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Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.

A Research Project Proposal Submitted in Partial Fulfilment for the Master of Science Degree in Medical Parasitology

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EXECUTIVE SUMMARY

Malaria is a vector-borne disease transmitted to human through the bite of an infected female *Anopheles* mosquito. It is endemic in the tropical and subtropical regions of the world with 94% of the disease burden found in Africa. Zambia contributes to 2% of the global burden of Malaria and is among the top 20 countries with the highest malaria disease burden in the world. The use of insecticide-based vector control has shown great success at controlling malaria from the 1950s to date and this has relied on a clear understanding of mosquito ecology and behaviour of the local malaria vectors.

Insecticide-based vector control is the main malaria elimination strategy identified to achieve a malaria free Zambia. However, the extensive use of insecticide-based vector control results into an increase in the population of resistant malaria vectors and behavior modification for mosquito survival. Therefore, this study will assess the malaria vector bionomics and phenotypic resistance to insecticides used in vector control in Ndola.

This will be a cross sectional study conducted in Ndola district and mosquitoes will be collected using CDC light traps, Pyrethrum Spray Catches, Aspirations and larval collection. Multiplex PCR will be used for determination of sibling species of the *An gambiae* and *An funestus* complex and for determination of blood meal sources. Data analysis will be done in Microsoft excel and stata version 14. A zero-inflated negative binomial regression model will used to analyze the mosquito count against the independent variables.

Contents

1.0 Introduction

1.1 Background

Malaria is one of the diseases of public health importance in the tropical regions. In 2019, the burden was estimated at 229 million malaria cases and 409, 000 deaths. The highest malaria disease burden (94%)of these cases and deaths) was from Africa and Zambia contributed 2% of this global malaria disease burden (WHO, 2020). This disease is caused by a protozoan parasite of the genus *Plasmodium* and is transmitted to humans through the bite of an infected *Anopheles* mosquitoes. There are about 40 species from the *Anopheles* genera known to transmit malaria (WHO, 2019). In Zambia the two main malaria vectors are *An funestus s.l* and *An gambiae s.l* (Das *et al.*, 2016). However, other *Anopheles* mosquitoes *An coustani* and *An squamosus* have shown to be more anthropophilic but tested negative for circumsporozoites (Fornadel *et al.*, 2011).

Vector control has played a significant role in reducing the global malaria disease burden (Wilson *et al.*, 2020). An estimated 1.5 billion malaria cases and 7.6 million malaria related deaths have been averted since 2000. Africa has recorded a reduction in the number of malaria cases and malaria deaths from 362.8 to 225.2 per 1000 and 121.1 to 40.3 per 100, 000 respectively (WHO, 2020). This has to a greater extent been attributed to concerted efforts in vector control (Wilson *et al.*, 2020).

To achieve a malaria free Zambia, the National Malaria Elimination Centre (NMEC) has adopted the use of Indoor Residual Spraying (IRS) and use of long-lasting insecticide treated nets (LLINs) as the main vector control strategies (Chipoya and Shimaponda-Mataa, 2020; NMEC, 2017). These interventions are aimed at reducing the human to vector interactions by creating a barrier and killing host seeking mosquitoes that rest on a sprayed wall surface or on LLINs. By doing this malaria infected host seeking mosquitoes die without making contact with the human host, thus

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reducing the population of malaria vectors infected with the parasite as well as the humans getting infected.

The successful implementation of vector control strategies is hinged on a clear understanding of the ecology and behavior of malaria vectors in different geographical locations (Wilson *et al.*, 2020). Once their ecology and behaviour are known, the vector control strategies employed target one or more vulnerabilities in the behavior of the vector. Further, mosquito behaviours have been shown to change when exposed to elements that threaten their survival (Sougoufara *et al*., 2020). Therefore, routine vector surveillance studies should be conducted to ensure that malaria vector behaviours and their ecology are well understood prior to deployment of any vector control strategy. It is against this background that this study is proposed, to generate knowledge on the behavior and phenotypic resistance of malaria vectors in Zambia.

1.2 Statement of the Problem

Zambia is among the top 20 countries with a high malaria disease burden contributing 2% of the global malaria burden (WHO, 2020). These malaria cases are mainly in the provinces closer to the Democratic Republic of Congo (DRC) and in the northern parts of the country (MOH, 2019).

The government has identified vector control as the main malaria elimination strategy. This strategy heavily relies on spraying at least 80% of eligible structures through IRS and universal coverage of LLINs (NMEC, 2017). However, the extensive use of insecticide-based vector control leads to mosquito behavior modification and increase in the selection of resistant malaria vectors (WHO, 2019). Therefore, vector surveillance should be conducted to monitor the malaria vector behaviour and selected resistant malaria vectors. However, the last entomological activity conducted in Ndola was 8 years ago to determine the susceptibility status of malaria vectors and it was found that *An gambiae s.l* was resistant to carbamates (Thomsen *et al.*, 2014). As it stands, there is no data on mosquito species composition, their behavior and insecticide resistance status of the malaria vectors. Therefore, it is uncertain whether the vector control interventions implemented target the malaria vectors and their current behavior.

1.3 Rationale of the Study

The IRS and LLINs strategy adopted for malaria control should be guided by data generated on the current malaria vector behaviour and their susceptibility status to insecticides used in vector control. Therefore, the proposed survey will identify the mosquitoes responsible for malaria transmission in Ndola to subspecies level, assess their current behavior and determine their phenotypic resistance status to the different insecticides used in malaria control.

The results generated will be useful in ensuring that vector control planning is based on updated information on the species, behavior and susceptibility status of local malaria vectors to insecticides used in malaria control. Further, the study findings will serve as baseline entomological survey to guide the possible resumption of regular surveys.

2.0 Research Questions

1. What is the species composition and density of malaria vectors in Ndola?
2. What are the biting and resting behaviours of mosquitoes in Ndola District?
3. What are the blood meal sources for mosquitoes in Ndola?
4. What is the insecticide resistance status of primary malaria vectors?

3.0 Objectives

3.1 General Objective

The main objective is to evaluate malaria vector behavior and their phenotypic resistance status to insecticides used in vector control in Ndola.

3.2 Specific Objectives

1. To determine species composition and vector density of malaria vectors in Ndola
2. To assess the biting and resting behaviour of malaria vectors in Ndola District
3. To assess host preference of blood feeding mosquitoes in Ndola
4. To evaluate the insecticide resistance status of primary malaria vectors in Ndola.

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4.0 Literature Review

4.1 Global Malaria Situation

Diseases transmitted by vectors account for 17% of all infectious diseases in the world (Eder *et al.*, 2018). About 700,000 deaths occur annually from pathogens transmitted by different vectors including mosquitoes, ticks, triatome bugs, snails, fleas, sandflies, tsetse flies, lice and black flies (Benelli *et al.*, 2020). Mosquitoes alone are responsible for 8 infections affecting man and these are chikungunya, dengue, lymphatic filariasis, rift valley fever, yellow fever, malaria, Japanese encephalitis and west nile fever. In 2019, 229 million cases of malaria were recorded and there were 385,000 deaths as a result of malaria infections (WHO, 2020). The WHO African region recorded the highest malaria cases of 215 million cases accounting for 94% of the global malaria diseases burden. The majority of these cases where only in 29 countries with Nigeria (27%) and the Democratic Republic of congo (12%) having the most malaria cases in the world. The remaining WHO regions South East Asia, Eastern Mediterranean, Western Pacific and Region of the Americans contributed to 6% of the global malaria disease burden.

The implementation of malaria control interventions has seen a significant reduction in the malaria incidence from 80 per 1000 in 2000 to 58 per 1000 in 2019 (WHO, 2020). This has been to a greater extent been attributed to vector control and the World Health Organization continue to put great emphasis on the need for improving these tool in order to effectively achieve malaria control (Wilson *et al.*, 2020).

Zambia has also recorded a reduction in the malaria prevalence in under five children from 15% in 2015 to 9% in 2018. This reduction could be attributed to the increase in the number of household that have received a treated bed net and/or IRS. However, Luapula province recorded the highest malaria prevalence of 30%. Copperbelt on the other hand recorded a reduction in the prevalence from 15.2% in 2015 to 7.7% in 2018 despite having the lowest bed net ownership rate of 55.3% (MOH, 2015)(MOH, 2019).

Further, a study showed that in the first 10 months of 2020, Zambia recorded an estimated 3.7 million cases of confirmed malaria cases. This rise in the number of confirmed malaria cases could be associated to the effects that COVID-19 had on efforts aimed at curbing malaria (Chisaya *et al.*, 2020).

4.2 Introduction to Mosquitoes

Mosquitoes taxonomically belong to the class insecta, order diptera, family Culicidae. These belong to five mosquito genera namely *Anopheles, Culex, Aedes, Mansonia* and *Conquillettidia* (Service, 1996; WHO, 2014). There about 3500 different species of mosquitoes worldwide, 400 of these belong to the *Anopheles* genera and only 40 of them have been implicated as malaria vectors (WHO, 2019). Mosquitoes require blood for the maturation of their eggs and its this host seeking behaviour that makes them ideal vectors of disease (O'Donnell *et al.*, 2019; Barry, 1996).

4.2.1 Life cycle of *Anopheles* Mosquito

The mosquito undergoes four distinct stages in their development; the egg, larvae, pupa and the adult stage. The first three stages of the life cycle of the mosquito are aquatic and the adult stage is the only terrestrial (Durden and Mullen, 2018). The time taken for the mosquito to metamorphose from one development stage to the next is dependent on a number of factors but most importantly is temperature, humidity and diet (Service, 2012).

a) **Egg Stage**: *Anopheles* mosquitoes lay an estimated 200 eggs in one oviposition in water. These eggs are laid singly and have floaters (Service, 2012). These eggs hatch in 2 – 3 at optimum environmental conditions (27 °C ± 2, 80% ± 10 relative humidity (RH)) but can take as long as 14 days in cold temperatures (Mazigo *et al.*, 2019).

b) **Larval Stage:** The eggs hatch into larvae and the larvae will pass through 4 larval instars in this stage. This is the feeding stage of the immature stage of the mosquito and lasts for a period of 8 to 10 days at optimum environmental conditions. The larvae lay horizontal to the water surface and they breath through the spiracles along the body of the larvae. From this stage the larval will undergo metamorphosis and develop into pupa (Durden and Mullen, 2018).

c) **Pupa Stage:** This is the non-feeding stage in the life cycle of the mosquito and exist as a *cephalothorax.* The pupa breath through the respiratory trumpets on the apical end of the cephalothorax. The pupa speeds most of its time on the water surface taking in air and if disturbed they swim in a jerk fashion (up and down). This stage also takes 2 to 3 days at optimum environmental conditions (Service, 2012).

d) **Adult Stage:** The pupae then emerge into adult mosquitoes normally at dusk and then the females fly into a swam of males for mating. Once the female mosquito mate, they lay eggs throughout their lifespan. They have several blood meals depending on the length of their gonotrophic cycle (Service, 2012).

4.3 Malaria Vectors

4.3.1 Global Malaria Vectors

The distribution of malaria vectors in the world differ from continent to continent. In the Americans region *An darlingi* is the most efficient malaria vector of *P. falciparum* and *P. vivax.*. It is a highly anthropophilic malaria vector and its natural habitats are the shady areas of streams and ponds. This mosquito can breed in both clear and muddy water with floating vegetation. Other malaria vectors in this region are *An nuneztovaris s.l, An albitarsis* found in South America. (Alimi *et al.*, 2015). In Asia, the most efficient malaria vector is *An stephensi* found to thrive most in urban areas (Alimi *et al.*, 2015). The breeding sites for this mosquito include man made water habitats such as wells, water receptacles, overhead tanks, fountains, barrels and tins and polluted water in drainages (Thomas *et al.*, 2016). Other malaria vectors include *An culicifacies s.l, An darius s.l* and *An maculatus s.l* (Sinka *et al.*, 2012).

4.3.2 Malaria Vectors in Africa

In Africa *An gambiae* and *An funestus* complex are the main malaria vectors of malaria (Mzilahowa *et al.*, 2012). *An gambiae s.l* prefers to breed in small temporal water collections such as gardens, hoof prints, tire marks and a dirty road (Ndiaye *et al.*, 2020). *Anopheles funestus s.l* on the other hand breeds in semi-permanent water bodies such as dams created from road construction, swamps, marshes and in the edges of a stream where the water is not moving (Fillinger *et al.*, 2009) (Mattah *et al.*, 2017).

There is now a threat of *An stephensi* a primary malaria vector in Asia (Balkew *et al.*, 2020). This new malaria vector poses a threat of increased urban malaria in Africa as it thrives in urban areas (Sinka *et al.*, 2020). In African countries *An. funestus* complex has shown to be abundant throughout the year and *An. gambiae* complex has shown to be most abundant in the wet season (Mzilahowa *et al.*, 2012). These two *Anopheles* complexes have been well studied and understood and this led to discovery of sibling species within these complexes. The *An. funestus* complex has 9 sibling species in Africa and these include *An funestus s.s, An rivolurum, An leesoni, An vaneedeni, An aruni, An fuscivenous, An parensis* and *An brucei*. Studies further show that *An funestus s.s* is the predominant and widely distributed malaria vector in Africa. *An leesoni, An* *revolurum, An parensis* and *An vaneedeni* are the other sibling species of the *An funestus* complex in Africa (Kweka, et al, 2013).

On the other hand, there has been a reduction in the malaria vector densities and species composition of the *An gambiae* complex due to vector control interventions (Derua *et al.*, 2015). *An gambiae s.s, An arabiensis* and *An coluzzi* are the other malaria vectors in the *An gambiae* complex transmitting *Plasmodium falciparum* and *Plasmodium vivax* (Wiebe *et al.*, 2017). Other sibling species in the *An gambiae* complex are *An quadrianulatus A, An. amharicus, An merus, An melas* and *An bwambae* (Ebenezer *et al.*, 2014). *An gambiae s.s, An arabiensis, An coluzzi, An quadrianulatus A, An quadrianulatus B* are fresh water malaria vectors. *An melas* and *An merus* are salt water malaria vectors and *An bwambae* is only found in Uganda (Bartilol *et al.*, 2021).

The number of secondary malaria vectors in Africa vary with geographical locations and very little is known about these vectors (Mwangangi *et al.*, 2013b). Studies have shown that due to the lack of a proper understanding of their ecology and behavior they have contributed greatly to residual transmission in areas with low malaria transmission (Ayuya *et al* 2021). The secondary malaria vectors of public health importance in Africa include *An coustani, An Ziemani, An pharoensis, An maculipalpis* (Afrane *et al.*, 2016).

4.3.3 Malaria vectors in Zambia

An. funestus and *An. gambiae* complexes are the primary malaria vectors in Zambia (Cross *et al.*, 2021). *An. funestus s.l* is the predominant malaria vector in 8 of the 10 provinces in Zambia. The main malaria vector on the Copperbelt province is *An gambiae s.s* and *An arabiensis* in the Southern Province (NMEC, 2019). The provinces were *An funestus s.l* is the predominant malaria vector are the same provinces with the highest malaria prevalence. Other malaria vectors potentially identified as secondary vectors have been identified through their highly anthropophilic and endophagic behaviours. *Anopheles leesoni* a zoophilic mosquito mainly found in Luapula province assumes the role of a malaria vector when its preferred blood meal source is not available (Das *et al.*, 2016). Other anopheles mosquitoes that are mainly zoophilic and exhibits a high exophagic behaviour are *An squamosus, An rufipes, An coustani, An pretoriensis* and *An quadrianuratus* (Stevenson *et al.*, 2016) (Lobo *et al.*, 2015). Further, studies have shown that other than being more zoophilic and exophagic *An arabiensis, An coustani* and *An pretoriensis* are early outdoor bitters (NMEC, 2019) (Fornadel *et al.*, 2011).

4.4 Malaria Vector Bionomics

Vector bionomics refers to the ecology of malaria vectors and their resting, biting and host preference (Massey *et al.*, 2016). Mosquitoes have been known to modify their behavior when exposed to elements that do not favour their proliferation (Sougoufara *et al*., 2020). This to a larger extent has been attributed to vector control interventions that are implemented to control mosquito borne diseases (Thomsen *et al.*, 2017). The most efficient malaria vectors in the sub-Saharan Africa have been found to be highly anthropophilic. They feed indoors and rest indoors after having their blood meals (Sougoufara, *et al*., 2020). However, in Tanzania, *An arabiensis* a zoophilic mosquito was found to prefer biting outdoors and resting outdoors (Limwagu *et al.*, 2019). The deployment of LLITNs in Senegal was associated with a change in biting time from biting in the night to biting in broad day time. Further, there was no indication of the change in their behaviour from being highly anthropophilic and endophilic (Sougoufara *et al.*, 2014). Another study in Tanzania revealed a change in biting behaviour within *An gambiae* complex from 1997 to 2009. The night biting activity of the *An. gambiae* complex was high in 1997 but in 2009 there was a shift to only *An gambiae s.s* and *An arabiensis* being the only malaria vectors predominantly biting late in the night (Russell *et al.*, 2011). Another study in Benin revealed that there was an increase in mosquito biting in broad day light after the introduction of LLITNs (Moiroux *et al.*, 2012).

A study in the Pan American Health Organization region found that land use change, climate change, deforestation, loss of forest cover has affected distribution of malaria vectors causing a shift in behaviour thereby increasing human to vector contact (Alimi *et al.*, 2015).

4.4.1 Species Composition

A study in Kenya showed that there was a change in the predominant malaria vector from *An funestus s.s* and *An gambiae s.s* to *An arabiensis* and *An merus* over a period of 20 years. Further, a notable shift in the feeding behaviour from humans to animals of *An gambiae s.l* (99% to 16%) and *An funestus s.l* (100% to 3%). This could be attributed to the shift in the species composition recorded from *An gambiae s.s* as the predominant malaria vector to *An arabiensis* (Mwangangi *et al.*, 2013a). Further, another study in Uganda showed a change in the species composition of malaria vector after the implementation of IRS and LLITNs. Before the interventions the predominant malaria vector was *An. gambiae s.s* (76.7%) but after the intervention, 99.5% of the mosquitoes collected were *An arabiensis* and *An gambiae s.s* was 0.5% (Musiime *et al.*, 2019).

4.4.2 Malaria Vector Density

A study in Uganda revealed that the distribution of LLITNs was associated with a reduction in the *An funestus s.l* vector density from 0.07 per house per night to 0.02 per house per night but not for *An gambiae s.l*. This revealed that *An funestus s.l* was more affected as compared to *An gambiae s.l* (Mawejje *et al.*, 2021). The introduction of vector control interventions in Uganda led to the reduction in the vector density of *An. gambiae s.l* from 76.7% before the intervention to 0.5% post intervention (Musiime *et al.*, 2019). A study in Zambia revealed that there was a reduction in vector density of *An quariannulatus* from 95.1% to 69.7% following implementation of IRS. However, there was a proportionate increase in number of *An. arabiensis* collected from 3.9% to 95.1% from the total mosquitoes collected in the *An gambiae* complex (Chinula *et al.*, 2018).

4.4.3 Resting Behaviour

Following the deployment of LLITNS in Western Kenya, a study revealed that there was a higher indoor resting density for *An. gambiae s.l* and *An. funestus s.l*. The introduction of LLITNs did not have an effect on the indoor resting behaviour of malaria vectors (Machani *et al.*, 2020). The results from this study indicate that there is need to implement an intervention that will also target indoor resting malaria vectors. A study in Western Kenya were a large proportion of the malaria vectors rest indoors revealed a reduction in the indoor resting density post IRS implementation (Abong'o *et al.*, 2020).

4.4.4 Biting Behaviour

Use of LLINs is the most appropriate vector control tool to deploy where mosquitoes predominantly endophilic. However, a study conducted in Senegal revealed that the deployment of vector control had no effect on the endophilic behaviour of the malaria vectors (Sougoufara *et al.*, 2014). Further, a study revealed that 60% of *An. Arabiensis* were able to successfully have their blood meals and an estimated 50% of these blood meals were taken from outside the house (Killeen *et al.*, 2016).

4.4.5 Host preference

Malaria vectors have their preferred hosts for their blood meals and they will only feed on another host in the event that their preferred host is unavailable. *An. arabiensis* a highly zoophilic mosquito prefers to feed on cattle but in the event that there is no cattle nearby they will feed on humans (Killeen *et al.*, 2016). Another study in conducted in Kenya were human and domestic animals were sharing the house revealed that mosquitoes fed on any available blood meal source host human, goat or bovine. 53.1% of the *An gambiae s.s* fed on humans, 26.5% fed on goats and 18.4% fed on bovine (Ndenga *et al.*, 2016). In Honduras it was found that most of *Anopheles* mosquitoes collected had multiple blood meals and that only 24.9% had fed on a single host. The Anopheles mosquitoes preferred to feed on either chickens or bovine. The Human blood index in this study was found to be 22.1% (Escobar *et al.*, 2020).

4.5 Insecticide-Based Vector Control

Vector Control is the main method of controlling malaria and it has played a significant role in the reduction of vector-borne diseases from the 1800s. This was achieved through a clear understanding of the behaviour and ecology of the different vectors of disease. However, after the 1940s to date, malaria vector control has significantly depended on insecticide-based interventions and this has brought in the challenge of insecticide resistance (Wilson *et al.*, 2020). Historically, organophosphates, organochlorines, carbamates and pyrethroids were the four classes of insecticides approved by WHO for use in public health vector control (Corrêa *et al.*, 2019). Pyrrole (Chlorfenapyr 240 SC) and Neonicotinoids (Chlothianidin WG) have been prequalified by WHO for use in public health vector control (WHO, 2013). In Africa, the main vector control strategies employed target the adults mosquitoes and the immature stages to a lesser extent.

4.5.1 Control of Adult Mosquitoes

a) **Use of treated bed nets:** Pyrethroids and pyrroles are the only two classes of insecticides approved by WHO for use in LLINs. These classes of insecticides have shown to pose very low risk to humans yet providing the desired lethal effect to arthropods of public health importance (CDC, 2019). The use of bed nets has shown to significantly reduce malaria prevalence in areas where the malaria vectors predominantly bite indoors and late at night

(Ntonifor and Veyufambom, 2016; Steinhardt *et al.*, 2017). The treated bed net creates a barrier between the vector and the human host and produces a toxic effect to the malaria vectors that rests on the bed net (Paaijmans and Huijben, 2020).

b) Indoor Residual Spraying: Indoor Residual Spraying involves the application of a predetermined amount of insecticide with a residual effect on a wall surface (Tangena *et al.*, 2020). In this intervention organophosphates, organochlorines, carbamates, pyrethroids, pyrroles and neonicotinoid have been prequalified for use in IRS (WHO, 2013). This intervention targets host seeking mosquitoes that rest indoors after a bloodmeal. Once the mosquito rests on a sprayed wall surface, the mosquito absorbs the insecticide through its legs and the mosquito dies (Phiri *et al.*, 2015). This intervention protects the next person from getting an infectious bite by killing the infected mosquito (Pinchoff *et al.*, 2016).

c) **Use of symbionts:** The use of symbionts for the control of mosquito borne diseases still remain largely unexplored (Ricci *et al.*, 2012). Endosymbionts have been shown to interrupt transmission in their natural or engineered form. In Anopheles a Wolbachia strain (wAnga-Mali) significantly reduces the prevalence and intensity of sporozoites on field collected mosquitoes (Gomes *et al.*, 2017). Another bacteria in its engineered form *Pantoea agglomerans* successfully inhibits the development of plasmodium falciparum by 98% in *Anopheles gambiae s.l.*(Wang *et al.*, 2012) . In a more recent study, *Microsporidia MB* naturally occurring in *An arabiensis* was found to completely interrupt transmission of the malaria parasite (Herren *et al.*, 2020).

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4.5.2 Control of immature stages of the malaria vectors

a) **Use of predators:** The use of predator fish in malaria control has not shown any statistical significant result on the malaria infection rates, entomological inoculation rate or the adult vector density in the areas where they have been studied (Walshe *et al.*, 2017). However, a study in western Kenya revealed that biocontrol was able to reduce the density of larvae and pupae (Howard *et al.*, 2007).

b) **Pathogens and parasites:** This is a widely practiced larval source management intervention targeting the larval stages of the mosquito (Service, 2012). *Bacillus thuringiensis var. israelensis (Bti)* and *Bacillus spharicus* have been found to be effective at controlling larvae and was well received and accepted in communities. These pathogen has been found to be ecofriendly and ease to produce in large numbers (Derua *et al.*, 2019). This intervention has been shown to be effective especially with the rise of insecticide resistance to pyrethroids (Zhou *et al.*, 2020).

c) **Use of Oils and Surface films:** This is an ancient mosquito larvae control that was mainly practiced in the Americas and India. It involves the application of a petroleum oil or isotearyl alcohol on the surface of the water so to kill the larvae through suffocation or toxic effect of the oil (Service, 2012). The use of monomolecular films is a more recent larvicidal strategy which works by forming an ultrathin film on the surface of mosquito breeding areas thus suffocating the larvae to death (Antonio-Nkondjio *et al.*, 2018).

4.6 Vector Control in Zambia

In Zambia, Indoor Residual Spraying and use of LLINs are the main malaria elimination strategies identified to achieve a malaria free Zambia (NMEC, 2017). These two vector control interventions have received massive funding from both the Government and Cooperating partners (PMI, 2019).

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However, larval source management is mainly implemented by Local authorities and mining companies.

In the quest to have a malaria free Zambia, there has been wide spread scaling up of these insecticide-based vector control (LLINs and IRS) to all the Districts in the country. Some districts have received both treated bed nets and IRS whereas others have only received one of the two interventions (PMI, 2019). However, these interventions have the ability to increase selection pressure on the population of mosquitoes that have resistant alleles (Nkya *et al.*, 2013).

4.7 Insecticide Resistance

The ability mosquitoes have to withstand toxic effects of an insecticide through natural selection or mutation is referred to as insecticide resistance. Usually this arises from repeated exposure of mosquitoes to insecticides or selection of individual mosquitoes that are able to detoxify the insecticide takes place. The mosquitoes selected survive and pass their ability to detoxify insecticides to their progeny (Riveron *et al.*, 2018). The widespread in insecticide resistance world over has been attributed to the extensive use of insecticides in agriculture and public health (Mouhamadou *et al.*, 2019).

Insecticide resistance has been reported against the four different classes of insecticides used for public health vector control and these are pyrethroids, organophosphates, organochlorines and carbamates (Mint Mohamed Lemine *et al.*, 2018)(Fang *et al.*, 2019)(Ondeto *et al.*, 2017).

Insecticide resistance is widespread in Zambia and has been recorded against pyrethroids, organochlorines and carbamates. Organophosphates and neonicotinoids are the only two classes of insecticides used in public health where local malaria vectors are still susceptible to (NMEC, 2019).

4.7.1 Mechanism of resistance

Routine monitoring of the susceptibility status of malaria vectors is cardinal for the successful implementation vector control (WHO, 2018). Area earmarked for insecticide-based vector control should be preceded by resistance surveillance to help determine most appropriate insecticide for use in vector control based on the susceptibility status of local malaria vectors. This monitoring of malaria vector susceptibility should be conducted once every year and in the event that there are several malaria vectors in the area with seasonal variations, monitoring at the beginning and end of the control effort should be done (McAllister and Scott, 2020).

There are basically four mechanisms of resistance and reports in some African countries indicate that the frequency of the presence of multiple mechanism of resistance is on the rise (Kwiatkowska *et al.*, 2013).

The figure below illustrates the different mechanisms of resistance (1) Reduced Penetration- Changes to the cuticle of mosquito exoskeleton prevents absorption of the insecticide. (2) Target-site- When the target-site for the insecticide is modified, the insecticide will no longer bind. (3) Metabolic- enzymes breakdown the insecticide before they can have a toxic effect on the mosquito

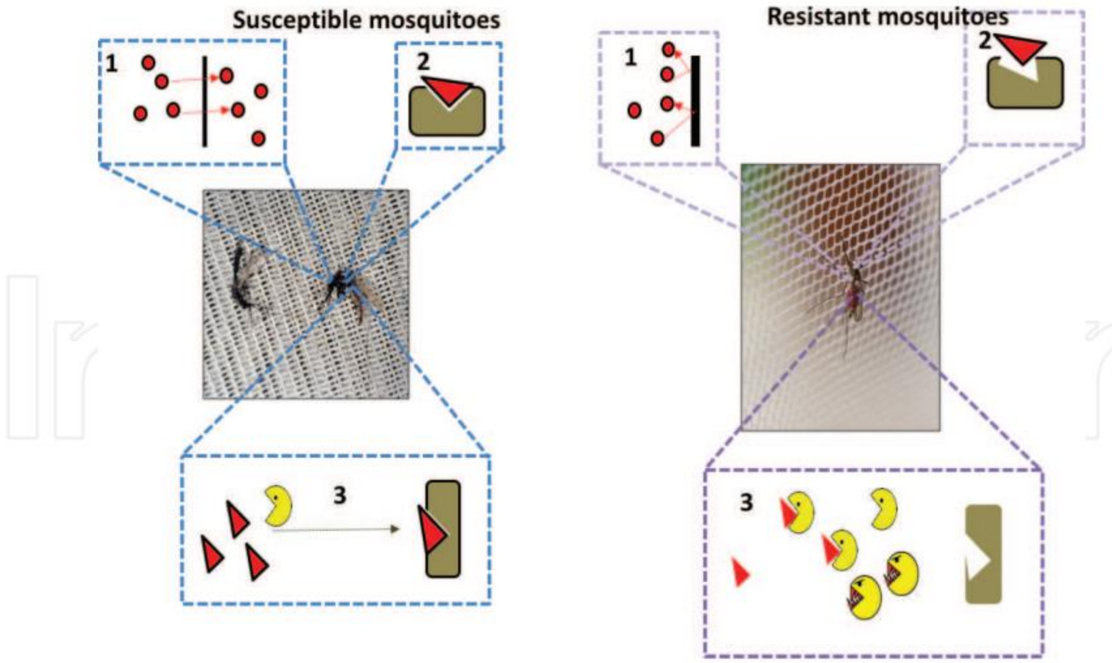

Figure 1: Summary of the 3 Main Mechanism of resistance

Source: (Riveron *et al.*, 2018)

Reduced Penetration: This type of resistance occurs when the cuticular structure composition is altered to reduce the amount of insecticide that is absorbed into the mosquito. Studies have shown that resistant mosquitoes have a thicker cuticle than susceptible mosquitoes (Riveron *et al.*, 2018). A study conducted in west Africa associated cuticle thickening to pyrethroid resistance (Yahouédo *et al.*, 2017). Additionally, the legs of resistant mosquitoes are sealed with large amounts of cuticular hydrocarbons compared to the susceptible mosquitoes (Balabanidou *et al.*, 2019). This mechanism of resistance is also one of the least studied yet it is possess a serious threat to vector control (Huang *et al.*, 2018).

Target-Site: In this mechanism of resistance, the target-site in the mosquito were the insecticide binds is modified and the insecticide can no longer bind. Insecticide molecules that enter the body of the mosquito fail to bind to the target-site because it has been altered. This is the most common mechanism of resistance in Africa and it has contributed greatly to the malaria disease burden (Yewhalaw and Kweka, 2015).

Metabolic Resistance: This type of resistance arises when the body enzymes digest the insecticide molecules before attaching to the target-site where it will produce the desired toxic effect. This is one of the most common type of insecticide resistance report in the sub-Saharan Africa (Riveron *et al.*, 2018). This mechanism of resistance is widespread across most African countries and has been reported in at least one of the four classes of insecticides used in public health vector control (Diouf *et al.*, 2020)

Behavioural Resistance: This type of resistance if not monitored and has the potential to negatively impact the vector control strategies employed (Sokhna *et al*., 2013). Mosquitoes in this type of resistance tend to avoid surfaces that contain insecticides including sprayed wall surfaces and treated bed net surfaces (Gatton *et al.*, 2013). Other studies conducted have shown that the use of insecticidal nets has led to the change in feeding behaviours of mosquitoes with a rise in the biting times at dusk and dawn (Killeen and Chitnis, 2014).

4.7.2 Insecticide Resistance Management

To slow down development of resistance, the NMEP has provided guidelines to rotate IRS products and implement a mosaic approach. This approach is based on the data generated from mosquito susceptibility assays conducted on mosquitoes from different locations benefitting from IRS (PMI, 2019). However, the NMEP has a limited number of sites were susceptibility data is collected from and these are mostly sites that are supported by partners including PMI sites, MACEPA sites and Global Fund coordinated by NMEP. On the Copperbelt there are only two active sites operated by PMI which are used as entomological surveillance sites (PMI, 2020).

5.0 Materials and Methods

5.1 Study Design

This will be a cross sectional survey involving the collection of mosquitoes from two catchment areas (one urban and one rural) of Ndola District both with a malaria high burden.

5.2 Study Site

The study will be conducted in Ndola District (12.9726° S, 28.6265° E), the provincial capital of the Copperbelt Province. The district shares a boundary with the Democratic Republic of Congo on the eastern side.

Chipulukusu Urban Health Centre and Kaniki Rural Health Centre are the two catchment areas that will participate in this study. According to the Health Management Information System (HMIS) data for 2020, there were 19618 malaria confirmed cases in Chipulukusu and 10,679 malaria confirmed cases in Kaniki catchment area (Ndola, 2020). Chipulukusu Urban Health Centre serves a low cost area with an estimated population of 49,730 people (CSO, 2020).

Chipulukusu is one of the catchment areas found at the centre of the District. The housing structure comprise of cement block, burnt brick and mud brick walled houses with both iron and grass roofing. Most families in Chipulukusu access kitchen and toilet facilities in standalone structures apart from the main house used mostly for sleeping (CSO, 2020).

On the other hand, Kaniki Rural Health Centre shares a border post with the Democratic Republic of Congo at Sakanya border post. The majority of the housing structure in the area are mud house roofed with grass or iron sheets. The catchment has an estimated population of 11, 716 people. The sleeping house, Kitchen and toilet facilities are stand-alone structures (CSO, 2020).

5.3 Study Population

The study population will include all the housing structures occupied by people in the selected catchment areas of Chipulukusu and Kaniki. The estimated number of household in Chipulukusu is 16000 and the total number of household in Kaniki is 4412.

5.3.1 Inclusion Criteria

The houses where mosquitoes will be sampled include those houses where people sleep in and there is an adult (16 years and above)

5.3.2 Exclusion Criteria

The houses in which occupants will not consent to participate and / or cook from inside the house using firewood will be excluded from this study.

5.4 Sample Size Determination

Total Collection efforts 225 (CDC LT 56 houses, PSC 30 houses, Aspirations 80 houses and 60 Larvae collection efforts)

Sampling for this study is based on vector sampling guidelines by WHO (WHO, 2019). However, the sample size has been determined based on the minimum number of structures per square meters and the predominant malaria vector. The minimum houses to be sampled for areas where *An gambiae s.l* is the predominant vector is 17 houses per square km and 42 houses per square km where *An funestus s.l* is the predominant vector. (Zhou *et al.*, 2004). A study further revealed that sampling 120 houses (120 collection efforts) gives precision equivalent to 200 houses for mosquito surveillance (Sedda et al, 2019)

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For vector susceptibility assays, a minimum of 100 adult female mosquitoes of a given species per insecticide is required. These mosquitoes will be tested in 4 replicates of 25 – 30 mosquitoes per WHO tube or CDC bottle (WHO, 2018).

5.6 Sampling Method

Purposive sampling will be used to select the two catchment areas that will participate in the study. Houses where adult mosquitoes will be collected will be randomly selected from each section maintaining at least 200 m between two participating houses. The collection tools that will be used for mosquito collection are;

5.6.1 CDC light traps

The traps will be set in randomly selected houses and mosquito collection will start at 18:00 hours and end at 06:00 hours the following morning. The traps will be set at a height of 1.5m from the ground next to a sleeping space. After every collection the mosquito collection cups will be properly labelled and transported to the laboratory for processing. At the laboratory, the mosquito collection cups will be put in a freezer at -4°C for 1 hour to kill mosquitoes that would still be alive.

5.5.2 Pyrethrum Spray Collections (PSC)

Adult mosquitoes resting indoors will be collected indoors using PSC from 05: 00 hours to 07: 00 hours in 20 houses per catchment area. The Collector will enter the house spread a 3m X 2m white linen over the floor, bed and furniture. The collector outside the house starts by spraying the eaves and other openings and once he is done the collector inside (wearing a nose mask) will saturate the house with insecticide to knockdown the mosquitoes. After the 10 minutes the collector will carefully fold the sheets towards the centre and take the sheets outside. The collector will pick the mosquitoes and place them in properly labelled petri dishes.

5.5.3 Aspirations

The live adult mosquitoes will be collected from 05:00 hours to 08:00 hours in houses where people sleep using mouth aspirator and prokopack. The live adult mosquitoes will then be put in bugdom cages and fed with a 10% sugar solution and transported to the laboratory for processing.

5.5.4 Larval collection

The larvae will be collected from breeding areas around houses where people live using dippers for larger breeding sites and Pasteur pipette for small breeding sites. The larvae will then be carefully transported to the laboratory where they will be reared into adult mosquitoes in a controlled microenvironment.

5.6 Variables

Variable type	Variable	Indicator
Outcome Variable	Mosquito Bionomics	Species Composition
		Malaria Vector Density
		Indoor Resting Density
		Man Biting Rate
Independent	Type of Wall	Mud Cement
	Roof type	Thatch Iron sheets
	Type of eaves	Open Closed
	Human Host	No. of People
	Animal Host	No. of animals
	Spray status	Sprayed Not Sprayed

	LLINs	No. of LLINs

5.7 Experimental Procedures

1. **To determine species composition and vector density of malaria vectors in Ndola:** Mosquitoes collected from houses using CDC Light traps and aspiration will be quantified by calculating total catches per household. All mosquitoes collected will be morphologically identified using a standard mosquito identification key for Afrotropical *Anopheles* mosquitoes (Coetzee, 2020). Confirmation of mosquito identification and determination of the subling species from the *An gambiae s.l* and *An funestus s.l* complexes will be done using multiplex PCR (Scott *et al.*, 1993) (Koekemoer *et al.*, 2002). DNA extraction will be done using the xygem DNA extraction kit. The PCR master mix prepared will include specific primers for sibling species. The PCR mix will then be run in the thermocycler and after amplification, the amplicons will be run on a gel.

   Malaria vector density: This is a very important indicator used in vector surveillance to assess the behaviour of malaria vectors or to assess the effectiveness of a vector control intervention. It is a mean of the malaria vectors collected from a define number of houses surveyed. It is calculated by dividing the total number of mosquitoes collected by the total number of houses surveyed.

   $$\textbf{Malaria vector density} = \frac{Number\ of\ malaria\ vectors\ collected}{Total\ number\ of\ houses\ surveyed}$$

2. **To assess the biting and resting behaviour of mosquitoes in Ndola**

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The Man Biting Rate helps to estimate the potential risk of exposure to infectious bites. The formular for calculating the human biting rate or man biting rate is

$$\text{Man biting rate} = \frac{Number\ of\ mosquitoes\ collected\ using\ light\ trap}{\text{Total Number of sleepers}}\ X\ HBI$$

The Indoor Resting behaviour is indicative of the mosquitoes that rest indoors. This indicator is an important one when considering whether to implement IRS or not. This is calculated by dividing the total number of mosquitoes collected using PSC over the total number of houses surveyed.

3. **To assess host preference of blood feeding mosquitoes:** Blood fed female anopheles mosquitoes collected will be analysed using multiplex PCR for the determination of blood meal sources (Kent and Norris, 2010). Extraction of DNA will be done using a xygen DNA extraction kit and the samples will be stored at -20°C prior to running the PCR. After PCR the amplicons will be run on a gel for determination of blood meal source. The Human Blood index will be calculated using excel using the formula;

   Human Blood Index $= \frac{No.of\ female\ mosquitoes\ with\ human\ blood}{Total\ number\ of\ mosquitoes\ analysed}$ (Escobar *et al.*, 2020). See appendi ????? for primers that will be used in multiplex PCR for determination of bloodmeal sources.

4. **To determine the insecticide resistance status of primary malaria vectors:** Live adult female anopheles mosquitoes will be exposed to various insecticides used in vector control using the using WHO Tube Bioassays and CDC bottle assays (WHO, 2018; Brogdon and Chan, 2010). The selected mosquitoes will be placed in WHO tubes lined with insecticide impregnated paper and left to sit for 60 minutes. Thereafter, knockdown will be observed

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at 60 minutes, 12 hours and final mortality will be read at 24 hours post exposure. For the CDC bottle assay, mosquitoes will be put in whatton bottles coated with insecticides and knockdown will be read every 15 minutes for 2 hours and final mortality will be determined 24 hours post exposure.

5.8 Data Analysis

The data collected will entered in excel for determination of species composition, vector density, Indoor Resting Density, Human Blood Index and Man Biting Rate.

Analysis of variance will be used to determine any statistical difference in the malaria vector density, indoor resting behaviour and man biting rate between the two areas. Susceptibility status of the local malaria vectors will be determined using WHO guidelines provided (98% - 100% means susceptible; 90% - 97% means suspected resistance and less than 90% means confirmed resistance) that is if mortality in the controls is less than 5%. A corrected mortality using Abbotts formula will be computed where mortality in the controls will be between 5% and 20%. However, if the mortality in the controls will be greater than 20%, the findings will be discarded and the assay will be repeated.

6.0 Ethics Considerations

Ethics clearance to undertake this study will be obtained from the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority. This study will assure protection of collectors and staff working during mosquito collection by ensuring that proper training is given to them and that they wear proper protective wear. It will also assure for preservation of ecology by sampling without total depletion. Permission to collect mosquitoes from the District will be sought from Ndola District Health Office. Additionally, informed consent will be obtained from the head of the house before collecting mosquitoes and larvae from their houses and their gardens respectively. This study will involve invading participants personal space during mosquito collection and assurance will be given that their health, safety and privacy will be preserved.

7.0 Work Plan

Description of Activity	Apr-21				May-21				Jun-21				Jul-21				Aug-21			
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12	Week 13	Week 14	Week 15	Week 16	Week 17	Week 18	Week 19	Week 20
Proposal Writing	■	■																		
Proposal Presentation			■																	
Ethics Clearance				■	■	■														
Data Collection							■	■	■	■	■	■								
Data Analysis								■	■	■	■	■	■	■	■	■				
Report Writing										■	■	■	■	■	■	■	■	■	■	
Dissemination of Findings																				■

8.0 Activity Based Budget

Description of Activity	Amount
Communication and Internet connectivity	ZMW 2,000.00
Mosquito collection	ZMW 5,000.00
Molecular Analysis of Mosquitoes	ZMW 45,000.00
Data Analysis	ZMW 3,500.00
Ethics Clearance	ZMW 1,000.00
Publication	ZMW 2,500.00
Total Amount	ZMW 59,000.00

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10.0 Appendices

Mosquito Collection Form

University of Zambia
School of Health Sciences
Department of Biomedical Sciences

Province: ______________________ District: ___________________ Study site: ____________________ Date of Collection: ______________ Collection Type: __________________ GPS Coordinates: Latitude _______________ Longitude ________________ Altitude (m): ____________

SN	Head of Household Name	No. of People	No. of Animals	Roof Type	Wall Type	Type of eaves	No. of LLINs	Brand of LLINs	Spray status	If Sprayed Insecticide used	Mosquito species	Abdominal Status		
												U	F	G

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PARTICIPANT INFORMATION SHEET

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Principal Investigator: **Mr Westone Hamwata**

Co-Investigators: **Dr N.M Shimaponda-Mataa**

Mr Mbanga Muleba

Introduction

You are invited to take part in a survey titled ***Malaria Vector Bionomics and Phenotypic Resistance Status to Insecticides Used in Vector Control in Ndola District.*** You will be provided with enough information about the study which will help you decide if you will participate or not.

Purpose of the Study

This study will evaluate the behaviour of malaria vectors and their susceptibility status to insecticides used in vector control. Specifically, species composition, malaria vector density, resting behaviour, biting behaviour, host preference and mosquito susceptibility status will be determined.

What will happen if you participate in this study

You must be provided with enough information about the study to help you understand why the research is being done and what it involves. This will help you decide whether to participate or not. Please take time to read or listen as I read the document to you.

Mosquito collection will be conducted at your premises and you will be asked questions which will just take 5 minutes of your time to complete the mosquito collection form.

How long will this study last?

This study will last 5 months. However, mosquito collection at your premise will only be conducted once.

What are the risks of participating in this study?

There are no major risks to being in this study. You will only be asked to move out of the house when conducting pyrethrum spray catches in a period of about 20 minutes then you can go back in between 05:00 hrs to 07:00 hrs. If you feel uneasy leaving investigators alone, you will be provided with a face mask so that you accompany the investigator inside house during saturation of the house with the pyrethroid insecticide.

The study involves invading your personal space and as such utmost confidentiality will be adhered to by the investigators. Further, necessary protective measures against COVID-19 are put in place during our interaction with you. We will ensure physical distancing, availability of hand sanitizer, strict use of masks by both you and the investigator.

What is the study Procedure?

By consenting to this study you will allow the investigators to access your premises to collect mosquitoes. Mosquitoes will be collected using four different collection methods. CDC light traps will be set in your house around 16:00 and retrieved the following morning around 06:00hrs. Mosquito collection using pyrethrum spray catches and aspirations will be done from 05:00 hours to 08:00hours. Larval collection will be conducted in breeding sites near your house. The mosquitoes collected will then be taken to the laboratory where they will be morphologically identified and molecular identification will be conducted for determination of sibling species in the *An gambiae* and *An funestus* complex.

What are the benefits of participating in this study?

There are no direct program benefits to you for participating in the study. You may find an indirect benefit in knowing you have participated in an important study that could help generate baseline data for informed vector control programming.

Compensation

Participants in the study will not be compensated for the time taken to participate in this study. Despite this, we would be grateful if you could allow the team to collect mosquitoes from your premises.

What freedom and rights do you have in participating in the study?

The decision to participate in this study is entirely yours, and no one else should make it for you. You are free not to join this study or to stop participating in the study at any time. You will not receive any punishment now or in the future because of this. We will respect your freedom of choice. We will not share any of your information with your family, friends, or parent.

How is your confidentiality protected in this study?

The information that is collected during the study will be kept private. No one will be told that you have participated in the study or what your answers are to the questions. The study team will make every effort to protect your privacy and maintain the confidentiality of all the information that you provide. Your name or other identifiers will not be included in reports from this study. The information will be stored on a secure computer system.

At the end of the study, the report from the study will be made available to researchers or others

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who are interested in using it to know more about the behaviour of malaria vectors and their phenotypic resistance status to insecticides used in vector control in Ndola. This means that others people besides the study staff will be able to see the information you provided.

What will happen to the results of the research study?

We will write a report combining all of the responses from all participants who have participated in this study. This report will be shared with The University of Zambia School of Health Sciences, Tropical Diseases Research Centre, Ndola District Health Office, National Malaria Elimination Centre and other key stakeholders.

Who has approved the research study?

This study has been reviewed and approved by the Tropical Diseases Research Centre Ethics Committee and National Health Research Authority.

Who else can you contact about the study and how do you contact them?

If you have any questions on the study or you being in the study, you or your selected relative/friend can contact Mr. Westone Hamwata, the Principal Investigator of the study on 0972790935. If you have a question about your rights as a research subject you or your selected relative/friend can contact the Secretary of the Tropical Diseases Research Centre Ethics Committee.

Declaration of Consent

I understand the contents of this Consent Form, and I agree to participate in this research study. I have had the opportunity to ask questions in an information session and all my questions have been answered to my satisfaction. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I understand that I am taking part in the study freely and

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that I can stop being part of this study at any time and for any reason. By signing this consent form, I agree to participate in the research study.

I agree to participate in this study Yes ☐ No ☐

_________________ _________________ ____/___/_______

Head of Household Signature Date

Witness (*if the participant was not able to read and understand the Consent Information Sheet and Informed Consent Document*).

I affirm that the Informed Consent Document has been read to the participant, and she understands the study and I have witnessed her consent to study participation.

_________________ _________________ ____/___/_______

Name of Witness Signature Date

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INFORMED CONSENT FORM

University of Zambia

School of Health Sciences

Department of Biomedical Sciences

Name of Head of Household: ____

Surveillance Activity Type: ____

House Number: ____

I can confirm that I have read (read to me) the information sheet and understand the objectives of this study. I had the opportunity to consider the information, ask questions and have had these answered fully.	Tick:
....(name of investigator) has explained to me the nature and purpose of the activities to be undertaken. I understand fully what is to be done.	Tick:
I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason.	Tick:
I agree to take part in the above activity.	Tick:

Signature of Head of Household: ___________________________ Date: ______________________

I confirm that I have explained to the person named above the nature and purpose of the activities to be undertaken.

Signature of Investigator: ________________________________ Date: _______________________

1 signed copy of this form is given to the head of household;

1 signed copy of this form is kept for the Principal Investigator