MOLECULAR CHARACTERISATION OF *MICROSPORIDIA MB* SPECIES AND CORRELATION WITH *PLASMODIUM* PRESENCE IN *ANOPHELES* MOSQUITOES IN MWEA AND MBITA, KENYA

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AUGUST 2018

DECLARATION

I declare that this thesis is a sole representation of my work and has not been reproduced elsewhere.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

ACTs	Artemisinin-Combination Therapies
BLAST	Basic Local Alignment Search Tool
CBT	Cattle-Baited Traps
CDC	Centre for Disease Control
Ct	Cyclic threshold
DALY	Disability-Adjusted Life Year
DNA	Deoxyribonucleic Acid
DVS	Dominant Vector Species
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FA	Formaldehyde
FISH	Fluorescence *In situ* Hybridization
HRM	High Resolution Melting
ICIPE	International Centre of Insect Physiology and Ecology
IRS	Indoor Residual Spraying
ITNs	Insecticide-Treated Nets
ITS2	Internal Transcribed Spacer Region 2
KEMRI	Kenya Medical Research Institute
LLINS	Long-lasting Insecticide-Treated Nets
MAb	Mouse Monoclonal Antibody
MB	*Mbita*
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
NP40	Nonidet P40
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline-Tween 20
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
rDNA	Ribosomal Deoxyribonucleic Acid
RDT	Rapid Diagnostic Tests
rRNA	ribosomal Ribonucleic Acid
SSC	Saline Sodium Citrate

SSU	Small Sub-Unit
WHO	World Health Organisation

ABSTRACT

Much of sub-Saharan Africa suffers from high malaria infection rates in spite of several vector-control strategies set up to control *Plasmodium* transmission. Currently, these approaches are only partially effective, compromised by the evolution of insecticide resistant mosquitoes and adaptive changes in mosquito feeding patterns. Therefore, there has been an expanding search for novel strategies to control both the vector densities and parasite transmission. Such techniques include the concept of using mosquito symbionts for transmission blocking. This study focused on a novel symbiotic *Microsporidia*n species (*Microsporidia MB)* isolated from *Anopheles gambiae s.l.* mosquitoes collected in parts of Mwea and Mbita, Kenya. It aimed at understanding the molecular biology of the strain, its phylogenetic classification and tissue distribution. The amount of *Plasmodium falciparum* sporozoites was established in each sample using Enzyme-linked Immunosorbent Assay (ELISA). Additionally, a highly sensitive and specific probe-based quantitative PCR assay was designed to quantify *Microsporidia MB* within each sample. Phylogeny studies using the highly conserved and variable 18S small subunit rRNA gene demonstrated the classification of *Microsporidia MB* within the same clade as *Crispospora chironomi*- a *Microsporidia*n species isolated from non-biting midges in Siberia. In field collected samples, a 5% prevalence of the microorganism was observed in both Mbita and Mwea areas. Fluorescence microscopy on infected larvae indicated that there was a cyst-like infection within the larval gut tissue. Furthermore, the correlation between the presence of the novel mosquito *Microsporidia MB* and the *Plasmodium* parasite was examined. Interestingly, a negative correlation between *Plasmodium falciparum* and *Microsporidia MB* quantity was revealed both in the field and laboratory colonized mosquitoes. These findings are promising as they point at *Microsporidia MB* being a plausible transmission-blocking agent against malaria in *Anopheles* mosquitoes.

CHAPTER ONE

INTRODUCTION

1.1 Malaria

The malaria disease burden still remains high in the tropics despite major breakthroughs in developing effective treatment and prevention strategies. In 2016 alone, the World Health Organisation (WHO) reported 216 million new malaria cases (1) with Africa recording 90% of these infections. There was a notable 5 million increase in malaria cases over the years (2010-2015) suggesting an impediment in its decline rate despite the control strategies already in place (1). In addition, approximately 445,000 of these infections were fatal with a staggering 91% of the deaths occurring in Africa. Children below the age of 5 years are the majority of the malaria victims. It is estimated that one child's life is lost every 2 minutes as a result of malaria infections (1).

1.2 Insecticide resistance

The main global strategies against malaria transmission have relied heavily on vector control (2) by the use of Insecticide-Treated Nets (ITN) and Indoor Residual Spraying (IRS) which have provided some level of relief from the malaria burden but transmission still remains high in many regions (3). Funding invested for inpatient and outpatient malaria casualties by non-governmental institutions like WHO, the World Bank Data and the Global Fund to Fight AIDS, Tuberculosis and Malaria have indicated a constant increase in costs which are now as high as 3 billion US dollars in 2015(1). WHO reported on the effectiveness of these interventions among at-risk populations and found an increase in use of ITNs and IRSs from 30% in 2010 to 54% in 2016 (1). However, these interventions have faced major challenges (4) with the evolution of insecticide-resistant mosquitoes as illustrated in Figure 1.1 (5).

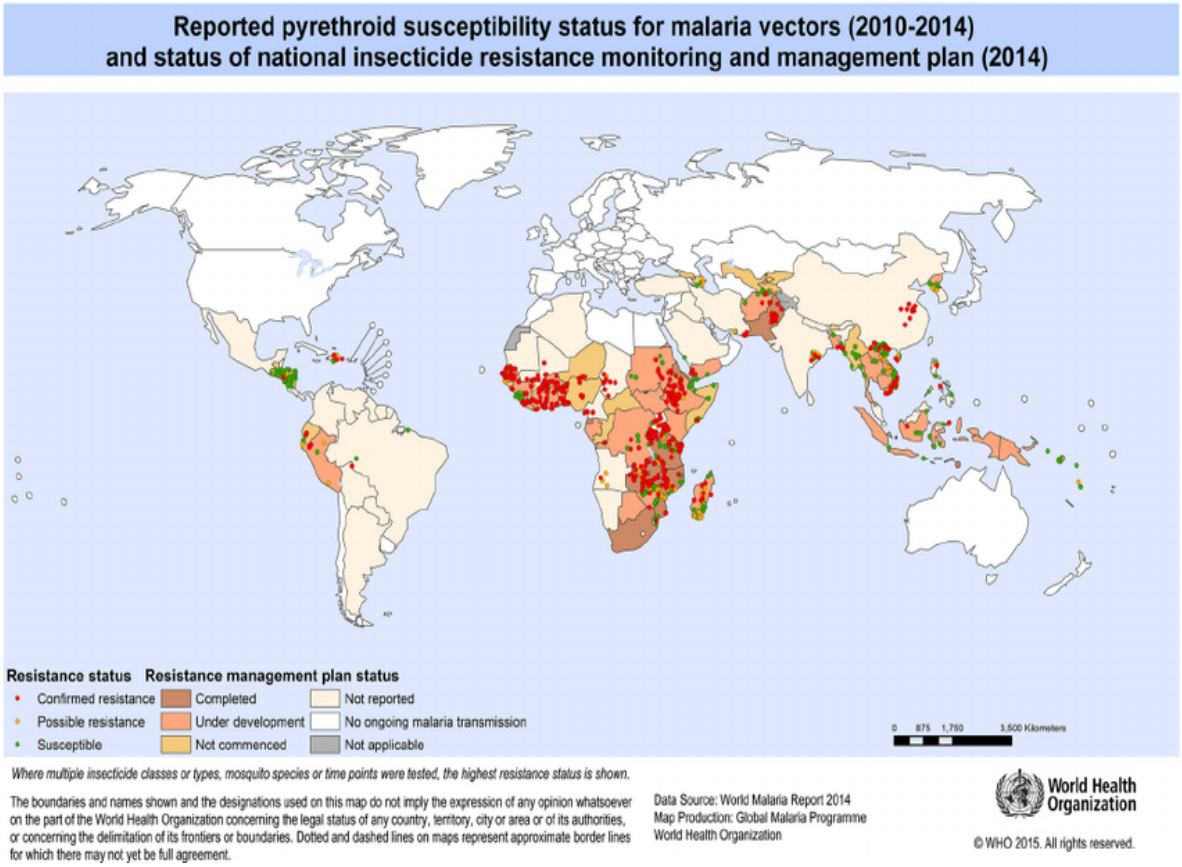


Figure 1.1: A map illustrating the status of pyrethroid resistance in the world and the status of national insecticide resistance monitoring and management plan. The areas with confirmed insecticide resistance are highlighted in red dots, most of which lie in the malaria endemic regions. The green dots indicate areas prone to resistance, while the orange dots illustrate regions with possible resistance (5).

Many mosquito populations are highly resistant to pyrethroids, a key component of insecticides used in ITNs. Carbamates, organophosphates and organochlorines have also registered some level of resistance (1,3,6). In Kenya alone, *Anopheles* species insecticide resistance in all four classes of insecticides has been reported as illustrated in Figure 1.2 (7). However, resistance to pyrethroids is predominant across most regions of the country. There is a notable increase in resistance in the Mbita region of Kenya where malaria occurrence is high (7). Insecticide resistance to organophosphates and organochlorines is not as widespread as pyrethroid resistance since they are not as widely used in Kenya as pyrethroids. The main challenges faced in controlling resistance have been the lack of new vector control tools, minimal or no funding and resources (both human and infrastructural) and the weak national surveillance of insecticide resistance in endemic regions (3,8).

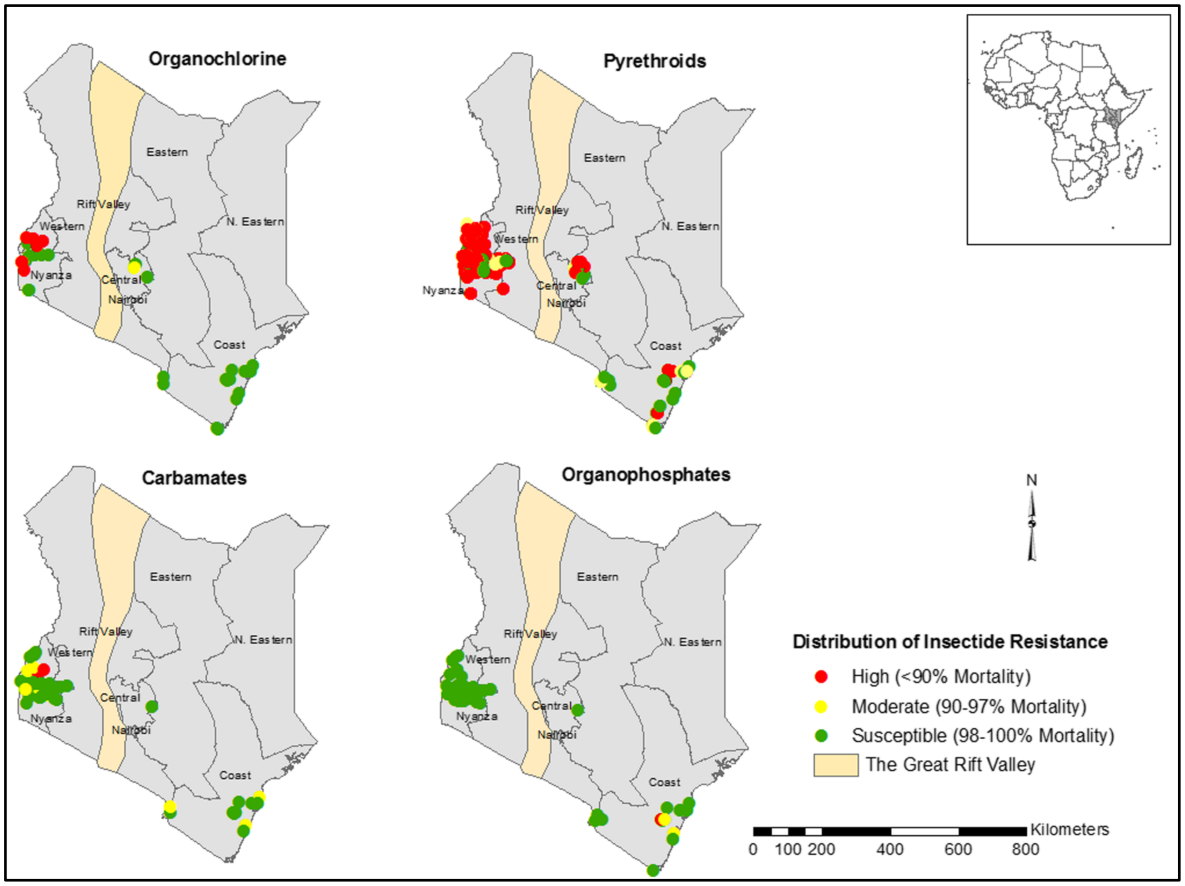


Figure 1.2: Comparison of the distribution of insecticide resistance in *Anopheles* mosquito species in Kenya (7). Red illustrates high levels of insecticide resistance, yellow mild resistance while green illustrates insecticide susceptibility of the vectors. Pyrethroid resistance is predominant across most regions of the country while organochlorines, organophosphates and carbamates indicate less resistance.

1.3 Alternative malaria control initiatives

In addition to the use of insecticides, malaria vaccine development is currently underway as a preventative measure (9–16). Nevertheless, this has not proven to be highly effective due to the complexity of the malaria parasite compared to viruses and bacteria for which vaccines have been developed (reviewed in 15).

Besides the already established malaria prevention and control strategies, advanced treatment options with improved drug combinations, such as Artemisinin-Combination Therapies (ACTs), have been developed to enhance treatment efficacy (1). Despite this, mutations within the *Plasmodium* parasite (12) have resulted in evolution of multidrug resistant strains (17–19).

1.4 Problem statement

Malaria is a widely studied disease that is a cause of high mortalities especially in the sub-Saharan Africa region (1). Despite all the work that has so far been set up to control malaria, an effective breakthrough has yet to be identified. Vector control has been a key feature in the control of malaria through the use of Long-Lasting Insecticide-treated Nets (LLINs) and IRS (20,21). However, the evolution of resistance among the major malaria vectors has become a deterrent to the already set up control initiatives, thus the need for newer, more innovative yet sustainable intervention strategies (3,22).

Research has now shifted focus to the isolating and studying of innate mosquito microbes with the aim of *Plasmodium* transmission blocking and or development *vis a vis* vector density control in totality (23,24). One such microorganism is *Microsporidia* which is spore-forming and can easily be propagated within the mosquito population therefore would be an ideal biological vector control tool (25–27). This study hence focused on a novel *Microsporidia* to this effect and aimed at determining its relationship with *Plasmodium falciparum.*

1.5 Justification

Mosquitoes are vectors of malaria, Dengue, Zika and many other disease-causing microorganisms. Malaria is one of the most disastrous illnesses in developing countries mainly affecting children and pregnant mothers. Some of the currently utilized vector control approaches range from IRS to LLINs. However, these strategies have been impeded due to several factors including; financial constraints, lack of knowledge and most important of all-insecticide resistance (5,8). Global initiatives to eradicate malaria have been faced with major challenges including; evolution of vector insecticide resistance and feeding time changes (28). Such problems have led to the need to study alternative biological methods to control mosquito populations in malaria-endemic countries (Figure 1.3).

Recent studies have revealed that mosquito symbiotic microbes could offer a potential solution to this problem (Figure 1.3) (29–31). Mosquitoes harbour a variety of microbes that have been shown to prevent *Plasmodium* transmission (31,32). *Microsporidia* is one such symbiont (27,33). Related studies have identified that *Microsporidia* -infected mosquitoes have lower mortality rates in larvae and pupae, reduced fecundity, lower female

biting rate and have even demonstrated that *Microsporidia* interfere with parasite development (25,26,34).

Pathogenic *Microsporidia* have been shown to effectively control several important malaria vectors. For example, *N. algerae* in *Anopheles stephensi* (35–37)*, Anopheles albimanus* (36,38) and *Anopheles quadrimaculatus* (39). There was a notable 50% survival rate in adult females in *A. albimanus* infected with *N. algerae,* a projected 97% potential control of vectors in the natural environment. Field trials in Panama (38) recorded an 86% reduction in larvae, although the persistence of the *Microsporidia* dropped to less than 1% after two months.

This study therefore aimed at understanding the biology of *Microsporidia MB* in the mosquito to provide a basis for its use as a biological vector-control method.

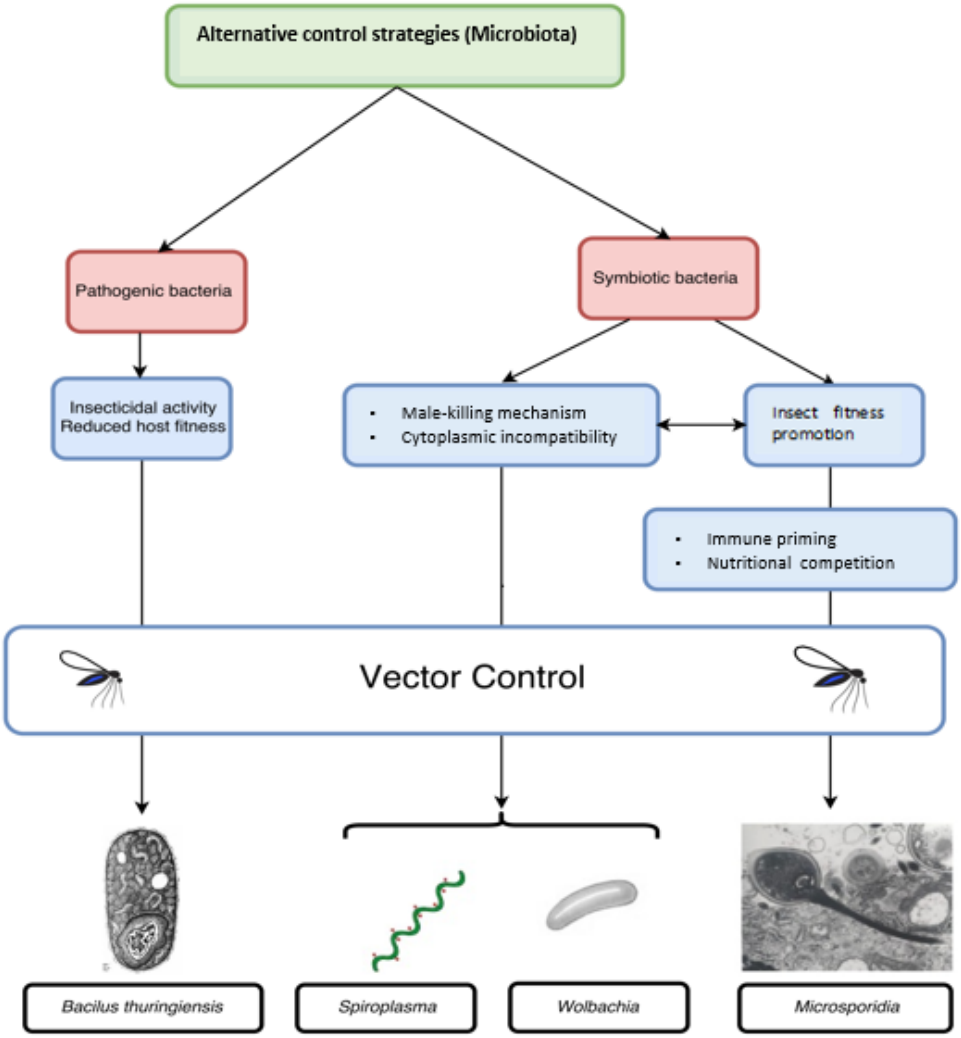


Figure 1.3: The potential use of microbes and microbial symbionts as biological vector control strategies. Pathogenic bacteria such as *B. thuringiensis* that have insecticidal capabilities against the vectors could be used to control vector population. Some commensal bacteria have an immune priming effect in their host, others compete with other pathogens for nutrients or produce inhibitory molecules against other foreign microorganism thereby protecting their host from parasite infection (23,24,31,40,41). Symbiotic microorganisms could also be used to control vector populations through reproductive manipulation such as the male-killing mechanism of *Spiroplasma* (42,43) and *Wolbachia* (44), and cytoplasmic incompatibility (45,46).

1.6 Research question

Does *Microsporidia MB* density reduce the capacity of *Anopheles* mosquito populations to vector *Plasmodium*?

1.7 Hypothesis

Microsporidia MB density reduces the susceptibility of *Anopheles* mosquito to *Plasmodium* infection.

1.8 Objectives

1.8.1 General objective

To isolate and characterise a novel *Microsporidia*n species (*Microsporidia MB*) and determine its correlation with *Plasmodium falciparum* parasite in wild-caught *Anopheles gambiae s.l.* mosquitoes in Mbita and Mwea, Kenya.

1.8.2 Specific objectives

i. To determine the prevalence of *Microsporidia MB* in *Anopheles gambiae s.l.* mosquitoes in Mbita and Mwea, Kenya.
ii. To determine tissue tropism from a laboratory-reared *Microsporidia MB*-infected *Anopheles gambiae s.l.* mosquito colony.
iii. To correlate the presence of *Microsporidia MB* and *Plasmodium* parasite in *Anopheles gambiae s.l.* mosquitoes in Mbita and Mwea, Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Dominant vector species of the human malaria in Africa

Mosquitoes are vectors of some of the worst diseases currently affecting developing countries. They have been shown to carry a wide range of agents of human disease including parasites, viruses, bacteria and filarials (23). Examples of diseases transmitted by the mosquito are malaria, yellow fever, dengue, Zika, Chikungunya and filariasis. Malaria is amongst the most devastating of these, recording the most cases of infection and deaths (1). Africa is one of the most malaria endemic regions hugely due to the presence of the *A. gambiae* complex which consists of some of the most capable human malaria vectors. This complex includes *A. gambiae, A. arabiensis, A. merus* and *A. melas* (47). Aside from these, other significant malaria vectors in Africa include *A. funestus, A. moucheti* and *A. nili* (47,48).

Anopheles gambiae is the most well-researched and amongst the most efficient malaria vector in sub-Saharan Africa (22). *A. gambiae* have been characterised as highly anthropophilic, preferring human hosts for blood source (49–52). Larvae are often found in areas associated with human activity such as old tyres, hoof prints, unused pots or rice cultivation. Adults have been shown to be both endophagic and endophilic (49,50,53,54), biting and resting indoors thereby increasing effective malaria transmission. Additionally, females commonly feed during late nights when there is less human disruption (47).

Anopheles arabiensis is another important vector of the malaria disease. It has been described to be zoophilic, exophilic and exophagic as compared to *A. gambiae* (55), preferring domestic animals as blood source, biting and resting outdoors. They display great behavioural plasticity and have variable predatorial traits based on their localities (56–58). These attributes of *A. arabiensis* enables them to adapt rather fast to counter IRS by demonstrating 'insecticide avoidance' depending on the insecticide used (59,60).

Anopheles melas, on the other hand, is considered a vector of lesser importance especially in areas with both *A. gambiae* and *A. arabiensis* due to their lower sporozoite rates (This is the proportion of female anopheline mosquitoes of a singular species that bear the *Plasmodium* sporozoites in their salivary glands) as compared to the latter (0.35% sporozoite rate in *A. melas* while 3.5% in *A. arabiensis* or *A. gambiae* in a study done in Cameroon

(58,61)). *A. melas* are commonly associated with brackish waters, mainly found in the coastal regions where they can occur in high densities making them problematic malaria vectors. Their biting behaviour is described as being opportunistic (both anthropophilic and zoophilic) (62), blood feeding continuously throughout the night (62).

There is limited knowledge on the *A. merus* since it is considered an incompetent vector in sustaining malaria transmission (58). Similar to *A. melas,* this species prefers brackish water and are mostly found along the coast. They also display opportunistic biting behaviour and prefer to blood feed and rest outdoors (63).

A. moucheti is another poorly studied malaria vector in the *A. gambiae* complex. It is restricted to forested areas (64). However, unpublished work has detected *Plasmodium falciparum* in this species (65). *A. moucheti* is highly anthropophilic and endophilic (49,52,66) with biting peaks from midnight to dawn (49), thus its classification as a Dominant Vector Species (DVS).

The *A. nili* complex also consists of several highly efficient yet overlooked vectors. These include: *A. nili, A. carnevalei, A. ovengensis* and *A. somalicus* (67). Of these, *A. nili* is the most important vector (66,68) since it is strongly anthropophilic and bites both indoors and outdoors in the early evening (49,50) whereas *A. carnevalei* and *A. ovengensis* are considered as secondary vectors (66,68). *A. somalicus* is characterised as zoophilic and exophilic, reducing its efficiency in human malaria transmission.

Aside from the *A. gambiae* complex, another major malaria vector is the *A. funestus.* This is a member of the *A. funestus* complex, including *A. rivulorum*, *A. confusus, A. parensis, A. vaneedeni* and *A. aruni* (69). There is a variation in vectorial capacity within this subgroup. Of the five species, *A. funestus* shows the most efficiency in malaria transmission (48). Studies have described *A. funestus* as highly anthropophilic with late night biting peaks (49,66,70–73), depicting endophilic resting behaviour (49,66) and relatively high longevity (22,56). All these characteristics render this species a better malaria vector than the rest in the *A. funestus* complex.

2.2 Lifecycle of *Plasmodium* in the mosquito

Plasmodium is a blood borne apicomplexan protozoa with a complex lifecycle (Figure 2.1). The anopheline vector is crucial to the parasite's life cycle (74). Effective *Plasmodium* transmission requires transformation from asexual to sexual stages in the mammalian host and the mosquito vector, respectively. Asexual blood stages of *Plasmodium* do not effectively infect the vector therefore sexual differentiation (formation of male and female gametocytes) in the blood is necessary for transmission from the host to the vector (75). *Plasmodium* development highly relies on the mosquito's midgut.

The *Plasmodium* parasite requires to surpass the vector's immune system and competition with gut microbiota population to successfully develop into its infective stage (76). Once the gametocytes are ingested in the mosquito, several biochemical factors including temperature drop, the presence of xanthurenic acid and pH rise, trigger gamete maturation in the midgut (77). Flagellation of the male gamete followed by fertilization leads to the formation of the zygote within the gut lumen **(**Figure 2.1**).** The zygote then develops into an ookinete. The highly motile and invasive ookinete then crosses the midgut epithelial layer from the gut. Ookinetes subsequently transform into oocysts and sporogenic replication occurs. The infective sporozoites are released from the oocysts and later relocate to the salivary glands where they are injected into a new host for the re-initiation of the *Plasmodium* lifecycle (75,78–80).

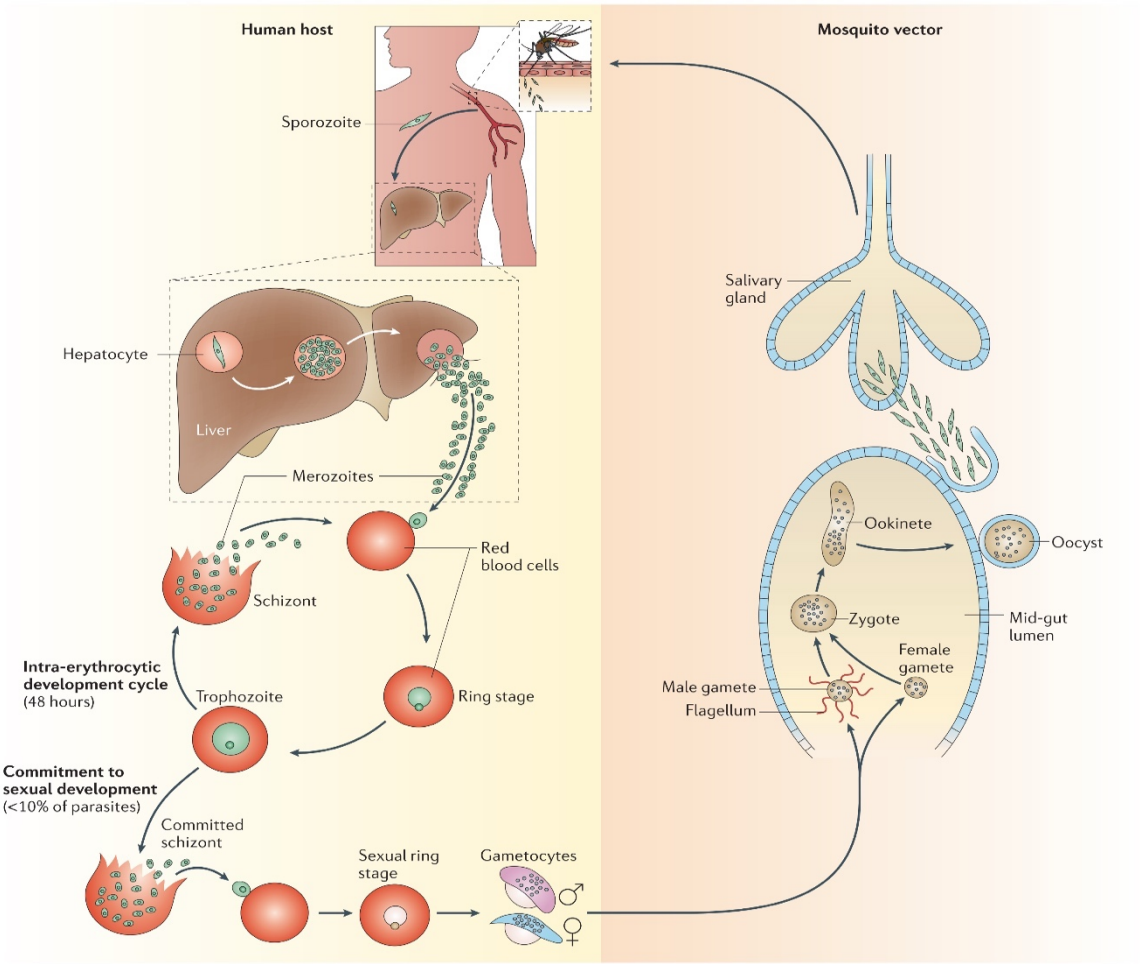


Figure 2.1: The asexual blood stages and sexual vector stages of *Plasmodium falciparum* development, in the human host and mosquito vector respectively, illustrating the importance of both stages for effective *Plasmodium* transmission (74).

2.3 Insect microbiota

Most animals harbour a diversity of microbes within their bodies (81). These microorganisms can be broadly classified into two categories: endosymbiotic and exosymbiotic.

Classical endosymbionts have a variety of effects on their insect hosts ranging from embryonic male-killing, slowed down larval and pupal development, reduced blood feeding, cytoplasmic incompatibility to mosquito sterility (30,82–84). *Wolbachia* and *Spiroplasma* are the two most widely studied endosymbionts whose prevalence in insect population is approximately 40% and 5-10%, respectively (82,85,86). These facultative

endosymbionts are known to confer protection against parasites and pathogens therefore making them good candidates for vector control (87–91).

Currently, *Wolbachia* is being used to control populations of *Aedes* mosquitoes that vector RNA viruses such as Zika, Chikungunya, Dengue, and several others (92,93). A study on *A. coluzii* (a major vector of malaria in West Africa) demonstrated the protective nature of *Wolbachia* against *Plasmodium* (83). Studies have artificially introduced *Wolbachia* strains (*w*AlbB and *w*MelPop) in *Anopheles gambiae* showing significant malaria parasite downregulation (82,85). Currently, none of the *Wolbachia*-infected *Anopheles* lines exhibit stable transmission of *Wolbachia* hence are not yet useful in the context of transmission blocking.

On the other hand, exosymbionts, which include a wide range of gut microbiota, are generally found in the insect's gut, reproductive tract and cuticle. It is increasingly being appreciated that many symbionts have complex life-cycles that involve extracellular and intracellular phases. The effects of this type of symbiont on host biology are not well understood.

Microsporidia is an example of such symbionts (94–96) classified as either endosymbiotic or exosymbiotic since it can be transmitted transovarially or horizontally through spores (95).

2.4 *Microsporidia*

Microsporidia are characterised as obligate intracellular eukaryotes which are widely thought to belong to the fungal kingdom (94,97). They are uniquely distinguished via the morphology of their spore which features a long, coiled polar filament used to inject the sporoplasm into the host cell during germination. This feature of the *Microsporidia* is key to their success as it enables them to infect a huge range of organisms (27,94).

Classification criteria of *Microsporidia* includes the following: nuclear condition (diplokaryotic and uninucleate), polar filament thickness and the number of coils in the filament around the periphery of the spore. However, these classification criteria cannot be used for higher levels of taxonomical classification such as species and genus since they are highly variable at these levels of classification (97). Therefore, the better mode of taxonomical classification is based on habitat and host. A comparative study of the phylogenies of 125 different *Microsporidia*n species (whose ssrDNA sequences are relatively complete) done (97) using neighbour-joining and maximum parsimony revealed that *Microsporidia* is grouped into 5 major groups/ clades (Figure 2.2) with three distinct classes- Aquasporidia from freshwater organisms, Marinosporidia isolated from marine hosts and Terresporidia from land (97). Within this classification, *Microsporidia* were also noted to have varying levels of co-evolution with their respective hosts (97).

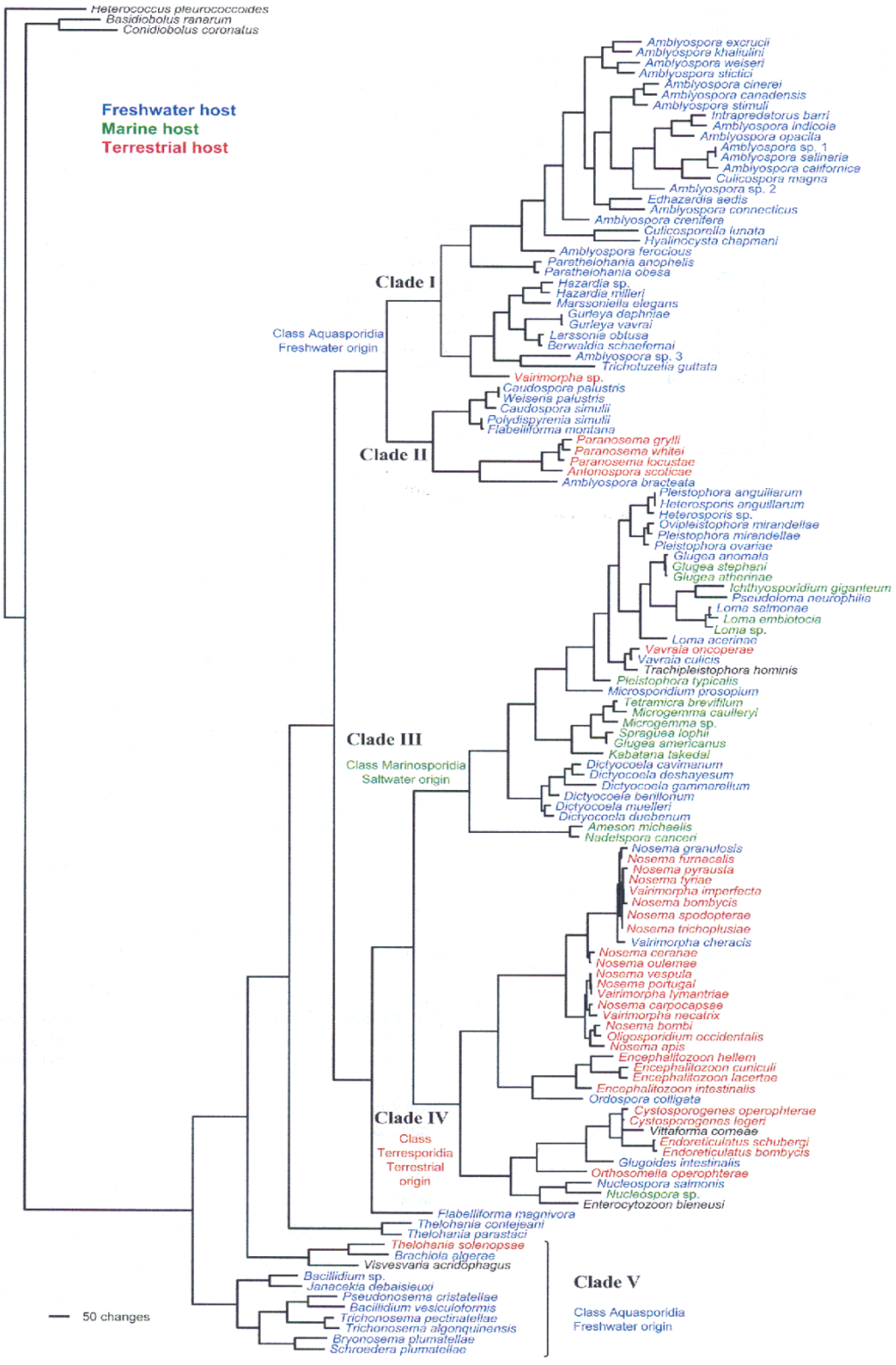


Figure 2.2: Phylogenetic classification of phylum *Microsporidia* highlighting the five major clades and three classes (*Aquasporidia*, *Marinosporidia* and *Terresporidia*) (97).

Microsporidia are lower eukaryotic organisms lacking mitochondria (98). The spore is the infective stage of this microorganism and is well-adapted with highly-developed injection apparatus suitable for host penetration (97). There are two classical ways for its dissemination in the natural environment: horizontal and transovarial (Figure 2.3).

Horizontal transmission takes place when the spores are ingested and germinate in the gut of the host and the sporoplasm (the infective germ) is injected into the host cell through the polar tube (Figure 2.3). On the other hand, transovarial transmission takes place when the spore germinates on the periphery or inside the ovaries and the infective germ injects itself inside the developing egg (99).

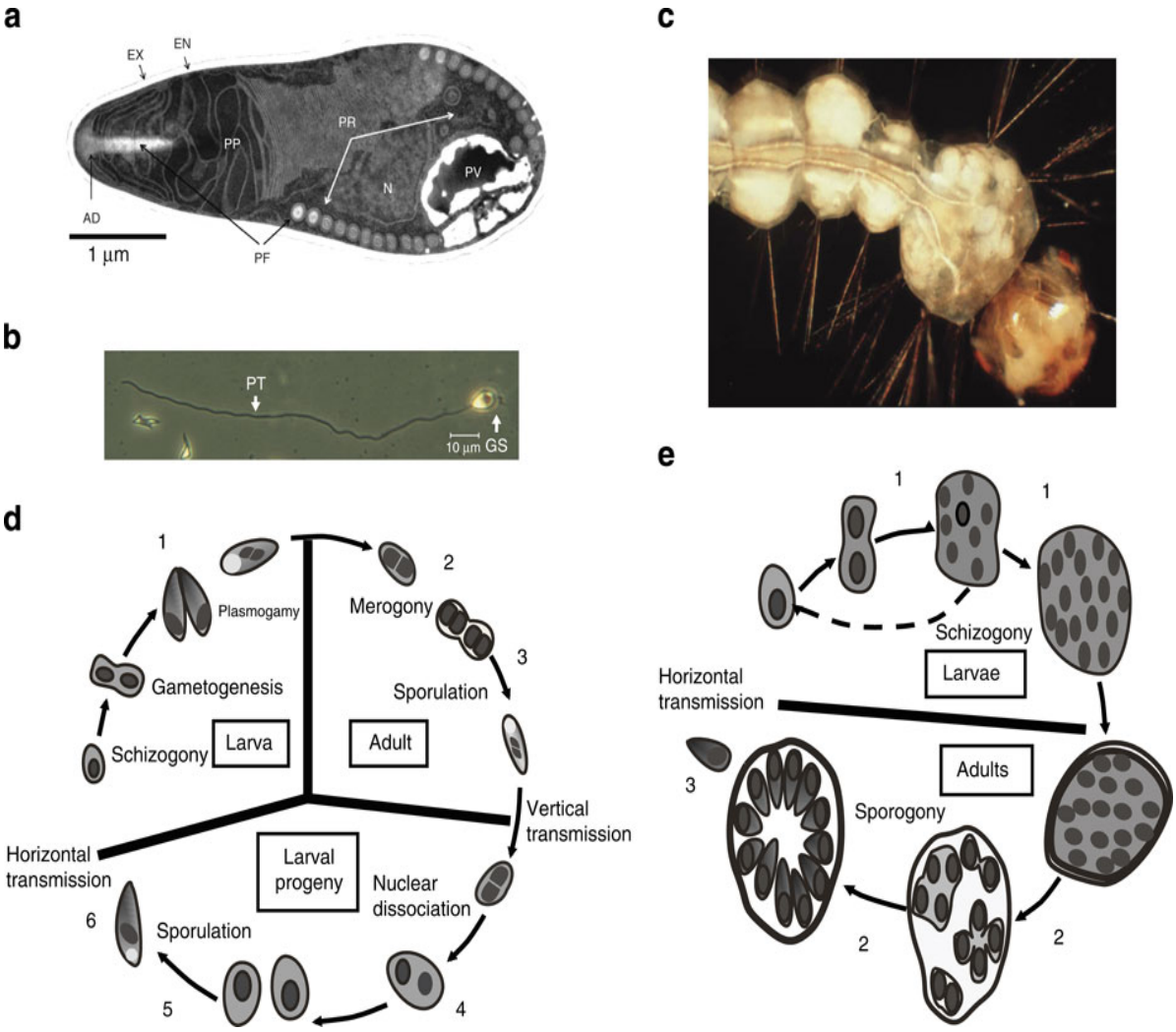


Figure 2.3: Biology of the *Microsporidia*n spore. a) An electron microscopy image of a mature *Microsporidia*n spore illustrating the unique microinjection apparatus containing an anchoring disk (AD) at one end, a polaroplast (PP) and the posterior vacuole (PV). The nucleus (N) is centrally located in the spore and enveloped with polyribosomes (PR). Also demonstrated here is the characteristic thick cell wall composed of an exospore (EX) and an endospore (EN) containing chitin. b) The spore appears to germinate as illustrated using light microscopy. A polar tubule (PT) can be observed through which the poroplasm is injected into the host cell. c) *Microsporidia*-infected *Aedes aegypti* mosquito larvae in its fourth instar. This image illustrates *Edhazardia aedis* infection localized around the larvae's fat body tissue. d) Lifecycle of *E.aedis* demonstrating both vertical and horizontal transmission. e) The lifecycle of *V. culicis* which are transmitted horizontally. The spores are taken up from the environment when the larvae are feeding which then embed into the midgut wall thereby infecting the host as it develops from larvae to adult. Consequently, *V. culicis* produces spores to be released into the environment (99).

2.4.1 *Microsporidia* of the mosquito

There are two broad categories of *Microsporidia*n species infecting mosquitoes and their classification is based on their lifecycle and host-parasite interaction- the simple unispore and the complex multispore categories (27). The simple unispore primarily depends on horizontal transmission and hence infects only one generation of mosquitoes and is non-host specific. Examples include *V. culicis* and *Nosema algerae.* In contrast, the complex multispore transmission relies on both horizontal and transovarial transmission. This ensures that it is effectively transmitted across two generations of mosquitoes with the assistance of intermediate hosts such as copepods in some *Microsporidia*n species. Contrary to the previous group, it is host and tissue specific. Examples in this category include; *Edhazardia aedis (Aedis aegypti)* and *Amblyospora connecticus* (*Aedes cantator)* (29, 30).

Figure 2.4 highlights some important *Microsporidia*n parasites isolated from various mosquito species. A study by Andreadis and colleagues (100) suggested that the *Microsporidia*n species infecting mosquitoes have largely co-speciated and they demostrated high host specificity levels especially within the *Amblyospora* group (Figure 2.4)**.** *Microsporidia* species associated with the *Ochleratus/Aedes* mosquitoes are phylogenetically set apart as monophyletic groups while those infecting the *Anopheles* species have been shown to cluster as a sister group to those affecting the culicine mosquitoes (100). *Anopheles* and *Aedes* have been shown to host two or more *Microsporidia*n species of shared ancestry, illustrating that host-switching is possible (100).

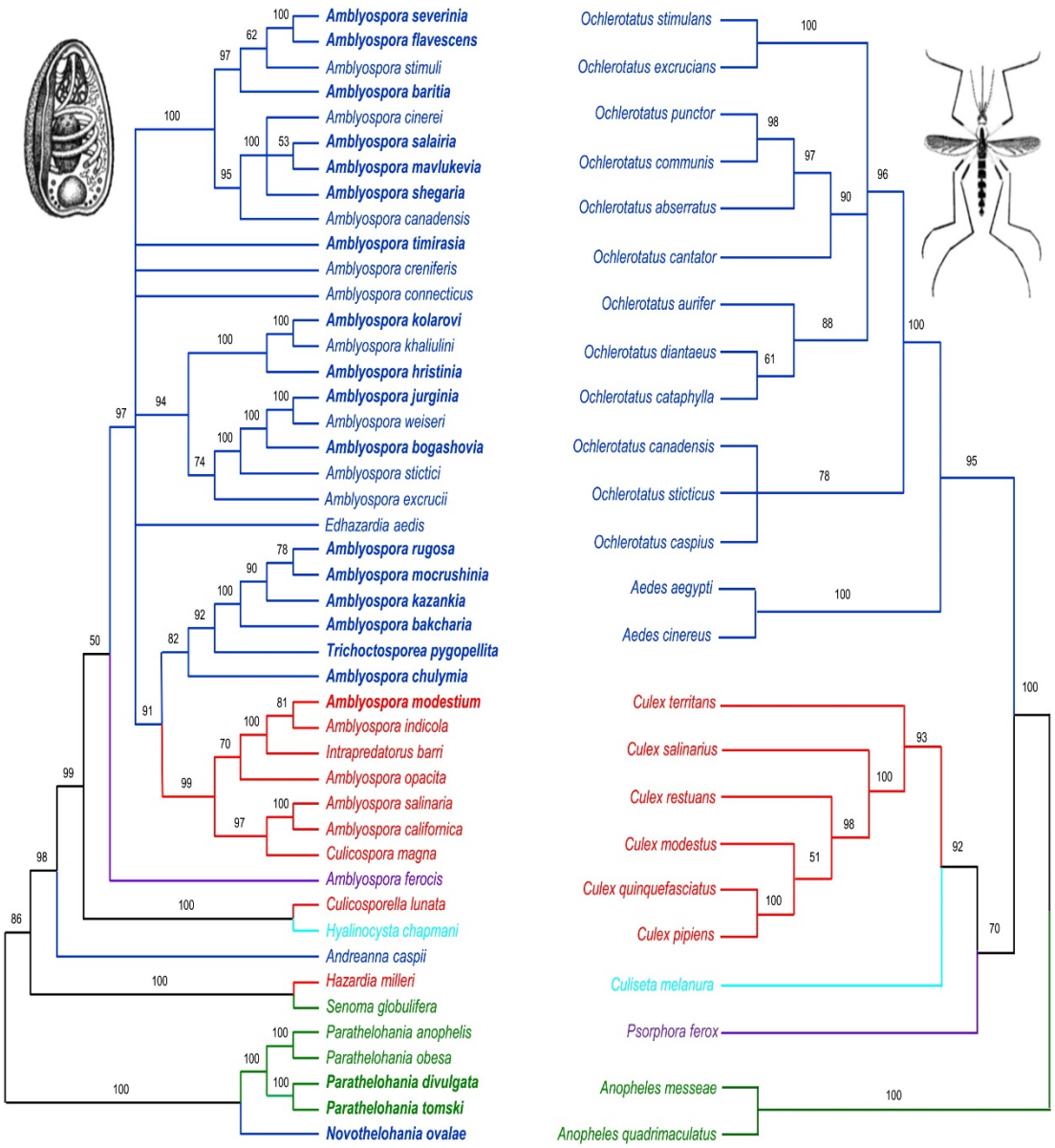


Figure 2.4: *Microsporidia*n species isolated from different mosquito species. *Microsporidia* color-coded based on respective host (100). In blue are those generally isolated from the *Ochleratus* species, red represents *Microsporidia* found in *Culex,* while in green are those affecting the *Anopheles* species.

2.4.2 *Microsporidia* as potential biological vector control agents

Microbes harboured by mosquitos have important consequences for the successful transmission of *Plasmodium*. Several environmental factors affect the microbial population within the mosquito. Notably, different microbes can either enhance or reduce *Plasmodium* transmission capacity of *Anopheles* mosquitoes (24,29–31,101).

Studies have observed that *Microsporidia* infections in mosquitoes have a range of both positive and negative effects on their host (25–27,33,99,102,103). They can affect their hosts directly by causing larval and adult mortality or by resulting in reduced host fitness, survival and fecundity. Simple unispore (monosporogenic) *Microsporidia*n species such as *V. culicis* mainly affect the reproductive capacity and lifespan of adults while having very little mortality in the larvae. *V. culicis*, for example, prevents malaria development in the mosquito by priming the host's immune system (25) and is able to protect its host from other pathogens (99,104). The complex multispore/polymorphic *Microsporidia* such as *E. aedis* also lower the reproductive capacity in their host by reducing female longevity and fecundity (26,105).

Microsporidia -infected mosquitoes have been shown to have an altered blood-feeding behaviour. *Edhazardia aedis* infecting *Aedis aegypti* mosquitoes (34) was shown to increase its host's blood-feeding rate if infected with the vertically transmitted binucleate spores. This would theoretically help to augment the microorganism's vertical transmission to the host's progeny. Interestingly, the horizontally transmitted uninucleate spores of *Edhazardia aedis* reduced the mosquitoes' blood meal uptake (34).

Competition for nutrients between the *Microsporidia* and *Plasmodium* parasite within the gut epithelial tissue is thought to reduce *Plasmodium* fitness and hinder its development, which could prevent the transmission of the malaria parasite (25,39).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling sites and sample collection

The main sampling sites used in this study were areas within Mwea (Central Kenya) and Mbita (Western Kenya), which have been shown from previous studies to have high prevalence of *Anopheles* mosquitoes (106–108).

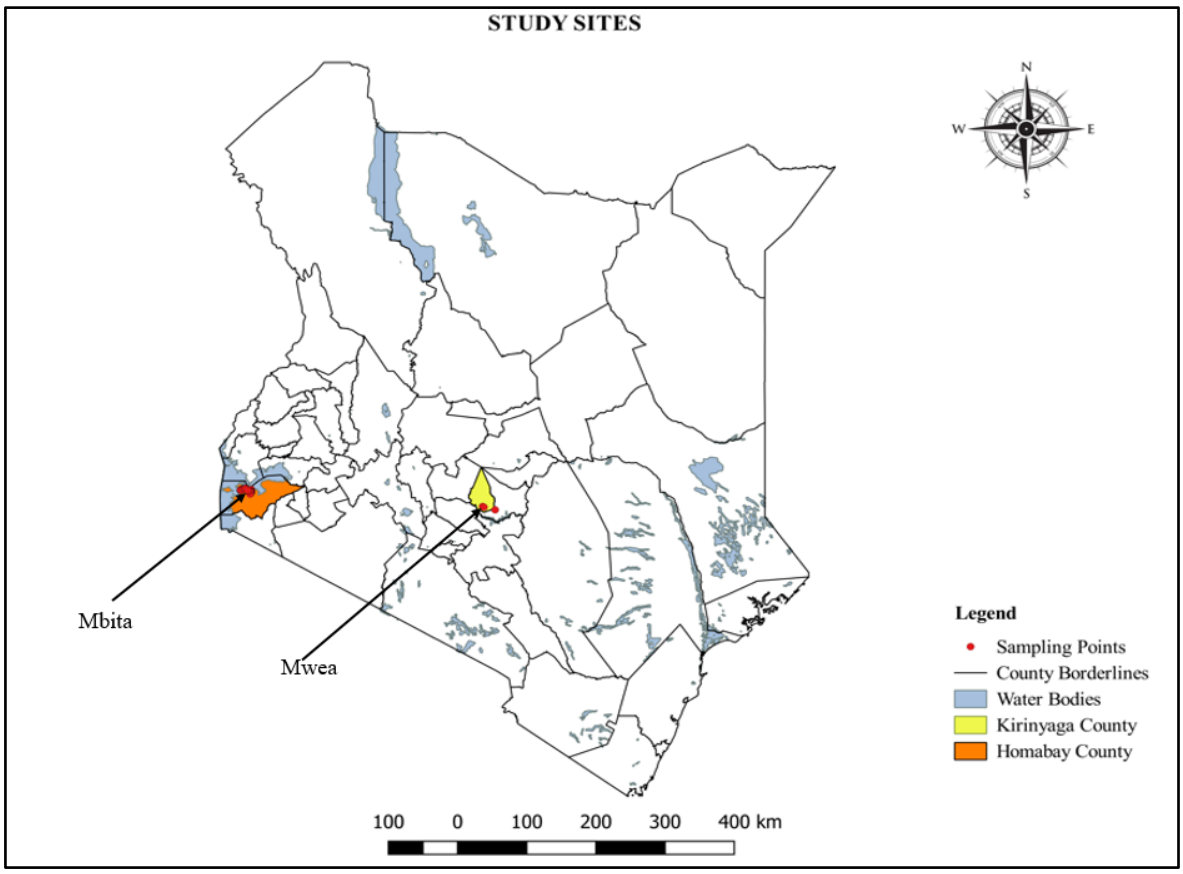


Figure 3.1 Map illustrating the two sampling sites- Mwea and Mbita.

Wild-caught adult mosquitoes were collected from various areas around Mbita (Nyawiya, Mageta and Kirindo) and Mwea (Mbui-Njiru and Karima) using Cattle-Baited Traps (CBT) and Centre for Disease Control (CDC) light traps. Indoor mosquito collection was done using aspirators and pyrethrum spray catches. The adult mosquitoes were then stored in 1.5 ml Eppendorf® tubes and transported in dry ice to ICIPE Duduville Campus, Nairobi. In contrast, the larvae were transported in water collected in well-ventilated specimen bottles and bags.

3.1.1 Mosquito rearing

Larvae collected from the field were transferred into water baths containing double-distilled water and reared in a separate room away from the adults under controlled laboratory conditions of 30.5 °C and 30% humidity. Larvae were maintained on a daily diet of TetraMin™ baby fish food and fresh double-distilled water added into their tubs every day to maintain oxygen levels.

Adult mosquitoes were reared at 28 °C and 70% humidity and day and night times manipulated by providing 12 hours of both white and red light, respectively. The adult mosquitoes were fed on 6% glucose soaked in cotton wool. To maintain colonies at the insectary, female mosquitoes were blood-fed using blood from laboratory mice. This was done by placing the donor's exposed forearm in the mosquito cages and allowing the mosquitoes to feed for an hour.

3.1.2 Mosquito membrane-feeding assays

Plasmodium screening of human subjects was done in areas around Mbita using Rapid Diagnostic Test (RDT) kits. Microscopy was further done on the RDT positive samples. Blood samples collected from malaria infected donors were used for the membrane feeding assays. The blood used was mixed with anticoagulant (heparin). Five hundred microliters of this blood was put into each of the mosquito minifeeders. The top of the minifeeder was then covered with a stretched out parafilm to enable easier injection of the proboscis during feeding. The feeder was then inverted and placed on top of the mosquito cages. To simulate normal human body temperature, 100 ml water at 37 °C was placed on the feeder (109). Fully engorged females were separated from the unfed ones after 30 minutes of feeding. These mosquitoes were then reared for a period of 7-11 days post-infection and dissected to check for oocysts.

3.2 Isolation and molecular characterisation of *Microsporidia MB*

3.2.1 DNA extraction

High-quality DNA was extracted using the protein precipitation technique. Extraction reagents were prepared in the following concentrations: cell lysis buffer (5 mM EDTA, 0.5% Sodium Dodecyl Sulphate (SDS) and 10 mM Tris (pH 8.0)), protein precipitation solution

(8 M Ammonium acetate and 1 mM EDTA), molecular-grade isopropanol (99.8%), 70% ice-cold ethanol and de-ionised water for DNA resuspension.

Each mosquito sample was placed in a 1.5 ml Eppendorf® tube with four 0.5mm extraction beads and 50 µl of 1× PBS. Bead beating was done for 15 seconds using the BioSpec® Mini-Beadbeater-16. Two hundred and fifty microliters of cell lysis buffer was then added to the crushed samples on ice. This was followed by incubation on an Eppendorf® dry bath incubator at 65 °C for 15 minutes. After incubation, 100 µl of protein precipitation solution was added to the solution and vortexed in three 10-second pulses, followed by a 5-minute incubation on ice with subsequent centrifugation at 16400 rpm at 4 °C for 5 minutes. The supernatant was then carefully pipetted into a clean pre-labelled 1.5 ml Eppendorf® tube and 300 µl isopropanol added to this. Mixing was done by inverting 100 times and the solution centrifuged at 16400 rpm at 4 °C for 1 hour.

This was followed by disposing of the supernatant and addition of 300 µl of ice-cold 70% ethanol in each tube. Mixing was also done by inverting 100 times and the samples were centrifuged for 30 minutes at 16400 rpm speed at 4 °C. Finally, 70% ethanol was discarded and the tubes left inverted overnight to allow for air-drying. The following day, each DNA pellet was dissolved in 50 µl of distilled water and left to dissolve at 4 °C overnight.

3.2.2 Primer design

Primers and probes for *Microsporidia* PCR assays were designed using Geneious Software v11. *Microsporidia* primers were designed to target the highly conserved 18S Small Sub-unit (SSU) rRNA and RNA polymerase β (*rpoB)* genes of the microorganisms (Appendix 1**)**. These primers were then checked for hairpin formation and primer dimers using the OligoAnalyser 3.1 online tool within the Intergrated DNA technologies website (https://eu.idtdna.com/pages) (110). Designed primers specificity was confirmed using the NCBI Primer-BLAST tool [https://www.ncbi.nlm.nih.gov/tools/primer-blast/] (111) and synthesized at Macrogen, Inc. , South Korea.

3.2.3 Optimisation of PCR conditions

PCR amplification conditions were optimised via Veriflex PCR amplification of controls using the Applied Biosystems SimpliAmp™ Thermocycler. To determine the specificity of the *Microsporidia MB* primers, these were tested on a variety of *Microsporidia* species

controls (*Hazardia, Parathelohania, Takaokaspora* and *Crispospora*) across six different annealing temperatures (50 °C, 52 °C, 54 °C, 56 °C, 58 °C and 60 °C). This was followed by gel electrophoresis to confirm amplification and determine the optimal primer annealing temperature.

3.2.4 Polymerase Chain Reaction (PCR)

Mosquito Species Identification

Primers for the identification of host species were adapted from a study by Santolamazza and colleagues (112) targeting the SINE S200 X6.1 locus to distinguish between the *Anopheles gambiae* species; *gambiae s.s.* and *arabiensis*. This was done via standard PCR on the Applied Biosystems® SimpliAmp™ Thermal Cycler. A 10 µl reaction volume was prepared consisting of 2 µl Solis BioDyne FIREPol® Master Mix Ready to Load (Mix Composition: 12.5 mM magnesium chloride, 1 mM of each dNTP, blue and yellow dyes, FIREPol® DNA polymerase and 5× reaction buffer containing 0.4 M Tris-HCl, 0.1 M ammonium sulphate and 0.1% w/v Tween-20), 0.5 µl of 10 pmol/µl of both forward and reverse primers (Appendix 1), 1 µl of the template and topped up to 10 µl with UltraPure™ nuclease-free distilled water (Invitrogen, UK). The PCR cyclic conditions used were: initial denaturation at 95 °C for 15 minutes, further denaturation at 95 °C for 30 seconds, followed by annealing at 58 °C for 45 seconds and extension at 72 °C for 45 seconds all done for 45 cycles. Final elongation was done at 72 °C for 5 minutes.

Microsporidia MB detection

Standard qualitative amplifications were done to screen for *Microsporidia MB.* The 490F/CRISPGEN primer set was used for this assay (Appendix 1). A 10 µl reaction volume was prepared consisting of: 2 µl HOTFirepol® Blend Mastermix Ready-To-Load (Mix composition: 7.5 mM magnesium chloride, 2 mM of each dNTPs, HOT FIREPol® DNA polymerase, proofreading enzyme, BSA, blue and yellow dyes that increase sample density during gel loading), 0.5 µl of 10 picomoles/µl of each primer (forward/reverse primers), 1µl of the template and topped off with 6 µl UltraPure™ nuclease-free distilled water (Invitrogen, UK). PCR cyclic conditions used were: initial denaturation at 95 °C for 15 minutes, further denaturation at 95 °C for 30 seconds, followed by annealing at 59 °C for 45 seconds and extension at 72 °C for 45 seconds all done for 38 cycles. Final elongation was

done at 72 °C for 7 minutes. Thereafter, agarose gel electrophoresis was used to resolve PCR amplicons.

3.2.5 Gel electrophoresis

Agarose gel electrophoresis technique was used for the visualization of PCR products. A 2% gel was prepared with 2 g agarose in 100 ml of 1× Tris-Acetate-EDTA (TAE) buffer. Preparation of 50× TAE buffer stock solution was prepared by adding 242 g of Tris base and 18.6 g disodium EDTA in 600 ml distilled water which was then followed by the addition of 57 ml glacial acetic acid, filling up to the 1 L mark with more distilled water. The agarose gel solution was microwaved until it completely dissolved, and the solution was clear. Ten percent of ethidium bromide (0.5 µg/mL) was stirred into the agarose mixture. This solution was poured onto a pre-levelled mounting tray loaded with the desired well-combs for solidification at room temperature (approximately 15 minutes). The first wells in each row were loaded with 5 µl of 1Kb plus Invitrogen™ DNA ladder. Five microliters of the amplicons were then loaded into each of the remaining wells. Electrophoresis was conducted on a Thermo Fisher Geltable, and allowed to run for 45 min (90V, 300 Amps). DNA bands were visualized using VWR® Genoplex gel documentation system with GenoCapture and Genosoft 4.0 software.

3.2.6 Purification of PCR products for sequencing

Microsporidia MB positive samples were selected for Sanger sequencing. Cleaning of the amplicons was done using the USB® ExoSAP-IT® PCR Product Cleanup kit. The ExoSAP-IT® reagent was directly added to the amplicon in a ratio of 2:5, respectively. Purification protocol was adapted from the kit. Briefly, to purify 20 µl of the PCR products, 8 µl of the ExoSAP-IT® reagent was added, making a total volume of 28 µl. Incubation was then done at 37 °C for 15 minutes to degrade remaining nucleotides and primers in the amplicon. Further incubation was done at 80 °C to inactivate the ExoSAP-IT® reagent. The purified amplicons were then sent to Macrogen Inc Company (Amsterdam) for Sanger sequencing.

3.3 Tissue Tropism using Fluorescence *in situ* Hybridisation (FISH)

Dissection of adults' gut was done in Phosphate Buffered Saline (PBS) solution 5-days post emergence and immediately transferred into a tube containing 4% Paraformaldehyde (PFA) solution and fixed overnight at 4 °C. Samples were rinsed in PBS and then transferred into

6% hydrogen peroxide in ethanol for 72 hours at 4 °C. This was followed by incubation in a hybridization solution containing: 25% 20× Saline Sodium Citrate (SSC), 1.2% Phosphate Buffered Saline- Tween20 (PBST), 2.5% sonicated salmon sperm, 1% Denhardt's solution (50×), 50% formamide and 200 µg/100 µl of *Microsporidia* -specific probe (10 µl). Activation of the probe in the RNA hybridisation was done at 100 °C for 5 minutes. The activated probe solution was added to the sample and left overnight in an incubation chamber at 60 °C.

The hybridised sample were then placed on a slide containing a drop of Syto 9® green fluorescent nucleic acid stain (ThermoFisher Scientific, MA, USA) and were visualised immediately using a Leica DM 500B confocal microscope (Leica Microsystems Inc., Illinois, USA). Imaging was done in the green and red channels to allow discrete visualization of the bound probe. Images were analysed with ImageJ (NIH, USA).

3.4 Correlating the density of infection of *Microsporidia* and *Plasmodium falciparum* in Anopheline mosquitoes

A *Microsporidia* probe-based qPCR assay was developed to investigate the levels of *Microsporidia MB* infection. Additionally, quantitative *Plasmodium falciparum* sporozoite ELISAs were simultaneously conducted to establish *Plasmodium* density within the vector.

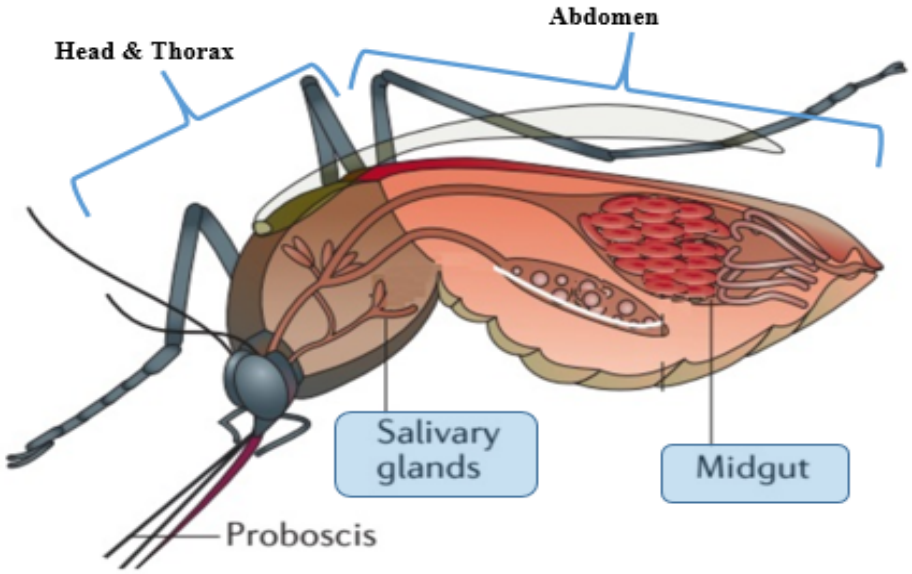


Figure 3.2: A diagrammatic representation of the internal anatomy of the mosquito (adapted from 113) highlighting tissues of importance during infection by microorganisms- the midgut and salivary glands. The head and thorax were used for ELISAs while abdomen used for *Microsporidia MB* molecular assays.

3.4.1 *Microsporidia MB* Quantitative PCR Assay

Relative quantification of *Microsporidia MB* was done in relation to host gene quantity. These quantitative assays were done concurrently in the Rotor- Gene Q HRM real time PCR thermocycler machine (QIAGEN, Hannover, Germany).

Quantitative PCRs were optimized as follows: host gene primers targeting the ribosomal S7 protein were used as published (115). Amplification efficiency was determined using clean unexposed mosquitoes from the Duduville insectary (both *gambiae* and *arabiensis*) which were extracted using the protein precipitation technique previously described in section 3.2.1. DNA quantity and quality from each sample was assessed using the Thermo Scientific™ NanoDrop™ 2000c Spectrophotometer. Two standards were prepared for both *gambiae* and *arabiensis*, by decreasing their individual DNA concentrations to 5ng/µl using nuclease-free water. Serial dilutions of each sample were then done up to the sixth dilution. Calibration runs were then done to check efficiency. The qPCR mastermix was composed of 2 µl HOT FIREPol® EvaGreen® HRM no ROX Mix (Mix Composition: 12.5 mM Magnesium chloride, EvaGreen® dye, BSA, dNTPs, HOT FIREPol® DNA Polymerase and 5× EvaGreen® HRM buffer), 0.5 µl of 5 pmol/µl of both forward (S7F) and reverse (S7R) primers (Appendix 1), 2 µl of the template and topped up to 10 µl UltraPure™ nuclease-free distilled water (Invitrogen, UK). The PCR profile for host gene amplification included an initial denaturation at 95 °C for 15 minutes, further denaturation at 95 °C for 30 seconds, followed by annealing at 61 °C for 45 seconds and extension at 72 °C for 45 seconds repeated for 40 cycles. Final elongation was performed at 72 °C for 7 minutes. After which a melt curve was generated including temperature ranges from 65 °C to 95 °C.

Similar optimization procedures were followed on the *Microsporidia MB* infected samples and tested on the probe-based quantitative PCR assay. Each primer-set had a specific probe labelled with a quencher (TAMRA) and reporter (FAM) (Appendix 1**)**. Optimal qPCR conditions were adapted as follows: a 10 µl reaction volume was made consisting of 2 µl Solis BioDyne 5× HOT FIREPol® Probe Universal qPCR Mix (Mix Composition: 15 mM

magnesium chloride, dNTPs, HOT FIREPol® DNA polymerase and 5× HOT FIREPol® Probe Universal qPCR buffer), 0.5 µl of 5 pmol/µl of both forward (490F) and reverse (CRISPGEN) primers (Appendix 1), 0.1 µl of 5 pmol/µl of the CRISPGEN probe, 2 µl of the DNA template and topped up to 10 µl with UltraPure™ nuclease-free distilled water (Invitrogen, UK). PCR thermocycling conditions used were: initial denaturation at 95 °C for 15 minutes, denaturation at 95 °C for 30 seconds, followed by annealing at 61 °C for 30 seconds and extension at 72 °C for 45 seconds all done for 40 cycles. A quantitative melt curve was generated simultaneously during the cyclic extension step including temperature ranges from 65 °C to 95 °C. Final elongation step was performed at 72 °C for 7 minutes.

3.4.2 *Plasmodium* Sporozoite ELISA

Sporozoite ELISA assays were conducted on freshly sectioned head and thorax of mosquito samples. ELISA protocol was adapted from (116) with slight modifications. Fresh reagents were prepared prior to the experiment. Each sample was homogenized in 50 µl blocking buffer: NP40 (1:1) solution in a 1.5 ml Eppendorf® tube using a pestle after one-hour incubation in this solution. Ground samples were then placed in -20 °C overnight. ELISA plates were coated with 50 µl of MAb capture antibodies (0.5 mg/ml Capture MAb Pf2A10-CDC). Dilution specifications for *Plasmodium falciparum* adapted from (116,117) and an overnight incubation step was performed at room temperature. After this incubation period, the MAb capture was removed and plates were dried by gently hitting on clean paper towels. Two hundred microliters of blocking buffer solution was pipetted into each well followed up by a one-hour incubation at room temperature. During this incubation, samples were removed from -20 °C and allowed to thaw at room temperature. Serial dilutions of the positive control were made using blocking buffer.

After one hour of incubation, the blocking buffer was discarded, and the plate dried by patting gently on clean paper towels at least five times. Thereafter, 50 µl of the samples were added to each of the wells. The first row of the plate was reserved for the serial-diluted *Plasmodium* positive control while the second row was set for the negative control (a clean insectary male mosquito ground up in blocking buffer: NP40 solution). This was followed by a 2-hour incubation period at room temperature to allow binding of the *Plasmodium* antigen to capture antibody. One hour into this incubation, substrate and conjugate solutions were prepared in separate tubes. ABTS® Substrate Component consisting of solutions A and B were mixed in a ratio of 1:1 to a final volume of 20 ml/plate. MAb peroxidase conjugate

(0.5 mg/ml Peroxidase Labelled Mouse Ab Pf2A10-CDC) was prepared in specified concentrations for *Plasmodium falciparum* at a concentration of 40 µl in 10 ml blocking buffer per plate. These solutions were then tested for efficiency by mixing 5 µl of the MAb peroxidase conjugate with 100 µl of the substrate solution. An immediate color change from clear to blue indicated that the solutions were working quite efficiently.

After the 2-hour incubation, solutions were discarded off into the sink and plates washed automatically using the BioTek® ELx50® ELISA washer, using specified conditions on a preset program (4 times wash using 200 µl PBS-Tween/well). After washing each plate, the aspirators were rinsed with distilled water to avoid cross contamination between plates. The plates were then inverted on clean paper towels at least 5 times and 100 µl of the MAb peroxidase conjugate added. An aluminium foil was used to wrap each ELISA plate. This was proceeded by a one-hour incubation in the dark at room temperature. Plates were then washed 4 times with PBS-Tween, dried on paper towels by inversion and 50 µl of substrate solution added. The plates were covered with clean aluminium foil, then finally incubated at room temperature for 30 minutes.

Finally, the plates were read at room temperature on the BioTek® ELx808® ELISA reader using Gen 5® Software. Absorbance was read at 405 nm and exported to an excel sheet for analysis. Qualitative cut-offs of absorbance was determined by calculating the mean negative from each plate plus three standard deviations (118). Optical Density (OD/Absorbance) correction was done on each plate by pooling all negative controls, calculation of the pooled negative mean and subtraction of this pooled mean from the mean negative per plate to obtain the specific correction value per plate. The unique correction value was then added/subtracted from all OD readings in each respective plate to normalize readings across plates. Standard curves of absorbance against sporozoite concentration were generated for each plate using the serially diluted positive controls. Quantification of samples was then established using the standard curve equation and their respective absorbance.

3.5 Ethical consideration

Ethical clearance (approval KEMRI/RES/7/3/1) was obtained prior to human blood sample collection. Parents and guardians of the children involved in this study signed written consent forms. Furthermore, consent was also obtained from heads of families to allow indoor mosquito collection (Appendix 3 and 4).

3.6 Data analysis

Data collected in this study was analyzed and figures generated using R-programming software version 3.3.3 (119). Results from standard PCR screening of the wild-caught samples for *Microsporidia MB* enabled the determination of its prevalence in the two sites. R software version 3.3.3 was used to compare the frequency of occurrence of *Microsporidia MB* infection in *Anopheles* between Mwea and Mbita regions of Central and Western Kenya, respectively.

All sequence analyses were done under the Geneious Software v11 (58, 59). Analyses involved trimming of sequences to increase quality and selecting the best quality sequences (that is, good sequence chromatograms with distinct peaks) for BLAST search on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Multiple sequences alignment was done using the Multiple Sequence Comparison by Log Expectation (MUSCLE) algorithm (113) alongside reference sequences of other *Microsporidia* species obtained from NCBI to determine similarity among the sequences (Appendix 2). Tamura-Nei genetic distance model alongside Neighbour-joining tree building algorithm was used in the creation of phylogenies and evaluated with 10000 replicates bootstrap support and 50% support threshold. Rooting was done using *Orthosomella operophterae* (Accession number: AJ302317.1) as an outgroup.

Plasmodium qualitative analysis cut-off was determined by mean negatives plus 3 standard deviations. Analysis was done per plate to ensure accuracy. Optical density reading corrections per plate were done using averaged pooled negatives to normalize values across plates. Standard curves were drawn using serial dilutions of the positive control thereby enabling sporozoite quantification.

Microsporidia MB relative quantification (this is the number of *Microsporidia MB* per host cell) was done versus host gene (endogenous control) using delta Ct values (difference in Ct of *Microsporidia MB* run and host gene run) which was further quantified as host Ct $^{(\text{host PCR efficiency})}$: *Microsporidia* Ct $^{(\textit{Microsporidia}\ \text{PCR efficiency})}$ ratio.

CHAPTER FOUR

RESULTS

4.1 Mosquito species identification

Identification of *Anopheles* sub-species using host gene primers targeting the SINE S200 X6.1 locus (120) as illustrated in Figure 4.1. A total of 805 mosquitoes were sampled from both sites. Seven hundred and thirty-one of this were confirmed to be of the *A. gambiae* complex. In Mbita, 513 *A. gambiae s.l.* were sampled. Out of this, 369 (71.9%) were *A. arabiensis* and 144 (28.1%) were *A. gambiae*. Moreover, a total of 218 *A. gambiae s.l.* were collected from Mwea, all of which were *A. arabiensis*. One hundred and four samples did not amplify with these *Anopheles gambiae s.l.* primers indicating that they were of a different species.

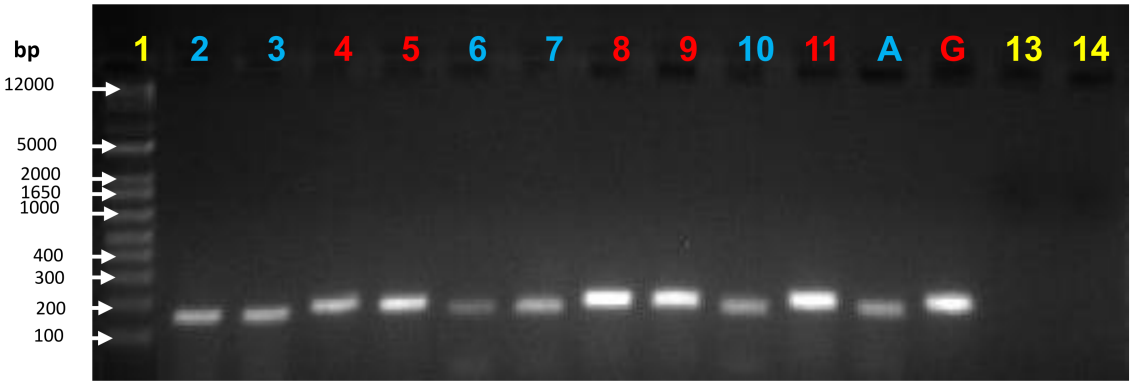


Figure 4.1: A gel representation of amplified products using *Anopheles gambiae* species-specific primers. Lane 1 represents TrackIt™ 1 Kb Plus DNA Ladder (Invitrogen™); lanes 2-11 represent the DNA samples; lane A is an *arabiensis* positive control (164 basepairs) while lane G is an *A. gambiae s.s.* control (194 basepairs). Samples labelled in blue were scored as *arabiensis* and those in red represent *gambiae* samples. Lanes 13 and 14 represent *A. funestus* control and no-template control (NTC), respectively.

4.2 *Microsporidia MB* prevalence in Mwea and Mbita, Kenya

A total prevalence of *Microsporidia MB* of 5% was demonstrated in both Mwea and Mbita (Table 1). A total of 731 *A. gambiae s.l.* samples were processed to determine the frequency of occurrence of *Microsporidia MB* in Mwea and Mbita; 218 and 513 respectively. In Mbita, 26 were *Microsporidia MB* positive whereas Mwea recorded a total of 11 positives.

Table 1: *Microsporidia MB* prevalence in both Mbita and Mwea. A comparison of the frequency of occurrence of *Microsporidia MB* mosquito infection between the two sites indicating a relatively similar prevalence in both sites.

Location	Total Number Infected	Total Number Sampled	Prevalence (%)
Mwea	11	218	5.04587156
Mbita	26	513	5.068226121
Total	37	731	5.061559508

4.3 Molecular phylogenetic analysis of *Microsporidia MB*

Primers targeting different flanking regions of the highly conserved 18S gene enabled the phylogenetic classification of the novel *Microsporidia MB* as shown in Figure 4.2. A BLAST search revealed that the amplified sequences were close hits to *Crispospora chironomi,* a *Microsporidia* isolated from non-biting midges in Siberia (121).

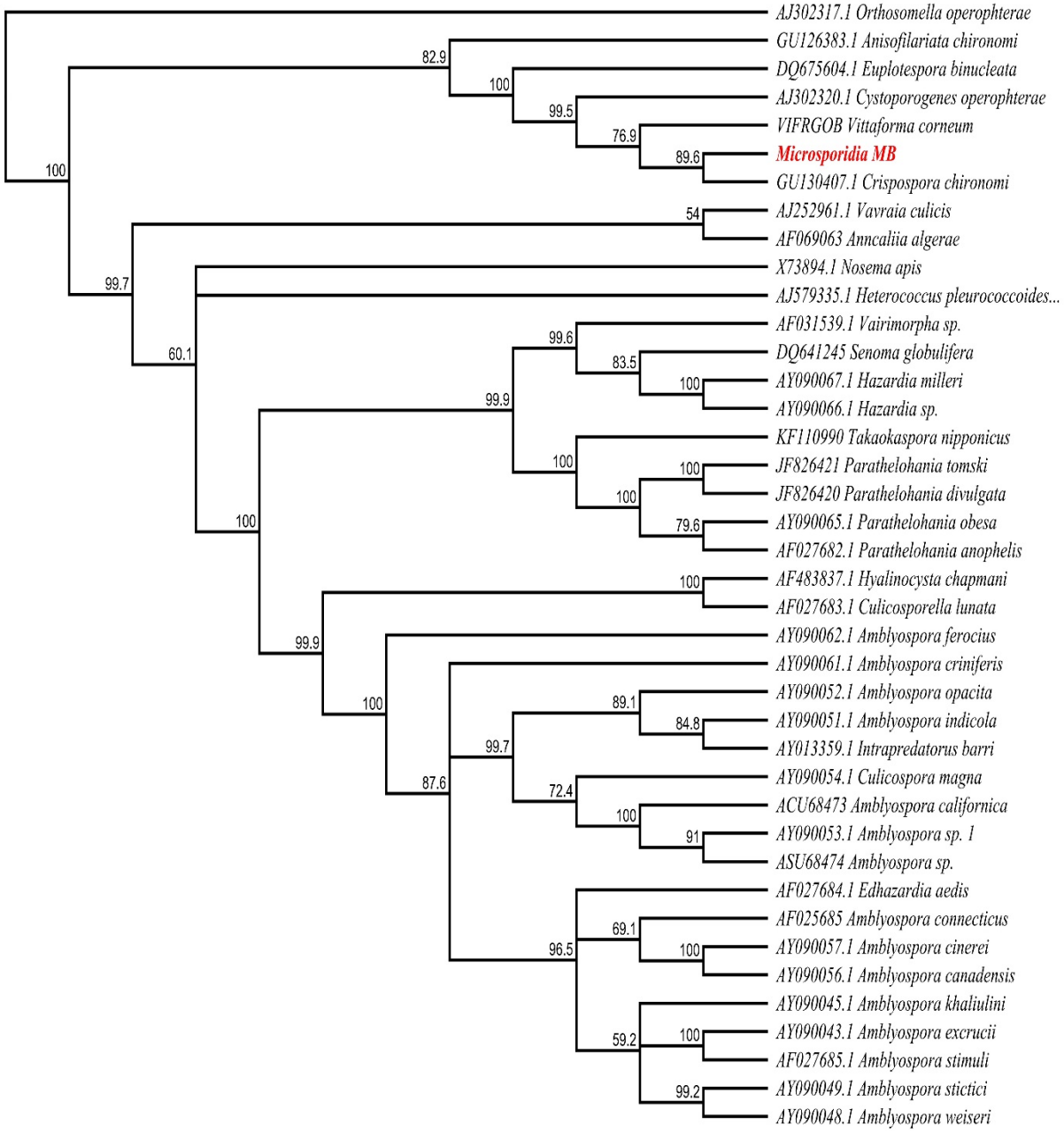


Figure 4.2: A neighbor-joining phylogenetic tree illustrating the close relationship between the novel isolated *Microsporidia*n microorganism (*Microsporidia MB*), seen here in red, and *Crispospora chironomi*. The numbers preceding the species identification denote the official NCBI accession numbers. Numbers on each node represent percent bootstrap values at 10000 replicates and 50% support threshold.

4.4 *Microsporidia MB* tissue tropism using Fluorescence *in situ* Hybridisation

This study demonstrated the stages of the spore maturation cycle of *Microsporidia MB* within the larval gut tissues as illustrated in Figure 4.3. In summary, figures (i) and (ii) illustrate the diplokaryotic stages of merogony development, figure (iii) highlights the capsulation of the spore and finally, figure (iv) is a representation of the mature spore ready to rupture. Rows (a) and (b) indicate images obtained from this study while row (c) show similar images from *Crispospora chironomi* spore development using Giemsa staining as viewed by Tokarev (121). The arrows indicate the direction of spore development.

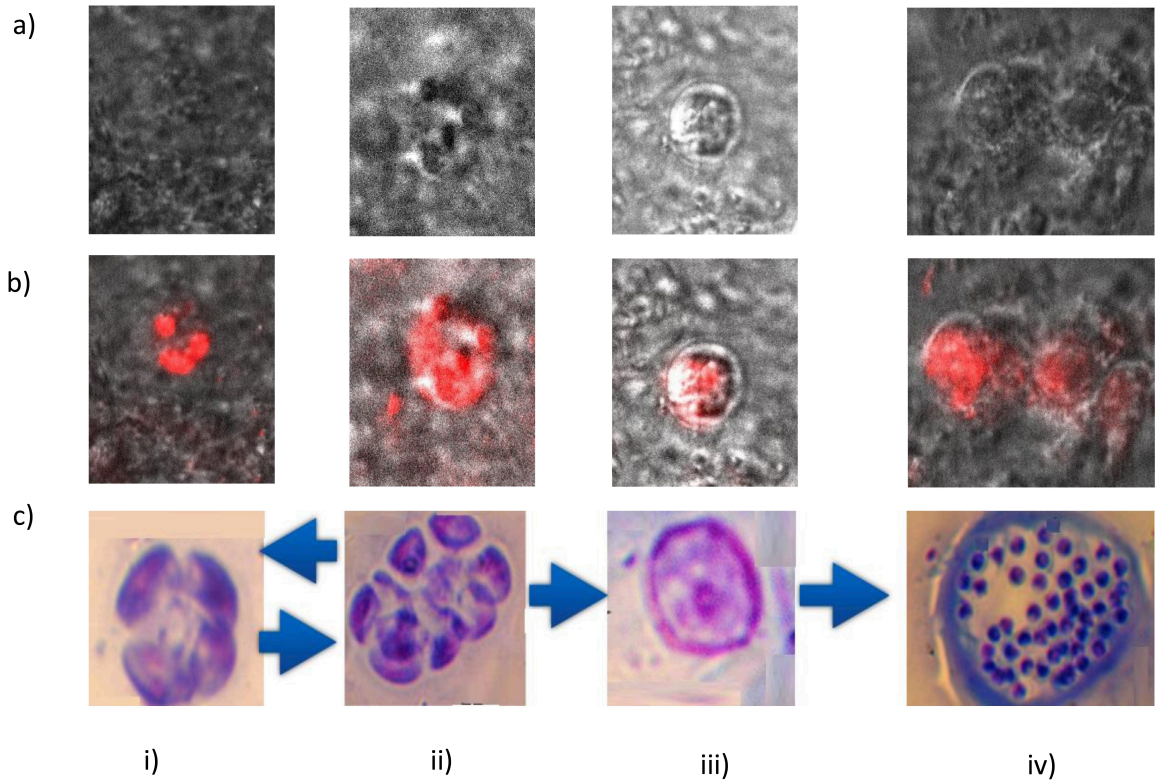


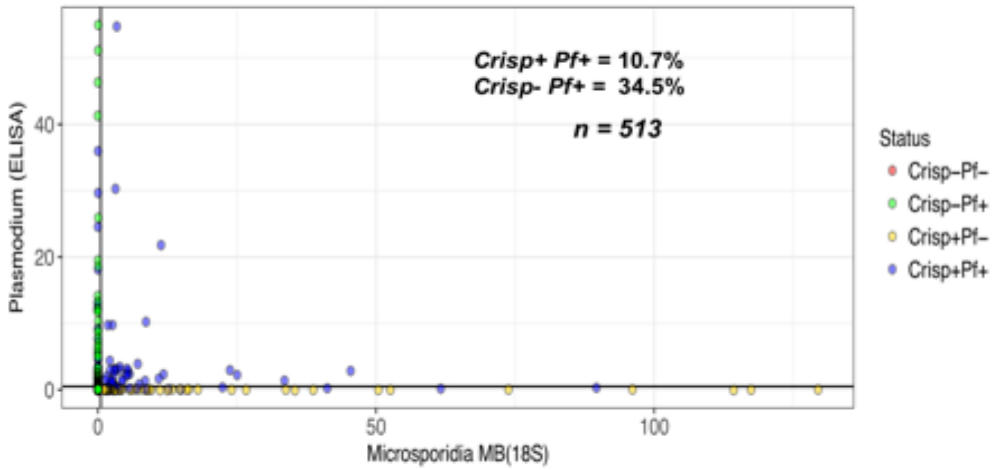
Figure 4.3: Fluorescence *in situ* Hybridization staining of the different lifecycle stages of *Microsporidia MB* isolated from mosquito gut tissues. a) Bright field microscopy images of the *Microsporidia MB* spore formation. b) An overlay of the red confocal microscope channel with the light-field channel. c) Images from Tokarev *et al.* 2010 corresponding to the *Microsporidia MB* spore images obtained in this study. i) & ii) The diplokaryotic stages of *Microsporidia* merogony. iii) Spore capsule formation. Distinct thick cell wall is illustrated. iv) The final mature spore stage.

4.5 Correlation of *Microsporidia MB* and the *Plasmodium* parasite in both wild-caught and lab-reared Anopheline mosquitoes

A negative correlation between *Microsporidia MB* and *Plasmodium* was demonstrated in both field and lab-reared *Anopheles* mosquitoes (Figure 4.4). In total 513 field and 1341 laboratory-reared samples were screened for both *Plasmodium* and *Microsporidia MB*.

Quantification data collected from both experiments plotted separately (*Plasmodium* versus *Microsporidia* density) revealed a consistent negative correlation (Figure 4.4 a and b). The number of field samples that were co-infected with *Microsporidia* and *Plasmodium* was 55 out of 513 (10.7%), whereas 177 samples out of the 513 were positive for *Plasmodium* and uninfected with *Microsporidia* (34.5%). In comparison, 2.1% of the insectary samples showed coinfection (29/1341), while 12.3% (165/1341) were *Plasmodium* positive and *Microsporidia* negative. Additionally a Spearman's Rank correlation test done in both tests indicated a negative correlation between *Microsporidia MB* and *Plasmodium falciparum* in both wild-caught (Spearman's correlation coefficient -0.008476 (p-value = 0.4275)) and insectary-reared (Spearman's correlation coefficient of -0.02592 (p-value= 0.2886)) mosquitoes.

a) Correlation of *Microsporidia MB* and *Plasmodium falciparum* densities in Wild-caught *A. gambiae s.l.* samples



b) Correlation of *Microsporidia MB* and *Plasmodium falciparum* densities in laboratory-reared *A. gambiae s.l.* samples

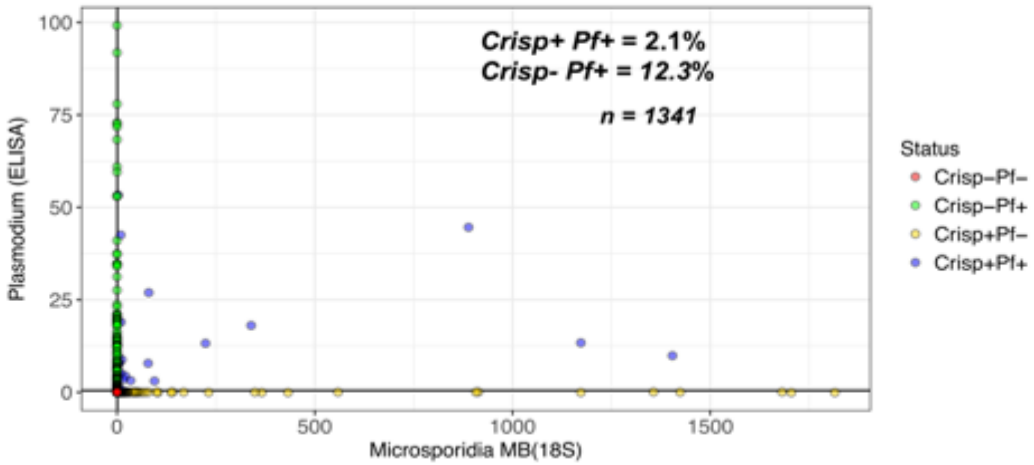


Figure 4.4: *Microsporidia MB* densities against *Plasmodium* infections in (a) field and (b) insectary samples. Co-infected samples are highlighted in blue and their relative abundance indicated in percent (*Crisp+Pf+).* The yellow data points represent *Microsporidia* positive samples that were *Plasmodium* negative *(Crisp+Pf-)* while the green indicate the *Plasmodium* positives that were *Microsporidia* negative *(Crisp-Pf+)*. The red dots on the other hand indicate samples that were negative for both microorganisms *(Crisp-Pf-)*. This correlation was done using the Spearman's rank correlation test. a) Illustrates the correlation of *Microsporidia MB* and *Plasmodium* sporozoite density in field-caught samples (n=513) indicating a negative correlation. b) Similarly demonstrates a negative correlation of *Microsporidia MB* and *Plasmodium* sporozoite density in laboratory-reared samples.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Vector biology has been a focal point in the control of malaria transmission in place of the already overwhelmed strategies. This has generated a thriving interest in microbiomes within the mosquito. Several microorganisms including *Wolbachia, Spiroplasma* and *Microsporidia* have been studied to this effect. A naturally-occurring *Wolbachia* strain, *wAnga-Mali,* was recently reported to have a refractory effect on *Plasmodium falciparum* (122). However, its mechanism of action is not yet fully understood. Moreover, a novel *Spiroplama* symbiont of *A. gambiae (Spiroplasma insolitum GAMB)* isolated from mosquitoes in Mwea and Mbita Kenya (123) showed a good prospect although further comprehensive research is needed.

On the other hand, there have been several reports of *Microsporidia* infecting *Anopheles* mosquitoes. *Nosema algerae,* a *Microsporidia*n species, has been suggested to reduce *Plasmodium* transmission levels in the vector *Anopheles stephensi* (35) by reducing adult life span. Another interesting *Microsporidia* in *Anopheles* is *Parathelohania anophelis,* a vertically transmitted male-killing microorganism (124). Nevertheless, these all belong to different clades of *Microsporidia* and are considered pathogens rather than inherited symbionts (27). Furthermore, a major shortcoming of these two *Microsporidia*n species as vector control candidates, is their reduced transmission rates and inability to maintain infections through generations (27). Notably, there has been diverse research on *Wolbachia* and *Spiroplasma* as compared to *Microsporidia.* In this regard, this scientific research aimed at identifying a new *Microsporidia*n strain that would meet these limitations as a potential biological vector control.

Therefore, this study reports the discovery of a native and novel *Microsporidia*n species (hereby termed *Microsporidia MB)* from *Anopheles* mosquitoes collected from Mwea and Mbita regions in Kenya. Additionally, it clearly outlines the prevalence of *Microsporidia MB* in these two regions and its effect on *Plasmodium falciparum* transmission. The discovery of *Microsporidia MB* in this study brings the total of *Microsporidia*n species isolated from *Anopheles* mosquitoes within these localities to four; including the *Parathelohania*-like*, Hazardia*-like and *Takaokaspora*-like strains, isolated and

characterised prior to this study using the 18S rDNA locus. Of the four *Microsporidia* strains isolated in these sites, *Microsporidia MB* was observed to be the most prevalent and therefore the focus of this research.

Previous literature has demonstrated the detection and molecular characterization of *Microsporidia* strains using the 18S rDNA locus resulting in reclassification of already existing *Microsporidia*n species, such as the recent renaming of *Nosema algerae* to *Brachiola algerae* (97). With reference to this, this study reported the effective design and development of optimal molecular-based assays thus enabling the fundamental understanding of the phylogeny of *Microsporidia MB*. The results from this study demonstrated the taxonomic classification of *Microsporidia MB,* placing this new *Microsporidia*n species within the same clade as *Crispospora chironomi*- a terrestrial *Microsporidia* isolated from *Chironomus plumosus,* a species of biting midges found in Siberia (121). A study of the biological structure of *Crispospora chironomi* by Tokarev *et al.* (121) demonstrated its different spore maturation stages. In line with this, FISH images of *Microsporidia MB* obtained in this study demonstrated similar spore maturation stages within the larval gut. This observation conforms to previous studies which have illustrated that larvae harbor the highly proliferative infective stages (spores) of the *Microsporidia* (27). The *Microsporidia MB* mature spore morphology was characterized by a thick capsular cell wall. Additionally, this experiment clearly demonstrated the diplokaryotic stages of the first and second merogony stages. These images suggest the polymorphic nature of *Microsporidia MB* via its diplosporoblastic and polysporoblastic forms similar to most terrestrial *Microsporidia* species in which *Crispospora chironomi* is also grouped (121).

This study furthermore suggests that *Microsporidia MB* is likely to be highly infectious, since a relatively high prevalence of infection is observed in the Mbita and Mwea samples. This concurs with previous reports which explain that the ubiquity and spore forming nature of *Microsporidia* strains enables them to be highly proliferative and adapt to varying environmental conditions (26,94,95). The similarity in prevalence of *Microsporidia MB* in these two areas indicate that the incidence of this microorganism is not solely dependent on geographical disparity and/or different ecological factors such as humidity, temperature and larval diet (81,82,104,125). Important to note, however, is the contrast in sample size between these two areas that could also play a role in the overall distribution.

Most importantly, this study illustrated that the presence of *Microsporidia MB* inhibits *Plasmodium falciparum* infection in *Anopheles gambiae s.l.* mosquitoes. Studies demonstrate that the insect's immune system is activated once they are infected by certain microorganisms, therefore rendering protection against other invasive parasites (82,84,126,127). Therefore, the inverse correlation between the two microorganisms could be caused by *Microsporidia MB* priming the host's immune system (25,104). Alternatively, *Microsporidia MB* could directly block *Plasmodium* invasion within the host's midgut thereby arresting *Plasmodium* development. This notion could be supported by the presence of *Microsporidia MB* infection within the gut tissue as demonstrated here by fluorescence microscopy. Besides this, nutritional competition might be another mechanism by which *Microsporidia MB* confers host protection against *Plasmodium.* Essential nutrients in the hosts, such as lipids, could be taken up by the rapidly reproducing *Microsporidia MB* thereby causing retarded growth of oocysts (30,128). All these possible mechanisms of action of *Microsporidia MB* need to be further studied to understand the nature of the protective phenotype.

5.2 CONCLUSION

This study developed a highly-specific molecular-based assay for the isolation and phylogenetic classification of the novel *Microsporidia MB*. Furthermore, fluorescence microscopy was used for visualization of the developmental stages of its infective spore. The observations made in this study altogether demonstrated the ability of *Microsporidia MB* to interfere with the fidelity of transmission of *Plasmodium falciparum* within the *Anopheles gambiae* mosquito.

5.3 RECOMMENDATIONS

i. Based on the interesting results obtained from this study, additional research needs to be done to fully understand the biology of *Microsporidia MB*. It would be of much interest to conduct whole genome sequencing to further improve the molecular taxonomic characterization of this species. Additionally, gene expression studies would be interesting to understand the host-microbe dynamics.

ii. Fluorescence *in situ* Hybridisation on whole body tissues of the infected larvae and adult mosquito, as outlined by Koga and colleagues (129), could also be effective in further unveiling the localisation of *Microsporidia MB* infection to determine the main reservoir tissues/organs. This is necessary for spore isolation and culturing of the *Microsporidia* to enable more molecular studies.

iii. Finally, the effect of co-infection of *Microsporidia MB* and other microorganisms within its host and how this affects the *Plasmodium* transmission blocking capabilities of this native species should be studied. Furthermore, there is need for more biological studies that carefully assess the effect of the host's environment on the host-parasite relationship dynamics within the field.

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APPENDICES

APPENDIX 1: Table 2 Primer details

Primer Name	Primer sequence (5' -> 3')	Expected product size (basepairs)	Gene target	Citation
S7F	TCCTGGAGCTGGAGATGAAC	153	Ribosomal S7 protein	(115)
S7R	GACGGGTCTGTACCTTCTGG	153	Ribosomal S7 protein	(115)
490F	GGACGAAGGCTGGAGTATCG	632	18S rRNA	This study
SS149RFORVITTA	CGCAACCTTGTTACGACTT	632	18S rRNA	This study
CRISPGENR (paired with 490F)	CCTGGTAAGTTTCCTCGCGT	222	18S rRNA	This study
CRISPGENPROBE	CCACTCCTTGTGTAGCTCCGT	222	18S rRNA	This study
forward	TCGCCTTAGACCTTGCGTTA	164 (*arabiensis)* and 194 (*gambiae*)	SINE S200 X6.1 locus	(112)
reverse	CGCTTCAAGAATTCGAGATAC	164 (*arabiensis)* and 194 (*gambiae*)	SINE S200 X6.1 locus	(112)
JFor	CACCAGGTTGATTCTGCCTGAC	350	18S rRNA	This study
JRevForCrisp	CCTCTCCGGTATCAAACCCTA	350	18S rRNA	This study

SS18SF (paired with **SS149RFORVITTA)**	CACCAGGTTGATTCTGCC	1200	18S rRNA	(96)
JKHF2	CAATCAGGGACGAATAGCTCAG	500	18S rRNA	This study
JKHR4	TCTCCCTGTCCACTATACCTAATG	500	18S rRNA	This study
RPOBCRISP29F	ACAGTAGGTCACTTGATTGAATGTC	230	*rpoB*	This study
RPOBCRISP262R	TACCATGTGCTTAAGTCTTTGGT	230	*rpoB*	This study

APPENDIX 2: MULTIPLE SEQUENCE ALIGNMENT OF 41 *MICROSPORIDIA*N SPECIES USING GENEIOUS V.11 INDICATING REGIONS OF PRIMER ALIGNMENT

APPENDIX 3: ETHICAL CLEARANCE

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1 October 31, 2016

TO: **DR. JEREMY HERREN, (PRINCIPAL INVESTIGATOR),**
INTERNATIONAL CENTRE OF INSECT PHYSIOLOGY AND ECOLOGY (ICIPE),
P.O BOX 30772-00100,
NAIROBI, KENYA

Dear Sir,

RE: PROTOCOL NO. NON-KEMRI 545 (*RESUBMISSION OF INITIAL SUBMISSION*): SYMBIOTIC MICROBES AND VECTOR COMPETENCE: CHARACTERIZATION AND ISOLATION OF CANDIDATES FOR MALARIA TRANSMISSION BLOCKING

Reference is made to your letter dated 13th October 2016. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the October 19, 2016.

This is to inform you that the Committee noted that the issues raised during the 255th Committee A meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **13th September 2016** have been adequately addressed.

Consequently, the study is granted approval for implementation effective from **31st October 2016** for a period of one year. Please note that authorization to conduct this study will automatically expire on **October 30, 2017.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to the SERU by **September 18, 2017**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the SERU and you should advise the SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

for **DR. EVANS AMUKOYE,**
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

APPENDIX 4: CONSENT FORM

International Centre of Insect Physiology and Ecology (ICIPE)

PO Box 30, Mbita Point, Kenya

Mobile number: 0722 369 254

Email: psawa@icipe.org

Who should I contact if I have questions on my rights as a volunteer in this research study: If you have any question on your rights as a volunteer, you or your parent should contact.

The Secretary, Kenya Medical Research Institute, Scientific and Ethical Review Unit.

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541 or mobile 0717719477

Email: seri@kemri.org

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

Subject's or Parent/Guardian's Signature: _____Date: _____

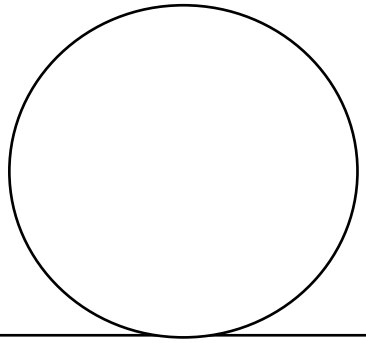
Permanent Address: _____

Witness's Name: _____

Witness's Signature: _____ Date: _____

Study Number: _____

Thumbprint of Volunteer or Volunteer's Parent/Guardian if Unable to Sign



Person Administering Consent:

Name:_____

Signature: _____

Informed consent agreement (blood collection)

What is the study called: **Symbiotic microbes and Vector Competence: Isolation and Characterization of Candidates for Malaria Transmission Blocking**

What is this study about: Malaria is transmitted by mosquitoes, however some mosquitoes are better at transmitting malaria than others. Scientist have found that mosquitoes can also be infected by other microbes and that these microbes may make mosquitoes unable to transmit malaria. We are trying to identify microbes that infect mosquitoes and make them unable to transmit malaria. We therefore need malaria infected blood to feed mosquitoes and determine if they can transmit malaria after being infected with these microbes.

Who is running the study: The study is being run by Dr Jeremy Herren, Dr. Daniel Masiga, Jandouwe Villinger and, Dr. Patrick Sawa (Head, St. Jude's Clinic, Thomas Odhiambo Campus, icipe). We will collaborate with other scientists at icipe, and MoPHS.

Do I have to participate: Participation in this study is voluntary. There is no penalty for refusing to participate. If you start the study you (your child) may discontinue your (your child's) participation at any time without any explanation. The principal investigators and co-investigators from ICIPE and MOPHS may decide to withdraw you (your child) from the study if we are unable to obtain a blood sample from you (your child).

What will happen to me if I participate in the study: You will be asked some questions, then tested for malaria. If you are found to have malaria in your blood, blood will be taken from a vein in your arm. We will then treat you for malaria at no cost to you. We will not test for HIV.

Are there any risks if I participate in the study: There is the possibility of mild discomfort, bruising and very rarely infection at the site where the blood is taken. But, should you (your child) be injured as a direct result of participating in this research project, you (your child) will be provided medical care, at no cost to you (your child), for that injury. You (your child) will not receive any injury compensation, only medical care. You should also understand that this is not a waiver or release of your or your child's legal rights. You should discuss this issue thoroughly with the principal investigator before you or your child enroll in this study.

Are there any benefits from the study: The study can lead to a better understanding of the microbes that prevent malaria transmission. The information obtained will be used to

develop new tools to control malaria transmission and hopefully contribute to a decline in malaria in the community and the continent as a whole. Volunteers will also find out if they are infected with malaria and if so receive treatment free of charge.

Will there be any compensation for being in the study: There is no compensation to volunteers for their participation.

How long does the study last: This study requires completion of a short questionnaire and a rapid malaria test. If you are found to be infected with malaria you will be requested to go to the icipe TOC St. Jude's clinic to for a blood draw and to receive treatment. The entire process will take about 30 minutes.

Who can participate in this study: Children with the consent of a parent / legal guardian.

Who will be able to see my information or lab results: Any information about you (your child's) will remain confidential. Only the people involved in the study will be able to see your information. We will keep all files in locked cabinets when they are not in use, and all blood stored in locked freezers. Your (your child's) name will not be used in any report resulting from this study. Any report from this study will refer to you/your child only by a study identification number and not by a name. All blood samples collected will be labeled with a study identification number; no names will be used.

What will happen to my blood: Your (your child's) blood will be tested for malaria. If positive a small amount of this blood will be drawn for to study ways to block mosquitoes from transmitting malaria. A sample of your blood will be kept frozen in case we want to do more testing on it in the future. These samples will be labeled with only your study number. They will be secured in freezers at ICIPE facilities and only study investigators and their authorized staff will have access. All safeguards ensuring privacy and confidentiality that are in place during this study period will also continue to be in place for the long-term storage of samples and if samples are sent outside of Kenya, no personal identifiers will be included. If you do not wish for your blood to be stored you can opt out of this.

If we do need to use the stored blood in the future we will first get permission from the Kenya National Ethical Review Committee.

Who can I contact about the study or my rights as a volunteer in this research study: If during the course of this study, you have questions concerning the nature of the research or you believe you have sustained a research-related injury, you should contact:

Who can I contact if I need information on the conduct of the study:

If you have any question you or your parent should contact:

Dr. Jeremy Herren

Molecular Biology and Bioinformatics Unit

International Centre of Insect Physiology and Ecology (ICIPE)

Duduville, Kasarani

P.O. Box 30772-00100

Nairobi, Kenya

Mobile number: 0716660160

Email: jherren@icipe.org

or

Dr. Patrick Sawa

St. Jude's Clinic

Thomas Odhiambo Campus,

International Centre of Insect Physiology and Ecology (ICIPE)

PO Box 30, Mbita Point, Kenya

Mobile number: 0722 369 254

Email: psawa@icipe.org

Who should I contact if I have questions on my rights as a volunteer in this research study: If you have any question on your rights as a volunteer, you or your parent should contact.

The Secretary, Kenya Medical Research Institute, Scientific and Ethical Review Unit.

C/o Kenya Medical Research Institute

P.O. Box 54840, Nairobi, Kenya

Tel. 254-20-2722541 or mobile 0717719477

Email: seri@kemri.org

IF THERE IS ANY PORTION OF THIS CONSENT AGREEMENT THAT YOU DO NOT UNDERSTAND, PLEASE TALK TO SOMEONE ON THE STUDY TEAM BEFORE SIGNING.

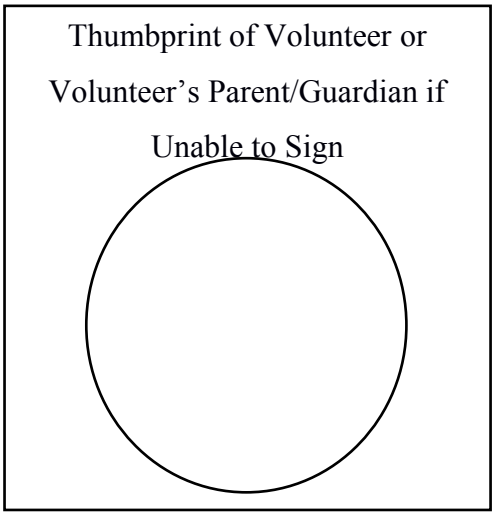
Subject's or Parent/Guardian's Signature: _____Date: _____

Permanent Address: _____

Witness's Name: _____

Witness's Signature: _____Date: _____

Study Number: _____



Person Administering Consent:

Name:_____

Signature: _____

INDIVIDUAL INFORMED CONSENT

Individual informed consent agreement

I, _____(name of parent/legal guardian) being the legal representative of (name of the participant)_____, certify giving hereby my consent for the child to participate in the research project titled: Symbiotic microbes and Vector Competence: Isolation and Characterization of Candidates for Malaria Transmission Blocking. I understand that I may feel some discomfort during the procedure and I understand that although the risks are minimal. I am aware that there will be no benefit apart from those described in the first part of this form to either my child or myself personally.

I have read the foregoing information, or it has been read to me. I have been given the opportunity to ask questions concerning this project. Any such questions have been answered to my full satisfaction. I have been provided with the name of the Project Coordinator who I may contact if any further questions arise concerning the rights of the child.

I consent voluntarily to participate in this study and I understand that I have the right to withdraw from the study at any time without penalty or loss of benefits.

Print Name and Signature _____

Date_____

Day/month/year

To opt out of blood storage for future use:

I consent voluntarily to participate in this study and I understand that I have the right to withdraw from the study at any time without penalty or loss of benefits. I do not wish to have my blood stored for future use

Print Name and Signature _____

Date_____

Day/month/year

If illiterate

I have witnessed the accurate reading of the informed consent form to the parent/legal guardian of the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name and Signature of Witness_____ Thumb print of parent

Date_____

Day/month/year

I have accurately read or witnessed the accurate reading of the informed consent form to the parent/legal guardian of the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Researcher_____ Signature _____

Date_____

Day/month/year

A copy of this Informed Consent Form has been provided to the parent or legal guardian of the participant _____ (initialed by researcher/assistant)

INDIVIDUAL RECRUITMENT INFORMATION

Site/Location ---------- Date of screening: ---------- Serial Number ----------

Identification code: ----------

IDENTIFICATION

First Name: ---------- Surname: ----------

Sex: --------- Age: ---------- or Date of Birth ----------

District: ---------- Locality: ----------

Parent/Guardian: ----------

CLINICAL PRESENTATION

ID number: ---------- Weight: ---------- Body temperature ----------

I-Major complains and symptoms: (yes = 1, No = 0)

Shiver ------- Abdominal pain -------- Vomiting -------- Dhiarrea -------- Headache --------

Constipation ------- Convulsion ------- Aching pains -------- Cough ------- Asthnia ---------

Fever --------- Initial date of feverache ---------- Anemia ----------

Previous drug history: date ---------- type of drugs ---------- dosage ----------

(within the last two weeks)

Other complains and signs ----------

II- Parasitological presentation

Thick blood film (Positive = +, Negative = 0) ---------- Species ----------

Asexual parasitemia (relative) ---------- (absolute) ---------- parasite/mm3

Gametocytemia (relative) ---------- (absolute) ----------gcts / mm3

TREATMENT

Drug: 1) ---------- dosage ----------

2) ---------- dosage ----------

Rendez-vous: ----------

LABORATORY EXPERIMENTS

Blood collection (yes = ok, No) ---------- Volume collected: ----------ml

Experimental infections ----------

Observations ----------