EXPERIMENTAL EVALUATION OF ENTOMOPATHOGENIC FUNGI FOR THE BIOLOGICAL CONTROL OF PHLEBOTOMINE SAND FLIES (DIPTERA: PSYCHODIDAE).

By

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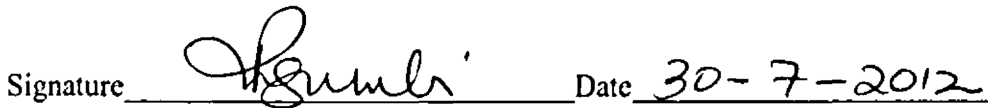
DECLARATION

This is my original work and has not been presented for a degree in /or any other University.



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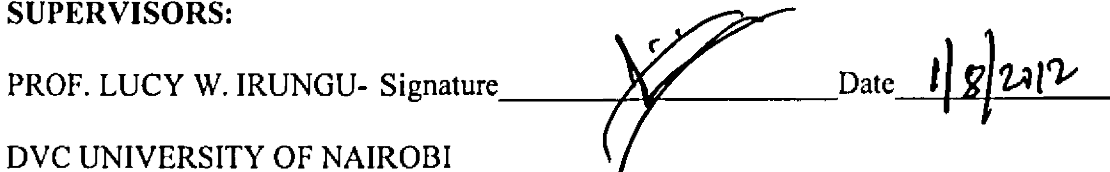

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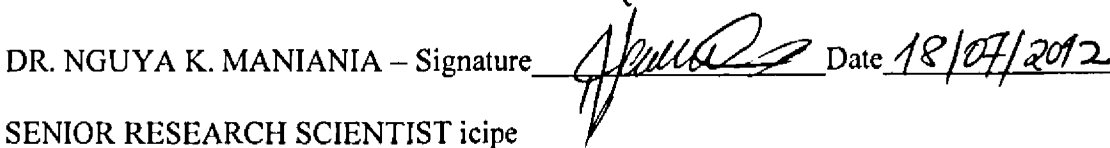

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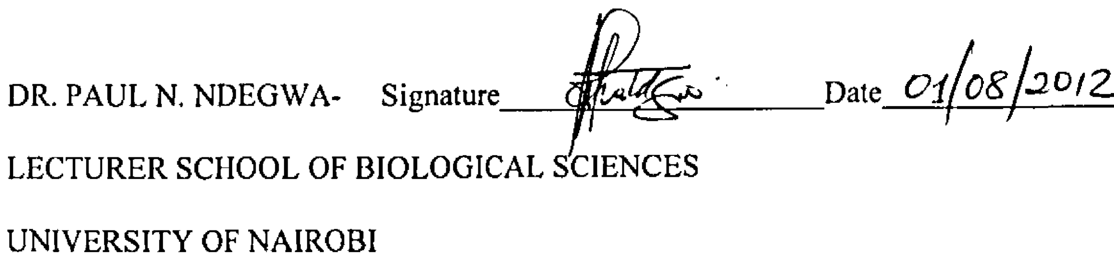

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ABSTRACT

The leishmaniases are some of the WHO's neglected tropical diseases which pose threat to human life in both the New and Old World. They are mostly transmitted through the bite of infected sand flies. The disease affects the poor from the third world. Disease control through chemotherapy is logistically difficult, toxic, expensive and out of reach for many. Entomopathogenic fungi (EPF) hold great potential as alternative vector control. These fungi infect the host through the cuticle. They are friendlier to non-target organisms than synthetic chemical insecticides. Although EPF are widely used to control agricultural pests, few attempts have been made to develop them as biocontrol agents of disease vectors like phlebotomine sand flies. The study had two phases: laboratory and field based studies. Isolates were cultured on Sabourand dextrose agar (SDA) in Petri dishes and incubated at room temperature (22-28 ^{0}C). In the laboratory, sand flies were exposed to 0.3 g of dry conidia evenly spread on a cotton velvet cloth covering the inner side of a cylindrical plastic tube (95x48 mm diam). Control flies were not exposed to any conidia. Mortality was recorded daily until all the sand flies died. The study tested 16 isolates of *Metarhizium anisopliae* and 3 of *Beauveria bassiana* to adult sand fly spp of *Phlebotomus duboscqi* in the laboratory. All the fungal isolates were pathogenic to the test-insect, causing mortality of between 76.8 and 100%. The (LT_{50}) and (LT_{90}) ranged from 3.0–7.8 days and from 5.3–16.2 days, respectively. One of the most virulent isolates of *M. anisopliae* was tested in the field at Rabai in Marigat Division, Baringo County. Dry conidia of the fungi (2.0 x 10^{14}) were introduced into ten termite mounds through the shafts using a modified foot pump. Preliminary results indicate that application of the fungus in termite mounds resulted in three to ten-fold reduction in the population of sand flies after 8 weeks. Two weeks after introduction of the conidia, the longevity of sand flies collected from treated termite mounds was shorter than those in the control mounds. Sand flies from the treated mounds died within 9 days whereas over 90% of sand flies collected from the untreated control termite mounds survived for more than 21 days. These results clearly demonstrate the potential of EPF as a biological control of phlebotomine sand flies. Further studies to determine the best methods for delivery and application in the diverse ecological settings of various leishmaniasis vectors are therefore recommended.

CHAPTER ONE

1.0 Introduction

Global distribution and estimates of incidences of visceral and cutaneous leishmaniases were given by national and international experts (WHO, 2007 and 2011). The figures state that, 350 million people are at risk, and 12 million people are infected annually. Based on these estimates, approximately 0.2 to 0.4 cases and 0.7 to 1.2 million VL and CL cases, respectively, occur each year. Every year 500,000 new cases are reported of visceral leishmaniasis (VL) and of these, 5,000 have resulted in deaths (WHO 2011). More than 90% of global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil. Infection with *L. donovani* is particularly rampant in India. Unlike other countries endemic with visceral leishmaniasis, India is unique in that transmission may occur without an animal reservoir. A human reservoir and competent sand fly vector is sufficient to transmit the parasite and cause disease (WHO, 2006). Although the information is very poor in a number of countries, this is the first in-depth exercise to better estimate the real impact of leishmaniasis. These data should help to define control strategies and reinforce leishmaniasis advocacy (PLoS one. 2012; 7(5): e35671.Epub 2012 May 31).

Cutaneous leishmaniasis is more widely distributed, with about one-third of cases occurring in each of three epidemiological regions, the Americas, the Mediterranean basin and western Asia from the Middle East to Central Asia. The countries with the highest estimated case counts: Afghanstan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Northern Sudan, Costa Rica and Peru, together account for 70 to 75% of global estimated CL incidence. Mortality data are extremely sparse and generally represent hospital-based deaths only (WHO, 2006).

Control of leishmaniasis remains a major public health problem worldwide and use of intergrated approaches seems necessary. Although vector control using insecticides is thought to have reduced the number of kala-azar cases in India (WHO, 2006), it has limitations in disease control, if used alone. Control of leishmaniasis by chemotherapy is expensive, involves long drug treatment regimens, and is often associated with serious side effects. Sand flies caught indoors are not necessarily disease vectors. It thus follows that accurate species identification and vector incrimination is done so as to avoid the use of limited resources against non-vectors.

Pyerthroids are the principal insecticides used for public health because of their low mammalian toxicity. However, pyrethroid resistance has been detected in many insect pests including leishmaniasis and malaria vectors. The occurrence of insecticide resistance to all classes of insecticides available for use in public health as well as mounting criticism concerning the use of chemical insecticides (due to their safety and environmental impacts on non-target beneficial invertebrates and humans), has provided an impetus for the development of alternative forms of vector control. Vaccine development is still at experimental stage but appears to be a promising strategy for leishmaniasis control since individuals who recover from the disease become refractory to further infection (Kedzierski, 2010).

Biological control is defined as the use of biopesticides to control pest populations. Several biological control agents including fungal pathogens have historically been used as larvicides for decades (Lacey *et al.*, 2004). However, targeting only larvae or

intermediate developmental stages of insect vector species is not sufficient to reduce disease transmission because malaria or leishmaniasis is transmitted by adults even if they exist in low numbers. The potential of using *M. anisopliae* and *B. bassiana* as biological control agents against adult mosquitoes is well described (Scholte *et al.*, 2005 and 2006; Achonduh and Tondje, 2008). Under laboratory conditions it has been shown that *M. anisopliae* and *B. bassiana* are effective in controlling insect vectors of both malaria and leishmaniasis (Achundoh and Tondje, 2008; Ngumbi *et al.*, 2011). A semi-field study in a rural village in Tanzania revealed that *An. gambiae* can be controlled using formulated conidia of *M. anisopliae* with 100% infected by day 11 (Scholte *et al.*, 2005). Use of biological agents to control sand flies as vectors is an area of interest, and if exploited, it could prove to be a useful strategy in controlling leishmaniasis.

As mentioned, insecticide resistance is a growing problem in insect control. Blanford *et al.*, (2005) reported that it is unlikely that cross resistance between fungal pathogens and insecticides will develop in the same insect vector (mosquito or sand fly). Scholte *et al.*, (2006) also showed that the development of fungal resistance will be slowed by the exponential killing of mosquitoes.

An integrated approach can be used to manage insecticide resistance in mosquito and sand fly disease vectors (WHO, 2006). Although a combination of insecticides and fungal pathogens showed mixed results, it was meaningful in terms of raising the mortality of infected insects (Delgado *et al.*, 1999; Pachamuthu *et al.*, 1999; Pachamuthu and Kamble, 2000; Ericsson *et al.*, 2007). Entomopathogenic fungi and chemical insecticides may act

synergistically allowing for the use of lower concentrations of either chemical insecticides and decreasing the likelihood of resistance to either entomopathogenic fungi (Ferron, 1971; Richter and Fuxa, 1984; Quintela and McCoy, 1997; Blanford *et al.*, 2005). Studies have revealed high mortality rates in cockroaches when exposed to boric acid and a low dose of *M. anisopliae* (Zurek *et al.*, 2002). Further, a recent development using entomopathogenic fungi revealed that the susceptibility of greater wax moth caterpillars to fungal pathogen increased following pre-exposure to an insecticide synergist (an enzyme inhibitor) (Serebrov *et al.*, 2006).

CHAPTER TWO

2.0 Literature Review

2.1 Leishmaniasis

Leishmaniasis is caused by diphasic protozoa of the genus *Leishmania* and *Viannia* (WHO, 2002; Desjeux, 2004). The disease is endemic in many parts of the world including Central and South America, Africa, Asia and the Mediterranean basin.

Leishmania and *Viannia* subgenera are grouped into complexes of species and subspecies based upon molecular, biochemical and immunological similarities. To further confuse the issue, there are several forms of the disease named by their clinical presentation including, cutaneous, mucocutaneous or visceral leishmaniasis. Each of these forms of disease is caused by different species of sand flies found in different regions of the world (WHO, 2002).

Cutaneous leishmaniasis of humans is associated with members of *L. aethiopica, L. major* and *L. tropica* complexes in the Old World and *L. mexicana* and *L. braziliensis* complexes in the New World. Visceral leishmaniasis is caused by *L. donovani* and *L. infantum* in the Old World regions, while *L. chagasi* is primarily responsible for visceral disease in the New World. Because *L. infantum* is the primary agent associated with the canine leishmaniasis, infections in dogs are often regarded as visceral even though they tend to cause both visceral and cutaneous disease (Slappendel and Ferrer., 1998). The disease is zoonotic all over the world with the exception of the Indian sub-continent, from

where no animal reservoirs have so far been reported (Bhattachrya and Gosh, 1983; Prasad, 1999; WHO, 2002). The most common animal reservoirs, where they exist (Table 1), are the domestic dog and some wild carnivores followed by rodents, marsupials and reptiles (Bray, 1974; Soulsby, 1982). According to the World Health Organization (2002), leishmaniasis is one of the six most important vector-borne diseases worldwide. The disease occurs in warm tropical and sub-tropical climatic conditions, and has been reported in 88 countries of the world, 66 of them being in the Old World (Asia, Africa and Europe) and the remaining 22 in the New World (South and Central America) (WHO, 2002) (Fig. 1).

Sand flies of the genus *Phlebotomus* (Old World) and *Lutzomyia* (New World) are primary vectors responsible for disease transmission. Currently these are the only known vectors capable of spread; fleas, ticks and other arthropods have not been shown to be competent vectors (Lindsay *et al.*, 2002; Mandell *et al.*, 2005). However, rare cases of leishmaniasis have been contracted through exchange of blood or body fluids, direct contact, congenital and sharing of needles by intravenous drug users are other reported forms of transmission (Cruz *et al.*, 2002).

Although it is not as common and widespread as malaria, the diseases caused by infection with *Leishmania* parasites continue to have a major impact on much of the world's population and is currently considered to be an emerging disease with high morbidity and mortality in the tropics and sub-tropics (Santos *et al.*, 2008). The disease which has been neglected by researchers and funding agencies, is endemic in 88 countries of the World

and 350 million people are considered at risk of infection (Handman, 2001; Desjeux, 2004; WHO, 2007). In these countries, it is estimated that 80% of the population earn less than $2 a day (Davies *et al.*, 2003).

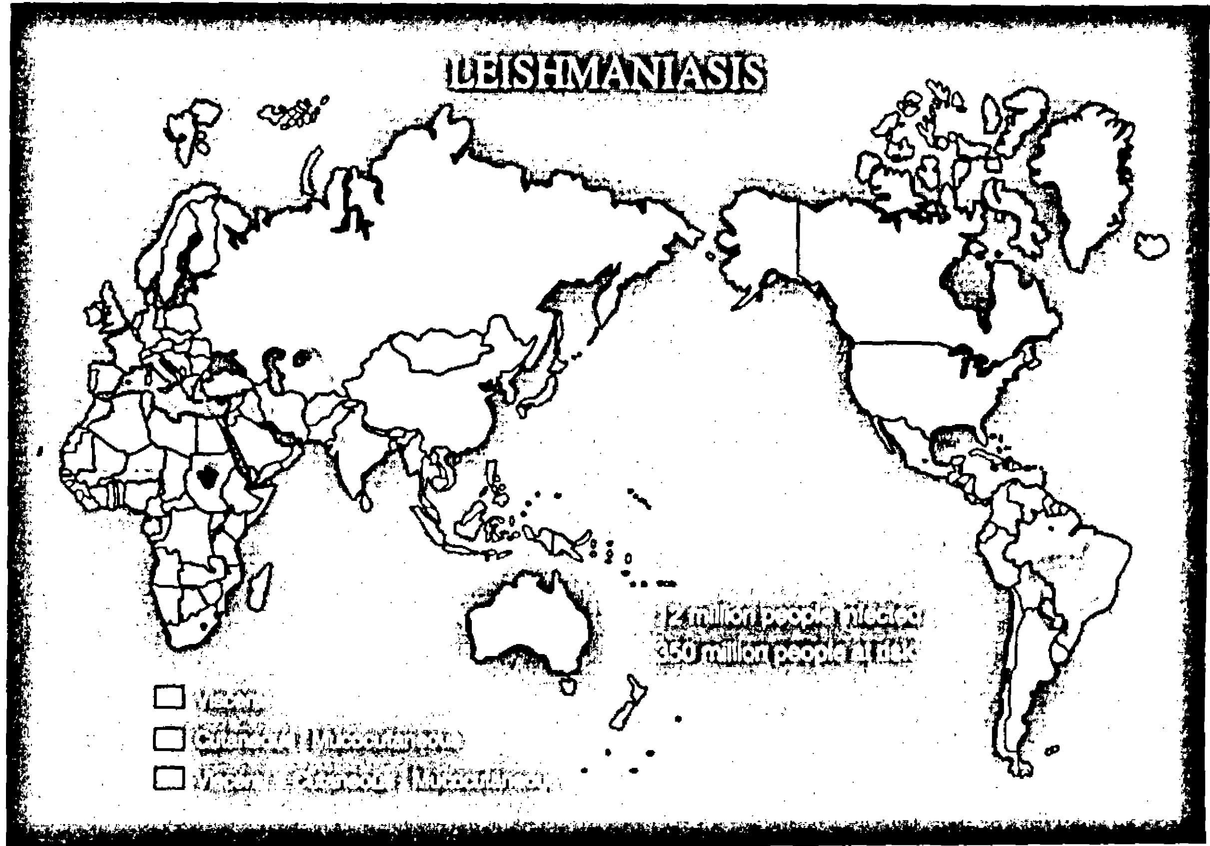


Figure 1: Geographical distribution of cutaneous and visceral leishmaniasis adopted from Handman 2001

The disease is characterized by both diversity and complexity (Herwaldt, 1999). The disease is diverse in the way it manifests itself clinically, types of *Leishmania* species, geographical location, and sand fly species transmitting the disease and incubation period taken by different *Leishmania* species to produce the disease in different countries of the world. The *Leishmania* species are complex in both visceral and cutaneous leishmaniasis in the Old and New World regions (Kaye, P and Scott, P., 2011).

A pressing need is for the technological advances in the understanding of the immune response to *Leishmania* and the pathogenesis of leishmaniasis to be translated into field-applicable and affordable methods for diagnosis, treatment, and prevention of this disease (Herwaldt, 1999). In the absence of a vaccine, the global disease burden has remained stable for some years, causing morbidity and mortality loss of 2.4 million disability adjusted life-years (DALYs) and approximately 70,000 deaths, a significantly high rank among communicable diseases (Davies *et al*, 2003; Reithinger *et al.*, 2007). There are 2 million new cases of leishmaniasis annually and 14 million infected people worldwide (WHO, 2007). An increase in the incidence of leishmaniasis is associated with urban development, destruction of forests, environmental changes and migrations of people to areas where the disease is endemic (Ashford, 2000; Kolaczinski *et al.*, 2008).

Strange phenomena such as wars, civilian migration, immunosuppression caused by medication and viral infections, globalization of work and leisure and transmission outside endemic areas contribute to the spread and increase of the disease (Reithinger *et al.*, 2007; Kolaczinski *et al.*, 2008).

In the decade, *Leishmania*-Human Immunodeficiency Virus (HIV) co-infection has surged as a major complication of leishmaniasis and has ignited calls for the recognition of leishmaniasis as an Acquired Immunodeficiency Syndrome (AIDS) defining illness (Singh, 2006). In Africa, particularly Ethiopia and Sudan, it is estimated that 70% of

adults with visceral leishmaniasis (VL) also have HIV infection (Desjeux, 2001; Guerin *et al.*, 2002).

2.2 Epidemiology of Leishmaniases

The epidemiology of visceral leishmaniasis is changing due to the increasing rate of co-infection with HIV. Spain, Italy and Southern France are experiencing a high incidence of co-infection with HIV and VL among the youth who have had previous exposure to *L. infantum* or use intravenous drugs. For the HIV-infected individuals in Spain, the fourth most common HIV-related infection is VL. Individuals with HIV have a lower immunity to infection. They are more susceptible to VL which requires a strong immunological response to contain the infection (WHO, 2006). With the spread of HIV/AIDS, co-infection with leishmaniasis has risen to epidemic proportions. Recently, visceral leishmaniasis, a disease typically found in rural areas, is now commonly seen in urban areas among the HIV-infected population. Co-infection with *Leishmania* is reported in 34 countries in: Africa, Asia, Europe, and South America (WHO, 2006). According to WHO, over 70% of HIV cases in southern Europe are also co-infected with visceral leishmaniasis (www//who.int/leishmaniasis/burden/hiv co-infection/en/index.html).

The species of *Leishmania* known to cause disease in humans are very similar morphologically but produce strikingly different pathological responses (Handman, 2001). The only feature common to all is the chronicity of disease manifestations (Handman, 2001). The degree or level of infection depends on the species of *Leishmania* parasites and the state of host's immune responses (Robert, 2006). The leishmaniases produce a spectrum of clinical manifestations ranging from: ulcerative skin lesions at the site of sand fly bite (localized cutaneous leishmaniasis (LCL); multiple non-ulcerative

nodules (DCL); destructive mucosal inflammation (mucosal leishmaniasis, MCL); and disseminated visceral infection (visceral leishmaniasis, VL) (Reithenger *et al.*, 2007). The world geographical distribution of various forms of leishmaniases is shown in Figure 1.

Cutaneous leishmaniasis starts as a small papule at the site of sand fly bite, which after some time increases in size, crusts and eventually ulcerates (Piscopo and Mallia, 2006). The victim presents with one or several ulcer(s) or nodule(s) in the skin (Chappuis *et al.*, 2007). It may take 3-18 months to heal in more than 90% of cases (Mandell *et al.*, 2005). The incubation period may last from 2 weeks to several months and cases of up to 3 years have been reported in the Old-World cutaneous leishmaniasis (Smith, 1955). In the New-World cutaneous leishmaniasis, the incubation period is usually 2-8 weeks (Marsden and Nonata, 1975). Different species of *Leishmania* can infect macrophages in the dermis, with varying clinical presentations and prognoses (Arevalo *et al.*, 2007). Here the etiologic agents are: *L. major, L. tropica* and *L. infantum.* The ulcers heal spontaneously although slowly in immuno-competent individuals, but cause disfiguring scars (Chappuis *et al.*, 2007). *Leishmania major* is the etiological agent of cutaneous leishmaniasis (Tonui *et al*, 2004).

In MCL, patients suffer from progressively destructive ulcerations of the mucosa, from the nose and mouth to the pharynx and larynx (Reithenger *et al.*, 2007). This causes difficulty in eating and an increased risk of secondary infection, which is fatal if not treated in good time. Although the incubation period is 1-3 months, mucocutaneous leishmaniasis may occur many years after the initial cutaneous ulcer has healed (Piscopo

and Mallia, 2006). These lesions are not self-healing and are usually seen months or years after the first episode of mucocutaneous leishmaniasis, when the macrophages of the naso-oropharyngeal mucosa become colonized (Chappuis *et al.*, 2007). *Leishmania braziliensis* is responsible for most of mucocutaneous leishmaniasis (Reithinger *et al.*, 2007).

Visceral leishmaniasis is a disease that is fatal if left untreated and is caused by *Leishmania donovani* complex- *L. donovani sensu stricto* in East Africa and the Indian subcontinent and *Leishmania infantum* in Europe, North Africa and Latin America (Lukes *et al.*, 2007). There are two types of VL, which differ in their transmission characteristics: zoonotic VL is transmitted from animal to vector to human and anthroponotic VL is transmitted from human to vector to human (Chappuis *et al.*, 2007). In the former, humans are occasional hosts while animals, mainly dogs, are the reservoirs of the parasite (Alvar *et al.*, 2004). Zoonotic VL is found in areas of *L. infantum* transmission whereas anthroponotic VL is found in areas of *L. donovani* transmission (Chappuis *et al.*, 2007).

Visceral leishmaniasis also known as 'kala-azar' is a systemic disease characterized by a range of symptoms including fever, hepatosplenomegaly, weight loss, weakness, hair loss and if untreated, may lead to death (Murray *et al.*, 2005).

For visceral leishmaniasis, the pathogenesis is complex. Three species of *L. donvani* cause visceral leishmaniasis: *L. donovani, L. infantum* and *L. chagasi.* Initially the

infection is asymptomatic. If the infection spreads, the severe symptoms of kala-azar disease become increasingly apparent. Once the species causing VL parasitize reticuloendothelial cells (RE), the disease, if left untreated, may result in 75% to 95% mortality rate within the first two years of infection (WHO, 2006). As the disease progresses, the spleen and liver may become enlarged with the invasion parasites in the (RE) cells. As a consequence of infection in the bone marrow, individuals may become anaemic and further destruction of red and white blood cells may occur. Although eye complications are rare, they sometimes manifest as retinal hemorrhages, keratitis, and central retinal thrombosis to name afew (WHO, 2006).

Post kala-azar dermal leishmaniasis (PKDL) is characterized by maculo-papular or nodular rash on the skin and is a complication of VL which is observed after treatment in Sudan and more rarely in other East African countries and in the Indian subcontinent (Zijlstra *et al.*, 2003). It can also occur in immunosuppressed individuals in *L. infantum*-endemic areas (Chappuis *et al.*, 2007). The interval between treated VL and PKDL is 0-6 months in Sudan and 6 months to 3 years in India (Musa *et al.*, 2002). Post kala-azar dermal leishmaniasis cases are highly infectious because the nodular lesions contain many parasites (Addy and Nandy, 1992), and such cases are the putative reservoir for anthroponotic VL between epidemic cycles.

Table 1: The species of *Leishmania* of public health importance (from Ralph 1982; Kreier and Baker 1987)

Species	Sub-species	Geographical distribution	Vector	Reservoir	Disease produced
Leishmania donovani Complex	*L.d. donovani*	Asia (mainly Nepal) Africa, Bangladesh	*Phlebotomus argentipes, P. martini, P. chinensis*	Only man	Visceral leishmaniasis (kalaazar)
	L. d. infantum	Central Asia Southern Europe & Mediterranean	*P. ariasi P. perferliwi P. tobbi, P.chinensis*	Usually dogs, wild canids, rarely rodents	Visceral leishmaniasis
	L. d. chagasi	Mexico, Central & South America	*Lutzomiya longipalis*	Mainly dogs rarely foxes	Visceral leishmaniasis
Leishmania mexicana Complex	*L.m. mexicana*	USA, (Texas), Mexico, Central & South America	*L. olmecaolmeca*	Rodents	Cutaneous leishmaniasis
	L.m. amazonensis	Amazon Basin Brazil	*L.flaviscutella*	Rodents, Fox Marsupials	Cutaneous leishmaniasis
	L. m. pifonoi	Venezuela	*L. flaviscutella*	Rodents	Cutaneous leishmaniasis
	L. m. garnhami	Venezuelan Andes	*L. townsendi*	Marsupials,	Cutaneous leishmaniasis
	L. m venezuelensis	Venezuelan Andes	(??)	(??)	Cutaneous leishmaniasis
Leishmania brazilensis Complex	*L.b. brazilensis*	Brazil	*L. intermedia, L. pessoai Psychodopygys welcomei*	(??)	Muco-cutaneous leishmaniasis
	L. b guyanensis	Amazon basin Brazil	*L. umbratilis L. whitmani L. anduzei*	Sloth Marsupials Rodents Primates Procyonids	Cutaneous leishman
	L. b panamensis	Panama and Costa Rica	*L. trapidoi L. yelbhiletor L. gomezi P. anamensis*	Sloth, Dogs Procyonids Primates	Cutaneous leishmanias
Leishmania tropica Complex	*L. t tropica*	Asia, India, Africa & southern Europe	*P. perferliwi P. papatasi P. sergenti P. chaubadi*	Dogs, (?) Rodents	Cutaneous leishmaniasis
	L.t. aethiopica	Africa	*P. longipes P. pedifer*	Hyraxes	Cutaneous and Diffuse cutaneous Leishmaniasis

L. peruviana	Peru.	(??)		Dogs Uta
L. major	Asia, (including India), Africa & south Europe	*P. papatasi* *P. caucasicus* *P. andrejevi* *P. mongolensis*	Rodents, Dogs	Cutaneous leishmaniasis

(??) = unknown

2.3 Pathogenesis

2.3.1 Life cycle of *Leishmania* parasites

The life cycle as described by Lindsay *et al.*, 2002 begins when an infected female sand fly inoculates a vertebrate host with flagellated promastigotes during a blood meal (Lindsay *et al.*, 2002) (Fig. 2). A typical inoculum contains around 100-1000 metacyclic promastigotes which quickly become engulfed by leucocytes, particularly macrophages, neutrophils and dendritic cells (Roberts, 2006). Macrophages are the first line of defense and promptly phagocytose the invading organisms (Roberts, 2006). Unfortunately, *Leishmania* organisms are capable of surviving within the macrophage where they undergo transformational change from flagellated promastigotes to non-motile amastigotes (Fig.2). In the vertebrate host, the amastigotes (contained within Macrophages) are capable of binary fission. Division continues until the macrophage lyses and amastigotes are released to infect neighbouring phagocytic cells. Infected macrophages or individual amastigotes enter the systemic circulation and subsequently disseminate to visceral organs leading to internal disease. Once the organism has entered systemic circulation, it can once again be taken up during a blood meal by the female sand fly. The ingested amastigotes travel to the gut of the sand fly and are once again transformed into promastigotes. In the vector, it is the promastigote stage within the gut of the sand fly that is capable of binary fission. These flagellated organisms subsequently

migrate to the hypostome of the sand fly and are inoculated into another vertebrate host completing the life cycle

2.3.2 Classification

The taxonomy of *Leishmania* parasite is complicated and ambiguous, however widely accepted classification is based on the position of promastigote development of *Leishmania* in the gut of sand fly relative to the position of pyrolus. Among these three groups: hypopylaria, peripylaria and suprapylaria, with the most species infecting humans found in the latter group while a few within in the peripylaria region (Ralph, 1982). The general species differentiation is based on the clinical signs and pathology, vectors and reservoirs involved, as different species are indistinguishable based on morphology. Biochemical (e.g isoenzyme analysis) and immunological (e.g monoclonal antibody) characteristics and molecular tools have been used for differentiation at species and sub-species level. Among them, isoenzyme analysis (a biochemical technique) remains the current gold standard technique (Anon, 2004), while DNA-based techniques are being used increasingly. The most common group/complex of species and sub-species of *Leishmania* having public health significance are summarized on Table 1.

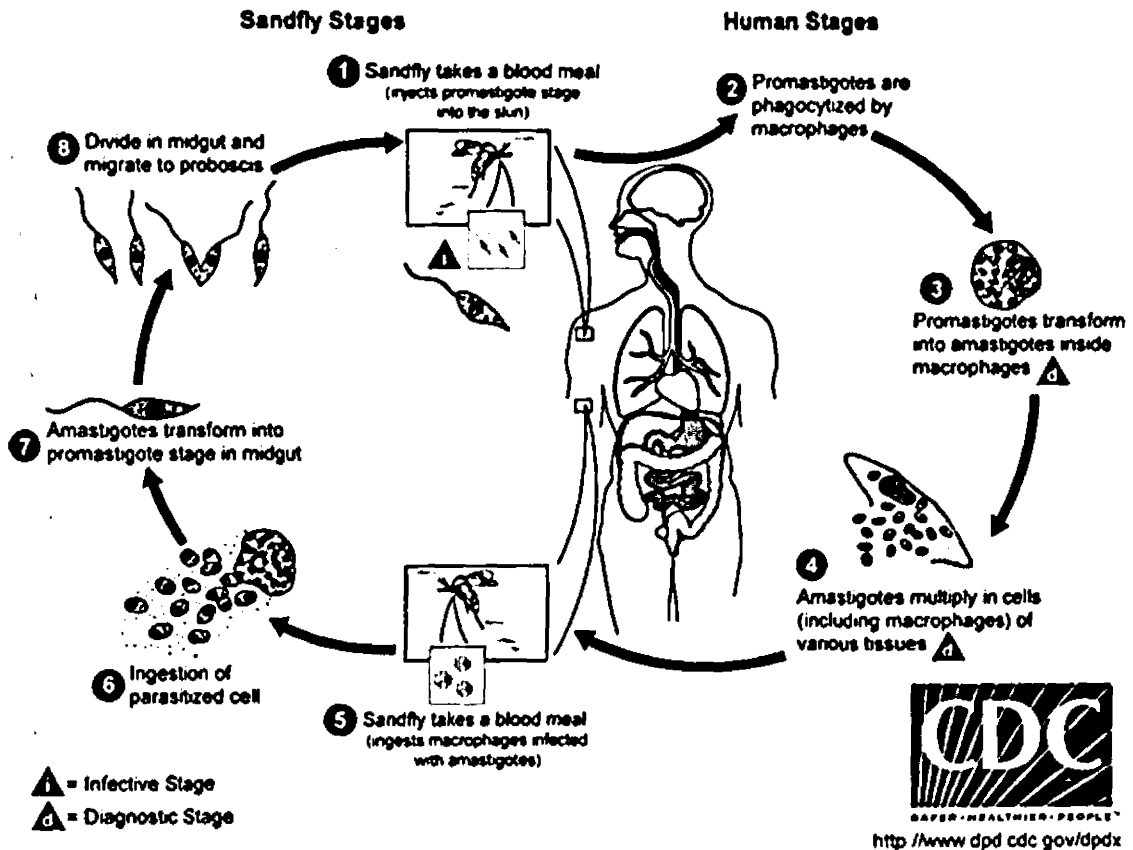


Figure 2: Diagram of *Leishmania* parasites as adopted from CDC website

2.4 Leishmaniases in Kenya

Visceral leishmaniasis and cutaneous leishmaniasis are endemic in Kenya while PKDL has also been reported (Muigai *et al.*, 1987; Muigai *et al.*, 1991; Tonui, 2006). The leishmaniases were reported in Kenya as early as in the 1930s (Fendall, 1961). Geographical distribution of leishmaniasis in Kenya is shown on (Fig. 3).

2.4.1 Visceral leishmaniasis in Kenya

Visceral leishmaniasis was first reported in Kenya following an outbreak in the Kings African Rifles troops who had camped north of Lake Turkana at the border of Kenya and Ethiopia during the second World War in the 1940s (Cole *et al.*, 1942). The disease in Kenya is caused by *L. donovani* and the vector is *P. martini* though other vectors like *P. orientalis* have been reported and could be transmitting the parasites (Young D G, 1979; Perkins *et al*, 1988; Johnson *et al.*, 1993; Ngumbi *et al.*, 2010). In Kenya man is the only known reservoir of VL (Wijers and Kiilu, 1984; Tonui, 2006). Since then, Turkana, Baringo, Kitui, West Pokot, Machakos, Mwingi, Meru, Isiolo, Wajir, Kajiado, Mandera, Keiyo and Marakwet districts have been considered to be endemic for kala-azar (Wasunna *et al.*, 2005; Tonui, 2006). Baringo and the neighbouring districts such as West Pokot were first identified as leishmaniases foci in 1955 (Mutinga, 1975). The first case of VL in Baringo district was recorded in 1948 at Kabarnet District Hospital (Mckinnon and Fendall, 1956). Baringo district is the only focus reported where both VL and CL are known to occur in Kenya (Muigai *et al.*, 1987; Perkins *et al*, 1988; Tonui, 2006). Some scientists believe that nomadic Turkanas may have introduced the disease into the area from the north. Others think that Kenyan soldiers returning from North Africa after the World War II were responsible for introduction of the parasite (Ryan *et al.*, 2006). The disease occurring in Baringo County has a focal distribution in the dry, hot areas with an altitude of below 1500 metres and the infections may be characterized as follows: 1) asymptomatic 2) sub-clinical and self- limiting (not medically identifiable), and 3) clinically manifesting disease (that is medically identifiable) (Tonui, 2006). Half of the reported VL patients are between 5 and 14 years of age and 66% of them are males because boys tend to remain playing late at night thereby increasing the chances of

interacting and exposing themselves to sand fly bites more than the girls, who tend to help with cooking chores thereby minimizing chances of interacting with sand flies. A human case of mixed infection of *L. donovani* and *L. major* has been reported in this dual focus of VL and CL (Tonui, 2006).

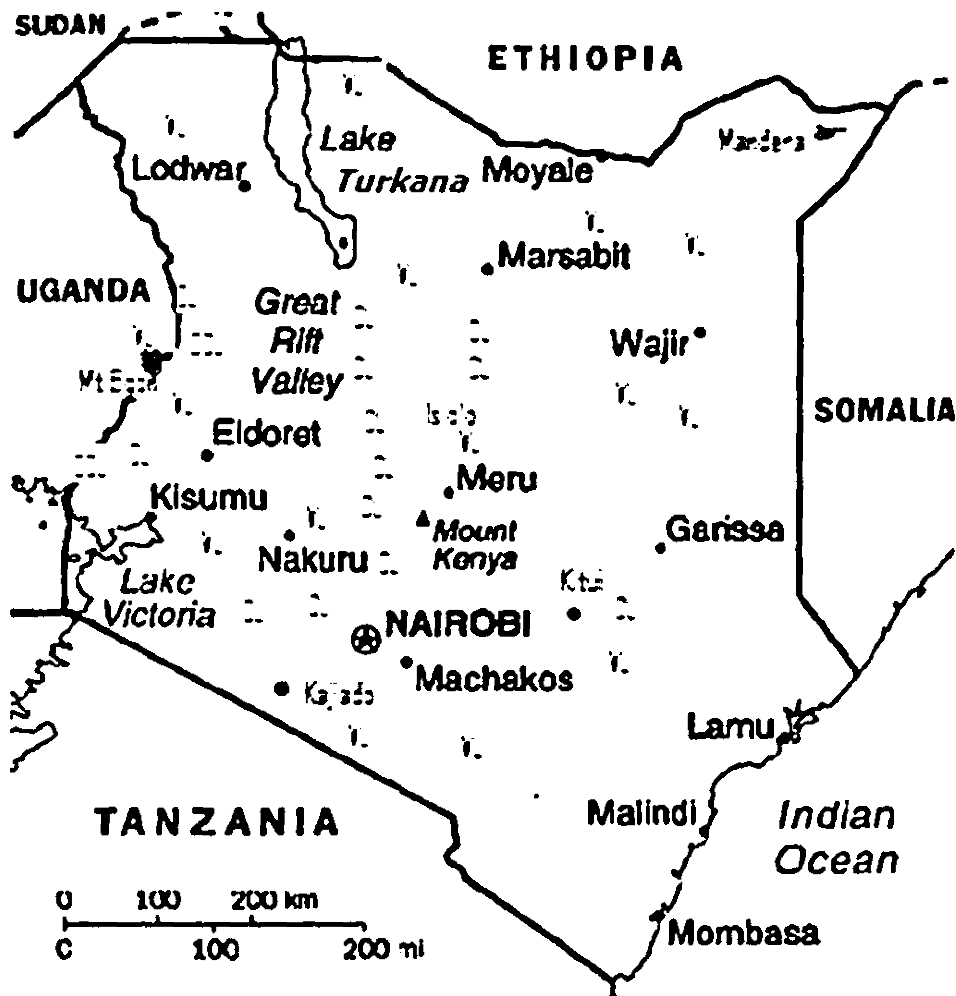
Periodic outbreaks of VL have been reported in Kenya since the disease was reported in the 1940s. Some of the VL outbreaks were reported in Kitui district in 1952 with 303 cases and peaked in 1953 with 2,142 cases (Fendall, 1961). More outbreaks have since been reported in 1966 in Meru with 1,500 cases (Wijers *et al*, 1966), Machakos in the 1970s and Kitui again in the 1980s (Ngoka *et al.*, 1978; Wijers *et al.*, 1984), Kajiado District in the early 1990s (Johnson *et al*, 1993), Baringo district in 1999 (Mbati *et al.*, 1999). In 2000, an outbreak of VL was reported in the previously non-endemic Wajir and Mandera districts of North Eastern Kenya where between May 2000 and August 2001, 904 patients were diagnosed with VL, with some of them coming from as far as southern Somalia and southeast Ethiopia (Marlet *et al.*, 2003). Visceral leishmaniasis seroprevalence in Kenya is unknown because of the lack of practical and accurate diagnostic test or surveillance system (Ryan *et al.*, 2006). Moreover, most VL infections occur in remote geographical areas where health facilities are not well established and where the infections often co-exist with malaria and other debilitating parasitic infections (Hailu *et al.*, 2005). Recent studies show that low socio-economic status and treating animals with insecticide are risk factors for VL, (because the insects avoid the smell of insecticide) while on the other hand sleeping near animals, owning a mosquito net and

knowing about VL symptoms are associated with a reduced risk of the disease in Kenya and Uganda (Kolaczinski *et al.*, 2008).

Visceral leishmaniasis affects not only the weakest in the community, such as children and those already affected by diseases such as HIV and tuberclosis, but also healthy adults and economically productive social groups (Hailu *et al.*, 2005).

2.4.2 Cutaneous leishmaniasis in Kenya

Cutaneous leishmaniasis in Kenya is caused by three species of parasites; *L. major, L. tropica* and *L. aethiopica* (Mutinga, 1975; Muigai *et al.*, 1987; Mebrahtu *et al*, 1993). In Kenya, CL due to *L. major* which is transmitted by *P. duboscqi* is rare in humans, but underreporting is likely (Tonui, 2006). *Phlebotomus duboscqi* is mainly found living in animal burrows where it feeds on small rodents which are frequently infected with *L. major* parasites (Schaefer *et al.*, 1994; Githure *et al.*, 1995). In Africa south of the Sahara, the presence of *L. tropica* (*sensu stricto*) was not suspected until a new focus was discovered in the Rift Valley in Kenya (Mebrahtu *et al.*, 1987; Lawyer *et al.*, 1991; Sang 1991). The proven vector for *L. tropica* in Kenya was shown to be *P. guggisbergi.* In a focus of *L. tropica* in Laikipia district, Kenya, *P. guggisbergi* was found to be attracted to domestic animals like: goats, sheep, dogs, cats, rabbits and hamsters. Wild animals caught in the caves where *P. guggisbergi* inhabit included: hyraxes (*Procavia capensis*); giant rat (*Cricetomys gambianus*), crested rat (*Lophiomys imhausi*) (Johnson *et al*, 1993; Jacobson, 2003). Hyraxes have been suspected to be the reservoirs of *L. tropica* and *L. aethiopica* parasites in Kenya (Mutinga, 1975; Sang *et al*, 1992).



Key: VL – Visceral leishmaniasis CL – Cutaneous leishmaniasis

DCL – Diffuse cutaneous Leishmaniasis

Figure 3: Geographical distribution of leishmaniases in Kenya

2.4.3 Diffuse cutaneous leishmaniasis (DCL)

Diffuse cutaneous leishmaniasis (DCL) was first reported in Kenya in 1969 in Bungoma district and the Mount Elgon area (Kungu *et al.*, 1972). *Leishmania aethiopica* has been identified as etiological agent, rodents are the animal reservoirs and *P. pedifer* Lewis, is the vector of DCL in the Mt. Elgon region (Mutinga and Ashford, 1972; Mutinga, 1975; Sang and Chance, 1993).

2.4.4 Post-kala-azar dermal leishmaniasis in Kenya (PKDL)

In Kenya, PKDL was first described by Manson-Bahr in 1959 (Manson-Bahr, 1959). Reported PKDL rates in Kenya which show up after VL treatment, show considerable variability in four studies of 0.05% (Southgate and Oriedo, 1967), 1% (Manson-Bahr, 1959), 6% (Muigai *et al.*, 1991) and 30% (Cole, 1944). A study involving twelve patients with diagnosis consistent with PKDL who were seen at the Centre for Clinical Research (CCR) of KEMRI from 1981 to 1985 indicated a wide range of clinical manifestations from macular hypopigmented lesions to generalized nodular lesions. All lesions cleared by self-cure or by treatment with sodium stibogluconate (Muigai *et al.*, 1991).

2.5 Vectors of leishmaniasis

Leishmaniasis is transmitted by the bite of phlebotomine sand flies which belong to the family Psychodidae, sub-family Phlebotomidae, and genera *Phlebotomus* (in the Old World), and *Lutzomiya* and *Psychodopygys* (in the New World). Thirty species of these sand flies are proven vectors of *Leishmania* species (Desjeux, 2004). A summary of the world distribution of vectors is shown on Table 1.

2.5.1 Life cycle of a sand fly

Unlike most biting Diptera, development of sand flies takes place in terrestrial rather than aquatic microhabitats. Although there have been relatively few successful attempts to identify breeding sites in nature, eggs are laid in soil rich in organic matter and the larvae pass through four instars before pupation and emergence. The difficulty of finding breeding sites is an important constraint to vector control measures available to

leishmaniasis control programmes, where application of larvicides is not a practical alternative.

The eggs are elongated oval-shaped, pale at first and darkening on exposure to air with a single black 'eye spot'. The larvae emerge through fissure and are legless and whitish with a dark capsule (Fig. 4). Those of the first instar can be distinguished by the presence of two caudal bristles, all the subsequent instars bearing four caudal bristles (Fig. 4). Fourth instar larvae also have a prominent sclerite on the dorsum of the penultimate segment. The pupae are golden brown and are affixed to the surface of the substrate in which they developed by the final larval exuvium. Shortly before emergence, the wings and eyes turn black. Male sand flies emerge about 24 hours before the females, allowing their external genitalia time to rotate 180° to the correct position for mating before females have emerged. Although there have been no studies of sand fly development time in nature, the time recorded from laboratory studies shows that the time from oviposition to adult emergence at ambient temperature is around 4-6 weeks (Beach et al., 1983). Some Palaearctic species diapause as larvae while those from arid and semi-arid regions diapause as eggs. The eggs take about 12-15 days to hatch, depending on the sandfly species and environmental conditions (temperature and relative humidity).

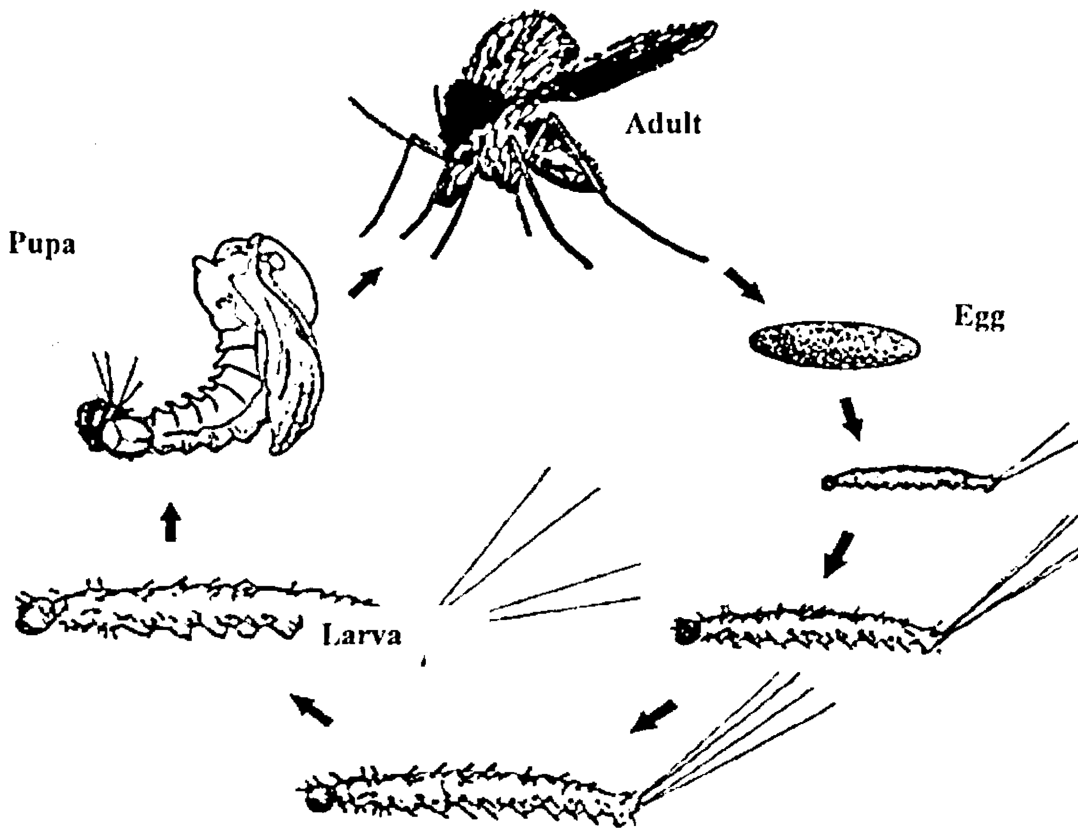


Figure 4: Diagram of sand fly life cycle courtesy of CDC website

2.6 Control of leishmaniases

Leishmaniasis is one of the most neglected tropical diseases, in terms of the few tools available for control and the lack of clear criteria for methods of control (WHO, 2006). The main control strategy of leishmaniasis is case finding and treatment plus, when feasible, vector control and in zoonotic foci, animal reservoir control (Neouimine *et al.*, 1996; WHO, 2004). These control strategies are tailored to the two main epidemiological entities: anthroponotic, when human beings are the sole reservoir, and zoonotic, when dogs are the major source of infection for the vector (Guerin *et al.*, 2002). In either

situation, efficient case management based on early diagnosis and treatment is the key to limit morbidity and mortality (WHO, 2007).

2.6.1 Vector control

Indoor residual spraying is a simple and cost effective method of controlling endophilic vectors and DDT remains the insecticide of choice for the control of leishmaniasis (Kishore *et al.*, 2006). Insecticides are still very effective in killing sand flies but very expensive to be practically accessible to the poor residents of the endemic areas and therefore, integrated alternative approaches must be sought to combat the menace (WHO, 2004). For example, a recent study in Sudan indicates a potentially strong reduction in VL incidence following the distribution and use of Insecticide Treated Nets (ITNs) (Ritmeijer *et al.*, 2007). A previous study indicated that the use of impregnated bed nets and going to bed early could provide a high degree of personal protection against VL (Elnaiem *et al.*, 1999).

However, the potential use of bed nets for sand fly control in North West Africa is complicated by the fact that during the hot season it is considered too hot to sleep under the fine-mesh nets, which cut down ventilation (Ritmeijer *et al.*, 2007). Furthermore, during the dry season people prefer to sleep outdoors and may be reluctant to use nets due to the daily routine of setting up nets before going to bed. In these resource restrained countries, vector control by spraying houses with insecticide is not sustainable due to logistical constraints and high cost (WHO, 2004). In Kenya, most vector species such as *P. duboscqi* are exophilic and exophagous, thus negating common strategies such as Indoor Residual Spraying (IRS) and ITNs (Clive *et al.*, 2003). Furthermore, the use of

DDT is prohibited in Kenya since it is one of the banned chemicals due to its potential for environmental damage.

Reservoir control involves the testing and culling of seropositive dogs in zoonotic visceral leishmaniasis endemic areas (Davies *et al.*, 2003). Impregnated collars, a novel method of topical application of repellents such as Deltamethrin, have been used to break the transmission of leishmaniasis in zoonotic visceral leishmaniasis (ZVL) foci (Mutinga 1975b); Shrech *et al.*, 1982). Animals' reservoir control for CL is based on the use of poison baits and environmental management to control rodents (Maroli and Khoury, 2004). On the overall, control through environmental management is expensive and difficult to implement and the efficacy of dog culling is questionable (WHO, 2004). The success of vector control strategies in developing countries is limited by the lack of well-trained technical personnel, weak delivery systems, low levels of political and financial commitments and implementation (WHO, 2004).

2.6.2 Vaccines against leishmaniasis

Abundant clinical and experimental evidence indicates that leishmaniasis can be prevented by vaccination (Handman, 2001; Davies *et al.*, 2003; Requena *et al.*, 2004; Coler and Reed, 2005). A vaccine to prevent the disease has been the goal for nearly a century based on the knowledge that a cured infection protects the individual from re-infection (Selvapandinyan *et al.*, 2006). However, prevention of leishmaniasis with an effective vaccine has to date not materialized (Piscopo and Mallia, 2006).

The completion of sequencing of *L. major* genome has added impetus to attempts to identify the genes that are responsible for resistance or susceptibility to leishmaniasis

(Ivens *et al.*, 2005). Many vaccine strategies have been pursued, including the use of whole lysate, killed avirulent or irradiated parasites. Additionally, DNA vaccines and purified or recombinant parasite antigens have also been tested. Most of these strategies have shown some degree of success in animal models but little or no protection in humans (Requena *et al.*, 2004). In general, the only successful immunization strategy in humans has been leishmanization, which is based on the development of durable immunity after the recovery from infection at a chosen site, usually the arm, with viable non-attenuated parasites (Breton *et al.*, 2005).

2.6.2.1 Leishmanization

The only proven vaccine agent in human beings is live *L. major* (leishmanization) (Ghalib and Modabber, 2007). In the Middle East, the deliberate infection with *L. major* was a common and effective practice for immunization against subsequent infections, but a fraction of the vaccinated persons produced lesions that required medical treatment (Khamesipour *et al.*, 2005). Leishmanization as a prophylactic vaccine was used on a large-scale in the Soviet Union and Israel with a high percentage of successful lesion development (Greenblatt, 1980; Kellina, 1981). Leishmanization was also employed in Iran in the 1980s and then in a massive programme covering over 2 million people during the Iran-Iraq war of 1982-1985 (Nadim and Javaidan, 1988).

The concept of leishmanization has had many problems, including the development of large uncontrolled skin lesions, exacerbation psoriasis and other skin diseases, and even immunosuppression as determined by low responses to the diphtheria, pertussis, and tetanus triple vaccine (Khamesipour *et al.*, 2006). In addition, *Leishmania* parasites are

believed to persist for a long time. As a result, leishmanization cannot be used on a large scale or in HIV pandemic areas (Ghalib and Modabber, 2007). At present, there is only one prophylactic vaccine in use in Uzbekistan (Khamesipour *et al.*, 2006).

2.6.2.2 Killed parasites

The earliest trials with killed *Leishmania* as a vaccine were conducted in Brazil in the 1940s. Later, from 1970s onwards Mayrink and colleagues developed a killed vaccine composed of five isolates of *Leishmania* containing four different species which was later simplified to a single *L. amazonensis* vaccine and tested for prophylactic potential in Colombia and Ecuador and as an adjuvant to chemotherapy in Brazil (Modabber, 1995; Genaro *et al.*, 1996).

In Venezuela, Convit and his group used a combination of autoclaved *L. mexicana* or *L. brazilliensis* promastigotes and *M. bovis* BCG either prophylactically, immunotherapeutically and or immunochemotherapeutically against South American leishmaniasis (Castes, 1989). Cure was associated with the development of Th1-type immune responses in the recipients, with the production of IFN-γ and absence of IL-4 (Cabrera *et al.*, 2000). In Venezuela, autoclaved killed *L. mexicana* is used to treat patients with CL (Convit *et al*, 2003), while in Ecuador, two doses of vaccine composed of *L. amazonensis* and *L. mexicana* mixed with BCG was shown to induce 73% protection (Armijos *et al*, 2003).

In the monkey model of CL, protective immunity was achieved using killed *L. amazonensis* co-administered with recombinant IL-12 as adjuvant while another study

using *L. major* with recombinant human IL-12 resulted in a skewed Th1 immune response but did not protect the primates against challenge infection with the parasite (Kenney *et al.*, 1999; Gicheru *et al.*, 2001). In general, considering all trials, based on the immunogenicity of various killed *Leishmania* preparations, it seems a better adjuvant than BCG would be required to produce a potent vaccine Khamesipour *et al.*, 2006).

2.6.2.3 Live attenuated vaccines

Recent advances in the ability to manipulate the *Leishmania* genome by introducing or eliminating genes has the potential to make live-attenuated vaccines a reality. The idea of live-attenuated vaccine is to expose the recipient to complex antigens in the right context over time without producing pathology (Selvapandiyan *et al.*, 2006). Using gene-targeting tools, it is now possible to generate parasites lacking genes essential for long-term survival in the mammalian host, such as the gene encoding the enzyme dihydrofolate reductase-thymidylate synthetase (*dhfr-ts*) (Titus *et al.*, 1995). In a mouse model, *L. major* parasites lacking *dhfr-ts* induced protection against *L. major* and *L amazonensis* but no protection conferred to Rhesus monkeys (Titus *et al.*, 1995; Veras *et al*, 1999; Amaral *et al.*, 2002).

Other genetically engineered and tested mutant parasites include the null mutants for the glucose transporter gene family in *L. mexicana* that exhibited reduced infectivity to BALB/c mouse macrophages, demonstrating that a single gene deletion can render a parasite virulent (Burchmore *et al*, 2003). Additionally, although *L. major* mutants deficient for leishmanolysin genes showed normal development in macrophages *in vitro*, they showed delayed lesion development in susceptible BALB/c mice (Joshi *et al*, 1998,

2002). *Leishmania major* mutants that lack LPG1 (the gene encoding a galactofuranosyl transferase) showed attenuated virulence in mice whereas the parasites that lacked LPG2 (the gene encoding a golgi GDP-mannose transporter) persisted indefinitely at low level in mice without displaying disease and provided protection from virulent *L. major* challenge (Uzonna *et al.*, 2004). Such protection from virulent challenges in mice has also been achieved after gene knockout for other genes: cysteine protease in *L. mexicana* and biopterin transporter in *L. donovani* (Alexander *et al.*, 1998; Papadopoulou *et al.*, 2002).

Attempts to develop avirulent parasites as vaccine candidates against leishmaniasis by irradiation, temperature sensitive mutations or random mutations induced by chemical agents has been hampered by reversion to virulence (Selvapandiyan *et al.*, 2006). These challenges can be met with the use of live attenuated strains which possess genetically defined mutations, can persist in the host without being virulent, have less chance of reversion to the virulent phenotype and can be produced in large quantities (Selvapandiyan *et al.*, 2006).

2.6.2.4 Recombinant and synthetic vaccines

The development of a defined vaccine against leishmaniasis has been accelerated by advances in the understanding of immunological mechanisms that mediate protection in animal models and to a lesser scale by supporting data from the characterization of immune responses in *Leishmania* infected individuals (Khamesipour *et al.*, 2006).

Newer vaccines under consideration for leishmaniasis comprise recombinant DNA-derived antigens and peptides. Some of the target antigens are species and life cycle stage specific while others are shared by promastigotes and amastigotes (Handman, 2001). Since T cells recognize peptides derived from cytosolic proteins bound in the major histocompatibility complex (MHC) class I groove or peptides derived from lysosomal compartment bound in the MHC class II groove on the antigen-presenting cell (APC) surface, all parasite proteins can function as antigens regardless of their location in the parasite (Handman, 2001).

Recombinant antigens can be delivered as purified proteins, as the naked DNA encoding them, or as bacteria manufacturing proteins *in situ* (Matzinger, 1998). Manipulations now allow targeting of the antigen to specific locations or to particular APCs, such as dendritic cells or langerhans cells, which are considered essential for the initiation of primary T-cell responses. Injection of bacteria or naked DNA may have the added advantage of providing adjuvant effect, which may 'activate' or 'licence' these APCs (Matzinger, 1998).

2.7 Diagnosis of leishmaniasis

Diagnosis and treatment follow-up of leishmaniasis pose a challenge to physicians working in endemic areas (Guerin *et al.*, 2002). Clinically, the diagnosis of visceral leishmaniasis is confirmed by demonstration of the parasite by microscopy (WHO, 2007). Intracellular *Leishmania* can be identified or cultured from aspirates of spleen, bone marrow, lymph node, or liver (Singh, 2003; Hailu *et al.*, 2005). The diagnostic yield is highest, about 98%, for spleen aspirates which have been used for routine diagnosis in

the field, for example in Kenya and Sudan (Herwaldt, 2002). However, where there are problems, precautions are necessary, and complications, though rare, may be serious (Guerin, 2002). Culture of the *Leishmania* parasite in Novy-MacNeal-Nicole (NNN) or Schneider's insect medium supplemented with 10% v/v foetal bovine serum, if properly performed is a more sensitive technique (Hailu *et al.*, 2005).

Serological tests based on the detection of specific humoral antibodies are less invasive (Hailu *et al.*, 2005). Such tests include Direct Agglutination Test (DAT), Enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), and rK39 dipstick test (Williams, 1995; WHO, 2007). In Kenya, ELISA was 98% sensitive and 100% specific, but there is no commercial kit (Boelaert *et al.*, 1999).

Direct Agglutination test (DAT) is easy to use in the field, and cost-effective, but there is no commercial source of antigen and results are not always reproducible (Boeleart *et al.*, 1999b). Testing with a commercially available immunochromatographic strip that uses recombinant leishmanial antigen K39 has proved 100% sensitive and 98% specific in India (Sundar *et al.*, 1998).

Many research centres have been evaluating the use of Polymerase Chain Reaction (PCR), especially on peripheral-blood samples. Polymerase Chain Reaction is now sensitive to the level of one parasite (Salotra *et al.*, 2001) and has been used successfully for diagnosis of VL in children in Italy (Cascio *et al.*, 2002) and for monitoring of relapse in HIV-co-infected patients (Pizzuto *et al.*, 2001). But PCR is still not easily usable in the

field, where confirmation of clinical diagnosis commonly remains a problem, and patients may not seek medical attention for many months (Guerin *et al.*, 2002). The mean delay from the onset of symptoms to definitive diagnosis was 7.7 months (SD 6.0) in a study in India, and 27.6% of cases were diagnosed longer than 9 months after onset of disease (Sundar *et al.*, 1991). Clinical follow-up is generally adequate to detect relapse in immunocompetent patients, but in immunosuppressed patients a non-invasive method of detecting parasite persistence or relapse would be useful (Guerin *et al.*, 2002). In such cases, PCR might be suitable. There is a clear need to strengthen both active case detection of CL and VL and diagnostic capacity at peripheral health centres where patients are usually treated on the basis of a presumptive diagnosis (WHO, 2007).

2.8 Treatment of leishmaniasis and their challenges

Treatment of leishmaniasis is difficult because of the intramacrophagic location of the infectious form (Rocha, 2005). In the absence of a potent vaccine against human leishmaniasis, the control of this disease relies primarily on chemotherapy (Handman, 2001). Until 1990, Pentostam was the only form of treatment for VL (Murray, 2004). Pentavalent antimonials and specifically sodium stibogluconate at a dose of 20 mg/kg /day administered intravenously or intramuscularly daily for 28 to 30 days is still the treatment of choice for VL in Africa today (Zijlstra and El-Hassan, 2001). However, this drug is not recommended for use in Bihar, India where relapse reached 65% by 1999 and in Europe where increasing deployment of the highly effective and efficient lipid formulations of amphotericin B has replaced it (Murray, 2004). Pentostam acts by interfering with the metabolism of the parasite (Conjivaram *et al.*, 2007).

The following drugs are currently in use for the treatment of leishmaniasis worldwide: 1) Sodium stibogluconate (Pentostam), 2) Amphotericin B, 3) Sitamaquine (WR6026), 4) Paromomycin sulphate (Aminosidine), 5) 4, 4'-Diamidino-a,o-diphenoxypentane di-isothionate (Pentamidine), 6) Hexadecylphosphorylcholine (Miltefosine).

Antimonials have several disadvantages: patients have to be admitted to hospital for 3-4 weeks for parenteral therapy; toxic effects such as athralgia, nausea, abdominal pain, and chemical pancreatitis; may limit the drugs' use. HIV co-infected patients are particularly prone to clinical pancreatitis (Pintado and Lopez-Velez, 2001). Long-term use at high doses to combat resistance is restricted by cardiotoxicity (Sundar *et al.*, 2000); brand-name products are expensive; there is a general problem of quality and batch-to-batch variability for both branded and generic drugs; and the poor quality of some generic formulations of the drug in India has led to serious toxicity (Sundar *et al.*, 1998). The efficacy and safety of generic (Albert David Ltd, Calcutta) and branded sodium stibogluconate (Pentostam, GlaxoSmithKline) were compared in randomized trials under field conditions in Sudan and Kenya under the auspices of the non-governmental organization Medicins Sans Frontieres (Guerin *et al.*, 2002). No difference was detected, and the investigators concluded that this generic antimonial could be used safely and effectively for the treatment of VL (Guerin *et al.*, 2002). However, the epidemiology of the disease in Sudan suggests that resistance to antimonial should be expected there soon (Guerin *et al.*, 2002).

Amphotericin B is the current alternative treatment of choice. Its drawbacks are the cost, limited availability in some areas, and toxicity, notably infusion-related side-effects (fever, chills, bone pain, throbophlebitis) and hypokalaemia, renal impairment, and anaemia (Croft *et al*, 2006). These problems are generally tolerable at the doses used in Bihar, where conventional amphotericin B is now the first-line drug of choice in kala-azar treatment (Guerin *et al.*, 2002). Though more expensive than the Indian antimonials, amphotericin B has cure rate of more than 97%, and resistance has not been reported (Guerin *et al.*, 2002). It is unfortunate that although liposomal amphotericin B was shown to be effective and less toxic for treatment of VL in Sudan and Kenya (Berman *et al.*, 1998), the drug is prohibitively expensive, and its use in African patients is limited (Berman *et al.*, 1998).

Paromomycin (aminosidine) has shown good efficacy against VL in Africa and is in phase III studies in India under the aegis of the institute of One World Health and East Africa managed by the Drugs for Neglected Diseases initiative (DNDi) (Croft *et al.*, 2006). However, this agent also requires parenteral administration (WHO, 2007).

Miltefosine (taken orally, 2.5mg/kg; 100mg/day for four weeks), is an antileishmanial drug recently approved for use against VL in India (Guerin *et al.*, 2002). Data on miltefosine use in East Africa are restricted to one study that was conducted in northern Ethiopia, in which it was found to be as safe and effective as sodium stibogluconate in HIV-negative patients and safer, but less effective, in HIV co-infected patients (Ritmeijer *et al.*, 2006).

Sitamaquine (WR6026) is an orally administered 8-aminoquinoline in development for the treatment of VL (Croft *et al.*, 2006). Preliminary clinical studies in Kenya have shown encouraging efficacy against various species of *Leishmania* (Wasunna *et al.*, 2005). The studies further demonstrated that sitamaquine was generally well tolerated in Kenyan patients with VL (Wasunna *et al.*, 2005). Although resistance to pentavalent antimonials is not yet a problem in Africa, in India, primary resistance to antimonials is common (Sundar *et al.*, 2000). However, there are *in vitro* studies suggesting that an Ethiopian strain, MHOM/ET/67/L82 of *L. donovani* exhibited synergy with Pentostam (Croft *et al.*, 2006).

Given the problems associated with the currently available drugs for the control of leishmaniasis, new strategies and improved treatments to replace or supplement existing therapy are needed urgently (Croft *et al.*, 2006). In the absence of a vaccine, there is an urgent need for alternative and effective control measures to complement the current ones. Some of the recent but very promising control measures are by use of biological agents to control sand flies (vectors of leishmaniasis) through entomopathogenic fungal infections (Scholte *et al.*, 2004).

2.9 Biological control agents

Biological control is defined as the use of biopesticides to control pest populations. Several biological control agents including fungal pathogens have historically been used as larvicides for decades (Lacey et al, 2004). The selection of candidate biological control

agents depends on factors including efficacy, environmental impact, cost consideration and compatibility with other intervention methods (Lacey and Orr, 1994).

2.9.1 Bacteria

Although large numbers of bacteria species have been isolated from disease vectors like mosquitoes, only a few are pathogenic. Those currently in use to control arthropods are spore-forming, rod-shaped bacteria of the genus *Bacillus* which commonly occur in soils. They include the most successful of biological control agents, namely *Bacillus thuringiensis (B.t)* and *B. sphaericus (B.s)* (Service, 1983; Lacey and Undeen, 1986). These bacilli have no ill-effects on non-target organisms (Lacey and Mulla, 1990; Lacey and Siegel, 2000) including humans and other vertebrates (Lacey and Siegel, 2000). Bacteria must be ingested to be effectively pathogenic. They are not contact poisons (Lacey and Undeen, 1986; Lacey and Siegel, 2000). Entomopathogenic bacteria kill their hosts by releasing one or several insecticidal proteins following ingestion (Lacey and Undeen, 1986). Although the mode of action of these bacterial toxins is not well understood, it is suggested that larvicidal toxins secreted by *B.t* kill their hosts through a series of cascade events (Hofte and Whiteley, 1989; Lee *et al.*, 2003).

2.9.2 *Bacillus thuringiensis*

The gram positive, spore-forming *Bacillus thuringiensis* was first discovered in silkworms in 1901 in Japan by Ishawata who called it *Bacillus soto*. It was only in 1911 in Germany that Ernst Berliner isolated the bacterium from dead flour moths and named it *Bacillus thuringiensis* (Milner, 1994). *Bacillus thuringiensis* is the most popular and widely used microbial insecticide because it is broadly pathogenic against larvae of

Lepidoptera, Coleoptera and Diptera (Lacey and Undeen, 1986). However, *B.t* is target specific depending on formulation. *Bacillus thuringiensis* var. *darmstadiensis* and *B. thuringiensis* var. *morrisoni* have no effect on Lepidoptera (Padua *et al.*, 1980 & 1984). Generally, formulations are prepared with respect to the area or habitat to be treated (Lacey and Undeen, 1986). The discovery of a single *Bacillus*, *Bacillus thuringiensis israelensis (B. t. i*), also called *B. t* (H-14), with highly insecticidal effects on multiple insect targets (Goldberg and Margalit, 1977), has revolutionalized the use of *B. t* as a biological control agent. Improvements in the formulation and application methods followed the first productions of *B. t. i* against insect targets in the early 1980s. *Bacillus thuringiensis israelensis* is principally effective against the larvae of mosquitoes, gnats and black flies (Lacey *et al.*, 2001).

2.9.3 Bacillus sphaericus

Found in soils and aquatic habitats, *Bacillus sphaericus* was first recognized as an insect pathogen in 1965 in California. It is specifically toxic against larvae of species of *Culex*, *Psorophora* and *Culiseta* (Lacey and Undeen, 1986). Although its spectrum of activity is not as broad as that of *B. t. i*, *B. s* appears to be particularly effective against the larvae of certain culicine species and phlebotomine sand flies (Lacey and Singer, 1982; Robert *et al.*, 1997). It is effective in polluted water and vegetation near animal burrows. The short life span of *B. s* in non-polluted environments in addition to its narrowed host range as compared to *B. t. i*, makes it a poor candidate for biological control (Lacey *et al.*, 2001; Fillinger and Lindsay, 2006). However, in a study conducted by Robert *et al*, (1997) in which the vegetation near animal burrows and eroded termite mounds was sprayed with sucrose solution with in-cooperation of the larval toxicant *B. sphaericus*, 40% of the

female sand flies fed *in situ*. Dispersing *B.s* carrier sand flies caused significant larval mortality in resting and breeding sites in animal burrows 10-30m from the sprayed vegetation for 2-12 weeks post treatment. Also adult sand fly populations breeding and resting inside animal burrows were significantly reduced, following direct application of *B.s* to the burrow entrances. This approach may be useful as biological control agents against phlebotomine sand flies in biotopes where larvae and adults use the same habitat (Robert *et al.*, 1997). The principal drawback with entomopathogenic bacteria for biological control is the cost of production and delivery which makes them less accessible to developing countries (Lacey *et al.*, 2001).

2.9.4 Viruses

Numerous viruses have been found to be responsible for epizootic deaths in many insect species, the most affected being dipterans of the Suborders Culicidae and Simulidae (Payne, 1982). Viruses, like bacteria, must be ingested to infect their hosts. Some viruses like, nucleopolyhedrosis viruses (NPV) and granuloviruses (GV) are the most pathogenic viruses against the larvae of many insect species including caterpillars and sand flies (Clark *et al.*, 1969; Lacey *et al.*, 2001). These viruses do not appear to present any safety concerns with respect to non-target invertebrates and vertebrates (Groner, 1990). However, they require live insect hosts for production (Lacey *et al.*, 2001).

2.9.5 Protozoa

Numerous protozoa are found in natural populations of insect species, but few can play an important role in infecting and reducing mosquito and sand fly populations. Species of Microsporida (Phylum: Microspora) are among the most commonly observed pathogenic

protozoans in insect populations (Chapman *et al.*, 1972; Legner, 1995). Entomopathogenic protozoa kill their host by the chronic effects of parasitism (Chapman *et al.*, 1972).

2.9.6 Entomopathogenic fungi

Entomopathogenic fungi were amongst the first organisms to be used for biological control of pests (Hajek, 1997). Mycoinsecticides or mycopesticides have been known since the eighteenth century with 47 species reported against mosquitoes in the early 1960s (Chapman, 1974). Entomopathogenic fungi are effective against eggs, larvae, intermediate stages and adults of a variety of insects which include locusts, grasshoppers, mosquitoes, sand flies and others (Chapman, 1974; Bateman *et al.*, 1998; Shi and Feng, 2004; Scholte *et al.*, 2005; Blanford *et al.*, 2005; Furlong and Pell, 2005; Achonduh and Tondje, 2008).

Entomopathogenic fungi are common and widespread in almost all classes of insects. To date, approximately 750 species of fungi from 90 genera have been documented to be pathogenic, but only a few of these species are currently being developed as pathogens against insect pests (Chapman, 1974; Roberts and Hajek, 1992; Fukatzu *et al.*, 1997; Hajek, 1997).

Most species are either from the fungal divisions; Zygomycota in the order Entomophthorales or Ascomycota of conidial Hyphomycetes which do not reproduce sexually (Hajek, 1997). Unlike Hyphomycetes fungi which infect and colonize insects after death, entomophthorales fungi do not produce major toxins for the progression of an

infection and are thus obligate pathogens (Roy *et al.*, 2006). Although these two groups of fungi vary in terms of history, they both produce asexual spores (commonly called conidia) representing the infective component as well as sexual spores (chlamydospores) enabling survival in the absence of new hosts (Hajek and Leger, 1994; Roy *et al.*, 2006). These two groups of fungi are also reportedly responsible for epizootic or natural epidemics within susceptible hosts (Powell *et al.*, 1986; Hajek and Leger, 1994), but this phenomenon is most commonly observed in association with the Hyphomycetes (Pell *et al.*, 2001). Two types of pathogenic fungi have been studied for use as biological control agents against mosquito species. These are aquatic fungi that comprise species of *Coelomyces* (Chytridiomycetes fungi), *Lagenidium giganteum* (an Oomycete fungus) and *Culicinomyces clavosporus* (a Deuteromycetes fungus), and the terrestrial fungi; *Metarhizium anisopliae* and *Beauveria bassiana* (Chapman, 1974; Roberts and Hajek, 1992). Most research has focused on *Lagenidium giganteum*, followed by *M. anisopliae* and *B. bassiana*.

2.10 The entomopathogenic Deuteromycetes- *B. bassiana* and *M. anisopliae*

Within the Class Deuteromycetes a morphological group of fungi known as Hyphomycetes exists. These are filamentous fungi that reproduce by conidia generally formed aerially on condiophores arising from the substrate. Many genera of entomopathogenic fungi occur in this group of fungi and have some of the widest of host ranges among entomopathogens, including several mosquito species. The most common route of host invasion is through the external integument, although infection through the digestive tract is possible (Goettel and Inglis, 1997). Conidia attach to the cuticle, germinate, and penetrate the cuticle. Once in the hemocoel, the mycelium grows

throughout the host, forming hyphal bodies called blastospores. Death of the insect is often due to a combination of the action of fungal toxins, physical obstruction of blood circulation, nutrient depletion and /or invasion of organs. After the host has died, hyphae usually emerge from the cadaver and, under suitable abiotic conditions conidia are produced on the exterior of the host. These are then dispersed by wind or water (Goettel and Inglis, 1997).

Also known as imperfect entomopathogenic fungi, *B. bassiana* and *M. anisopliae* belong to Ascomita, Class Deuteromycetes (Hyphomycetes), Order Hypocreales and family Clavicipitaceae (Roberts, 1970; McCoy *et al.*, 1988). For more than 120 years, *B. bassiana* and *M. anisopliae*, in addition to *B. brongniartii*, have been used to control pest insects (Zimmerman, 2007). These fungi are known to infect over 220 insect species (Roberts, 1970; McCoy *et al.*, 1988; Scholte *et al.*, 2003).

Beauveria bassiana (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin are widely distributed from the arctic to the tropics and can be isolated from insects as well as soils. They are anamorphic fungi (asexual reproduction) and infect various insect species through parasitism (Clark *et al.*, 1968; Robert, 1970; Roy *et al.*, 2006). The biological agents of *B. bassiana* and *M. anisopliae* are conidia whose toxic action is often specific to a single or group of insects (Rombach *et al.*, 1986, 1987; Furlong and Pell, 2005). The infected insect host is killed by multiple mechanisms involving mechanical damage of the cuticle, toxins produced by the entomopathogenic

fungi, water loss and food depletion following infection (Chapman, 1974; Clarkson and Charnley, 1996).

Fungal pathogens are currently commercialized and extensively used as biocontrol agents against agricultural pests and public health disease-vectors such as flies and cockroaches. Information concerning the commercial use of these fungicides can be obtained from United States Environmental Protection Agency and the Pesticide Action Network in North America (www.epa.gov, www.pesticideinfo.org).

2.10.1 *Beauveria bassiana*

The genus *Beauveria* contains at least 49 species of which approximately 22 are considered pathogenic (Kirk, 2003). *Beauveria bassiana* (Bals-Criv-) Vuill- is the most important of the commonly used fungi in the genus. Originally known as *Tritirachium shiotae*, this fungus was renamed after the Italian lawyer and scientist Agostino Bassi, who first implicated it as the causative agent of a white (later yellow or occasionally reddish) muscardine disease in domestic silkworms *(Bombyx mori* L.), (Furlong and Pell, 2005; Zimmerman, 2007). Bassi's work led to innovative vector control strategies, which benefited the European silkworm industry. *Beauveria bassiana* (Bals-Criv) Vuill is considered to be one of the most effective entomopathogenic fungi for various reasons: cosmopolitan distribution (Bidokha *et al.*, 1998), ability to infect any life stage of its host, wider host range including plants, can infect almost all orders of insects (Clark *et al.*, 1968; Roberts and Hajek, 1992) and can infect certain plant tissues (Bing and Lewis, 1992). *Beauveria bassiana* can be isolated from insect cadavers or from soil in forested areas by using sample media (Beilharz *et al.*, 1982), as well as by baiting soil with insects

(Zimmerman, 1986). In the laboratory it can be cultured on simple media (Robert and Hajek, 1992; Goettel and Inglis, 1997).

Conidia, which are the infective particles, measure 2-4μm in diameter and are globose to sublobose or ovoid in shape. However, the size of the thin-walled and smooth conidia varies with species (Samson and Evans, 1982). *Beauveria bassiana* is characterized by the denticulate and distinctive zig-zig appearance of conidiogenous rachis which is an extension of proliferating conidiogenous cell bearing conidia. The conidiospores are generally grouped in clusters (Samson and Evans, 1982).

2.10.2 *Metarhizium anisopliae*

Metarhizium anisopliae (Metschnikoff) Sorokin, initially known under the name *Entomophthora anisopliae*, was first described near Odessa in Ukraine from infected larvae of the wheat cockchafer *Anisopliae austriaca* in 1879, and later on, *Cleonus punctiventis* by Metschnikoff. It was later renamed as *Metarhizium anisopliae* by Sorokin in 1883 (Tulloch, 1976).

Metarhizium causes a disease known as 'green muscardine' in insect hosts because of the green colour of conidial cells. The genus *Metarhizium* is pathogenic to a large number of insect species, many of which are agricultural and forest insects (Ferron, 1978). In addition, *M. anisopliae* has been found to have potential as a biological control agent of mosquitoes (Scholte *et al.*, 2005). *Metarhizium*, like *Beauveria* is one of the most common entomopathogenic fungi, with wide-world distribution. The species is soil-borne and infects predominantly soil-dwelling insects. Taxonomy of *Metarhizium* is not

straightforward. The current classification of the taxon is mainly based on the morphology of conidia conidiogenous cells. Some authors combine these with biochemical and molecular characteristics (Riba *et al.*, 1986), and /or host pathogenicity, cold-activity and sporulation colour (Yip *et al.*, 1992; Rath *et al.*, 1995). Driver *et al.*, (2000) used 10 different clades, based primarily on molecular data, although this leaves room for debate. *Metarhizium anisopliae* consists of 4 varieties (Driver *et al.*, 2000), two of which are considered important, these being *Metarhizium anisopliae* var. *acridum* (previously known as *Metarhizium flavoviride*) and found mainly in Homoptera and *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) Sorokin, the latter being the known of the two species. *Metarhizium anisopliae* has a large host-range, including arachnids and five orders of insects (Boucias and Pendland, 1998), comprising over 200 species.

On terrestrial insects, the life cycle begins with a conidium attaching to the host cuticle, forming an appressorium, followed by penetration peg to enter the cuticle. After entering the hemocoel, the hyphae formed produce and release toxins, killing the host 4-16 days (depending mainly on the host species) after contamination (Ferron, 1981; Khachatourians, 1991; Boucias and Pendland, 1998). These toxins include Destruxins, Swaensinone, and Cytochalasin C (Strasser *et al.*, 2000). Histopathological studies of elaterid tissues infected by *Metarhizium anisopliae* suggest that toxins (destruxins) kill the host by inciting degeneration of the host tissues due to loss of the structural integrity of membranes and then dehydration of cells by fluid loss (Ferron, 1981). If the conditions are warm and moist, conidiophores will grow through the cuticle to cover the insect with

conidia. The cycle in mosquito larvae varies from the above. If floating conidia are applied, larvae contact them when they break the water tension with their perispiracular valves for air intake. The fungus germinates and penetrates into the respirical siphon, blocking the breathing mechanism (Daoust *et al.*, 1982; Lacey *et al.*, 1988). Plugging of the spiracles usually leads to death before significant invasion of the hemocoel has occurred, so hyphal body formation is minimal. Cadavers in the aquatic environment are overrun with bacteria rather than mycelium, and no new conidia are formed. Although much less frequently observed, larvae can also ingest dry conidia (Crisan, 1971; Roberts, 1974), where they apparently without germination, release lethal substances into the gut (Crisan, 1971; Roberts, 1970, 1974).

Metarhizium anisopliae has several characteristics that make it interesting as a microbial control agent. It causes high mortality of mosquito larvae in laboratory populations, the fungus can be grown in massive amounts on inexpensive artificial media, and conidia can be stored easily. Moreover, its failure to germinate in the mosquito environment until actual exposure to a host and its resulting persistence in the environment, as well as the fact that its effect is not limited to periods of host molting (as for *Beauveria bassiana*), make this fungus a very promising control agent (Roberts, 1970). The fungus is commercially produced by Biocare, Australia; BCP, South Africa; Bayer, Germany (BIO 1020), and several Brazilian companies, as control agents for German cockroaches and termites, black vine weevil, citrus root weevil and sugarcane pests (Khetan, 2001).

Zimmerman (1993) claims that because of no toxicological or pathological symptoms in birds, fish, mice, rats and guinea pigs after exposure to conidia of the fungus *Metarhizium anisopliae* was safe. Also Strasser *et al.*, (2000) concluded from a risk-assessment study that the fungus poses no obvious risk to humans, or the environment. However, it has been reported that the fungus may cause human keratitis (DeGracia *et al.*, 1997).

2.10.3 Mode of action of Entomopathogenic fungi (Deuteromycetes)

Unlike other biopesticides such as bacteria and viruses, entomopathogenic fungi do not have to be ingested to cause infection, making them valuable as biological control agents. Although some reports suggest a mode of infection through the siphon tips or gut of insect larvae (Lacey *et al.*, 1988; Goettel and Inglis, 1997), entomopathogenic fungi generally infect or penetrate their targets percutaneously (Charnley, 1989). This can occur by adhesion of spores to the insect integument, especially the intersegmental folds, or by simple tarsal contact (St. Leger *et al.*, 1986; Charnley, 1989; Scholte *et al.*, 2003). A schematic representation (Fig. 5) of how the infection develops in the insect from three to seventeen days post infection shows at least six identifiable stages from initial infection till death (Clarkson and Charnley, 1996; Scholte *et al.*, 2004).

Generally, fungi do not cause instant mortality but cause sublethal and late-life lethal effects, on different stages of the insect life cycle. Due to such properties, fungi can potentially be used as 'evolution proof' agents and overcome mosquito resistance unlike the currently deployed fast-acting chemical insecticides (Mnyone *et al.*, 2009). Increased mortality rate among older individual insects could be explained by various physiological properties that change with age of mosquitoes and possibly other insect species. Older

mosquitoes showed reduced immune function in experiments done by Mnyone *et al.*, 2009. Non-blood-fed mosquitoes were found to be more susceptible to fungus infection, than blood-fed ones. A digested blood meal increases the nutrient reserve of the host, thereby increasing the time required for the fungus to deplete nutrients and kill the insect. In addition, insects with digested blood meal can mount a stronger immune response. The fact that blood-fed mosquitoes are less susceptible to fungal infection could be beneficial in terms of evolution proofing against resistance development. Histopathological studies of tissues infected by fungus suggest that, the insect dies due to the combination of nutrient depletion, mechanical damage, and toxicosis (Ferron, 1981). Other factors contributing to deaths of insects infected with fungus include: age of the insects, melanization, enzymes such as phenol oxidases which are suggested to be important in melanization and sclerotizationin hemolymph, and numbers of immune competent hemocytes decrease with age. A change in number and morphology of circulating hemocytes was attributed to increased mortality in older *Aedes aegypti* (Mnyone *et al.*, 2009). Moreover, glycogen and total soluble proteins deteriorate with age (Mnyone *et al.*, 2009).

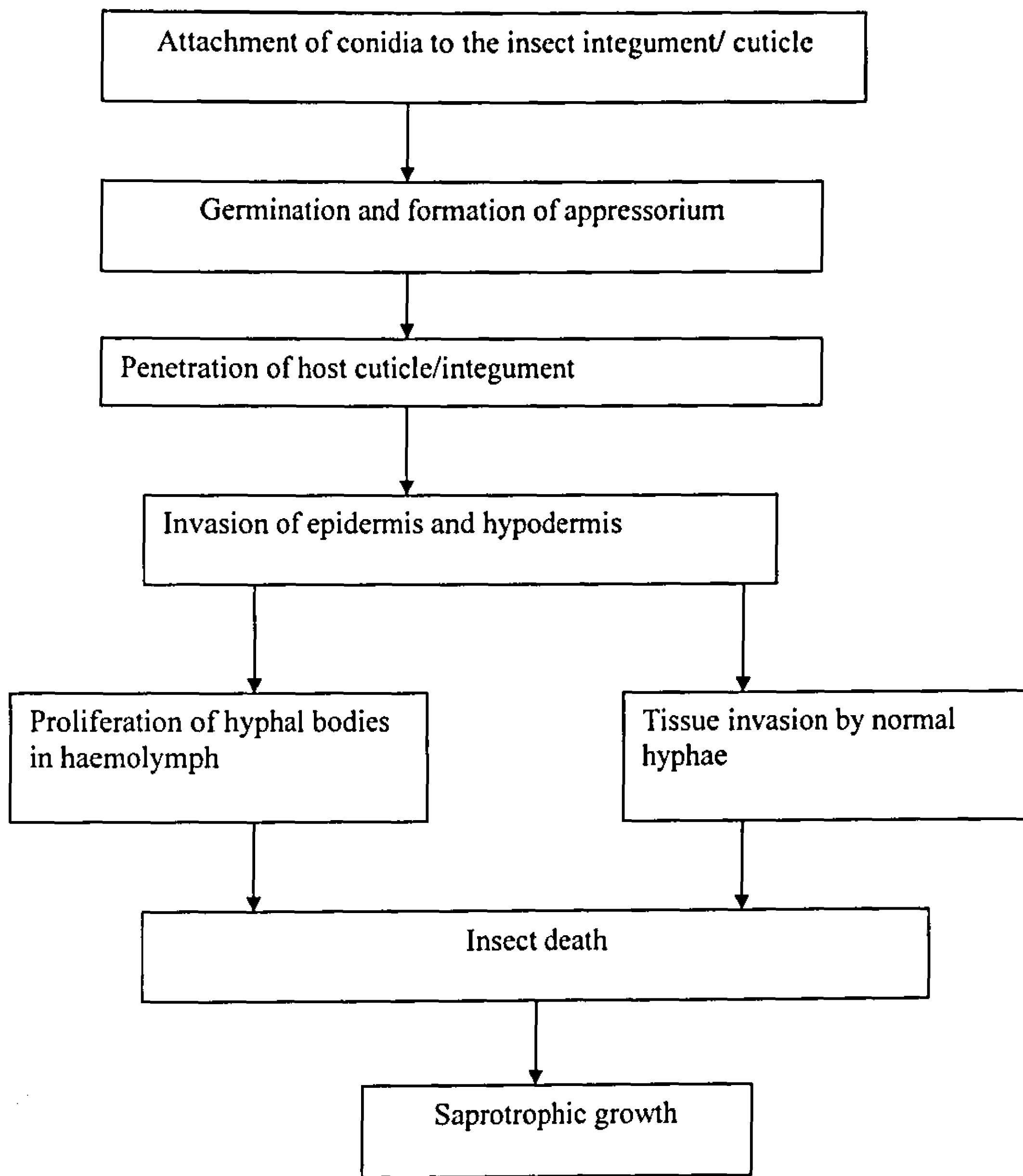


Figure 5: Schematic steps of the *in vivo* developmental cycle of fungal pathogen inside the insect host (Clarkson and Charnley, 1996).

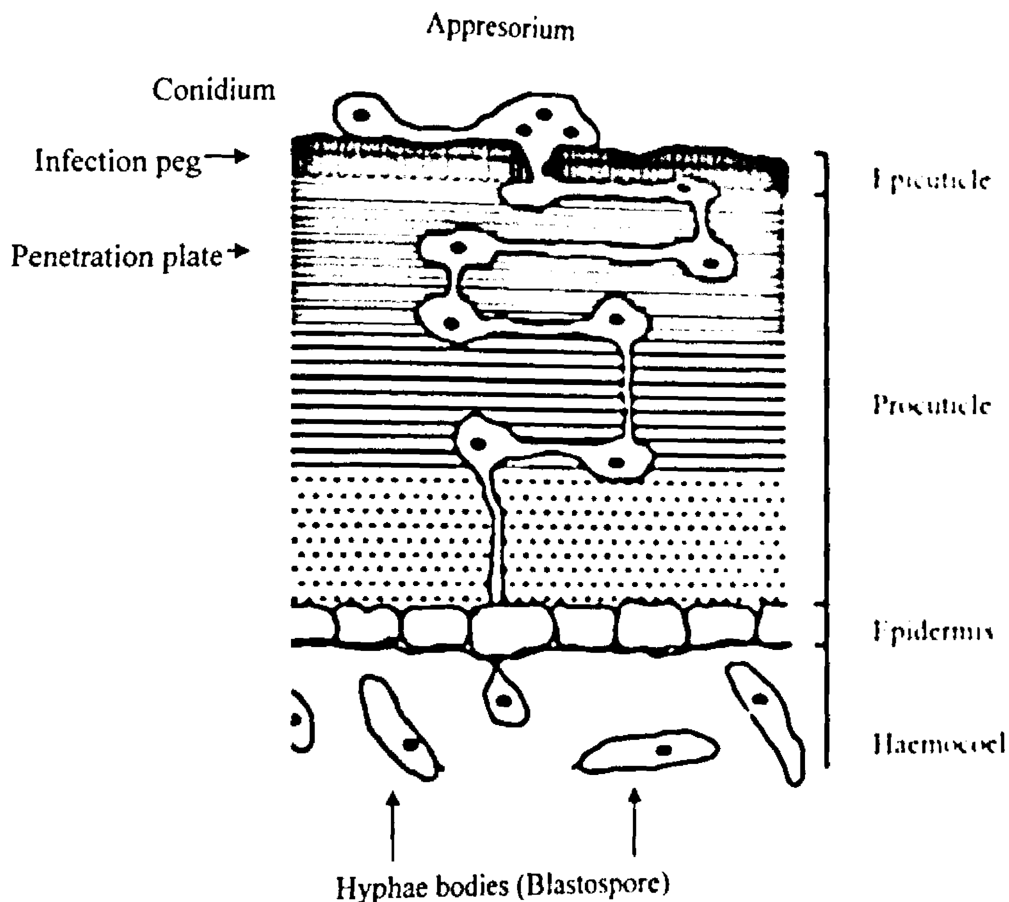


Figure 6: Structure of insect integument/cuticle and the mode of penetration by conidia (Clarkson and Charnley, 1996).

Figure 6 – Attachment of the conidia to the host cuticle which produces the germ tube is the first step and most important part of the fungal infection (Charnley, 1989). It is suggested that contact of conidia to the host integument occurs via non-specific forces exerted by rod-lets (straight and slim cylindrical structures) which are unique to the conidial stage of the infection (Boucias *et al.*, 1988; Bidochka *et al.*, 1995).

Alternatively, the attachment of conidia to the insect integument may be initiated by hydrophobic interactions between conidial hydrophobins and the waxy surface of the insect cuticle (Bidochka *et al.*, 1995 and 2000). Fungal hydrophobins are a class of hydrophobic- rich proteins that allow the fungus to attach to solid hydrophobic surfaces (Bidochka *et al.*, 1995).

Germination and successful infection depends on a number of factors including, environmental conditions (temperature, humidity and oxygen concentration), host susceptibility, host stage and age with younger hosts usually more susceptible than older ones, and chemical composition of the host's cuticle (Fig. 6). However, attachment and germination can fail, and the failure is generally attributed to inhibitory compounds like fatty acids, phenols and quinine (Ferron, 1980). Activities related to the attachments and germination of conidia and the formation of appressorium have been reported (Bidochka *et al.*, 1995; St. Leger, 1998).

However, literature on the exact mechanism and signal elements responsible for cuticle invasion are still unclear (Bidochka *et al.*, 2000). Generally, germinated conidia produce an appressorium, which then forms an infection peg and a penetration plate (St.Leger *et al.*, 1991). Some researchers reported the absence of the appressoria in certain insects infected with *B. bassiana*. However, these cells, which are apical swellings that function in the attachment of conidia on the host's cuticle, are generally formed after the penetration of germ tube through the host's cuticle (St. Leger *et al.*, 1991; (Clarkson and Charnley, 1996). The result is the production of a disease in the haemocoel of the insect

host (Charnley, 1989) (Fig. 6). The penetration of conidia through the host's cuticle involves both mechanical pressure and enzymatic degradation (St. Leger *et al.*, 1986; Charnley, 1989; Hajek and Leger, 1994; Bidochka *et al.*, 1995; Clarkson and Charnley, 1996). Enzymatic degradation involves the production of several and different amounts of cuticle degrading enzymes which vary according to the species and strains of the fungi. These enzymes will exhibit variable levels of pathogenicity towards their hosts. Following successful penetration of the cuticle, the fungus produces blastospores or hyphae bodies, which are passively distributed in the hymolymph and the fat body (Hajek and Leger, 1994; Clarkson and Charnley, 1996).

In order to kill their host, fungal pathogens release a wide range of secondary metabolic compounds, commonly called toxins, inside the insect host, particularly in the haemocoel. A plethora of toxins have been suggested but the most important are destruxins from *M. anisopliae* and *B. bassiana* (Clarkson and Charnley, 1996; Strasser *et al.*, 2000). Destruxins are cyclic hexadepsipeptides containing five amino acids (β-alanine, alanine, valine, isoleucine and praline) and α-hyroxy acid. The ionophore beauvericin is a toxic cyclic hexadepsipeptide and comprises a cyclic repeating sequence of three molecules of N-methyl phenylalanine altering with three molecules of 2-hydroxyisovaleric acid (Strasser *et al.*, 2000). During the initial penetration of host, *M. anisopliae* and *B. bassiana* secrete large amounts of single extracellular protease called chymoelastase protease or Pr1 to degrade the host cuticle (St. Leger *et al.*, 1987, 1992 and 1995). The endomoprotease Pr1, which is the major enzyme secreted by *Metarhizium* during the degradation process of the cuticle, also differs in terms of biochemistry among

strains. However, the cuticle degrading enzymes are produced in a sequential manner with the proteolytic enzymes and esterases first followed by chitinases, proteins surrounding the cuticle must be degraded before the action of chitinases begins St. Leger *et al.*, 1992 and 1995).

The death of an infected insect varies from 2-15 days post infection depending on the fungal strain and species, but more particularly on the characteristics of the host (Ferron, 1978; Boucia and Pendland, 1998). When the infection process which is followed by the death of host is complete, the fungus switches back to its hyphal mode and, under relatively humid conditions, the fungus subsequently grows out of the cadaver surface to produce new, external, infective conidial saprophytic growth (Ekesi *et al.*, 1999; Jianzhong *et al.*, 2003; Mitsuaka, 2004). However, under very dry conditions, the fungus may persist in the hyphal stage inside the cadaver where the conidia are produced inside the body (Daoust and Roberts, 1983; Hajek and Leger, 1994; Hong *et al.*, 1997). Under favourable conditions, sporulated cadavers can infect other individuals from the same target species through horizontal transmission (Meadow *et al.*, 2000; Quesada-Moraga *et al.*, 2004). Horizontal transmission can also occur during the mating. Scholte *et al.*, (2004) demonstrated that male mosquitoes can acquire fungal infection after mating with infected females. In addition, autodissemination of fungal pathogens was proven successful between infected and uninfected adult beetles in the laboratory and under field conditions (Kreutz *et al.*, 2004).

2.10.4 Host ranges of fungal species and strains

Although *B. bassiana* and *M. anisopliae* have a wide host range, *M. anisopliae* is more restricted than *B. bassiana*. In addition, certain strains and genotypes are more restricted within species, and even significantly between species (Rombach *et al.*, 1987; Bidochka and Small, 2005; Furlong and Pell, 2005). *Metarhizium anisopliae* var. *anisopliae* attacks almost all classes of insects including arachnids whereas *M. anisopliae* var. *majus* is mainly restricted to soil dwelling beetles (Mccoy *et al.*, 1988; Scholte *et al.*, 2003b). LuBiLoSa (Lutte Biologique contre les Locustes et les Sauteriaux) identified *M. anisopliae* var. *acridium* as the most effective biological control agent for adult locusts and grasshoppers but not for mosquitoes in environmentally safe areas (Prior Greathead, 1989; Price *et al.,* 1997; Lomer *et al.*, 2001). Furthermore, isolates are also more specific under field conditions compared to laboratory studies (Jaronski *et al.*, 2003) and the geographic occurence of the fungus seems to play a role in host preference. Rombach *et al.*, (1986) and Bidochka and Small (2005) showed that some genetic groups of *Metarhizium* from tropical and subtropical regions were more specific to particular classes of host insects. The virulence of entomopathogenic fungus varies within species and isolates (Prior *et al.*, 1995; Bateman *et al.*, 1996).

2.10.5 Influence of physiological state of the host

The physiological state of the insect host also plays a role in pathogenicity in the treated population. An increased rate of infection amongst insects already physiologically weakened increases the mortality rate amongst the healthy individuals following contact with infected individuals (Scholte *et al.*, 2004). The physiology of insect hosts can be weakened by the absence of sugar or blood (Nayar and Van Handel, 1971; Foster, 1995),

or by exposure to chemical insecticides where less resistant hosts will die by intoxication by a low dose of insecticide while the more insecticide resistant individuals will succumb to fungal infection (Delgado *et al.*, 1999; Pachamuthu and Kamble, 2000; Ericsson *et al.*, 2007).

2.10.6 Influence of temperature, humidity and solar ultraviolet radiation on fungal virulence

Temperature, humidity and light are critical components to the virulence of entomopathogenic fungi. Fungal conidia are very sensitive to high temperatures (over 34°C) and ultra violet light, depending on the strains of fungus (Fargues *et al.*, 1997; Jianzhong *et al.*, 2003). Their ideal temperature range is 20-30°C (e.g 25°C for *B. bassiana* and 27-28°C for *Metarhizium anisopliae*) (Thomas *et al.*, 1996; Jianzhong *et al*, 2003; Mitsuaki, 2004). High and low temperatures as well as humidity affect the speed of insect infection by inhibiting spore germination, which in turn affects the formation of a germ tube and penetration through the insect cuticle (Ekesi *et al.*, 1999; Fargues and Luz, 2000; Mitsuaki, 2004). Oscillating temperatures can also slow infection and colonization by fungal pathogens (Zimmerman *et al*, (1982); Morley-Davies *et al.*, (1995) and Inglis *et al.*, 1999) demonstrated that conidia can tolerate high temperatures associated with high relative humidity. However, the viability of conidia decreases significantly when exposed to sunlight (Zimmerman, 1982; Morley-Davies *et al.*, 1995).

Temperature and humidity are found to regulate sporulation, with high (over 34°C) or low (below 10°C) temperatures reducing or preventing sporulation (Ekesi *et al.*, 1999; Fargues and Luz, 2000). A relative humidity of between 75-100% is favourable for

optimal germination of *Beauveria* conidia (Soza-Gomez and Alves, 2000). Under favourable conditions, infection speed and sporulation can be reinstated, followed by a high infection rate where population densities are high (Thomas *et al.*, 1996). However, the delivery and application methods of fungal spores can contribute efficiently to high mortality of hosts in high temperatures (Bateman, 1992).

Although the longevity of conidia is a function of the fungus treated surface (Blanford *et al.*, 2005), it is evident that the moisture content of conidia (Hedgecock *et al.*, 1995; Hong *et al.*, 1997), and relative humidity also play an important role in the longevity of conidia (Zimmerman, 1982; Daoust and Roberts, 1983). The conidia from various fungi and strains within fungus species also showed varying longevities in relation to relative humidity and temperature (Daoust and Roberts, 1983; Hong *et al.*, 1997). Thus, different formulations of conidia are needed when using fungi against different hosts.

2.11 Justification of the study

Leishmaniasis is a major public health problem placing 350 million people from 88 countries in the tropics and subtropics at risk, with estimated 14 million new cases annually. It is estimated that 2 million deaths occur annually in the world. The disease has been neglected by researchers and funding agencies. Control of leishmaniasis by chemotherapy is expensive, involves long drug treatment regimens, and is often associated with serious side effects. Drug resistance has been reported in some countries like in India where sodium stibogluconate (Pentostam) cannot be used effectively to treat visceral leishmaniasis. Unless new strategies are developed to combat leishmaniasis, the

already enormous health and economic burden related to the disease in the tropical countries is bound to worsen.

The occurrence of insecticide resistance to all classes of insecticides available for use in public health as well as mounting criticism concerning the use of chemical insecticides (due to their safety and environmental impacts on non-target beneficial invertebrates and humans), has provided an impetus for the development of alternative forms of vector control. Vaccine development is still at experimental stage but appears to be a promising strategy for leishmaniasis control (Kedzierski, 2010). Under laboratory conditions it has been shown that *M. anisopliae* and *B. bassiana* are effective in controlling insect vectors of both malaria and leishmaniasis (Achundoh and Tondje, 2008; Ngumbi *et al.*, 2011). Use of biological agents to control vectors of leishmaniasis is a good option to be exploited in an effort to control the disease. This study was designed to produce a biological control strategy to control vectors of leishmaniasis.

2.11.1 Scientific Hypotheses

1) Isolation and culturing of isolates of entomopathogenic fungi (EPF) from endemic sand fly areas will yield virulent strains that are capable of infecting laboratory-reared phlebotomine sandflies.
2) Infection of sand flies with EPF will lead to high mortality rates in sand flies in the field.

2.11.2 General Objective

To characterize potential entomopathogenic fungi found in leishmaniasis endemic regions of Baringo district and ICIPE germplasm and use them to control sand fly populations.

2.11.3 Specific Objectives

1) Isolate and identify the most virulent strains of entomopathogenic fungi (EPF) in the laboratory from soil samples and ICIPE germplasm.
2) Test the ability of the entomopathogenic fungal isolates to infect and kill target sand fly species and their larval stages under laboratory conditions.
3) Undertake small scale field testing of selected fungal pathogens against field sand fly populations in the termite mounds.

CHAPTER THREE

3.0 Materials and Methods

3.1 Study sites

The study was conducted near two schools: Rabai and Perkerra Primary Schools in Marigat division, Baringo County, Rift Valley Province, Kenya (Fig. 7). The schools are approximately 5 km and 7 km south and south-east of Marigat town respectively. The town is 250 km north-west of Nairobi (0^0.27'30"N and 36^0E) and at an elevation of 1030m above sea level. The division is semi-arid with unreliable annual rainfall of between 300 and 600mm. The ground is mostly bare soil or rocky ridges with gullies in some areas. Average daily temperatures are usually above 33^0C in Marigat division. The area is a scrubland dotted with *Acacia* trees and the recently introduced *Prosopis juliflora* (locally known as 'Mathenge') to check desertification in the region. The area is inhabited by two main ethenic groups; Tugens and Maasai njemps. The main agricultural activities practiced here are growing of cash and subsistence crops such as maize, bananas, watermelons, onions and vegetables in the Perkerra irrigation scheme. Animal husbandry is also practiced with large numbers of sheep, goats and cattle kept in both Rabai and Perkerra. The main rainy season occurs between March and August, while the short rainy season occurs between November and December each year. The hottest months are between January and March with little or no rain falling down. Rodent burrows which are preferred breeding and resting sites for *P. duboscqi*, are numerous in both vegetation covered and bare grounds, with termite mounds being a common feature

in this area. The termite mounds act as the breeding and resting sites for sand fly species like *P. matini* and other *Sergentomyia* species.

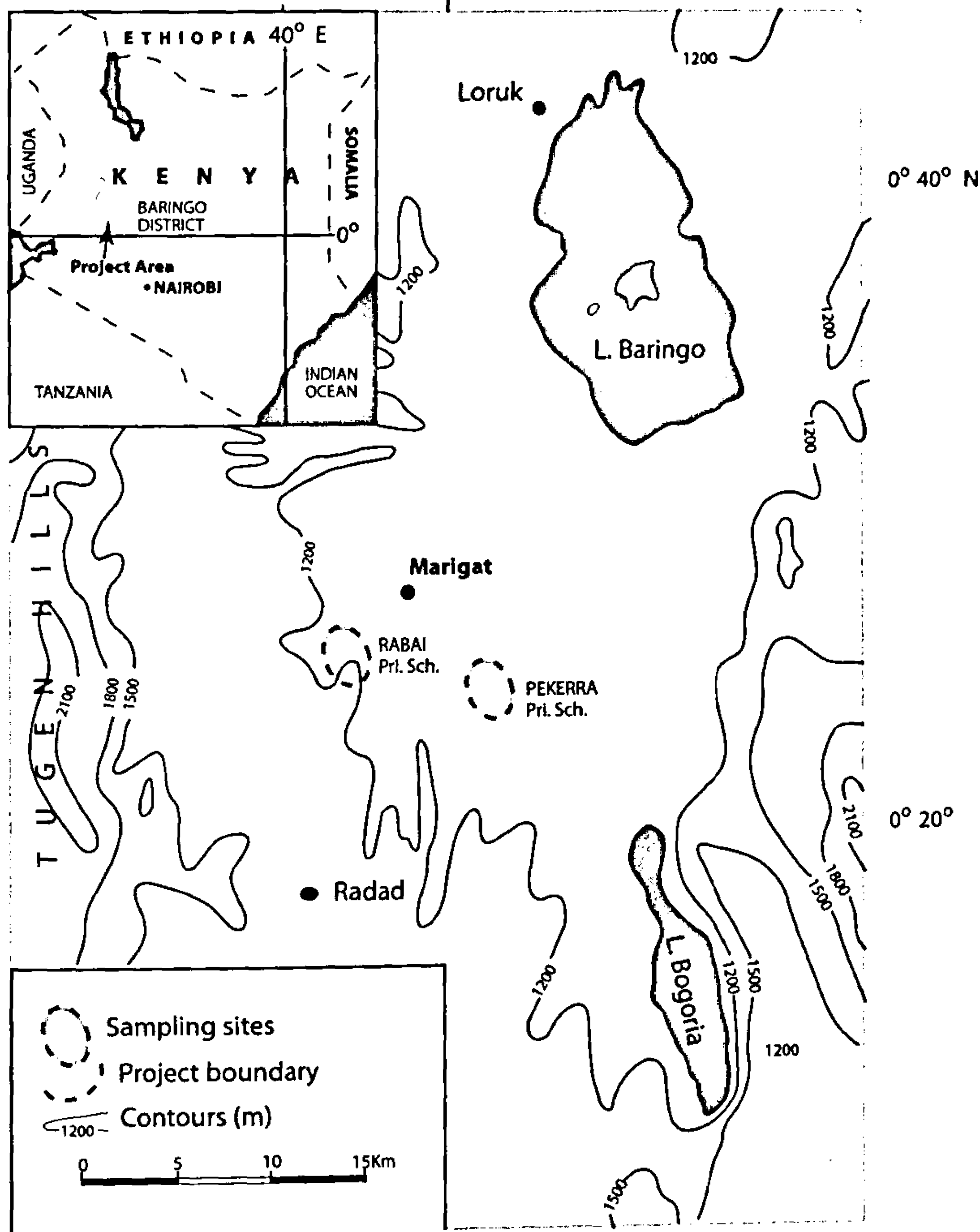


Figure 7: Map of Kenya showing the study sites in Baringo County

3.2 Study Design

The study was made up of two phases namely: Laboratory and Field- based studies. The laboratory-based studies involved isolation and identification of entomopathogenic fungi from soil samples collected from leishmaniasis endemic areas of Baringo County and ICIPE Germplasm Centre. The most pathogenic isolates to sand fly species of *Phlebotomus duboscqi* were selected for field trials. Isolation, culturing, identification, pathogenicity and virulence testing of the isolates were carried out in International Centre of Insect Physiology and Ecology (ICIPE) while the rearing of sand flies for the lab-based studies was done at the Entomology Department, Leishmaniasis laboratory of Kenya Medical Research Institute (KEMRI).

The field based studies were conducted in Baringo County. Inactive termite mounds in Rabai study site were selected for the experiments. The termite mounds were grouped into two categories: experimental and control. The experimental group was treated with conidia while the control group received no conidia. Sand fly catches were compared between the two groups of termite mounds. Survival curves were constructed to show the mortalities from the two groups.

The study was conducted initially under laboratory conditions, and subject to the outcome of lab-based studies, field studies at leishmaniasis endemic areas in Baringo County were undertaken. Peridomestic breeding sites (termite mounds and animal burrows) that are major habitats of sand fly vectors in arid and semi-arid areas of Kenya (Robert *et al.*, 1997; Ngumbi *et al.*, 1998) were therefore targeted for the validation of the proposed strategy. A 1 x 2 kilometer area was marked out and split into two blocks (A

and B). Twenty sand fly-positive termite mounds (preferably dormant or inactive mounds) were selected for use as control and experimental groups. Site A was used as control while site B was experimental and treated with the fungus.

3.3 Sand fly trapping and processing

Sand flies were trapped for four consecutive nights with Centres for Disease Control (CDC) and prevention light trap (J.W. Hock Ltd, Gainesville, FL, U.S.A.) and were operated by 6-volt rechargeable batteries from 1800 to 0600 hours. A collection net was tied at one end of the trap and suspended close to the mouth of the termite mound (Fig. 12C). Every morning the sand flies were aspirated using mouth aspirators, counted, recorded and put in vials for transportation to KEMRI, Nairobi for mounting and identification. Sand flies were mounted on microslides with gum chloral. Identification was done with the help of sand fly identification keys by Abonnec (1965).

The diversity and sandfly population densities in the termite mounds were assessed by trapping sandflies at the beginning of this study to establish the baseline data. The two study sites selected were Rabai and Perkerra, where the vectors of visceral and cutaneous leishmaniasis were trapped. *Phlebotomus martini* which transmits *Leishmania donovani*, the causative agent for visceral leishmaniasis and *P.duboscqi* which transmits *L. major* which causes cutaneous leishmaniasis were trapped. The two sand fly species of medical importance have apparently adapted themselves to the semi-arid climatic conditions quite well and are perennial species. While *P. martini* prefers living in the termite mounds, *P. duboscqi* prefers living in the animal burrows (Ngumbi *et al.*, 1998). These habitats are

usually ideal for the survival of the sand fly species, because they offer high humidity and are relatively cool inside even when the surrounding areas outside may be hot and harsh to the sand flies. The study site with more *P. martini* species was selected for the field studies.

3.3.1 Mass rearing of sand flies

The identity of the insect vector used in this study was *Phlebotomus duboscqi* Neveu-Lemaire, a vector of *Leishmania major* in Kenya. The sand flies used in the laboratory studies are laboratory stocks which were collected from Baringo County in 1983. The rearing techniques developed for 3 previously colonized species of phlebotomine sand flies were also suitable for maintaining this species. *Phlebotomus duboscqi* were comparatively easy to propagate in colony because, 75% of the females survived oviposition, took additional blood meals, laid 2nd and in a few cases 3rd egg batches, which substantially increased the productivity of each generation (Beach *et al.*, 1986).

The colony originated from animal burrows near Marigat town, Baringo County, Rift Valley Province, Kenya (Beach *et al.*, 1986). The following procedure was used to maintain and rear the colony in our insectary at KEMRI. Adult sand flies were released into (4 x 4 x 5) cm Plexiglass cage with a plaster of paris-coated floor and back wall to enhance fly visibility. A cloth sleeve, attached to the cage entrance, permited access to the inside while denying already caged flies an escape route. Included in the cage were fresh apple slices to provide the flies with sucrose and, as blood meal source, a hamster confined in a small wire mesh cylinder or anaesthetized with sagatal injection was offered to the female sand flies to feed on. The apples slices awere changed daily. Blood fed females were individually isolated in 7 dram vials or placed in groups of 50 flies in

larger vials which were partially filled with plaster of paris and fitted with screen tops. The plaster of paris substrate was moistened with distilled water to stimulate oviposition, and a small bead(s) of 30% honey solution, a sucrose source for the enclosed female(s) was suspended from the screen top. The top was cleaned with wet cotton wool or gauze to get rid of the old bead(s) and new ones placed on the screen. This was done for a period of 10 days during which time the females would have finished laying their eggs. Gravid females that survived long enough to lay their eggs died shortly afterwards or took a second blood meal if a hamster was availed to them. Eggs embryonated and hatched in the vials where they were laid. Larval development occurred in this multipurpose chamber, the only change being that the screen cap used during oviposition, was replaced by one of solid plastic with small air holes, a modification to prevent larval escape. Newly hatched larvae commenced feeding immediately. Therefore, a small amount of larval food was placed with the eggs as embryogenesis neared completion. Larvae were checked daily and additional food added as needed. The vials were uncapped after pupation and the adult flies released inside a Plexiglass cage, previously described, where the adult flies carried on with the entire cycle again. Rearing of our *P. duboscqi* colony was done in our insectary at KEMRI at 26 ^{0}C, 80-90% RH. Glass windows in the insectary permitted exposure of the colony to the naturally occurring photoperiod. The mean development times of various stages in the life cycle were: oogenesis (blood meal-oviposition) 7 days, embryogenesis (oviposition-egg hatching) 9 days, immature stages (1st instar – adult emergence) 55 days. Adults began feeding as early as 4 days post emergence. Total generation time which was computed as the mean

elapsed time between a female taking blood meal and the first of her offspring taking blood meal was approximately 75 days (Beach *et al.*, 1982).

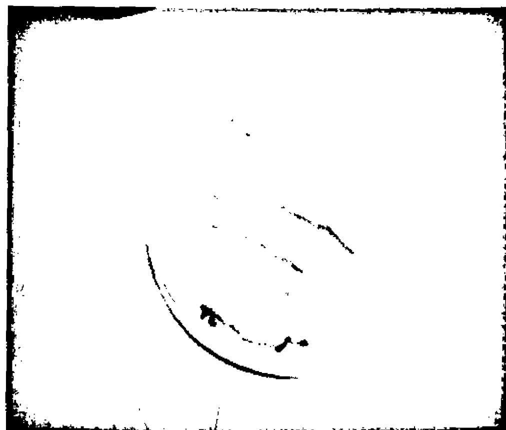
During the experiments, sand fly numbers were raised from approximately 1,000 females per week to over 5,000 females per week to cater for the increased demand for experimental flies.

3.4 Entomopathogenic fungi: *M. anisopliae* and *B. bassiana*

3.4.1 Isolates and culture maintenance

All the fungal isolates in the study (16 *Metarhizium anisopliae* and 3 *Beauveria bassiana*) were obtained from ICIPE's Germplasm Centre, except one isolate that was isolated from soil samples collected from Baringo County and given letter "O" (Table 2). Isolates were cultured on Sabourand dextrose agar (SDA) in Petri dishes and incubated at room temperature (22-28 ^{0}C). Conidia were harvested by scrapping the surface of three week old cultures. The viability of conidia was determined by spread plating of 0.1 ml of conidial suspension (titrated to 3 x 10^6 conidia ml^{-1}) on SDA plates. Sterile microscope cover slips were placed on each plate. The plates were incubated at 24-29^{0}C and examined after 12-18 hours (Fig.8). Immature cultures of *Metarhizium anisopliae* are whitish in colour and this colur changes to green as they get older and mature (Fig. 8). Percent germination was determined by counting approximately 100 spores for each plate at x40 magnification. Percent germination = (total germinated/ total number of spores counted x 100. Each plate served as a replicate with four replications per isolate. Conidia were produced on long white rice substrate in plastic bottles (130 x 130 x 230 mm). The

substrate was autoclaved for one hour at 121°C and inoculated with 3-day old culture of blastospores. The substrate was then incubated for 21 days under ambient conditions (20-26 °C, 40-70% RH) and then allowed to dry for 5 days at room temperature. Conidia were harvested by sifting the substrate through a sieve (295 µm mesh size) and were stored at 4-6 °C before being used in the experiments.



Immature culture (white) **Mature culture (green)**

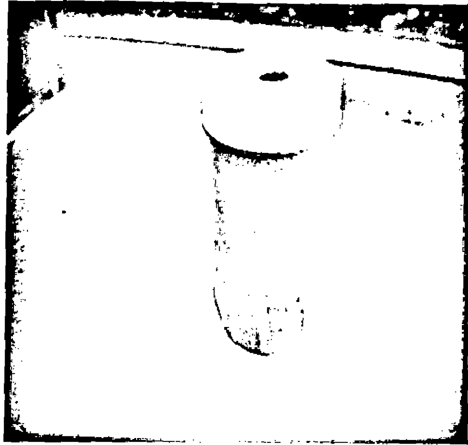
Figure 8: ***Metarhizium anisopliae*** **cultures**

3.4.2 Inoculation of adult sand flies with entomopathogenic fungi

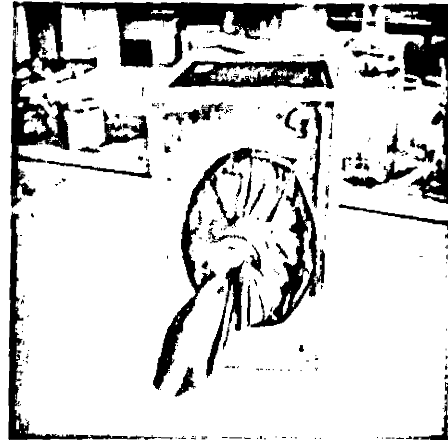
Adult sand flies were contaminated using the technique described by Maniania (1994) and Migiro *et al.*, (2010). Briefly, flies were exposed to 0.3 g of dry conidia evenly spread on a cotton velvet cloth covering the inner side of a cylindrical plastic tube (95 x 48 mm diam) (Fig. 9). In all, 25 sand flies were transferred to the tube and allowed to walk on the velvet for one minute after which 20 sand flies were transferred to clean ventilated Plexiglass cages (150 x 150 x 200 mm) (Fig. 9). The sand flies were maintained on apple slices which supplied them with sugar as source of energy. Control

sand flies were exposed to fungus-free velvet material before being transferred to similar ventilated Plexiglass cages. Each treatment consisted of 20 sand flies per replicate with four replications per isolate. Test sand flies were maintained at ambient conditions (25 ± 2 ^{0}C and 60-70 % RH).

The remaining 5 sand flies were used to estimate the number of conidia picked up by a single sand fly in each treatment. Insects were transferred individually to 2 ml cryogenic tubes containing 1 ml of sterile distilled water plus 0.05 % Triton X-100. The tube was vortexed for 2-3 min to dislodge conidia from the insect and the concentration of conidia was determined using a haemocytometer. Mortality was recorded daily until all the sand flies died. Dead sand flies were surface-sterilized in 70% alcohol followed by 3 rinses in sterile distilled water and transferred to Petri dishes lined with damp sterilized filter paper to promote fungal growth on the surface of the cadaver. Mycosis was confirmed by microscopic examination. Isolates that caused mortalities between 97.5 and 100 % were considered to be highly pathogenic and can be used for field experiments to control sand flies.



A-Contamination cylinder



B-Observation cage

Figure 9: Infection of adult sand flies

Figure 9 above shows the adult sand fly contamination cylinder in which 25 sand flies were put and left to walk on velvet-lined cylinder for 3 minutes, after which they were transferred into the observation cage for daily observation and maintenance until all the flies died.

3.4.3 Isolation and testing of the most virulent strains of entomopathogenic fungi (EPF) from soil samples and ICIPE germplasm

Soil samples for the isolation of entomopathogenic fungi were collected from Baringo County. Due to time factor, a decision was made to use fungal isolates for screening from ICIPE Arthropod Germplasm Centre. Only one isolate was cultured from the soil samples collected from Baringo County. Nineteen isolates of *Metarhizium anisopliae* and *Beauveria bassiana* were tested against adult sand flies (*Phlebotomus duboscqi*). Insects were contaminated following the technique described by Dimbi *et al.*, (2003) for fruit flies. In control treatments flies were exposed to fungus-free velvet. The adult sand flies

were kept for about 12 days within which time most of them would be dead. The environmental conditions were maintained at a relative humidity of 80 % and room temperature of 26^{0}C. Data were analysed using SAS.

The mortality rates were corrected from experimental to natural mortality using Abbott's formula (1925), a factor that makes them better and more reflective of the causes of deaths. The data on percentage mortality were arc sin-transformed before submitting to analysis of variance. From the laboratory results, six isolates qualified for use in the field trials as shown on Chapter 4, Table 2.

3.4.4 Testing the pathogenicity of the EPF isolates in infecting and killing target adult sand fly species and the larval stages under laboratory conditions

3.4.4.1 Laboratory evaluation of EPF pathogenicity in adult sand flies:

Nineteen isolates of *M. anisopliae* and *B. bassiana* were isolated and tested against female adult sand flies (*P. duboscqi*). The sand flies were infected using the technique described by Dimbi *et al.*, (2003) for the fruit flies. Briefly, 0.3 grams (3 x 10^{7}) of dry conidia were spread evenly onto velvet material lining the inside of a cylindrical plastic tube (95 x 48 mm) (Fig. 9). Twenty five sand flies were transferred to the cylindrical tube, and allowed to walk on the velvet for 3 minutes after which 20 sand flies were transferred to clean ventilated Plexiglas cages (150 x 150 x 200 mm) (Fig. 9). The remaining 5 flies were used to estimate the number of conidia picked up by a single fly in each treatment. To do so, insects were transferred individually to 2-ml cryogenic tubes containing 1 ml of sterile distilled water plus 0.05 % Triton X -100, vortexed for 2-3

minutes to dislodge conidia, and the concentration of conidia determined using a haemocytometer (Fig. 10B). In the control treatments flies were exposed to fungus-free velvet (Fig. 10A). All sand flies were maintained on slices of apple as a sugar source for energy and at a temperature of 26 ± 2^0C, 60-70% RH and 12L: 12D photoperiod, unless stated otherwise. Sand flies were observed daily for mortality over a period of 12 days. The experiment was replicated 6 times.

Mortality data were corrected for natural mortality in the controls (Abbott 1925) and arcsine-transformed to normalize the data before analysis of variance (ANOVA). Means were separated by Student-Newman-Keuls test at $P = 0.05$. Lethal time to 50% mortality (LT_{50}) and the lethal time to 90% mortality (LT_{90}) values were determined using probit analysis. All analyses were carried out using the SAS statistical package (SAS Institute).

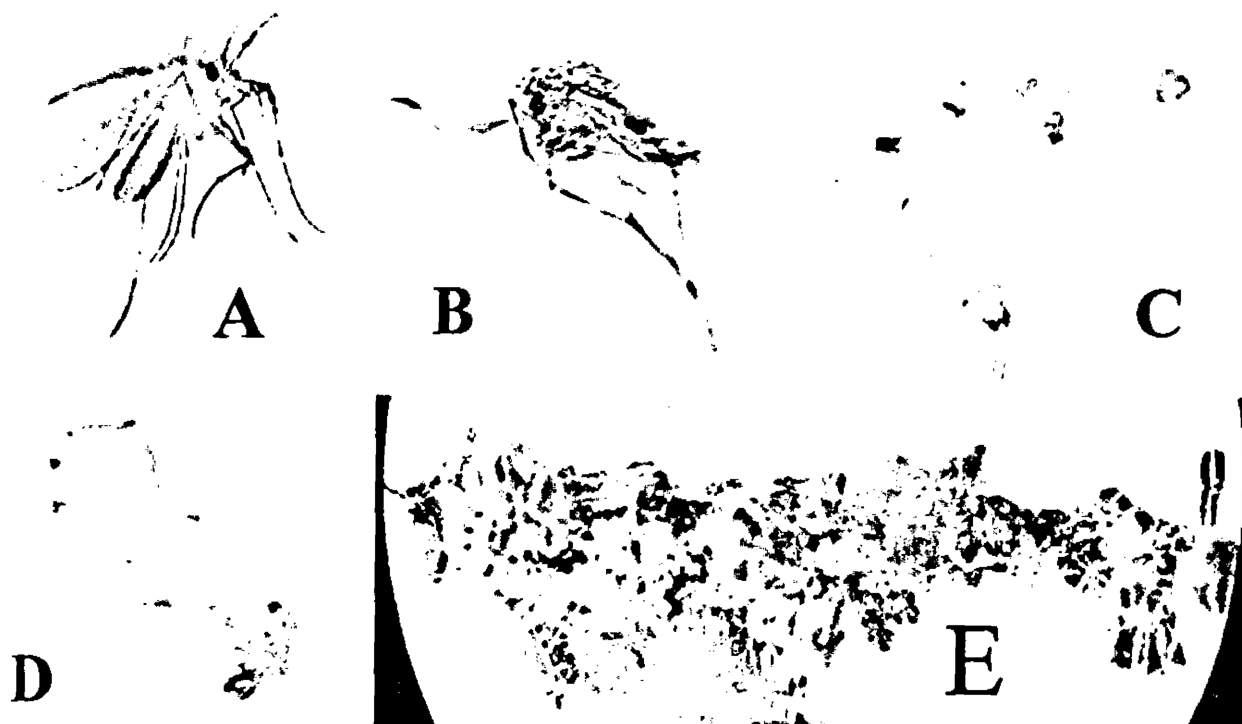


Figure 10: Pathogenicity of *M. anisopliae* in sand fly. A) Control sand fly; B) Cadaver of sand fly from *M. anisopliae* treated termite mound showing mycosis; C) 4th instar larva of *P. duboscqi*; D) Pupa of *P. duboscqi*; E) Cadaver of *P. duboscqi* 4th instar larva showing *M. anisopliae* mycosis, under a dissecting microscope at x40 magnification

3.4.4.2 Laboratory evaluation of EPF pathogenicity on 4th instar larvae and pupae: Selected EPF isolates (C, H, M, O, P and S) Table 2, were tested against 10 laboratory reared *P. duboscqi* 4th instar larvae each for preliminary evaluation of pathogenicity (Figs. 10C & 10D). Larvae and pupae were separately placed in groups of 10 per Petri dish and sprayed with 1.5ml of 1 x 10^8 conidia suspended in 0.2% sterile Triton X-100 solution of each EPF isolate (Fig. 11). For controls, larvae and pupae were sprayed with 0.2% sterile Triton X-100 solution without EPF conidia. Each isolate was replicated six times. The larvae were maintained at 26 ± 2°C, 60-70% RH and 12L: 12D photoperiod on a fungus rich mixture of decomposed commercial rabbit food and droppings prepared at KEMRI as described by Beach *et al.*, (1986). Larvae and pupae were observed daily

over a minimum period of 14 days for mortality and adult emergence (Table 3). Percentage mortality in the treatments was corrected for natural mortality in the controls (Abbott 1925). Mycosis on retrieved larvae cadavers (Fig. 10E) was determined by surface sterilizing of individual larva using 2% hypochlorite solution followed by incubating on damp, sterile filter paper at room temperature for 1 week. Development of hyphae and conidia on the cadaver was taken as confirmation of EPF infection.

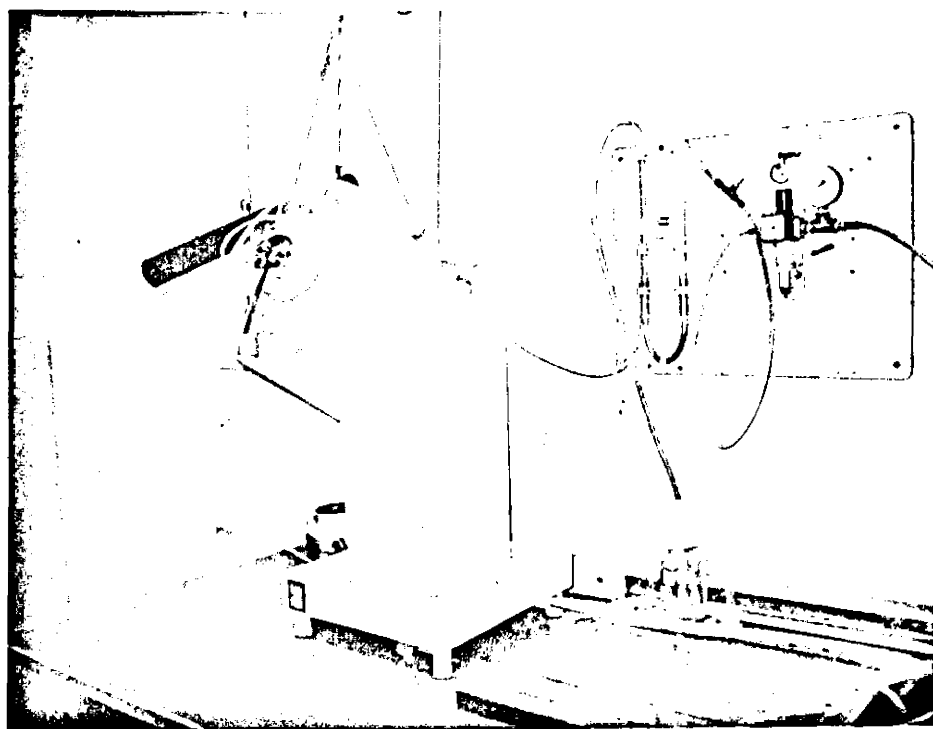


Figure 11: Contamination chamber of larvae and pupae

3.4.5 CDC light trap usage and operation

CDC light traps were used to trap sand flies originating from the termite mounds. The traps were suspended close to the mouth of the termite mound (approximately 50cm) above the mouth. The traps were turned on at 1800 hours and collected at 0600 hours in the following morning. Collection nets containing the sand flies were carefully removed from the CDC light traps and the sand flies aspirated with mouth aspirators. They were then put in small vials.

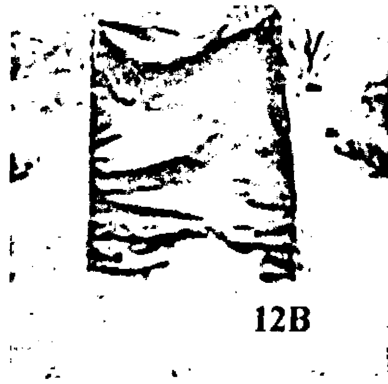
3.4.6 Testing of selected fungal pathogens against field populations of sand flies

3.4.6.1 Shielded and unshielded sand fly trapping

Sand fly trapping was carried out in two sites (A and B) at Rabai with equal numbers of termite mounds. The distance separating these two sites (A and B) was approximately 400 meters. Ten CDC light traps were set at the selected 10 termite mounds each night for four nights in which two nights were used to trap sand flies in shielded termite mounds while the other two nights were used to trap sand flies from the unshielded termite mounds. This arrangement was designed to show whether there was any difference between shielded and unshielded sand fly catches. In shielding the termite mounds, we were avoiding sand flies that were not coming from the treated termite mound. Sand flies trapped inside the shielded termite mounds had a higher chance of getting infected with the fungus than those caught with no shielding. Our interest in this experiment was to show shielding keeps out flies that are not from the treated termite mound. Figures 12A, 12B and 12C demonstrate how the experiment was carried out in the termite mounds. (Fig. 12A) shows an inactive termite mound which was unshielded

during the unshielded trapping. (Fig. 12B) shows a sheet of black polythene paper shielding a termite mound during the shielded trapping, while (Fig. 12C) shows a CDC light trap placed near the mouth of a ventilation shaft during sand fly trapping at night. The light trap was operated by a 6-volt rechargeable battery and was hang on a 2 metre metallic rod which was inserted into the ground to position the trap near the mouth of the termite mound. This arrangement was done before the start of the small-scale field evaluation of entomopathogenic fungi.

Unshielded termite mound



Shielded termite mound

CDC light trap collecting flies at night

Legend: 12A-Unshielded termite mound

12B-Shielded termite mound

12C-CDC light trap collecting fies

Figure 12: Sand fly trapping arrangement

3.4.6.2 Small-scale field testing of entomopathogenic fungi (EPF)

Mass production of the selected fungi isolate (H) for field trials was done by inoculating Saboraunds dextrose agarose liquid cultures into rice as a solid substrate for mass production. The study site selection was based on the past records of vector surveillance, which indicated that Baringo County (Rabai) offered the best opportunities for both vectors of VL and CL, *P. martini and P. duboscqi* respectively (Ngumbi *et al.*, 1998). Selection of sites in the field was done randomnly. The only factor considered during the selection of sites (A) and (B) was that the control site (A) should be on the windward side to avoid fungi sprayed at the treatment site (B) from getting blown to the control site (A).

The termite mounds were also randomnly picked to avoid any bias in the study. Twenty termite mounds around this locality were selected for inclusion in the study and divided into two groups of control and EPF treatment. A baseline of the sand fly population density was established by trapping for two consecutive nights using CDC light traps set at the main ventilation shaft of each termite mound (Fig. 12C). In order to ensure that the sand flies trapped in a particular termite mound originated from the termite mounds only, a 5 feet high barrier, made of a black polythene sheet measuring approx. 2 x 1.5 meters was erected around each termite mound (Fig. 12B). Additionally, this barrier acted as a wind shield that stabilized sand fly collections during the trial period, this is important because sand flies are weak fliers and trapping yields are known to fluctuate due to strong wind. The control group consisting of termite mounds which was assigned identification numbers 1-10, was located near human settlements. The experimental group consisting of termite mounds assigned numbers 13-21, 25 were scattered away from human settlements. In the latter group, 5 grams of dry conidia powder was delivered into the

inner recesses of the main ventilation shaft using a modified foot pump for maximum dispersal of conidia by diffusion. A follow-up treatment of 15 grams of conidia per termite mound was applied on the 2nd week post initial treatment after realizing that the amount of surface area sprayed with 5g of conidia was inadequately treated. For the control group, no EPF was applied. Sand flies from both control and treatment groups were trapped from 1800 hrs to 0600 hrs for at least two consecutive nights on weeks 2, 5, 9 and 13 post initial treatment and transported back to the laboratory where they were maintained on apple slices as a sugar source and observed for survival rates and for evidence of infection with fungi (mycosis). The latter was determined by surface sterilizing of individual sand fly using 2% hypochlorite solution followed by incubating on damp, sterile filter paper at room temperature for one week. Development of hyphae and conidia on the cadaver was taken as confirmation of EPF infection.

3.5 Data Analyses

Data collected from the experiments were subjected to analysis using Kaplan-Meier Kruskal-Wallis (KW) ANOVA (XLSTAT 2009.2.01 software for Windows), and Student's *t*-tests and Chi-square tests using STATISTIX 7.0 for Windows. Survival and mortality curves were analysed using Abbott's formula (1925) and ANOVA to compare fungal treated versus control groups. The mean survival time and lethal time required to reach 50% mortality (LT_{50}) per replicate was obtained from Kaplan-Meier analysis. The mean survival times of replicates within each trial were compared using 2 sample t-tests or One-Way ANOVA to measure variation between replicates. Replicates with no significant differences between them were pooled while outlier replicates showing significant differences were eliminated from the analysis. The LT_{50} -values of treated

versus control were compared using Student's *t*-tests. The means of each of the fungus treated *P. duboscqi* sand flies were compared using one-way ANOVA. The effects of exposure time on mortality or sand fly survival were analyzed using the Kruskal-Wallis ANOVA. Sporulation tests were also assessed by counting the number of spores in a given field under the microscope and expressing that number as a percentage. Mortality and sporulation data were corrected (Abbott, 1925) and compared using the Chi-square tests.

CHAPTER FOUR

4.0 Results

4.1 Isolation and identification of the most virulent isolates from soil samples and ICIPE germplasm.

Nineteeen isolates were identified and tested through laboratory assays for this study comprising: 16 *M. anisopliae* and 3 *B. bassiana.* The data on percentage mortality were arc sin-transformed before statistical analysis. The best isolate was C-10 with a mortality rate of 100% and an LT_{50} of 3 days and LT_{90} of 5.3 days. The mortality rates have been given letters like 'a', 'ab', 'abc,' etc to show some statistical closeness and significance. Mortality in the controls was approximately 16.8 ±1.7 %. All the isolates were found to be pathogenic to *P. duboscqi.* This was the only species existing as a colony in KEMRI, which was used to act as a representative of other sand fly species in the study. *Phlebotomus martini* which is the vector of the fatal VL in Kenya lives predominantly in the termite mounds which were our selected sites of attack with EPF. Mortality ranged between 76.8 and 100% in all the fungal isolates tested. The lethal time taken to 50% (LT_{50}) and 90% (LT_{90}) mortality ranged from 3.0–7.8 days and from 5.3–16.2 days, respectively. The virulent isolates (six in number and coded letters: C, H, I, M, O & P) causing mortalities of 97.5–100% were selected for further studies including; determination of pathogenicity against larvae and adult sand fly populations in the field trials in phase II. Due to logistical reasons concerning money and time, only one of the six isolates was picked for the field trials. This did not mean the other isolates were not good enough, but there was no time to test more than one in the field. The selection of the six isolates was purely based on their performance in terms of pathogenicity on *P.*

duboscqi. Laboratory results showing pathogenicity against adult *P. duboscqi* are shown in Table 2.

Table 2: Pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* isolates in adult sand flies

Fungal Isolate	% Mortality (± S.E)	LT_{50} (days) (95% Fiducial limits)	LT_{90} (days) (95% Fiducial limits)	Slope	χ^2	Probability
Control	16.8 ± 1.7	N/A	N/A	N/A	N/A	N/A
M. anisopliae						
A-icipe41	95.0 ± 4.1abcd	4.5 (4.3-4.6)	10.8 (10.4-11.3)	0.11	1103.1	<0.0001
B-gategi	89.2 ± 5.4cde	5.5 (5.3-5.7)	16.2 (15.3-17.3)	0.11	1080	<0.0001
C[*¶-mer10]	100.0 ± 0.0a	3.0 (3.0-3.1)	5.3 (5.2-5.5)	0.13	972.6	<0.0001
D-emb27	96.7 ± 2.1abcd	4.2 (4.1-4.3)	9.1 (8.8-9.4)	0.12	1194	<0.0001
E-icipe18	92.5 ± 1.7abcd	6.2 (6.1-6.4)	12.7 (12.3-13.3)	0.15	1319.7	<0.0001
F-icipe21	90.8 ± 2.0bcde	4.2 (4.1-4.3)	11.2 (10.7-11.7)	0.10	1039.6	<0.0001
G-icipe60	91.7 ± 2.1abcde	4.8 (4.7-4.9)	10.1 (9.7-10.5)	0.13	1269	<0.0001
H[*¶-icipe30]	100.0 ± 0.0a	4.1 (4.0-4.2)	7.7 (7.5-8.0)	0.14	1253.5	<0.0001
I[*¶-KR16]	100.0 ± 0.0a	5.0 (4.9-5.0)	8.7 (8.4-8.9)	0.17	1392.1	<0.0001
J-sudan4	80.2 ± 3.5e	6.8 (7.0-7.3)	15.7 (16.0-18.1)	0.16	1172.2	<0.0001
K-caterpillar	76.8 ± 4.5f	6.0 (6.-6.8)	14.8 (14.9-16.3)	0.11	1027.3	<0.0001
M[*¶-icipe62]	98.3 ± 1.1ab	4.5 (4.4-4.6)	9.6 (9.3-10.0)	0.13	1248.6	<0.0001
N-icipe20	91.7 ± 3.1abcde	3.7 (3.6-3.9)	10.2 (9.7-10.7)	0.10	943.4	<0.0001
O[*¶-Sfly18]	97.5 ± 1.7abc	3.7 (3.6-3.8)	8.3 (8.0-8.6)	0.11	1067.3	<0.0001
P[*¶-emb26]	100.0 ± 0.0a	5.0 (4.4-4.6)	7.6 (7.4-7.8)	0.17	1347.4	<0.0001
R-Ri/Ra	88.3 ± 3.8de	7.6 (7.4-7.7)	15.5 (14.8-16.4)	0.18	1132	<0.0001
B. bassiana						
L-mbita	90.0 ± 3.7bcde	5.0 (4.9-5.1)	11.6 (11.1-12.1)	0.12	1218.2	<0.0001
Q-gpk	89.4 ± 3.8de	5.7 (5.5-5.8)	13.3 (13.9-15.4)	0.12	1192.2	<0.0001
S[¶-kericho]	96.7 ± 1.7abcd	5.6 (5.4-5.7)	13.2 (12.6-13.9)	0.12	1226.8	<0.0001

Means followed by the same letter are not significantly different (Student-Newman-Keuls test, P>0.05)
* indicates isolates (causing mortalities of 97.5–100%) selected for field trials; ¶ indicates selected for larvae bioassays

NB: All the isolates used in the experiments were acquired in Kenya except one from Sudan.

4.2 Data Analysis

The numbers of dead sand flies per treatment replicate were recorded daily and the mean cumulative proportion surviving each day in the 6 replicates was calculated. Standard errors were also produced across all replicates. Assuming there were no variations amongst and between treatments (using one way ANOVA), the mean cumulative proportion surviving each day and the standard errors were calculated across all the replicates. Graphs of the percentage cumulative sand fly survival against time (days) after exposure were constructed using Microsoft™ Excel.

4.3 Laboratory evaluation of EPF pathogenicity in 4th instar larvae and pupae

Control of emergence due to infection with entomopathogenic fungi ranged from (32-60 %) for all the six selected isolates as demonstrated in the 4th instar larvae assay results. This is indicative of EPF's potential as a control agent targeting sand fly breeding sites (Table 3). Computation of the performance of the fungal isolates was arrived at through the following formular: 100(*Mt-Mc/Mc*) in which '*Mt*' stands for mortality due to treatment and '*Mc*' is for mortality in the control group.

The selection of 4th instar larvae was based on their bigger size than the other three instar larval stages which are smaller in sizes and are difficult to work with, and more so when larval food is added to the petri dishes. Percentage mortality in the treatments was corrected for natural mortality in the controls (Abbott 1925).

There was a 98-100% pupae hatching to adult flies observed in the controls and all treatments, indicative of poor EPF pathogenicity (Fig.10D). However, the duration of sand fly development in the pupal stage is relatively short (approx. 2 days) with shedding of the hardened cuticle during adult emergence likely to occur before successful penetration and establishment of EPF infection in the sand fly.

Table 3: Pathogenicity (effectiveness) of *M. anisopliae* and *B. bassiana* isolates in sand fly larvae control

Fungal Isolate	% Emergence	% Mortality (n-emergence)
Control	77	23
M. anisopliae		
C	55	45
H*	40	60
M	47	53
O	65	35
P	68	32
B. bassiana		
S	63	37

*Indicates selected for preliminary field evaluation

4.4 Species composition at Rabai and Perkerra

Seven sand fly species were trapped in two days to help select the best site to carry out the entomopathogenic fungi treatment. Two *Phlebotomus* species of medical importance (*P. martini* and *P. duboscqi*) were trapped at the two sites. *P. martini* were represented by 40 (57.1 %) at Rabai and at Perkerra by 6 (5.3 %). *P. duboscqi* were represented by 1 (1.4 %) at Rabai and 7 (6.1 %) at Perkerra Table 4. The others were *Sergentomyia* species that do not transmit any parasites harmful to humans. The results revealed Rabai as the best site to conduct the sand fly control experiment with entomopathogenic fungi.

Table 4: Relative abundance of sand fly species at Rabai and Perkerra

	Species	Rabai F	M	T	%	Perkerra F	M	T	%
1.	*P. martini*	22	18	40	(57.1)	4	2	6	(5.3)
2.	*P. duboscqi*	1	0	1	(1.4)	5	2	7	(6.1)
3.	*S. schwetzi*	20	1	21	(30.0)	84	7	91	(79.8)
4.	*S.squamipleuris*	2	1	3	(4.3)	3	1	4	(3.5)
5.	*S. bedfordi*	2	0	2	(2.9)	3	2	5	(4.4)
6.	*S. clydei*	2	0	2	(2.9)	0	0	0	(0)
7.	*S. antenata*	1	0	1	(1.4)	1	0	1	(0.9)
	Totals	50	20	70	(100)	100	14	114	(100)

Legend: F = Females, M =Males, T = Total, % = Percentage in parenthesis.

4.5 Determination of numbers of sand flies from shielded and unshielded termite mounds

To determine whether an ordinary black plastic paper sheet could be effectively used as a "shield", we constructed structures around the termite mounds. The four days trapping of sand flies at site A produced a mean collection of 44.0 sand flies from unshielded while the shielded gave a mean collection of 9.9 sand flies. Site B produced a mean collection of 47.0 sand flies from the unshielded termite mounds while the shielded had 8.0 sand flies (Fig. 13). Shielding was done as shown on (Fig. 12B). A tentative analysis of sand fly catches from the two groups (shielded and unshielded nights) returned a *p*-value less than 0.05 indicating that there was a significant difference in sand fly densities. A light drizzle and winds in the evening of one of the trapping days did not seem to have

affected the outcome of these analyses. Sampling sand flies from a particular mound was therefore shown to be possible through the use of plastic paper sheets as light shields.

The results of trapping sand flies in shielded and un-shielded termite mounds are shown on (Fig. 13).

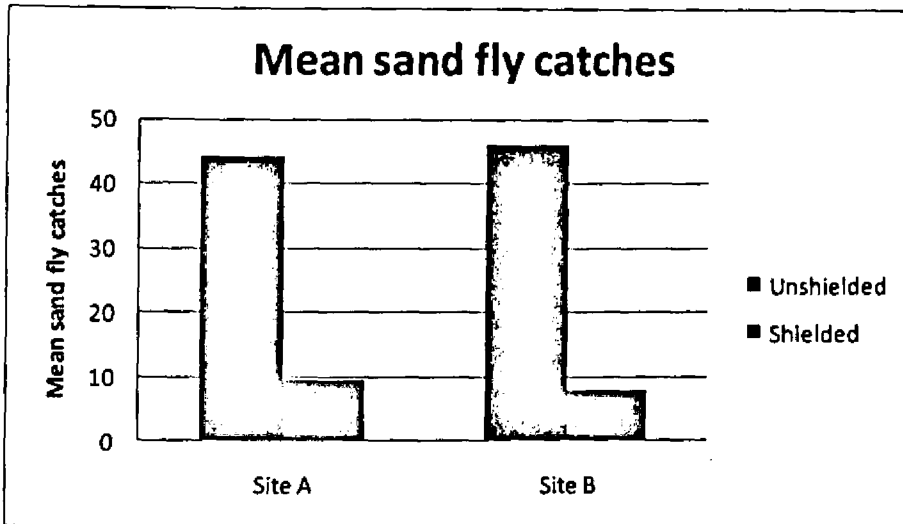


Figure 13: Mean sand fly catches from shielded and unshielded termite mounds

4.6 Evaluation of entomopathogenic fungi (EPF) in the field

Sand fly population densities at the two sites (A & B) were very different in terms of numbers (Fig.14). Site A had fewer sand fly catches per trap/ night than site B. Because of this difference in numbers, it was not possible and logical to compare the two sites as would have been the case in a laboratory setting. However, the two sites were kept throughout the study to give a reflection of the environmental changes which occurred during the study period. These changes can be seen on the two graph lines showing control and EPF treatment. The discovery of uncomparable sand fly catches from the control site A and EPF treatment site B, resulted into use of baseline sand fly catches

before and after treatment for assessment of the success in controlling sand fly numbers in the field.

The measurement of efficacy of *M. anisopliae* was based on percentage reduction of the sand fly population density, which was calculated by comparison of sand fly abundance per termite mound in the treatment group versus their baseline abundance. Percentage of reduction was calculated using the following modification of Abbott's formula: % reduction = $100(Y_b - Y_t)/Y_b$, where Y_b and Y_t is the mean number of sand flies collected for baseline and post treatment, respectively. Differences between sand fly populations in the individual termite mounds before and after treatment EPF were also compared by Mann-Whitney test using SPSS statistical software, version 9.0.

There was an increase in the sand fly population of *M. anisopliae* treated termite mounds from week 0-5 followed by a sharp decline in week 9 post initial treatment. This conformed to the overall trend in the sand fly collections in the control group (Fig. 14).

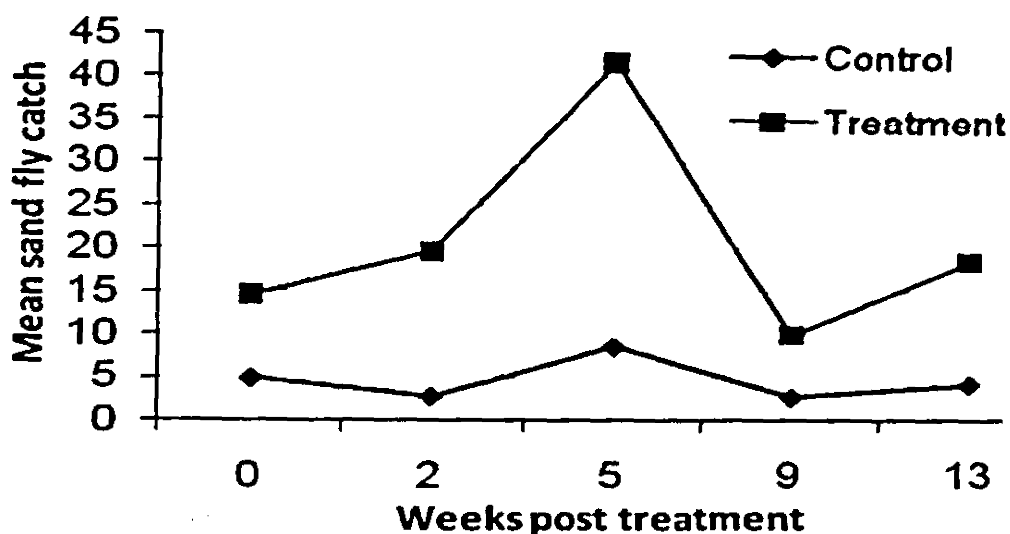


Figure 14: Mean sand fly collections in control and *M. anisopliae* treated termite mounds over the duration of the field trial.

4.7 Reduction of sand fly densities in the *M. anisopliae* treated termite mounds

Ten (10) termite mounds which were marked with numbers: 13, 14, 15, 16, 17, 18, 19, 20, 21 and 25 were treated with *M. anisopliae* fungus in the treatment site. Sand fly trapping was carried out before the intervention with *M. anisopliae* for each individual termite mound to establish baseline data. After intervention, sand fly catches were carried out after every two weeks for a period of three months. Percentage reduction of sand fly population densities were calculated and recorded for each termite mound. Successful control of sand fly populations using *M. anisopliae* was demonstrated in week 9 with significant difference between control and treatment groups, Mann-Whitney test $U = 18.0$, $z = -2.425$, $p = 0.015$. 6 out of 10 of the treated termite mounds demonstrated a

percentage reduction of between 4.63-71.53% (Fig. 15). No reduction was observed in 1 out of 10 (termite mound 21) which did not register a drop in numbers of flies caught, probably due to poor spraying of the inner recesses of the termite mound, and failure in 2 of the treated termite mounds (13 and 17) whose batteries and traps failed to run and operate a number nights. Results for termite mound number 14 are unavailable due to loss of the trapping equipment through stealing.

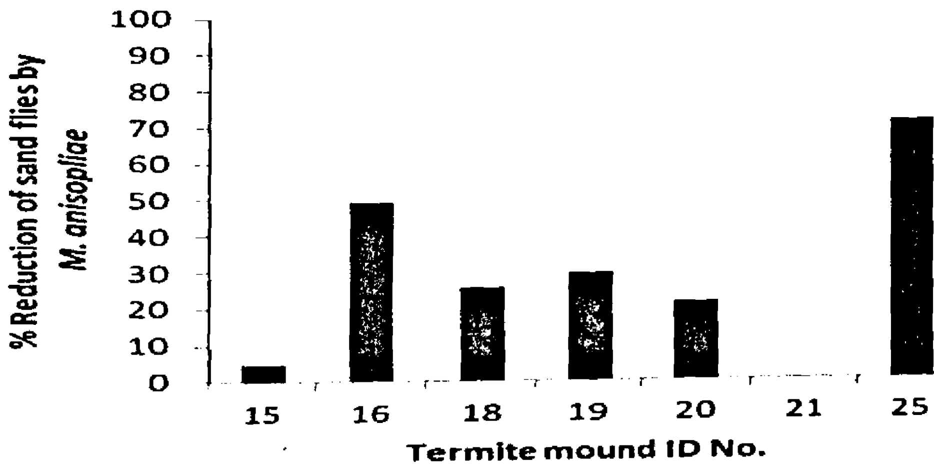


Figure 15: Percentage reduction of sand fly population density in *M. anisopliae* treated termite mounds at 9 Weeks post initial treatment

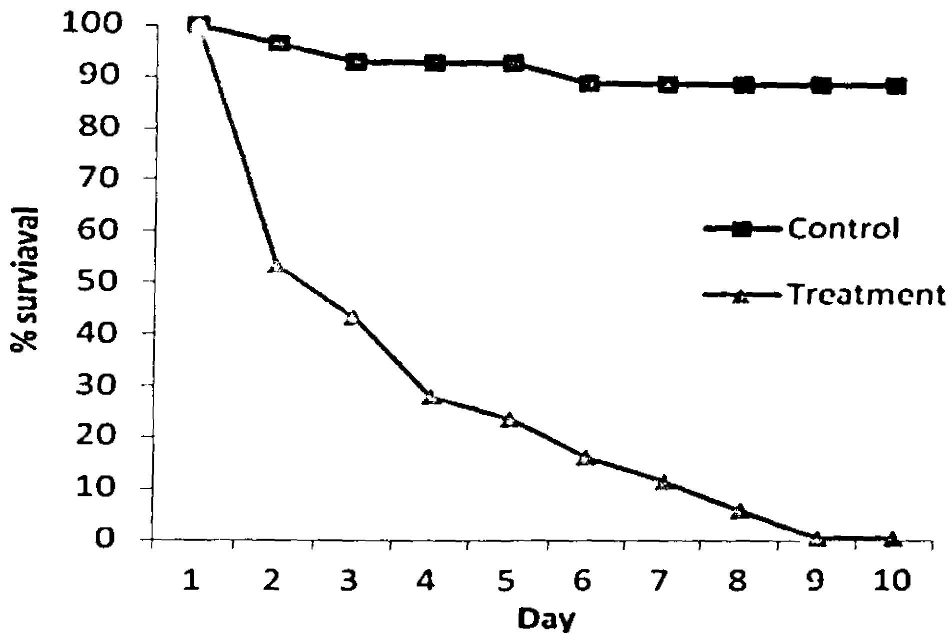


Figure 16: Survival of sand flies collected from *M. anisopliae* treated and control termite mounds at week 9 post initial treatment

4.8 Survival rates of sand flies collected from *M. anisopliae* treated and control termite mounds at week 9 post initial treatment

Survival rates of sand flies trapped from *M. anisopliae* treated and the control groups were followed from the first day of treatment upto day 10. Entomopathogenic fungus which is a slow acting biopesticide produced remarkable results on sand flies collected at week 9 post initial treatment. Mortality was 100% in the sand flies collected from the treated termite mounds in week 9 and died within 9 days in the laboratory compared to sand flies collected from the control termite mounds that had a mortality rate of 10.7% over the same duration (Fig. 16). *M. anisopliae* infection was observed in 42% of the sand fly cadavers collected from the treated termite mounds and 0% in the control group

(Fig. 10B), indicating that the reduction in sand fly population density observed in week 9 can be attributed to the EPF intervention.

4.9 Control of termites with *M. anisopliae* in their natural habitats

Observations on termite mounds treated with *M. anisopliae* 10 months post treatment showed that 7/10 (70%) of the termite mounds had no sign of termites existing in them and neither were there signs of reconstruction taking place. Only 3/10 termite mounds were found to be active and reconstruction of the termite mounds was going on Table 5. Sand flies trapped in the treated termite mounds six months post treatment were found to be positive for *M. anisopliae* fungus.

Table 5: Control of termites (*Macrotermis subhalynus*) with entomopathogenic fungi (*M. anisopliae*) in the field

Termite mound Numbers	Initial status of Termite mound	Status of Termite mound after 10 months post treatment
13	Presence of termites in TM	All termites dead
14	Presence of termites in TM	All termites dead
15	Presence of termites in TM	Termites present
16	Presence of termites in TM	Termites present
17	Presence of termites in TM	Termites present
18	Presence of termites in TM	All termites dead
19	Presence of termites in TM	All termites dead
20	Presence of termites in TM	All termites dead
21	Presence of termites in TM	All termites dead
25	Presence of termites in TM	All termites dead

Legend: TM= Termite mound

7/10 termite mounds had their termites controlled by *M. anisopiliae* 10 months post treatment.

CHAPTER FIVE

5.0 Discussion

Considering the global prevalence of vector-borne diseases such as leishmaniasis (WHO, 2008), the increasing incidence of resistance to chemical insecticides in target sand fly populations and problems associated with environmental contamination by these compounds (Osoria *et al.*, 2007; Bouman and Kylin, 2009), alternative control methods of sand fly control are becoming increasingly important (Murphy *et al.*, 1994; Zaim and Guillet, 2002). The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* are regarded as good candidates for biological control agents for managing insecticide resistance in leishmaniasis vector control.

Infection with entomopathogenic fungi has been shown to lower disease transmission by malaria vectors and other insect pests by reducing their numbers and vector abundance (Scholte *et al.*, 2003a; 2004b; Dimbi *et al.*, 2004; Achonduh and Tondje, 2008; Kikankie, 2009). Previous studies on *P. duboscqi* showed that sand flies are susceptible to *M. anisopliae* and *B. bassiana* (Ngumbi *et al.*, 2011). A semi- field trial carried out in Tanzania by Scholte *et al.*, (2005) showed that *An. gambie* adults can be infected with *M. anisopliae* spores using spore treated black cloths strategically placed inside human dwellings. Farenhorst *et al.*, (2008) demonstrated in the laboratory that clay pots treated with oil formulated fungal conidia can be used to deliver *M. anisopliae* spores to adult anopheline mosquitoes.

This study considered the potential for using Hyphomycetes such as *Metarhizium* or *Beauveria* for biocontrol of vectors of leishmaniasis high: These fungi can be cost-effectively mass-produced, even locally, and many strains are already commercially produced, circumventing the time-consuming and costly process of registration, including risk- assessment of new fungal control agents. *B. bassiana* and *M. anisopliae* are produced by 14 and 10 companies respectively, (including some in Africa), aimed at controlling various insect pests including termites, cockroaches, black vine weevil, white flies, aphids, corn borers, cockchafers, and other insects (Strasser *et al.*, 2000).

Concerning the future of myco-insecticides, Burgess (1998) points out that: 'Improvement in shelf-life duration and formulations is the key to the future and should enable fungi to compete in efficacy with chemical insecticides on nearly equal terms and should increase projected market size towards industrial viability'. Research should target three areas that need improvement: application, storage and production.

The ideal fungus for sand fly control should have the following characteristics: a) kills both larvae and adult stages, b) requires one or a few applications per season, c) is easily dispersed by adult females to previously unoccupied breeding sites, d) shows residual activity and persistence in the sand fly population after introduction, e) kills selectively sand flies and no other organisms, f) is effective over a large range of temperatures, relative humidity and breeding sites, g) is easily and cost-effectively mass-produced and formulated, h) retains prolonged activity during storage (long shelf-life) and i) is not harmful to humans and other non-target organisms. None of the insect-pathogenic fungi

presently known exhibit all these characteristics, but they all exhibit at least some (Burgess, 1998).

Laboratory reared *P. duboscqi* species used in the lab-based experiments were found to be susceptible to infection by unformulated conidia of *B. bassiana* and *M. anisopliae* (Table 2). These fungi induced significantly higher mortality rates compared to those of the control groups. This difference in mortality between fungus treated and untreated groups should be attributed to the presence of fungal conidia which overpower the immune response of the infected host, and proliferate by penetrating the cuticle and invading the internal organs ultimately causing the death of the host (St Leger *et al.*, 1991; Clarkson and Charnley, 1996). Mortality rates in the lab-based experiments on *P. duboscqi* ranged from 76-100% from *B. bassiana* and *M. anisopliae* infections.

Approximately 90% of the fungus infected sand flies died within 12 days of acquiring their infection, which is encouraging in terms of *Leishmania* transmission (which takes 8-10 days to reach metacyclic stage that is infective) because of the time it takes for the parasite to mature in the sand fly gut before transmission can take place (Lawyer *et al.*, 1990). Death occurring within 12 days post fungus infection should lead to a significantly reduced potential for leishmaniasis transmission, an effect that should be further enhanced by indications that the lifespan of a sand fly infected with both entomopathogenic fungus and *Leishmania* parasites is likely to be even shorter than otherwise (Blanford *et al.*, 2005).

Sand flies exposed to fungal spores in the termite mounds within the first 2 weeks showed significantly lower rates of mortality than those exposed at 9 weeks post initial treatment under similar conditions.This shows that infection is a function of time and that a longer potential exposure time leads to the acquisition of greater numbers of infective spores. Clark *et al.*, (1968) demonstrated that continuous exposure in a field environment did induce an exponentially increasing mortality rate in a relatively short period. This compares well with our results in the field trial when we increased our field dosage from 5 to 15 g. Although the viability of conidia may decrease in time, the effectiveness of fungal treatment showed high infectivity rates in the field. It has been suggested that the slow killing speed of entomopathogenic fungi could lead to them being evolution-proof against resistance (Read *et al.*, 2009). This is because any resistance-related genes would be diluted by the genes of susceptible individuals passed onto the next generation before they have succumbed to the fungal infection. For this strategy to work, the fungi should kill the vector before the parasite completes its life cycle inside the sand fly or mosquito.

Low mortality was observed in the pupa stage assays with 98-100% adult emergence observed in the controls and all treatments, indicative of poor EPF pathogenicity and penetration of conidia. Dry conidia have been shown to kill mosquitoes faster than oil formulated conidia (Scholte *et al.*, 2003) and this could be one of the reasons behind the poor EPF pathogenicity in the pupal stages because the conidia was in a suspension. When dry conidia were used to control malaria vectors (Scholte *et al.*, 2003), *M. anisopliae* was more virulent to mosquitoes than *B. bassiana* after a three day exposure, although it is unclear what the viabilities of the conidia were. These results are

comparable to our laboratory reared *P. duboscqi* where *M. anisopliae* performed better than *B. bassiana* in registering four isolates with 100% mortality among the group of 19 isolates used in the experiment. In this study we used 4th instar larvae and pupae because they are larger than the other larval stages and are easy to see and observe what is happening to them after treatment. The other reason behind the choice of these stages was that they have more developed cuticle surfaces that can be ideal for conidia attachment. However, the duration of sand fly development in the pupa stage is relatively short (approx. 2 days) with shedding of the hardened cuticle during adult emergence likely to occur prior to successful penetration and establishment of EPF infection in the sand fly. The percentage mortality of larvae after exposure to fungi ranged from 32- 60 % control among the six isolates tested, as demonstrated in the 4th instar larvae assays indicative of EPF's potential as a control agent targeting sand fly breeding sites. Isolate H which was one of the six isolates used, killed 60% of all the larvae which were sprayed with this particular fungus, while only 40% of the larvae managed to emerge as adult flies.

Mortality was 100% in the sand flies collected from the treated termite mounds in week 9 and died within 9 days in the laboratory compared to sand flies collected from the control termite mounds that had a mortality rate of 10.7% over the same duration Figure 16. *Metarhizium anisopliae* infection was observed in 42% of the sand fly cadavers collected from the treated termite mounds and 0% in the control group, indicating that the reduction in sand fly population density observed in week 9 can be attributed to the EPF intervention.

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Observations on termite mounds treated with *M. anisopliae* 10 months post treatment showed that 7/10 (70%) of the termite mounds had no sign of termites existing in them. This was similar to the work of Maniania *et al.*, (2001) in which he controlled termites in a maize cropping system with *M. anisopliae* fungus. EPF can be used to control termites as observed on the termite mounds treated with *M. anisopliae*.

The measurement of efficacy of *M. anisopliae* was based on percentage reduction of sand fly population density, which was calculated by comparison of sand fly abundance per termite mound in the treatment group versus their baseline abundance. It was found that in the field we could not find equal numbers of sand flies, both in the control and treatment termite mounds to compare them after application of the conidia. As can be seen on Fig. 14, collections sand flies from the control group were fewer than the sand flies trapped in the treatment site. However, we kept the two sites to keep track of the environmental changes during the study.

In a small-field trial, treated termite mounds produced significant reduction in sand fly population 9 weeks post infection. Sand flies trapped 6 months post treatment in two of the ten termite mounds treated with conidia were positive for the fungi used in the field experiments. This showed that the fungi used are capable of tolerating high temperatures experienced in this dry region and can also persist for a long time in the environment which agrees favourably with the conditions put forward by Burgess (1998). Radiation was not able to destroy the fungi and sand flies continued dying from the fungal infections existing in their habitats. This is a good sign of the potential of

entomopathogenic fungi in controlling sand flies that transmit *Leishmania* parasites. Since no resistance has been reported in entomopathogenic fungi concerning the vectors of leishmaniasis, it is hoped that EPF will be a good alternative to chemical insecticides in controlling insect pests. Moreover entomopathogenic fungi are friendlier to the environment than chemical insecticides and cheaper to acquire (Scholte et al., 2003).

The results of the current project provide significant proof of concept that the pumping of dry conidia powder is a viable delivery method for *in situ* targeting of visceral leishmaniasis vector, *P. martini* breeding sites.

5.1 Conclusion and Recommendations

- Successful control of sand fly populations using *M. anisopliae* was demonstrated.
- Indigenous free living EPF is a potential biological control for sand fly control.
- Added advantage of EPF is being larvicidal (Ngumbi *et al.*, 2011). However, targeting larvae for sand fly control has been a challenge.
- *M. anisopliae* infection was observed in sand fly cadavers collected from the treated termite mounds indicating that the reduction in sand fly population density observed in week 9 can be attributed to the EPF-intervention. The increase in sand fly numbers in weeks 1 and 5 can be attributed to the fact that it takes time for the EPF infection to establish itself and kill the sand flies.
- Entomopathogenic fungi infect their hosts through the cuticle and do not need to be ingested like other entomopathogens (viruses, bacteria and protozoa).

Therefore, they offer great potential for the control of hematophagous insects like sand flies.

- Determination of effective treatment dose for termite mounds of unspecified and varying sizes presented a challenge in the EPF intervention.
- In conclusion, EPF is a feasble candidate for development as a sand fly control strategy in the control of leishmaniases in endemic areas. Further studies to determine the best methods for delivery and application *vis* a *vis* the diverse ecological settings of various leishmaniasis vectors is therefore recommended.

APPENDICES

APPENDIX I: PRODUCTION OF FUNGI ON RICE SUBSTRATE

STARTER CULTURE

50ml of sterile LB media is inoculated with spores from clean culture plate and incubated for three days in a shaker incubator- 100rpm at 37°C. The culture is examined microscopically to check for any contamination. More LB media flasks are then inoculated (depending on the amount of production). The cultures (Inoculums) are incubated for 4-5 days.

SUBSTRATE PREPARATION

Untreated rice is washed and precooked in hot water to soften it. Two kilogram of rice is then packaged into each polythene (Milner) bag, sealed and autoclaved for 1hr at 121°C. The sterile rice is inoculated with the fungal inoculums under sterile conditions. The inoculated bags (cultures) are then incubated at 28°C-30°C in the culture room for three weeks. The fungal spores are harvested by sieving them through the microspore sieves (45µ θ). The spores are then dried in a dedicator to remove excess moisture and parked in air tight polythene bags and kept in a cool dry place.

LB media-

45g yeast extract

45g glucose

15g peptone

Dissolved in 1L of distilled water

The media is distributed in 50 ml into 250ml flasks and autoclaved.

APPENDIX II: Gum-Chloral mountant
Puri's medium

1) Distilled water 10mls
2) Gum acacia (powder)...8gms
3) Chloral hydrate (crystals) 70gms
4) Glycerine 5mls
5) Glacial acetic acid 3mls

Ingredients should be dissolved in the above order at room temperature. A magnetic stirrer may be used to help in mixing them well. Filter the fluid through cotton wool.

After Minter: *Bulletin of Entomological Research*, 54: 483 (1963).

APPENDIX III: SOP FOR SOLUTIONS USED IN SAND FLY DISSECTIONS.

Solution B

These sand fly dissection solutions have a short shelf-life and therefore need to be prepared a fresh after every month.

Formular for solution B: (Soak solution)

1) Combine the following ingredients adding them to 111.8mls of sterile water.
2) Measure and add 6mls of freshly mixed Penicillin-Streptomycin solution.
3) Measure and add 1.2mls Gentamycin of sulfate (50mg/ml)
4) Weigh and add 60mg of 5-Flurocytosine.
5) Weigh and add 1ml (12mg of Amph-B powder in 20ml sterile water) of Amphotericin B.
6) Filter through 0.22 micron filter and put in a sterile bottle, add a label with the following information: Solution B, Soak solution, Date prepared, and your initials.
7) Store in a refrigerator for use in dissections of sand flies.

Solution D.

Formular for Solution D: (Dissection solution)

1) Combine the following ingredients adding them to 111.8mls of sterile saline.
2) Measure and add 6 mls of freshly mixed Penicillin-Streptomycin solution.
3) Measure and add 1.2ml of Gentamycin sulfate (50mg/ml).
4) Weigh and add 60mg of 5-Flurocytosine.
5) Filter through sterile 0.22 micron filter and put in sterile bottle(s).
6) Label bottle, Solution D, Dissecting solution, date prepared and your initials.
7) Store the bottle (s) in a refrigerator for use during sand fly dissections.

APPENDIX IV: List of abbreviations used.

ZCL	= Zoonotic Cutaneous Leishmaniasis
ACL	= Anthroponotic Cutaneous Leishmaniasis
ZVL	= Zoonotic Visceral Leishmaniasis
VL	= Visceral Leishmaniasis
CL	= Cutaneous Leishmaniasis
ICIPE	= International Centre for Insect Physiology and Ecology
EPF	= Entomopathogenic Fungi
CDC	= Centre for Disease Control
TM	= Termite mound
LT	= Light trap
KEMRI	= Kenya Medical Research Institute
ANOVA	= Analysis of Variance
NCST	= National Council for Science and Technology
SDA	= Sabourand dextrose agar
TDR	= Research on Tropical Diseases
DALYs	= Disability adjusted life-years
HIV	= Human Immunodeficiency Virus
AIDS	= Acquired Immunodeficiency Syndrome
LCL	= Localized cutaneous leishmaniasis
MCL	= Mucosal leishmaniasis
PKDL	= Post Kala-azar dermal leishmaniasis
DCL	= Diffuse cutaneous leishmaniasis
CCR	= Centre for Clinical Research

ITNs	= Insecticide Treated Nets
IRS	= Indoor Residual Spraying
NNN	= Novy-MacNeal-Nicole
DAT	= Direct Agglutination Test
ELISA	= Enzyme linked Immunosorbent Assay
IFAT	= Indirect fluorescent antibody Test
PCR	= Polymerase Chain Reaction
DNDi	= Drugs for Neglected Diseases Initiative
BCG	= Bacillus Calmette-Guerin
DDT	= Dichloro-diphenyl-trichloroethane

APPENDIX V: Publication arising from this study

PEER-REVIEWED PUBLICATION:

Ngumbi, P. M., Irungu, L. W., Ndegwa, P. N. & Maniania, N. K. (2011). Pathogenicity of *Metarhizium anisopliae* (Metch) Sorok and *Beauveria bassiana* (Bals) Vuill to adult *Phlebotomus duboscqi* (Neveu-Lemaire) in the laboratory. *Journal of Vector-Borne Diseases*, **48**: 37-40.

APPENDIX VI: Overview of Fungal Taxa (Kingdom to Genus)

Table of Overview of fungal taxa (Kingdom to Genus) showing classification and nomenclature after Kirk *et al.*, 2001

1) Kingdom	2) Phylum	3) Class	4) Order	5) Family	6) Genus
CHROMISTA 1)					
	Oomycota 2)				
		Oomycetes 3)			
			Saprolegniales 4)		
				Saprolegniaceae 5)	
					Leptolegnia 6)
			Pythiales		
				Pythiaceae	
					Pythium
					Lagenidium
			Myzocytiopsidales		
				Crypticolaceae	
					Crypticola
FUNGI					
	Chytridiomycota				
		Chytridiomycetes			
			Blastocladiales		
				Coelomomycetaceae	
					Coelomomyces
	Zygomycota				
		Zygomycetes			
			Entomophthorales		
				Ancylistaceae	
					Conidiobolus
				Entomophthoraceae	
					Entomophthora
					Erynia
		Trichomycetes			
			Harpellales		
				Legeriomycetaceae	
					Smittium
		Deuteromycetes (Hyphomycetes)			
					Culicinomyces
					Beauveria
					Metarhizium
					Tolypocladium

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