGGAAGAGGTGGTTGTCATTGGTGCTTT-T------TGGACTCTTGCCT
GH10                -TGTATA----TGGAAGAGGTGGTTGTCATTGGTGCTTT-T------TGGACTCTTGCCT
GH11                -TGTATA----TGGAAGAGGTGGTTGTCATTGGTGCTTT-T------TGGACTCTTGCCT
                                                    *

C.fasciculata       TTGTTCTGGTTTCAATGAC-------ATCTAGTGCGTTCACAACAAGCGTCTGCTCAACT
L.hertigi           TGGGGCTCCTTTCAATGAGGTGT--GCTATTCTAGTTGCTGAAAA-AACTTCCCCCCTTT
E.monterogeii       CGAGACTGCCTCAAAGG---GGG--GCGGTGTATCCTAGTGTAAG-CAGCCTCCAACTTG
L.colombiensis      CGAGACTGCCTCAAAGG---GGG--GCGGTGTATCCTAGTGTAAG-CAGCCTCCAACTTG
L.equatorensis      CGAGACTGCCTCAAAGG---GGG--GCGGTGTATCCTAGTGTAAG-CAGCCTCCAACTTG
L.braziliensis      CGCGGCTACGCCAAGTGAGCTGTGAGATGTTCCGCTTTGTCCCGA-CCGTCACCACCACT
L.panamensis        TGCGGCTACGCCAAGTGAGCTGTGAGATGTTCCGCTTTGTGCCGA-CCGTTACCACCACT
L.guyanensis        TTCGGCTACGCCAAATGAGCTGTGAGATGTTCCGCTTTGTGCCGA-CCGTTACCACCACT
L.adleri            TGCTGCTCCTTGAAGTAAGTCGA--CATACCCACGTTTCTGCAGT-TGCTTGCCTTTACT
L.hoogstraali       TGCTGCTCCTTGAAATAAGTCGA--CATACCCACCTTTCTGCGGC-TGCTTGCCTTTACT
L.tarentolae        TGCTGCTCCTTGAAATACGTCGA--CATACCCACCTTTCCGCAGC-TGCTTGCCTTTACT
L.gymnodactyli      TGCTGCTCCTTGAAATACGTCGA--CATACCCGCCTTTCTGCAGC-TGCTTGCCTTTACT
L.mexicana          TGCTGCCTCTTCAGATGCGTCGA--CATGTTGGACTTTGTGCAGC-TCCTCACCTTCACT
L.amazonensis       TGCTGCCTCTTCAGATGCGTCGA--CATGTTCGACTTTGTGCAGC-TCCTCACCTTCACT
L.donovani          TGCTGCTCCTTCAAATGCGTCGA--CATGTCCGATTTTCTGCAGC-TACTCACCTCCACT
L.infantum          TGCTGCTCCTTCAAATGCGTCGA--CATGTCCGATTTTCTGCAGC-TACTCGCCTCCACT
L.tropica           TGCTGTTCCTTCAAATGTGTCGA--CATGTCCGACTTTCTGCAGC-TACTCACCTCCACT
L.major             TGCTGTTCCTTCAAGTGTGTCGA--CATGTCCGACTTTCTGCAGC-TGCTCACCTCCACT
L.gerbilli          TGCTGTTCCTTCAAATGTGTCGA--CATGTCCGACTTTCTGCAGC-TGCTCACCTCCACT
L.turanica          TGCTGTTCCTTCAAATGTATCGA--CATGTCCGACTTTCTGCAGC-TGCTCACCTCCACT
AM-2004             GACCGCTCCTTCAAATGAGCCGA--GCTGTTCCGTTTCTTGCGGC-AGCTGACCTCCACG
L.martiniquensis    TGCATGTTCTTCAAAGGAGCTGA--ACTACCCCGCTTCTTTAAGC-AACTGAAATCCACT
L.enriettii         GACTGATCCTTCGAATTAGCCGA--GCTGTTCCGCTTCTTGCGGC-AGCTGACCTCTACT
GH5                 GGCTGCTCCTTCAAATGAGCCGA--GCTGTTCCGTTTCTTGCGGC-AACTGACCTCTATT
GH10                GGCTGCTCCTTCAAATGAGCCGA--GCTGTTCCGTTTCTTGCGGC-AACTGACCTCTATT
GH11                GGCTGCTCCTTCAAATGAGCCGA--GCTGTTCCGTTTCTTGCGGC-AACTGACCTCTATT
                                                    *

C.fasciculata       TCTTTCCG-TCTTTACATCTCCTCA-ATTTTTAAATTTCATCCTGGTCTCTGCTGCGGCT
L.hertigi           GCACTTTCATCTATTCCATCCTTGTCTTTGAGTGTTTGTTTTCGACTTTGGGTTTTGCAA
E.monterogeii       CCCCTAACATCTCTTCC-TTTCTGTCTTTGTGTTATTCTCTCCGACTCACTGTGCTGCCC
L.colombiensis      CCCCTAACATCTCTTCC-TTTCTGTCTTTGTGTTATTCTCTCCGACTCACTGTGCTGCCC
L.equatorensis      CCCCTAACATCTCTTCC-TTTTTGTCTTTGTGTTATTCTCTCCGACTCACTGTTTTGCCT
L.braziliensis      TCCCCCCCCCCATTTTTTCC-TTCCCTGTTCTTCTTTGCTTGGGACAATCTGCTTCGCAC
L.panamensis        TCCCCC----CCTTTTCTCC-TGCCCTGTTCTTCTTTGCTTGGGACAATCTGCTTCGCAC
L.guyanensis        TCCCC-----CCTTTTCTCC-TGCCCTGTTCTTCTTTGCTTGGGACAATCTGCTTCGCAC
L.adleri            TCTTTCT--CTTCCTCCTC-------CTCGCTGTGCTCTGTGCGTCCATGCGCCTCATGC
L.hoogstraali       TCTCTCT--CTTCCTCTTC-------CTCGCTGTTTTCTGTGCGTCCATGCGCCTCC---
L.tarentolae        TCTCTCCCTCGTCCTCTTC-------CTCGCTGTTTTCTGTGCGTCCATGCGCCTCC---
L.gymnodactyli      TCTCTCT--CGTCCTCTTC-------CTCGCTGTTTTCTGTGTGTCAATGCGCCTCC---
L.mexicana          TTCATCTCTCCCTCCTCGTTGTTGT---------TTTCTG--TGTCCATGTGGCTCGTGT
L.amazonensis       TTCATCTCTCCCTCCTCG---TTGT---------TTTCTG--TGTCCATGTGGCTCGTGT
L.donovani          TTCATCTCTCCCTCTTCCTTGTTGT---------TTTCTGTGTGTCCATGCGCCTTGTGT
L.infantum          TTCATCTCTCCCTCTTCCTTGTTGT---------TTTCTGTGTGTCCATGCGCCTTGTGT
L.tropica           TTCATTTCTCCCTCTTCCTTGTT------------TTCTGTGTGTCCATGCGTCTCGTGT
L.major             TTCATCTCTCCCTCTTCCTTTTT-T---------TTTCTGTGTGTCCATGCGTCTCGTGT
L.gerbilli          TTCATCTCTCCCTCTTCCTTTTT-T---------TTTCTGTGTGTCCATGCGTCTCGTGT
L.turanica          TTCATCTCTCCCTCTTCCTTTTT-T---------TTTCTGTGTGTCCATGCGTCTCGTGT
AM-2004             TCCATCTCTTCTTCCCTCC------TCACTTCTTCTTTTCGGTGACTCTCTGCCTCACAT
L.martiniquensis    TTCATTTATCTCCTTCGCCCCTGCTCATTTTTTATTCTTTGGCAACTCTCTGCCTCACAT
L.enriettii         TTTCA------TCTCTCCTACTCATTT------TTGTTTTTGGTGTTATCTGCCTCGTAT
GH5                 TTTCA------TCTCTCCTTTCCTACTTATTTTTGCCTTTGGTGATTATCTGCCTCACAT
GH10                TTTCA------TCTCTCCTTTCCTACTTATTTTTGCCTTTGGTGATTATCTGCCTCACAT
GH11                TTTCA------TCTCTCCTTTCCTACTTATTTTTGCCTTTGGTGATTATCTGCCTCACAT
                                                              *

C.fasciculata       TTATTTTCTCTTCTCTCTCAAACGCCCGTTCTTACCGCACAACACAAAACCAATAACACA
L.hertigi           ACC-----TTTCCACTTCCTGCATTTTCTTT-----TTATTTTTCATTCATCTCTATCGA
E.monterogeii       ACG-----GTTGCACTTACA----------A-----CCACTTCTCATTGGTCTTGGCACA
L.colombiensis      ACG-----GTTGCACTTACA----------A-----CCACTTCTCATTGGTCTTGGCACA
L.equatorensis      ACG-----GTTGCACTTACA----------A-----CCACTTCTCATTGATCTTGGCACA
L.braziliensis      GCC-----TCTACACGTGCGATAACG----C-----GCTCTTCTCGCGCGCCTCTGTCCA
L.panamensis        GCC-----TCTACACGTGCGATAACG----C-----GCTCTTCTCGCGCGCCTCTGTCCA
L.guyanensis        GCC-----TCTACACGTGCGATAACG----C-----GCTCTTCTCGCGCGCCTCTGTCCA
L.adleri            GCT-----CCTCAACTTGCACCAAAA----T-----TTCTTTGCCGCACACCGTTGTGGA
L.hoogstraali       ----------------------------------------------------------
L.tarentolae        ----------------------------------------------------------
L.gymnodactyli      ----------------------------------------------------------
L.mexicana          TTG-----TCTCAACTTGCGACAACG----T-----TTC----TTGCGCACCTCTGCGGA
L.amazonensis       TTG-----TCTCAACTTGCGACAACG----T-----TTC----TTGCGCACCTCTGCGGA
L.donovani          GCG-----TCTCAACTTGCGACAACT----T-----TTC----TTTCGCGTCTCTGTGGA
L.infantum          GCG-----TCTCAACTTGCGACAACT----T-----TTC----TTTCGCGTCTCTGTGGA
L.tropica           GCG-----TCTCAACTTGCGACAACT----T-----TGC----TTGCGCGCCTCTGTGGA
L.major             GCG-----TCTCAACTTGCGACAACT----T-----TTC----TTGCGCCCTTCTGTGGA
L.gerbilli          GCG-----TCTCAACTTGCGACAACT----T-----TTC----TTGCGCCCTTCTGTGGA
L.turanica          GCG-----TCTCAACTTGCGACAACT----T-----TTC----TTGCGCCCTTCTGTGGA
AM-2004             CTG-----TCTCCACTTGCAACA-------C-----CTCTCACTCGTGCTCGTCTGGCTA
L.martiniquensis    GAA-----TCTTTACTTGCCACAACT----T-----TTCCGACGCGCGCTCCTCTGGCAA
L.enriettii         GTG-----TATCCACTTGCCACAACG----T-----TTCCCACTCGTGCTCCTCCGACAA
GH5                 GTG-----TCTCCACTTGCTACAACG----T-----TTCCCACTCGTGCTCCTCCGACAA
GH10                GTG-----TCTCCACTTGCTACAACG----T-----TTCCCACTCGTGCTCCTCCGACAA
GH11                GTG-----TCTCCACTTGCTACAACG----T-----TTCCCACTCGTGCTCCTCCGACAA
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C.fasciculata          ACTTAACTTTGCAAGCTTCAAGAGTTCGTACGTAACGCATCATGCCCGCTG
L.hertigi              TCTCCACCTTGACTGCTTTAAGGTTTCATTCGCAAGGGATCATGCCCGCTG
E.monterogeii          TTTGCACCTTGGCAGTTTTAAGGTTTCTTTTGCAAGGGATCATGCCCGCTG
L.colombiensis         TTTGCACCTTGGCAGTTTTAAGGTTTCTTTTGCAAGGGATCATGCCCGCTG
L.equatorensis         TTTGCACCTTGACAGTTTTAAGGTTTCTTTTGCAAGGGATCATGCCCGCTG
L.braziliensis         AT-ATGGCCTGCCCAACTGAAGCAGTGT--TGTTTTCCACCATGGCTGCT-
L.panamensis           ATTATGGCCTGCCCAACTGAAGCAGTGT--TGTTTTCCACCATGGCTGCTG
L.guyanensis           AT-ATGGCCTGCCCAACTGAAGCAGTGT--TGTTTTCCACCATGGCTGCTG
L.adleri               CCGTTATCGGAACAGTTTTAGGGCTTCGTTTGCAACGAATTATGGCTCCTG
L.hoogstraali          --------------------------------------------------
L.tarentolae           --------------------------------------------------
L.gymnodactyli         --------------------------------------------------
L.mexicana             ATACCGTCTGCACAGCTTTAGGGCTTCATTTGCAACGGATCATGCCTCCTG
L.amazonensis          ATACCGTCTGCACAGCTTTAGGGCTTCATTTGCAACGGATCATGCCTCCTG
L.donovani             ATAACATCTGAACAGCTTTAGGGCTTCATTCGCAACGGATCATGCCTCCTG
L.infantum             ATAACATCTGAACAGCTTTAGGGCTTCATTCGCAACGGATCATGCCTCCTG
L.tropica              ATACCATCTGAACAGTTTTAGGGCTTCATTCGCAACGGATCATGCCTCCTG
L.major                ATACCATCTGAACAGCTTTAGGGCTTCATTCGCAACGGATCATGCCTCCTG
L.gerbilli             ATACCATCTGAACAGCTTTAGGGCTTCATTCGCAACGGATCATGCCTCCTG
L.turanica             ATACCATCTGAACAGCTTTAGGGCTTCATTCGCAACGGATCATGCCTCCTG
AM-2004                ATCCTGTCGGATCAGGCTTAGGGTTTCATTCGCAACGAATCATGGCTGCTG
L.martiniquensis       CCGCCGTTTGGACAGGCTTAGTGTTTCCTTGGCAACATATCATGGCTGCTG
L.enriettii            ATTCTGTTCGATCAGGCTTAGGGTTTCATTTGCAACGAACCATGGCTGCTG
GH5                    ATCCTGTTTGATCAGGCTTAGGGTTTCATTCTCAACGAACCATGGCTGCTG
GH10                   ATCCTGTTTGATCAGGCTTAGGGTTTCATTCTCAACGAACCATGGCTGCTG
GH11                   ATCCTGTTTGATCAGGCTTAGGGTTTCATTCTCAACGAACCATGGCTGCTG
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