MOLECULAR CHARACTERIZATION OF MALARIA VECTORS IN GONJA-SAME DISTRICT: THE IMPACT OF INSECTICIDE TREATED NET INTERVENTIONS

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR DEGREE OF MASTERS OF SCIENCE IN PARASITOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

An entomological survey was conducted in the lowland and highland of Gonja Maore and Bombo villages respectively in Same District north - eastern Tanzania. The aim of this survey was to estimate the malaria vectors abundance and to determine the malaria vector composition of *Anopheles gambiae* and *Anopheles funestus* sibling species. Mosquitoes were collected from two identified villages with houses preferred for mosquito resting. In each of the selected village, twelve houses were provided with CDC light traps and mosquito magnet traps to collect indoor and outdoors mosquitoes respectively. The collected mosquitoes were sorted morphologically and identified by using dichotomous taxonomic key followed by preservation for molecular genotyping. DNA extraction was done using modified Bender buffer method whereby each mosquito was treated individually. The DNA were analyzed by polymerase chain reaction and the resulting amplified DNA was run in the 2% agarose gel electrophoresis parallel with ladder marker and photographed in the ultraviolet trans-illuminator light (UVP) to visualize the bands which stained by ethidium bromide. The results of this study indicate that, *An. arabiensis* were predominant sibling species of the *An. gambiae* complex in the study area, followed by *Anopheles rivulorum* and *Anopheles leesoni* for the malaria vectors. Furthermore, it was found that there has been a marked change in sibling species composition whereby *Anopheles gambiae s.s.* changed from being the most abundant in the past, to become the most rare species and *An. arabiensis* had changed to become the most common species in the study area. It is concluded that the decline in population of *An. gambiae* complex has excessively affected the most important malaria vector and thus reducing its role in the transmission of malaria in the study areas. It is recommended to carry out longitudinal study which will provide more information on malaria vectors composition of the sibling species and seasonal variation of the vectors.

DECLARATION

I Edward Zakayo Sambu, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

Edward Zakayo Sambu **Date**

(MSc. Candidate)

The above declaration is hereby confirmed;

_____ _____

Prof. Elikira Ndeshilio Kimbita **Date**

(Supervisor)

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DEDICATION

I dedicate this work to my best friend, my wife Betty Kobelo and our three sons Peter, Denis and Brighton.

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LIST OF ABBREVIATIONS AND SYMBOLS

AR	*Anopheles arabiensis*
BP	Base Pair
CDC	Centers for Disease Control
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Tri_Phosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FUN	*Anopheles funestus*
GA	*Anopheles gambiae*
IRS	Indoor residual spray
ITN	Insecticides treated nets
KAc	Potassium Acetate
LEES	*Anopheles leesoni*
LLINs	Long-lasting Insecticide-treated Nets
M	Molar
ME	*Anopheles merus* and *Anopheles melas*
PAR	*Anopheles parensi*s
QA	*Anopheles quadriannulatus*
rDNA	ribosomal deoxyribonucleic acid
RIV	*Anopheles rivulorum*
RPM	Revolution Per Minute
S.l.	Sensu lato
S.S.	Sensu stricto
SDS	Sodium Dodecyl Sulfate
Taq	Thermus aquaticus

TDR	Tropical Diseases Research
UN	Universal
VAN	*Anopheles vaneedeni*

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Malaria transmission in most parts of Africa is carried out by *Anopheles gambiae* sensu stricto Giles 1902, *Anopheles arabiensis* Patton, 1905 (Coetzee *et al.,* 2000) and *Anopheles funestus* Giles, 1987 (Gillies and Coetzee, 1987). Members of each complex group are difficult to distinguish morphologically. *Anopheles gambiae* s.s. being the primary vector which is restricted to a 128-240 kilometers stop along the coastal belt of Tanzania (Mboera, 2000). However, the species was found in Kilombero valley which is about 500 km from the coast (Mboera, 2000). Apart from the morphological similarities among these *Anopheles gambiae* sibling species their biology, vectorial competence, and host seeking behaviour is different, and for *Anopheles funestus* sibling species is closely associated with human dwellings hence it plays a critical role in malaria transmission (De Meillon *et al*., 1977).

1.2 Mosquitoes Distribution

Anopheles funestus Giles complex consist of nine species that are distributed throughout Africa; these are *Anopheles parensis* Gillies, 1962 *Anopheles aruni* Sobti, 1968 *Anopheles confusus* Evans and Leeson, 1935, *Anopheles funestus*, *Anopheles vaneedeni* Gillies and Coetzee, 1987, *Anopheles rivulorum* Leeson, 1935, *Anopheles fusciveno-sus* Leeson, 1930, *Anopheles leeson* Evans, 1931 and *Anopheles brucei* Service, 1960; Gillies and Coetzee, 1987). *Anopheles gambiae* complex occurs widely throughout southern, eastern, and central Africa. The *An. gambiae* s.l. complex is currently comprised of eight sibling species namely *An. gambiae* sensu stricto Giles, 1902, *An. Arabiensis* Patton, 1905, *An. quadriannulatus* Theobald, 1911, *An. amharicus* (Coetzee *et al*., 2013). *An. bwambae*

White, 1985, *An. melas* Theobald, 1903, *An. merus* Dönitz, 1902 and *Anopheles culuzii* (Coetzee *et al.,* 2013).

1.2 Problem Statement and Justification

The Anopheline mosquitoes profile before long lasting insecticides treated nets interventions in Gonja villages were composed of *Anopheles gambiae* s.s., *Anopheles gambiae* complex, *Anopheles funestus* type form, *Anopheles rivulorum*, *Anopheles pharoensis*, and *Anopheles parensis* (White, 1969). Following the global warming and climatic changes, the ecological condition could favor the survival and availability of the malaria vectors species which were not available before or conversely, could eliminate all the pre-existing malaria vectors in the study area. Furthermore, there have been widespread use of insecticide treated mosquito nets and indoors residual spray, which could have an influence of the vector distribution. Although some studies reported decrease in malaria vectors, information on which particular species in the *An. gambiae* s.l. and *Anopheles funestus* complex have been mostly affected are still lacking. The sibling species vary greatly in their biology, behavior and would be expected to react differently to climate changes. Due to these facts, accurate and sensitive identification method such as Polymerase Chain Reaction is crucial to separate the sibling species of the malaria vectors. This study therefore was designed to determine the available vector species composition and abundance which will help in designing a cost-effective vector control tool.

1.2.1 Main Objective

To characterize malaria vectors in Gonja-Same district and determine the impact of Insecticides Treated Nets interventions.

1.2.2 Specific objectives

i. To determine the malaria vector composition (*Anopheles gambiae* and *Anopheles funestus* sibling species) in Gonja-Same district area.

ii. To estimate the malaria vectors abundance in the study area.

1.3 Research Questions

i. What are the sibling species available in this area?

ii. What is the composition of malaria vectors after ITN intervention in this district?

ii. What is malaria vectors abundance in the study area?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biology of a mosquito

Anopheles mosquitoes belong to the phylum Arthropoda, class Insecta, order Diptera, family Culicidae, subfamily Anophelinae and genus *Anopheles*. The importance of *Anopheles* mosquitoes in warmer parts of the world is their role in transmission of malaria, lymphatic filariasis and pathogenic viruses (Gillies and Coetzee, 1987).

During their life cycle mosquitoes undergo complete metamorphosis, which includes going through the following distinct stages: egg, larva, pupa and adult, the first three stages being aquatic. Mosquitoes, as other insects, are cold-blooded (poikilothermic) animals and, therefore, are highly dependent on environmental temperature for development and survival. Their growth rate and other aspects of their physiology are temperature-dependent. As the temperature increases, their development time shortens (Gillies and Coetzee, 1987).

Mosquitoes breed in standing water, a female mosquito lays 50-200 eggs per oviposition and the eggs are laid in or near water, eggs are laid singly directly and are unique in having floats on either side. Eggs are not resistant to drying. They hatch within 2-3 days, although hatching may take up to 3 weeks in colder climates. Mosquitoes have four larval instars stages, the first instars are hardly noticeable to the human eye. They have a well-developed head with mouth brushes which are used for feeding, a large thorax, and a segmented abdomen. *Anopheles* larvae lack a respiratory siphon and for this reason position themselves so that their body is parallel to the surface of the water. Larvae breathe through spiracles located on the 8th abdominal segment and therefore must come

to the surface frequently. Larvae move through the water in a twisting motion. When they sense a shadow or movement in their habitat, larvae quickly dive to the bottom to avoid predation. They feed on microscopic plant, algae and bacteria, At the end of each instar, the larvae moult, shedding their exoskeletons to allow for further growth and become pupae. The pupa is comma-shaped by side view and moves in a somersault fashion. The head and thorax are fused into a cephalothorax with the abdomen curving around beneath. As it is for larvae, the pupae must come to the surface frequently to breathe by using a pair of respiratory trumpets on the cephalothorax. The pupae are non-feeding stage which can last 2 to 3 days. After few days the dorsal surface of the cephalothorax splits and the adult mosquito emerges (Foster and Walker, 2002).

Adult mosquitoes have slender bodies with three distinct division head, thorax and abdomen. The head has appendages specialized for acquiring sensory information and for feeding. The head contains compound eyes and a pair of long, segmented antennae. The antennae are important for detecting host odors as well as odors of breeding sites where females lay eggs. The head also has an elongate, forward-projecting proboscis used for feeding, and two sensory palps. The thorax is specialized for locomotion. Three pairs of legs and a pair of wings are attached to the thorax (Foster and Walker, 2002).

The abdomen is a segmented body part which expands considerably when a female takes a blood meal. The blood taken is digested over time serving as a source of protein for the production of eggs, which gradually fill the abdomen (Foster and Walker, 2002). Adult mosquitoes usually mate within few minutes after emerging from the pupal stage. The male mosquitoes form large swarms, usually around dusk and the females fly into the swarms to mate. The female mosquito mates only once in its life and the sperms are stored in the spermatheca (Foster and Walker, 2002).

Males live for about a week, feeding on nectar and other sources of sugar. Females will also feed on sugar sources for energy but usually require a blood meal for the development of eggs. After obtaining a full blood meal, the female will rest for a few days while the blood is digested and eggs are developed. This process depends on the temperature but usually takes 2-3 days in tropical conditions. Once the eggs are fully developed, the female lays them and resumes host seeking. The cycle repeats itself until the female dies. Females can survive up to a month (or longer in captivity) but most probably do not live longer than 1-2 weeks in nature. Their chances of survival depend on temperature and humidity, but also their ability to successfully obtain a blood meal while avoiding host defenses (Foster and Walker, 2002).

As in most areas of Africa, *Anopheles gambiae* complex is the major vector species in Tanzania with the sibling species *An. gambiae* s.s. and *An. arabiensis* Paton being the most important within the complex (White, 1974). *Anopheles gambiae* s.s. predominates or is the only species in humid coastal and lacustrine areas, while *An. arabiensis* has been found to predominate in dry and semi-arid areas (Mnzava and Kilama, 1986). Despite several decades of malaria research, locality-specific information on the diversity, spatial and temporal distribution of *Anopheles gambiae* complex and *Anopheles funestus* group is still wanting. Species within the two complexes differ in host biting preference, abundance and vector competence. Therefore identification of the mosquito vectors to species level and mapping species distribution in heterogeneous environments is critical in understanding the role of each in malaria transmission (Coetzee, 2004). Moreover, strategies for malaria control require a solid understanding of vector dynamics and factors influencing their spatial and temporal distribution (Mbogo *et al.,* 2003). Such information would help to develop early warning systems for predicting malaria epidemics, and planning control programme based on accurate prediction of their effects (Thompson *et*

al., 1997). Given the need for locally adapted, integrated vector control strategies, a better understanding of the ecology, biology and population structure of the vectorial units is necessary (TDR, 2002).

2.2 Insecticide Treated Nets for Mosquito Control

2.2.1 Use of long lasting insecticides mosquito nets/Insecticides treated nets

Uses of mosquito nets help keep mosquitoes away from people, and thus greatly reduce the infection and transmission of malaria (Kulkarni *et al.*, 2010). Insecticide-treated nets (ITN) are estimated to be twice as effective as untreated nets and offer greater than 70% protection compared with no net (Kulkarni *et al.*, 2010). New technologies like Olyset allow for production of long-lasting insecticidal mosquito nets (LLINs), which release insecticide for approximately 5 years (Malima *et al*., 2008). ITNs have the advantage of protecting people sleeping under the net and simultaneously killing mosquitoes that contact the net. This has the effect of killing the most dangerous mosquitoes. Some protection is also provided to others, including people sleeping in the same room but not under the net (William *et al*., 2003). No large scale intervention of ITNs has been employed in Gonja area since termination of Pare-Taveta malaria scheme in 1959 until Tanzanian Government developed a subsidy (voucher) scheme to distribute long-lasting insecticide-treated nets (LLIN) to pregnant women and under-five children in a public private partnership called the Tanzanian National Voucher Scheme (TNVS) (Eze *et al*., 2014). This scheme which has a very active private sector involvement, started in 2004 followed by several projects like mass distributions of free LLINs throughout the country which took place between 2009 and 2011 (Eze *et al*., 2014).

Another protective measures is the use of mosquitoes repellents which have shown to play an important role in preventing humans from mosquito bites before retiring to bed and can

therefore be used as supplemental protective measures to insecticide-treated nets and indoor residual spray that can easily be adopted in rural communities of Africa (Curtis *et al.,* 1987; 1991; Seyoum *et al.,* 2003).

2.2.2 Indoor residual spraying

Indoor residual spraying (IRS) is the application of insecticide to the inside of dwellings, on walls and other surfaces that serve as a resting place for some of malaria vector mosquitoes which rest on or a nearby surface while digesting the blood meal. In this technology insecticide kills mosquitoes and thus prevents disease transmission (TDR, 2002). There are only four insecticide classes approved by the World Health Organization Pesticides Evaluation Scheme (WHOPES) for IRS. These are organochlorides (OC), organophosphates (OP), carbamates (C) and pyrethroids (PY)(WHO, 2013). In Tanzania IRS commenced in 2007 in two districts of Kagera Region, in northwest Tanzania and has since then been extended to cover eighteen districts. The pyrethroid lambda-cyhalothrin (ICON 10CS, Syngenta) has been used in 2007 and carbamate insecticide bendiocarb (Ficam 80% wettable powder, Bayer) were used in 2012 (West *et al*., 2014). This intervention reduced malaria prevalence from 41% in 2007/2008 to 8% in 2008/2009 with this and other data indicating success after blanket spraying multiple times (West *et al*., 2015).

However, in 2011 tests of mosquito susceptibility using standard WHO bioassays showed resistance to pyrethroids in *An. gambiae* sensu stricto; as a result, IRS policy was changed to use the carbamate insecticide bendiocarb (Ficam 80% wettable powder, Bayer) by the President's Malaria Initiatives in 2012 (Protopopoff *et al*., 2013).

2.3 Species Identification

Morphological identification is the key to all other advanced methods. Species identification is mainly been performed using morphological methods; for example in *Anopheles gambiae* sensu lato the apical pale band on palps usually much wider than other two pale bands compared to *Anopheles funestus* sensu lato in which three pale bands are approximately the same width. Also there is a pale spot on third main dark area of vein one in *Anopheles gambiae* s.l. while this spot is absent in *Anopheles funestus* s.l.

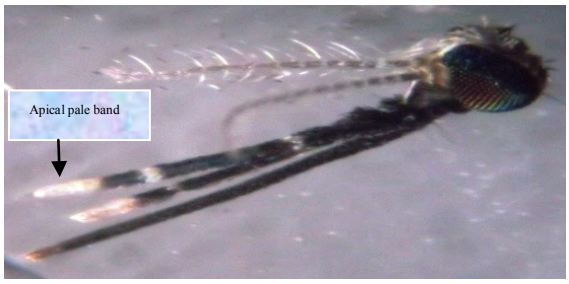

Figure 1: Palps of *An. gambiae*

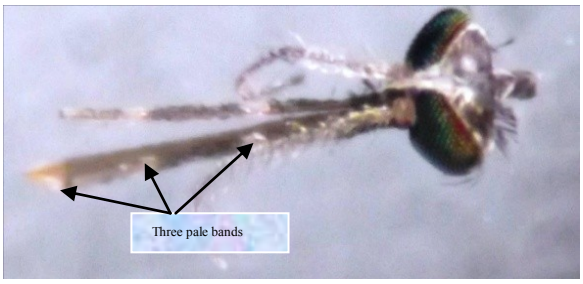

Figure 2: Palps of *An. funestus* s.l.

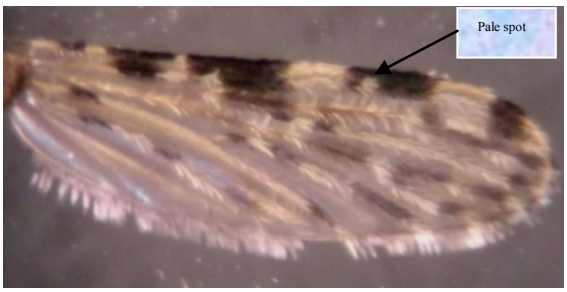

Figure 3: Wing of *An. gambiae* s.l.

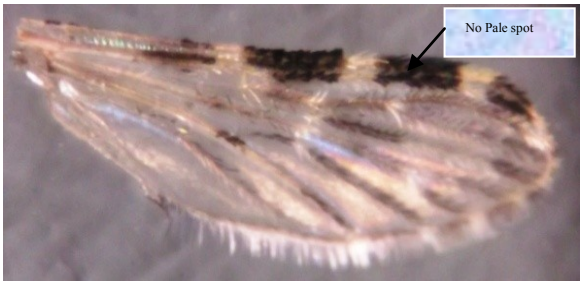

Figure 4: Wing of *An. funestus* s.l.

Keys for adult mosquitoes and larvae have been developed for most parts of the world. Taxonomy keys used in East Africa are Gillies and De Meillon (1968) and A supplement to the Anophelinae of Africa south of the Sahara by Gillies and Coetzee (1987). However morphological features cannot differentiate between sibling species in major malaria vector complexes. Morphological features overlap between species (e.g., *An. funestus, An. parensis,* and *An. vaneedeni*) or *Anopheles gambiae* complexes (Gillies and Coetzee, 1987).

2.4 Molecular Identification of Sibling Species

Molecular identification technique for *Anopheles funestus* sibling species and *Anopheles gambiae* complex has been developed and has made it possible for molecular differentiation among the complex groups. This reliable species identification method of the complex groups has increased the precision which lead to effective control method selection and implementation for sibling species (Koekemoer *et al*., 2002). The molecular identification of *Anopheles gambiae* sibling species techniques utilizes oligonucleotides primers to differentiate five members of the sibling species of *Anopheles gambiae*. This techniques uses species specific DNA sequences region, and the primers which consist of one universal primer that is complimentary to all five species and four species specific primers for *Anopheles arabiensis* Patton, 1905, *Anopheles gambiae* Giles, 1902, *Anopheles quadriannulatus* Theobald, 1911, *Anopheles melas* Theobald, 1903 and *Anopheles merus* Dönitz, 1902.The universal primer binds with one of the species specific primers to produce a DNA fragment of unique length for specific species (Scott *et al*., 1993).

Molecular identification methods for *Anopheles funestus* complexes have mainly used the rDNA locus because it is represented in multiple copies throughout the genome of mosquitoes, and it contains highly variable regions (Paskewitz *et al*., 1997). One transcription unit consists of three coding regions, 18S, 28S, and a small 5.8S gene, which are separated by non coding regions called the Internal Transcribed Spacer regions 1 and 2 (ITS_1 and ITS_2) (Paskewitz *et al*., 1997 and Koekemoer *et al.,* 2002). The transcription units are separated by intergenic spacer regions (IGSs). The ITS regions shows relatively high levels of intraspecies variation but not as high as the IGS region. Variation found in these regions makes it possible to design species-specific diagnostic assays (Koekemoer *et al*., 2002).

2.5 Malaria

Malaria is a disease caused by a protozoa belonging to the genus *Plasmodium* transmitted to human through the bites of infected female *Anopheles* mosquito (WHO, 2014). There are five species of *Plasmodium* can infect humans being. Most deaths are caused by *Plasmodium falciparum* whereas *P. vivax*, *P. ovale* and *P. malariae* generally cause a milder form of malaria. The species *P. knowlesi* has been shown to causes disease in humans (Caraballo, 2014). The mosquito bites and introduces the parasites from its saliva into a person's blood. The parasites travel to the liver where they mature and reproduce (WHO, 2014). Malaria causes symptoms which usually begin seven to fifteen days after an infective mosquito bite that typically include fever, fatigue, vomiting and headaches. In severe cases it can cause yellow skin, seizures, coma or death (Caraballo, 2014).

Malaria is typically diagnosed by the microscopic examination of blood using blood films, or with antigen-based rapid diagnostic tests (Caraballo, 2014). In Tanzania malaria has been diagnosed by using microscope for some decades, but currently the Ministry of Health, Social development, Gender, Elders and Children through the National Malaria Control Programme (NMCP) has introduced rapid diagnostic tests (RDTs) in all health facilities to scale-up malaria diagnosis and improve case management. Rapid Diagnostic Tests detect presence of parasite specific antigens and available RDTs detect either *Plasmodium falciparum* specific histidine rich protein 2 (PfHRP-2), *Plasmodium* lactate dehydrogenase (pLDH) or aldolase (Bell *et al*., 2006; Murray *et al*., 2008).

Other methods of malaria diagnosis including the use the polymerase chain reaction to detect the parasite DNA have been developed, but are not widely used in areas where malaria is common due to their cost and complexity (Nadjm and Behrens, 2012).

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Study Area

The study was carried out in Gonja area, Maore ward (4°20′S, 38°00′ E) of Same District, northeast Tanzania. Two villages were involved, Bombo village on the highlands (1210 m above sea level) and Maore Kadando village which is on lowland (500 m above sea level).

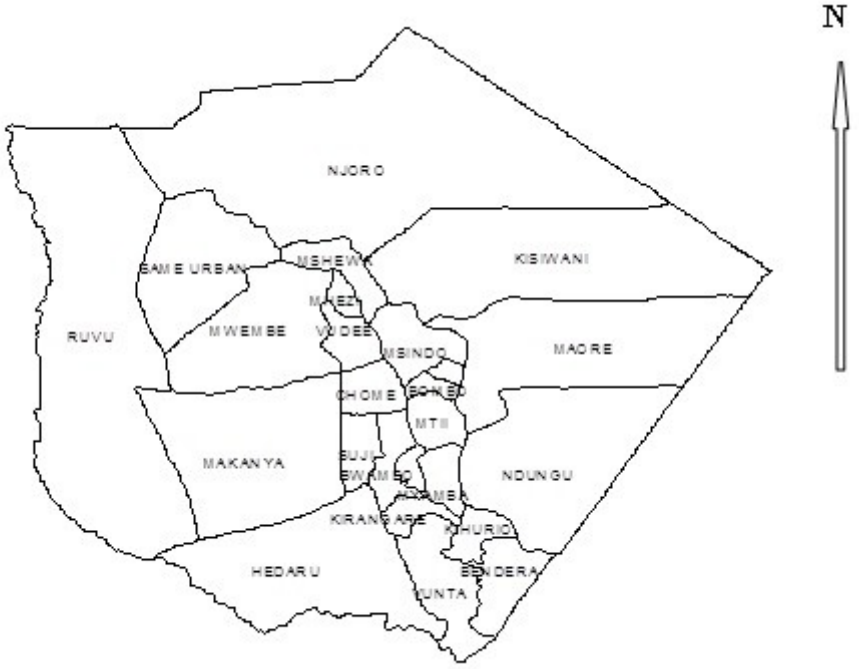

Figure 5: Map showing the location of surveyed villages in Same District

Topographically, Maore area lies in the lowland of Mkomazi valley (500-900 m) and Bombo on highlands of the Pare mountains (1200-1800 m). The average annual rainfall is 780mm occurring mainly between February and May with short rains in November to January. The main activities of residents in the low land (Gonja Maore) is growing rice in the swampy and irrigated area of the Mkomazi rice irrigation scheme. In the plain dry land, they keep cattle, sheep and goats, while in the highland (Bombo) they grow ginger,

bananas, maize, cassava, beans, tomatoes, vegetables and small scale livestock keeping (zero grazing system). Most of the houses in lowland were brick-walled with corrugated iron sheets with only a few having thatched roofs. In contrast, in the highland area, most houses were mud-walled with thatched roofs and only a few had brick walls and iron roofs.

3.2 Sample Selection and Sample Size

Before commencing mosquitoes collections in the study sites villages, leaders were informed about the study and verbal consent was obtained from the heads of households. Ethical approval for the study was provided by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania (Ref: NIMR/HQ/R.8a/Vol. IX/1858) and Sokoine University of Agriculture (SUA) (Ref:SUA/ADM/R.1/8/Sambu). In the two selected villages (Maore and Bombo) mosquitoes were collected from houses (such as those with thatched roofs) purposely selected because they are preferred for mosquito resting. In each village, twelve houses were provided with light traps, untreated rectangular bednet and Mosquito magnet.

3.3 Study Design and Data Collection

3.3.1 Indoors mosquitoes collection

A cross sectional survey was conducted in December and January; in each village traditional style houses (mud walls with thatched roofs) were purposively selected (non probability sampling) and each occupant in these houses were provided a rectangular un-impregnated bed net to protect the individual sleeping in the room, then standard Centers for Diseases Control light traps (CDC traps) with an incandescent light bulb (Model 512; John W. Hock Company) hung besides of bed occupied by a person sleeping in un-impregnated bed net with 150cm from the floor. The light trap was operated from 2000 h

to 0600 h and retrieved in the morning. To determine the malaria vectors abundances in the study area, the mosquitoes collected by CDC light traps were sorted, morphologically identified using Gillies and De Meillon (1968) key, and then preserved in the silica gel in Eppendorf tubes for further identification by PCR method.

3.3.2 Outdoor mosquito collection

The Mosquito Magnet® (MM) trap (American Biophysics Corporation, currently owned by Woodstream Corporation, Lititz, PA) was battery operated trap and runs on propane gas that is catalytically converted to produce carbon dioxide (CO_2), heat and water vapor. A thermoelectric generator uses excess heat from combustion to produce electricity to power the trap. The trap is based on counter flow geometry technology whereby a fan produces a down-flow plume of CO_2 through a central pipe and an updraft through a larger surrounding pipe that draws in mosquitoes attracted to the CO_2 and keeps them in the collecting bag within the trap.

The trap was run from 1800 hrs to 0600 hrs outside the house (outdoor) to collect exophilic, anthropophilic and zoophilic mosquitoes. In the morning the mosquitoes were collected in their respective labeled paper cups, and transported in a cool box to research laboratory for further processing. In the laboratory mosquitoes were sorted morphologically and identified by using dichotomous taxonomic key followed by preservation in the labeled Eppendorf tubes with silica gel (Gillies and De Meillon, 1968).

3.4 Molecular Identification of Collected Mosquitoes

3.4.1 DNA extraction

DNA extraction was done using modified Bender method (Scott *et al.,* 1993). Individually, mosquitoes were homogenized in a 1.5 ml Eppendorf tube in 100 µl Bender

buffer (0.1 M $NaCl_2$, 0.5 M Tris Hcl pH 7.5, 0.05 M EDTA pH 9.1, 0.2 M sucrose and 0.5% SDS). The homogenate were incubated at 65^{o}C for 30 minutes. 15µl of pre chilled 8M potassium acetate (KAc) was added. This was incubated at 4°C for 30 minutes followed by centrifugation at 14 000 rpm for 5 minutes. The supernatant was transferred into a fresh tube and 250µl absolute ethanol added, mixed and then incubated at -20°C for 3 hrs to obtain DNA precipitate. This followed by centrifugation at 14 000 rpm for 10 minutes and the supernatant discarded to obtain a DNA pellet which was dried by inverting the tube over paper towel. The dried pellet was then re-dissolved in 100µl of sterile double-distilled water, incubated at 4°C for one hour and thereafter kept at -21^{o}C for long storage (to avoid DNA degradation) until required for PCR amplification.

3.4.2 DNA amplification for *Anopheles gambiae* s.l.

DNA amplification was done by using thermal cycler machine (S1000^{TM} BIORAD) with initial cycle of denaturation at 94°C for 15 minutes followed by 35 cycles of annealing (94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds) and extension at 72°C for 10 minutes and final hold at 4°C until required for PCR amplification. The mastermix used were TEMPase hot start (Ampliqon III, VWRBie Berntsen, Denmark) (dNTP, $Mgcl_2$, Taq buffer, and hot start Taq) with the final concentration of one times.

The primers used (eurofins mwg/operon-16805692 D4) include a universal primer that matches ribosomal DNA for the members of *An. gambiae* complex (UN= 5'-GTG TGC CCC TTC CTC GAT GT- 3'), The primers which were used to identify both *An. merus* and *An. melas* are (ME=5'-TGA CCA ACC CAC TCC CTT GA-3') and specific primers for *An. quadriannulatus* (QA=5'-CAG ACC AAG ATG GTT AGT AT- 3'), *An. gambiae* (GA=5'-CTG GTT TGG TCG GCA CGT TT-3') and *An. arabiensis* (AR=5'-AAG TGT CCT TCT CCA TCC TA-3') (Scott *et al.,* 1993).

3.4.3 DNA amplification for *Anopheles funestus* complex

For *Anopheles funestus* complex the initial cycle of denaturation was 94°C for 15 minutes followed by 40 cycles of annealing (94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 40 seconds) and extension at 72°C for 10 minutes and final hold at 4°C until required for electrophoresis. The primers used for *Anopheles funestus* complex group were (UV =5'-TGT GAA CTG CAG GAC ACA T-3'), *Anopheles funestus* (FUN =5'-GCA TCG ATG GGT TAA TCA TG-3'), *Anopheles vaneedeni* (VAN= 5'-TGT CGA CTT GGT AGC CGA AC-3'), *Anopheles rivulorum* (RIV =5'-CAA GCC GTT CGA CCCTGA TT-3'), *Anopheles parensis* (PAR =5'-TGC GGT CCC AAG CTA GGT TC-3') and for *Anopheles leesoni* (LEES =5'-TAC ACG GGC GCC ATG TAG TT-3') (Scott *et al.,* 1993).

The resulting amplified DNA was run in the 2% agarose gel electrophoresis parallel with ladder marker and photographed in the ultraviolet trans-illuminator light (UVP) to visualize the bands which were stained by ethidium bromide.

CHAPTER FOUR

4.0 RESULTS

4.1 Mosquito Abundance

A total of 5856 mosquitoes were collected using CDC light traps and Mosquito Magnet™ traps in lowland (Kadando village) and highland (Bombo village) in Gonja. More mosquitoes (74.4%; N=4355) were collected indoors using light traps than the outdoor operated Mosquito Magnet™ traps. With respect to altitude, more mosquitoes (99.8%; N=5846) were collected in lowland than in the highland village. Of the collected mosquitoes, 3253 (55.5%) were *Culex quinquefasciatus*, 2587(44.2%) were *An. gambiae* complex, 7(0.1%) were *An. funestus* group and other non malaria vector mosquito species accounted for (N=9) 0.1 %. Mosquitoes trapped and proportion collected by each trapping method in different altitude is shown in table 1. The average number of mosquitoes collected per trap per night for light trap and mosquito magnet trap was 26 & 8, respectively.

Table 1: Abundance and composition of mosquitoes collected in the study areas

Species collected	No. (%) in lowland village		No. (%) in highland village		Total
	Light Trap	Magnet trap	Light Trap	Magnet trap	
Cx. quinquefasciatus	2930 (90.1)	316 (9.7)	7 (0.2)	0 (0.0)	3253
An. gambiae complex	1411 (54.5)	1173 (45.3)	3 (0.1)	0 (0.0)	2587
An. funestus group	6 (85.7)	1 (14.3)	0 (0.0)	0 (0.0)	7
An. pharoensis	8 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	8
An. coustani	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	1
Total	**4355 (74.4)**	**1491 (25.5)**	**10 (0.2)**	**0 (0.0)**	**5856**

4.2 Molecular Identity

Mosquitoes that did not belong to the *An. gambiae* complex or *An. funestus* group (non malaria vectors) were excluded from further molecular analysis. Thus, 2587 collected *An.*

gambiae complex and 7 *An. funestus* group were analyzed for sibling species composition using molecular techniques. Of 2587 specimens of *An. gambiae* complex analyzed for sibling species, 2587 (100%) were identified as *An. arabiensis* sibling species. Likewise, of 7 specimens of *An. funestus* group processed for species identity, 5 (71.4%) were *Anopheles rivurolum and* 2 (28.6%) were *Anopheles leesoni*. Based on molecular identification, 2587 (54.5%) of *An. arabiensis* were collected with light trap while 1176 (45.5%) were collected with mosquito magnet trap. The sibling species of *An. gambiae* complex and *An. funestus* group collected by each trapping method is shown in table 2.

Table 2: PCR testing of sibling species of *An. gambiae* complex and *An. funestus* group

PCR test	# tested	Sibling species identified	LT Catch (%)	MMT Catch (%)	Total
An. gambiae complex	2587	*An. arabiensis*	1411 (54.5)	1176 (45.5)	2587
An. funestus group	7	*An. rivulorum*	5 (71.4)[¥]	0 (0.0)	5
		An. leesoni	1 (14.3)[¥]	1 (14.3)[¥]	2

LT=Light Trap; MMT=Mosquito Magnet Trap; [¥] Denominator = tested *An. funestus* group (7)

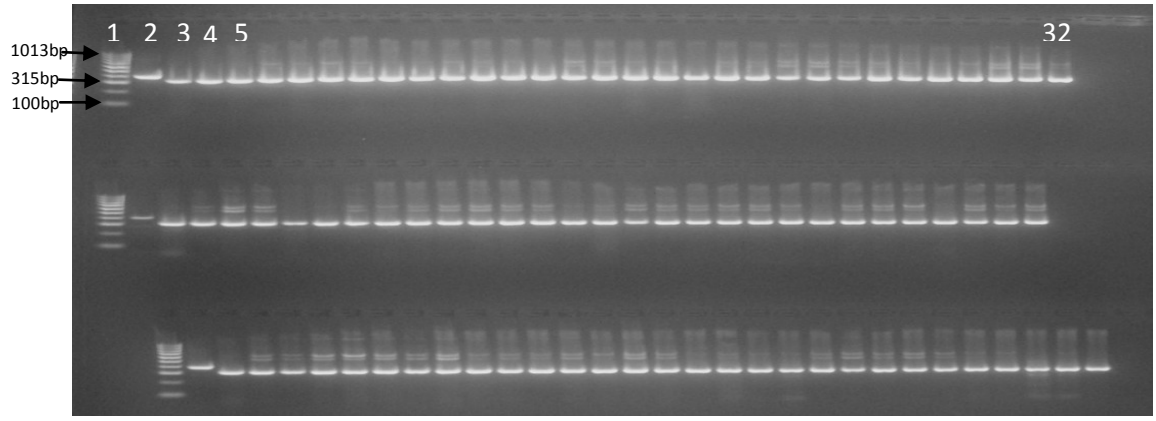

Figure 6 : PCR detection of members of the *An. gambiae* s.l. complex collected in Gonja Maore and Bombo villages of Same-District: Lane 1= 100 bp DNA ladder; Lanes 2 and 3 are control, Lane 4-32 are individual species of *Anopheles arabiensis*

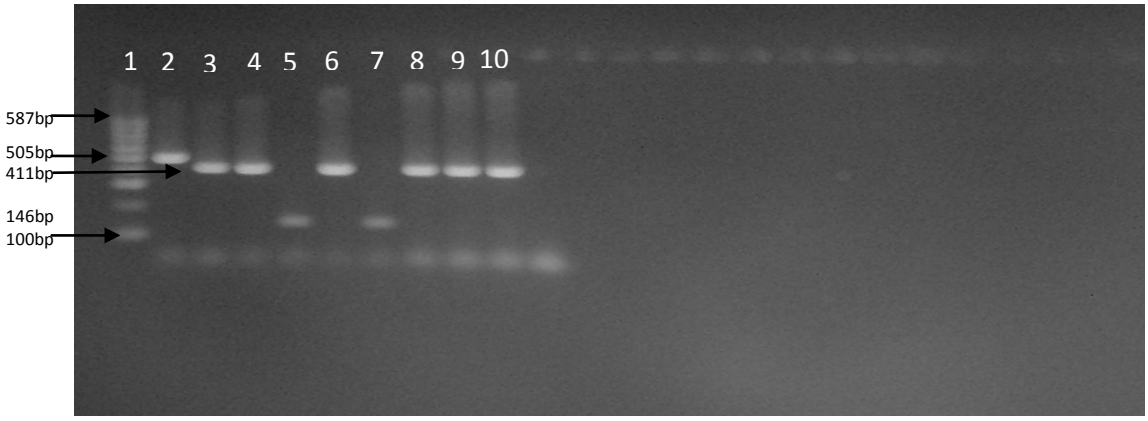

Figure 7: PCR detection of members of the *An.funestus* s.l. complex collected in Gonja Maore villages of Same-District: Lane 1=100 bp DNA ladder; Lane 2-3 are control. Lanes 4,6 and 8-10 are *Anopheles rivurolum* and Lane 5,7 are *Anopheles leesoni*

CHAPTER FIVE

5.0 DISCUSSION

Collection and identification of mosquito vectors provides the knowledge of their biology, ecology and distribution which is important in the control operations. Using CDC light and Mosquito magnet traps, this study reported relative abundance and distribution of sibling species of the *An. gambiae* s.l. complex and *An. funestus* group in a lowland and highland village of Gonja, Same district in north-eastern Tanzania. The study has shown that the relative abundance of members of *An. gambiae* complex and *An. funestus* group varied considerably in the lowland and highland village, with 99.8% and 100% of the former and later sibling species collected in the lowland village. This differential preference of the malaria vectors to breed in lowland areas agrees with findings of other studies which indicate that relative high temperatures and availability of suitable habitats are the main factors (Gillies and Coetzee, 1987). Of a particular relevancy to the current study, Mboera and his colleagues (2002) working in the same villages 15 years ago did not collect any mosquito in highland areas. However, with climate change, malaria transmission in highland has been reported in many areas of east Africa (Hay *et al*., 2002; Himeidan and Kweka, 2012).

Surveillance of malaria vectors revealed that light and mosquito magnet traps employed had relatively the same efficiency in collecting *An. gambiae* complex (54.6 and 45.3%, respectively). Since light traps and mosquito magnet traps were used inside and out of the houses respectively, In other study which compared the efficacy of Mosquito Magnet and CDC light traps, the Mosquito Magnet caught more mosquito than the CDC light trap (Hutchinson *et al.,* 2007). In contrast, Reusken found that the CDC trap performed better than the Mosquito Magnet in the Netherlands (Reusken *et al*., 2011). A limited study in

Germany compared the CDC traps and Mosquito Magnet, but did not find significant differences (Rose *et al*., 2006). The study indicates that there was an equal proportion of biting activities of *An. gambiae* complex between indoor and outdoor. However, members of the *An. funestus* group were only collected with light traps which were set indoors. Members of the *An. gambiae* complex and *An. funestus* group have been shown to have variable preferences for their breeding sites, host types and resting behavior as detailed by Gillies and Coetzee (1987). In members of the *An. gambiae* complex, *An. gambiae* s.s. has strong preference of feeding on humans and rest to complete gonotrophic cycle indoors. *An. arabiensis* is a more liberal vector, having fairly the same anthropophilic tendencies like *An. gambiae* s.s., but when other mammalian hosts are available it prefer feeding on them and rest outdoors to complete gonotrophic cycle (Gillies and Coetzee 1987). In members of the *An. funestus* group, *An. funestus* s.s. is the predominant species both in numbers and geographical distribution, and also the most anthropohilic (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

Analysis of the membership in the *An. gambiae* complex by molecular techniques revealed the presence of *An. arabiensis* as the only sibling species in the study area. In an earlier study conducted in a village of Buiko located near the current study site, Mnzava and Kilama (1986) documented the presence of *An. gambiae* s.s., *An. merus* and *An. arabiensis* as sibling species in the area. When comparing the current composition with what had been reported in the past, it is evident that there has been a change in sibling species composition over time, with *An. arabiensis* predominating. Studies have suggested that the scale up of insecticide treated nets may be one of the causes of change the composition of the *An. gambiae* complex (Russell *et al.,* 2011; Mutuku *et al*., 2011; Derua *et al.*, 2012). However, since the change in malaria vector dynamics has been reported in areas with no

insecticide intervention (Meyrowitsch *et al*., 2011; Derua *et al*., 2012) climate change has been reported to perpetuate change in malaria vector dynamics (Githeko *et al.,* 2000).

On the other hand, analysis of the *An. funestus* group by molecular technique recorded two sibling species namely *An. rivulorum* and *An. leesoni,* with the former species predominating. In areas of northeastern Tanzania, four sibling species of *An. funestus* group, namely *An. funestus* s.s, *An. parensis, An. rivulorum* and *An. leesoni* have been reported (Derua *et al*., 2015). Of particular relevancy to the current study, members of *An. funestus* group are known to exhibit species replacement when confronted with insecticide based interventions (Gillies and Smith, 1960; Gillies and Furlong, 1964). Due to insecticide intervention (IRS) in Pare area (the Pare-Taveta Scheme) the malaria vector *An. funestus* s.s was replaced by *An. rivulorum* (Gillies and Smith, 1960). In the current study, the predominance of *An. rivulorum* and absence of *An. funestus* s.s. suggest that the later species have not recovered since its replacement in the IRS intervention. Moreover, climate change has shown to have impact in the composition and distribution of *An. funestus* group (Githeko *et al*., 2000).

The absence of anthropophilic, endophilic *An. gambiae* s.s. and the predominance a more liberal vector *An. arabiensis* (biting both human and animals indoor and outdoor) might present significant challenges in mosquito control operations. Although *An. arabiensis* is considered a relatively a poor vector as compared to *An. gambiae* s.s. (Mutero and Birley 1987), its reduced contact with humans renders it less in contact with ITNs and IRS. This behavior is also likely to select *An. arabiensis* for resistance due to contact with sub-optimal doses of insecticides as it has been recently reported that long lasting insecticide treated nets are more effective in controlling *An. gambiae* s.s. than *An. arabiensis* (Kitau *et al.,* 2012). Moreover, the finding of high proportion of *An. rivulorum* which has been

suspected as a malaria vector (Wilkes *et al.*, 1996; Coetzee and Fontenille, 2004; Kawada *et al.,* 2012) call for more research for this species. In this research the *Culex quinquefasciatus* appeared to be the majority amongst the trapped mosquitoes which accounted for 55% which indicates the increase of *Culex quinquefasciatus* compared to the study conducted in the year 2002 in which *Culex* accounted for 9.8% of the trapped mosquito in the same study area (Mboera *et al.,* 2002). This could be contributed by the urbanization and modernization of houses in this study area in which cesspit and septic tanks increase in number thus providing favorable and stable breeding sites for the Culex mosquito.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

For the first time, this study have reported occurrence of malaria vectors in highland village of Gonja in Same district. Using molecular techniques, this study have shown that *An. arabiensis* is the only malaria vector identified in the study areas. As a result of change in climate, other members of the *An. gambiae* complex which were reported in the past (*An. gambiae* s.s. and *An. merus*) were not found in the study areas. With regard to members of the *An. funestus* group, this study suggests that *An. funestus* s.s which was replaced by *An. rivulorum* during the Pare-Taveta malaria scheme had not recovered. The absence of the most effective malaria vector, the *An. gambiae* s.s. is encouraging but predominance of a liberal vectors the *An. arabiensis* present yet another significant challenge due to the fact that this vector can feed indoor/outdoor and can switch its host from human to domesticated animals thus evading ITN/IRS intervention. This calls a different approach in the control of malaria in Gonja area.

6.2 Recommendations

This was a cross sectional study conducted for a relatively short time, hence a longitudinal study is recommended in the future as it will provide more information on malaria vectors composition and distribution. However, the findings of this study have shown that as the role of endophilic, anthropohilic *An. gambiae* s.s. is negligible in malaria transmission in the study areas while the role a liberal *An. arabiensis* is increasing, there is a pressing need to introduce outdoor mosquito control interventions to complement ITNs and IRS. Moreover, the finding of *An. rivurolum*, *An. leesoni* and *An. pharoensis* calls for further investigation to establish their role in malaria transmission in the study areas.

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APPENDICES

Appendix 1 : *Anopheles gambiae* complex PCR

TEMPase hot start master mix

An. gambiae	390bp
An. arabiensis	315bp
An. quadrianulatus	153bp
An. merus/melas	466/464bp

Date: _____/_____/_____

Run nr.1_ Purpose:_____

Samples:

1	9	17	25	33	41	49	57	65	73	81	89AG
2	10	18	26	34	42	50	58	66	74	82	90AR
3	11	19	27	35	43	51	59	67	75	83	91
4	12	20	28	36	44	52	60	68	76	84	92
5	13	21	29	37	45	53	61	69	77	85	93
6	14	22	30	38	46	54	62	70	78	86	94
7	15	23	31	39	47	55	63	71	79	87	95
8	16	24	32	40	48	56	64	72	80	88	96

Multiply: x100

Reagents	**Vol. pr sample**	**Vol. in mix**	**Final conc. per sample**
H_2O	4.0 µl	400	
Primer mix: (1.25uM) UN GA AR QD ME	4.0 µl	400	0.25 µM pr. Primer
TEMPase hot start mastermix	10 µl	1000	1x

Add. 18 µl Master Mix

2 µl Sample DNA

94°C 15 min

94°C 30 se
50°C 30 sec } x 35
72°C 30 sec

72°C 10 min

4°C until required for further analysis

Appendix 2*: Anopheles funestus* group PCR

An. funestus	505bp
An. rivulorum	411bp
An. vaneedeni	587bp
An. parensis	252bp
An. leesoni	146bp

Date:____/____/____

Run nr.2_ Purpose:_____

Samples:

1	**9**	**17**	**25**	**33**	**41**	**49**	**57**	**65**	**73**	**8**	**89**
2	**10**	**18**	**26**	**34**	**42**	**50**	**58**	**66**	**74**	**82**	**90**
3	**11**	**19**	**27**	**35**	**43**	**51**	**59**	**67**	**75**	**83**	**91FUN**
4	**12**	**20**	**28**	**36**	**44**	**52**	**60**	**68**	**76**	**84**	**92RIV**
5	**13**	**21**	**29**	**37**	**45**	**53**	**61**	**69**	**77**	**85**	**93PAR**
6	**14**	**22**	**30**	**38**	**46**	**54**	**62**	**70**	**78**	**86**	**94LEE**
7	**15**	**23**	**31**	**39**	**47**	**55**	**63**	**71**	**79**	**87**	**95NEG**
8	**16**	**24**	**32**	**40**	**48**	**56**	**64**	**72**	**80**	**88**	**96**

Multiply: x100

Reagents	**Vol. per sample**	**Vol. in mix**	**Final conc. per sample**
H_2O	16	**160**	
Primers: (uM) UN FUN RIV VAN PAR LEE	1	**100**	µM per. Primer
TEMPase hot start mastermix	5	**500**	

Add. 22 µl Master Mix 3 µl Sample DNA

94°C 15 min

94°C 30 sec
50°C 30 sec
72°C 40 sec
72°C 10 min
} x 40

4°C until required for further analysis

Appendix 3: Ethical clearance

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
P.O. Box 9653
Dar es Salaam
Tel: 255 22 2121400/390
Fax: 255 22 2121380/2121360
E-mail: headquarters@nimr.or.tz
NIMR/HQ/R.8a/Vol. IX/1858

Ministry of Health and Social Welfare
P.O. Box 9083
Dar es Salaam
Tel: 255 22 2120262-7
Fax: 255 22 2110986

25th November 2014

Edward Z Sambu
NIMR Muheza, P O Box 81
MUHEZA, Tanga

CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Molecular characterization of malaria vectors in Gonja-Same District: The Impact of Climate Change and Insecticide Treated Nets Interventions (Sambu E Z *et al*), has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health & Social Welfare and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine. NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Gonja in Same District, Kilimanjaro region.

Approval is for one year: 25th November 2014 to 24th November 2015.

Name: Dr Mwelecele N Malecela

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Dr Margaret E Mhando

Signature
Ag CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, SOCIAL WELFARE

CC: RMO
DED
DMO

Appendix 4: Clearance permit for conducting research

CLEARANCE PERMIT FOR CONDUCTING RESEARCH IN TANZANIA

SOKOINE UNIVERSITY OF AGRICULTURE
OFFICE OF THE VICE-CHANCELLOR
P.O.Box 3000, MOROGORO, TANZANIA

Phone: 023-2604523/2603511-4: Fax:023-2604651

Our Ref. SUA/ADM/R.1/8/Sambu **Date: 25th August 2014**

The District Medical Officer
SAME.

Re: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

The Sokoine University of Agriculture was established by Universities Act No.7 of 2005 and SUA Charter of 2007 which become operational on 1st January 2007 repealing Act No 6 of 1984. One of the mission objectives of the University is to generate and apply knowledge through research. For this reason the staff, students and researchers undertake research activities from time to time.

To facilitate the research function, the Vice-Chancellor of the Sokoine University of Agriculture (SUA) is empowered under the provisions of SUA Charter to issue research clearance to both, staff, students and researcher of SUA.

The purpose of this letter is to introduce to you **Mr. Sambu, Edward Zakayo** a bonafide **MSc. (PARASITOLOGY)** student with registration number **HD/T/SUA/VET/59/2013** of SUA. By this letter Mr. Sambu has been granted clearance to conduct research in the country. The title of the research in question is "M**olecular Characterization of Malaria Vectors in Gonja-Same District: The Impact of Climate Change and Insecticide Treated Nets Interventions"**

The period for which this permission has been granted is **from October 2014 to July 2015.** The research will be conducted in **Same District.**

Should some of these areas/institutions/offices be restricted, you are requested to kindly advice the researcher(s) on alternative areas/ institute/offices which could be visited. In case you may require further information on the researcher please contact me.

We thank you in advance for your cooperation and facilitation of this research activity

Yours sincerely,

Prof. Gerald C. Monela
VICE-CHANCELLOR

Copy to: Student – **Mr. Sambu, Edward Zakayo**

VICE CHANCELLOR
SOKOINE UNIVERSITY OF AGRICULTURE
P. O. Box 3000
MOROGORO, TANZANIA