PREVALENCE OF ASYMPTOMATIC AND SUBMICROSCOPIC MALARIA PARASITAEMIA IN KOROGWE, NORTH-EASTERN TANZANIA

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

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ABSTRACT

Despite the intensification in malaria control interventions, malaria still remains a major public health problem in malaria endemic settings. Asymptomatic infections that largely remain undetected act as reservoir of transmission jeopardizing the current control efforts. The aim of this study was to determine the prevalence of asymptomatic malaria infection, anaemia and submicroscopic parasitaemia in individuals of Korogwe District, a malaria endemic setting in north-eastern Tanzania. A community-wide cross-sectional malariometric survey involving participants aged 0-19 years was conducted in two villages of Korogwe District, a malaria endemic setting in north-eastern Tanzania. Parasite DNA was extracted from dried blood spots (DBS) collected during the survey. Detection of submicroscopic malaria infection from slide negative cases was done by *Plasmodium* genus and species-specific nucleotide amplification method. Out of 565 participants, 211 (37.41%, 95% CI: 33.99-41.49%) had malaria infection after testing using malaria rapid diagnostic test (mRDT), whereas only 81 (14.38%, 95% CI: 11.71-17.54%) were malaria positive by microscopy. Of the 565 participants, 130 (23.0%, 95% CI: 19.71-26.67%) and 33 (5.84%, 95% CI: 4.18-8.11%) had asymptomatic malaria based on mRDT and microscopy, respectively. Out of 565 participants, 135 (23.89%, 95% CI: 20.54-27.59%) were anaemic. Three out of 206 (1.5%, 95% CI: 0.47-4.46%) samples that were malaria negative by microscopy were found to be positive when analyzed by polymerase chain reaction (PCR), indicating the presence of submicroscopic malaria. Multivariate logistic regression analysis for predictors of anaemia showed that, participants aged between 5-9 and 10-14 years were significantly associated with asymptomatic malaria and anaemia (adjusted odds ratio (AOR) = 0.6, 95% CI: 0.37-0.98, p = 0.04) and (AOR = 0.32, 95% CI: 0.14-0.75, p = 0.01), respectively. The current study observed that asymptomatic malaria is much more prevalent in participants aged above five years. Furthermore, participants aged 5-14

years were at risk of asymptomatic malaria associated with anaemia. A low proportion of submicroscopic parasitaemia was observed in this study. The findings highlight the need for targeted interventions focusing on those participants aged above five years in malaria control interventions.

DECLARATION

I, Paul Martine, 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 it had neither been submitted to another Institution nor being concurrently submitted in any other Institution.

_____ _____

Paul Martine Date
(MSc. Candidate)

The declaration is here confirmed by;

_____ _____

Prof. Gerald Misinzo Date
(Supervisor)

_____ _____

Prof. John P.A. Lusingu Date
(Co-Supervisor)

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DEDICATION

My work is dedicated to my wife Elifrida Martine and son Stephen Paul for courage and patience during my entire study period. I also dedicate my work to my supervisors Professors Gerald Misinzo and John Lusingu for their profound supervision.

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LIST OF ABBREVIATIONS AND SYMBOLS

ºC	degree Celsius
µL	microlitre,
µM	micromolar
ACTs	artemisinin-based combination therapy
AIDS	acquired immunodeficiency syndrome
AOR	adjusted odd ratios
bp	base pairs
CI	confidence intervals
DBS	dried blood spot
DNA	deoxyribonucleic acid
Hb	haemoglobin
HRP2	histidine rich protein 2
ITNs	insecticide treated nets
LLINs	long lasting insecticide nets
MaReCa	Malaria Research Capacity Building for Clinical Trials in Tanzania
MRCC	Medical Research Coordinating Committee
mRDT	malaria rapid diagnostic test
NAA	nucleic acid amplification
NBS	National Bereau of Statistics
NIMR	National Institute for Medical Research
OR	odds ratio
PCR	polymerase chain reaction
pLDH	parasite lactate dehydrogenase
RBCs	red blood cells

RDTs	rapid diagnostic tests
rRNA	ribosomal ribonucleic acid
SUA	Sokoine University of Agriculture
TBE	TRIS borate ethylenediaminetetra acetic acid
TBS	thin blood smear
TF	thin film
TMIS	Tanzania Malaria Indicator Survey
WHO	World Health Organization

CHAPTER ONE

1.0 BACKGROUND INFORMATION

1.1 Malaria

Malaria is among the most life threatening infectious diseases affecting humans, accounting for approximately half a million deaths annually (WHO, 2016). The aetiological agents of malaria are parasites of the genus *Plasmodium* which are spread to people through bites of infected female mosquitoes of the genus *Anopheles* (WHO, 2017). There are five *Plasmodium* parasites that cause malaria in humans including *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* (WHO, 2017). Asymptomatic malaria is the presence of malaria parasites in the blood in the absence of symptoms (Gudo *et al.,* 2013). Asymptomatic infections are important as potential contributors to the infectious reservoir of malaria parasites (Manjurano *et al*., 2011). To interrupt malaria transmission, asymptomatic infections are important to be identified accurately (Sumari *et al*., 2017).

1.2 Global Situation of Malaria

Globally there was an increase of 3.5 million malaria cases in 2017 compared to 2016 (WHO, 2018). The estimated number of malaria deaths stood at 435 000 in 2017 compared to 451 000 in the preceding year 2016 (WHO, 2018). More than 90% of these cases were from Africa. Nearly half of the world's population is at risk of malaria with more cases occurring in sub-Saharan Africa. However the World Health Organization (WHO) regions of south-east Asia, eastern Mediterranean, western Pacific and the America are also at risk (WHO, 2017).

1.3 Malaria in Sub-Sahara Africa

In Africa south of the Sahara including Tanzania, malaria continues to claim lives of people (WHO, 2018). Malaria control intervention using effective antimalarial drugs such as, artemisinin-based combinational therapy (ACTs) is unlikely to reduce malaria transmission substantially in much of sub-Saharan Africa where individuals are rapidly re-infected (Khatib *et al.*, 2012). Malaria vector control interventions using insecticide treated nets (ITNs) and long lasting insecticide nets (LLINs) are also faced with challenges of multiple insecticides resistance thus hampering malaria elimination efforts (Kisinza *et al.*, 2017). The currently available interventions alone will not lead to malaria elimination in most of the poverty stricken rural areas (Tusting *et al.*, 2016).

1.4 Malaria in Mainland Tanzania

Malaria still continues to place a heavy burden in some parts of mainland Tanzania. A country wide school malaria parasitological survey done in Tanzania, shows that malaria is still a burden in Tanzania (Chacky *et al.*, 2018). According to the Tanzania Malaria Indicator Survey of the year 2017 (TMIS, 2017), malaria transmission in mainland Tanzania is markedly heterogeneous ranging from low in the central regions (1.1%) to high transmission in southern (13.6%), western (16.6%) and lake regions (10.6%). Majority of the regions are characterized by stable perennial transmission with peak transmission occurring shortly after long and short rains. A high magnitude of asymptomatic malaria in mainland Tanzania has been previous reported (Manjurano *et al.*, 2011; Sumari *et al.*, 2017).

1.5 Problem Statement and Justification

There is a lack of information on asymptomatic and submicroscopic malaria parasitaemia and its association with anaemia in Korogwe, north-eastern Tanzania. Despite the achievement presented by studies conducted in north-eastern Tanzania in controlling

malaria, the current study assessed the status of malaria parasite infection in an area of changing malaria epidemiology. The finding from this study is expected to fill the knowledge gap about low density malaria parasitaemia in the study area.

1.6 Research Questions

i. What is the proportion of asymptomatic malaria parasitaemia in the study area?
ii. What is the status of anaemia among study participants?
iii. Is there any association between asymptomatic malaria parasitaemia and anaemia?
iv. What is the proportion of *Plasmodium* species submicroscopic parasitaemia in dried blood spots (DBS) corresponding to blood slides negative?

1.7 Hypothesis

Microscopy underestimates malaria parasite infection compared to nucleic acid detection methods such as polymerase chain reaction (PCR).

1.8 Study Objectives

1.8.1 General Objective

To determine the prevalence of asymptomatic and submicroscopic malaria parasite infection in Korogwe District, a malaria endemic setting in north-eastern Tanzania.

1.8.2 Specific Objectives

i. To determine proportion of asymptomatic malaria parasitaemia among study participants by age groups.
ii. To determine anaemia status among study participants by age groups, gender and altitude.

iii. To investigate the association between asymptomatic malaria and anaemia among study participants by age groups.

iv. To determine the proportion of *Plasmodium* species submicroscopic parasitaemia in DBS corresponding to blood slide negative using nucleic acid detection tool.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Asymptomatic Malaria

Asymptomatic malaria is the presence of malaria parasites in the blood in the absence of symptoms (Njama-Meya *et al.*, 2004; Gudo *et al.*, 2013; Singh *et al.*, 2014). In asymptomatic infection treatment is not triggered by health care seeking for clinical illness (Lindblade *et al.*, 2013). Accurately identifying and targeting the human reservoir of malaria parasitaemia remains a critical step to reduce the burden of malaria worldwide. This is true not only in low transmission settings approaching elimination, but also in highly endemic countries, where repeated infections cause a partial non-sterilizing immunity to malaria and a large proportion of infections may be asymptomatic (Rek *et al.*, 2016).

The final stages of malaria elimination are often prolonged, with low-grade transmission sustained by a parasite reservoir present in asymptomatic individuals who may not readily engage or attend health care facilities to be treated. However, having low level parasitaemia is associated with a risk of anaemia (Lusingu *et al.*, 2004). In a longitudinal study conducted in Muheza, children who carried low density parasitaemia at the start of the study had a lower risk of contracting a febrile malaria episode but a higher risk of anaemia during the study period, than children who were slide negative at this point in time (Lusingu *et al.*, 2004). Novel strategies are needed to identify and eliminate the parasites in asymptomatic individuals (Cotter *et al.*, 2013).

The development of asymptomatic and low density malaria infection is related to an individual's tolerance to parasites (Schneider and Ayres, 2008). There are two factors

which play a role in asymptomatic malaria infections, human and parasite factors influencing duration of infection (Drakeley *et al.,* 2018). Human factors among others include genetically determined haemoglobinopathies and acquired immunity (Drakeley *et al*., 2018). Genetic analysis of malaria parasites in Zambia found that in some settings individuals with symptomatic infections had different parasite strains compared to asymptomatic individuals (Searle *et al*., 2017).

Transmission can occur from individuals with asymptomatic microscopic parasitaemia. These individuals are semi-immune and maintain low levels of parasitaemia and gametocytaemia for sustained periods of time (Roper *et al*., 1996). Carriage of asymptomatic parasitaemia potentially has profound implications for the health of individuals and for the control and elimination of malaria (Mosha *et al*., 2013; Imwong *et al*., 2015).

Asymptomatic malaria infection poses a real public health risk since health care seeking is not triggered by clinical illness and hence serves as a reservoir of malaria parasite with ongoing transmission (Ganguly *et al*., 2013). Moreover asymptomatic malaria infections are associated with recurrent episodes of symptomatic parasitaemia, chronic anaemia, co-infection with invasive bacterial disease and cognitive impairment (Chen *et al*., 2016). To make malaria elimination feasible, novel strategies are required to accurately identify asymptomatic malaria (Cotter *et al*., 2013). Recently Argentina and Algeria have managed to eliminate malaria (WHO, 2019). In mainland Tanzania a high magnitude of asymptomatic malaria has been reported posing a challenge to malaria control and elimination in this endemic setting (Manjurano *et al*., 2011; Sumari *et al*., 2017).

2.3 Submicroscopic Malaria Parasitaemia

Submicroscopic malaria parasitaemia, is the presence of malaria parasite densities below the detection threshold by microscopy (Okell *et al*., 2012; Rek *et al*., 2016). Submicroscopic malaria parasitaemia can only be revealed by use of molecular biology technique that target the detection of parasites' nucleic acids (Rek *et al*., 2016). One of the common methods used for nucleic acid amplification is PCR (Singh *et al*., 1999).

Recent advances in molecular techniques have allowed the development of highly sensitive methods for detecting peripheral parasitaemia at submicroscopic levels (0.1-10 parasites/ μL) (Bousema *et al*., 2014; Imwong *et al*., 2014; Hofmann *et al*., 2015). The application of molecular approaches in surveillance has resulted in an increase in the prevalence of detectable parasitaemia, often 2-10 fold higher than the prevalence obtained by conventional methods (Okell *et al*., 2012).

Parasite prevalence is a key metric used to quantify the burden of malaria and assess the impact of control strategies. Most published estimates of parasite prevalence are based on microscopy and likely underestimate true prevalence (Rek *et al*., 2016). The established standard for malaria diagnosis in the field remains to be blood film examination. Lower limits of detection range from 10-20 parasites per microlitre (μL) in research settings and to more than 100 parasites/ μL in clinical practice (Wongsrichanalai *et al*., 2007).

Submicroscopic malaria parasitaemia can be transmitted to the mosquito vector and hence contribute significantly to the overall infectious reservoir (Okell *et al*., 2012). In a systematic review study it has been reported that light microscope misses approximately 50% of malaria infections compared to PCR (Okell *et al*., 2009). Literature shows that in cross-sectional surveys, the proportion of low density infection among all detected malaria

infection is higher in low transmission areas than in high transmission areas for *Plasmodium* species, *P. falciparum, P. ovale, P. malariae and P. vivax* (Okell *et al*., 2012). For *P. falciparum*, it is estimated that in low to moderate settings low density infections accounts for 20-50% of transmission to mosquitoes (Okell *et al*., 2012).

Understanding the role of submicroscopic malaria as part of the transmission reservoir in malaria elimination can be achieved through active surveillance and the use of rapid diagnostic tests (RDTs) with detection limits are similar to microscopy (Sutcliffe *et al*., 2012). When these efforts failed to curb subsequent malaria transmission, the use of more sensitive molecular diagnostics is suggested (Ogutu *et al*., 2010; Sumari *et al*., 2017).

2.4 Anaemia

Anaemia is a condition in which the number of red blood cells (RBCs) or their oxygen-carrying capacity is insufficient to meet physiological needs, which vary by age, sex and altitude (WHO, 2011). Symptoms of anaemia include feeling tired, weakness and shortness of breath but they also depend on the underlaying causes. Major causes of anaemia are decreased RBCs production, blood loss and increased RBCs breakdown. Causes of decreased RBCs production includes iron deficiency, vitamin B12 deficiency thalassemia and neoplasm of the bone marrow. Causes of blood loss includes trauma and gastrointestinal bleeding. Causes of increased RBCs breakdown includes diseases such as malaria, other genetic diseases like sickle and autoimmune diseases.

2.5 Diagnosis of Asymptomatic Malaria

Asymptomatic malaria carriers in community based studies are clinically identified basing on study participant’s axillary body temperature, most published work shows a cut off

<37.5 ^{o}C (Mboera *et al*., 2015). Presence of malaria parasites in asymptomatic individuals is confirmed by mRDT and microscopy (Mboera *et al*., 2015; Nzobo *et al*., 2015).

2.6 Microscopic Diagnosis of Malaria by Thick Blood Smear (TBS) and Thin Film (TF)

Microscopic examination of thin blood film and thick blood film is the reference gold standard advocated by WHO. It allows rapid diagnosis and monitoring of treatment efficacy of malaria by monitoring parasitaemia. This techniques is inexpensive and remain the most widely used technique. However, its performance in terms of sensitivity and reliability are directly dependent on the experience of the microscopists and the level of parasitaemia of the infected. The smear allows better examination of the morphology of parasites and red blood cells and therefore make diagnosis of *Plasmodium* species more easer. The detection threshold of blood smear is 50 parasites/ µL. Its sensitivity is much lower than PCR which can detect low parasitaemia (10 parasite/ µL). The microscopic diagnosis may also encounter difficulties in identifying species particularly in the presence of parasites altered by presumptive treatment or in case of very low parasitaemia (Moody, 2002).

2.7 Detection of Malaria Parasites by RDTs

The RDT is based on the principle of immunochromatography using strips sensitized by specific monoclonal antibodies detecting malaria antigens. These tests are quick and easy to read and can be performed by moderately trained personnel. There are suitable especially in non-specialized structures where microscopy is not available. The RDTs are commercially available and they exists in different types of antigens used.

(i) Antigen detection histidine protein 2 (HRP2): This specific glycoprotein of *P. falciparum* is generated by all asexual erythrocytic stages of the parasite

and can persist in the peripheral blood more than 15 days after the disappearance of parasites. These tests are created with more than 96% sensitivity compared to conventional microscopic techniques, when evaluated on the blood smear parasitaemia is greater than 100 parasites/ µL**.** Their detection threshold varies from 100 to 300 parasites/ µL. Drawback of these test are the persistent of antigenemia after healing and monospecificity to *P. falciparum.* False positive associated with these tests are associated with cross-reaction and rheumatoid factor (Iqbal *et al*., 2000). False negative are possible and are due to mutation in the gene encoding the HRP2 or presence of antibodies HRP2 (Humar *et al*., 1997; Grobusch *et al*., 1999).

(ii) Antigen detection of Parasite Lactate Dehydrogenase (pLDH): These are glycolytic enzymes which have the advantage of being common to the four *Plasmodium* species, detected at all asexual and sexual stages of the parasites. These tests have a detection threshold identical to that of HRP2. Their clearance is fast and they do not persist in the blood after death of *Plasmodium* (Lee *et al*., 2014).

(iii) Antigen detection of aldolase. This is a key enzyme in the glycolytic pathways of all human malaria parasites. The sensitivity of detection of these antigens is however lower than that of tests detecting HRP2 and pLDH (Barber *et al*., 2013).

2.4 Diagnosis of Submicroscopic Malaria Parasitaemia

For epidemiological research and surveys that aim to map submicroscopic parasitaemia, the WHO recommends the use of more sensitive nucleic acid amplification (NAA) molecular techniques for detection of submicroscopic parasitaemia (WHO, 2014). The

most commonly used target for NAA methods in these surveys is amplification of the 18S rRNA gene that targets DNA (Snounou, 1993). Other molecular methods for detection of submicroscopic parasitaemia include reverse transcription PCR that targets RNA (Adams *et al*., 2015; Sumari *et al*., 2017).

2.5 Diagnosis of Anaemia

The diagnosis of anaemia is based on haemoglobin (Hb) measurements. The WHO recommends diagnoses of anaemia at sea level based on age adjusted Hb level, sex and status of individual (WHO, 2011). In addition WHO has categorized anaemia in three groups, Hb level < 7 g/ dL severe anaemia, between 7 and 9.9 g/ dL as moderate anaemia, between 10 and 10.9 g/ dL as mild anaemia. The normal level of Hb for all age groups and sex of individual is set at Hb > 11g/ dL (WHO, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in two villages of Korogwe district namely Mkokola (a village in lowland area) about 300 m above the sea level and Kwamasimba (a village in a highland area) about 700 m above the sea level (Fig. 1). The district is about 100 kilometers inland from the coastal town of Tanga. It is a tropical area with two rain seasons, March to June and October to December. Normally January to February is dry but recently there have been climatic changes that have led to merging of these two rainy seasons. Entomological surveys in the study areas have shown that *Anopheles gambiae* is the most prevalent vector in lowlands while *A. funestus* predominates in the highlands (Bødker *et al*., 2003). The prevalence of *P. falciparum* malaria parasitaemia from the community in lowland villages has decreased from 78% in 2003 to 13% in 2008, whereas in the highland villages, the decrease was from 25% to 3% during the same time (Mmbando *et al*., 2010). Population censors of 2012 reported 242 038 people living in Korogwe, with 118 544 male and 123 494 female (NBS, 2013). The majority of the inhabitants reside in rural settings, practicing subsistence farming and informal trade (Korogwe District Council, 2017). The most common diseases in Korogwe are respiratory tract infection, pneumonia, diarrhoea, malaria, bacterial infection and acquired immunodeficiency syndrome (AIDS) (Mahende *et al*., 2015).

Figure 1: Map of Korogwe showing the study area. The present study was conducted in the villages of Mkokola and Kwamasimba (indicated by a dot). Mkokola is located in lowland area while Kwamasimba is located in a highland area.

3.2 Study Design and Population

The present study was a retrospective cross-sectional study that analyzed data and DBS collected by malaria research project entitled "Malaria Research Capacity building for field clinical trials in Tanzania (MaReCa- Project)" in June 2018. The study populations were villagers of Mkokola and Kwamasimba villages of Korogwe District whose demographic information exists in the database of the National Institute for Medical Research (NIMR) in Korogwe field station. Study participants in each village were distributed in different age groups as follows: 0-4 years: n= 119, 5-9 years: n= 72, 10-14 years: n= 59 and 15-19 years: n= 32.

3.3 Sample Size

The sample size for this study was obtained conveniently from the information of 565 participants who took part during the malariometric survey. However for objective four of this study (submicroscopic parasitaemia), samples for PCR were estimated using the formula by Kirkwood *et al*. (2003) $n = z^2p\ (1\text{-}p)/\ d^2$, where: n is the total number of samples required, z is a standardized normal deviate value that correspond to a level of statistical significance of $p \leq 0.05$ which is 1.96, p is the proportion of malaria in Korogwe (Mahende *et al*., 2016), d is the margin of error on p which is 0.05. The sample size for PCR was 188 basing on the formula and was adjusted to 206 to take care of any sample that might be disqualified based on their quality or loss.

3.4 Proportion of Asymptomatic Malaria Parasitaemia among Study Participants

Proportions of asymptomatic malaria parasitaemia among the study participants were computed using mRDT and microscopy results. Asymptomatic malaria carriers were defined as individuals with axillary body temperature $<37.5^{o}C$, with no clinical signs and were malaria case positive by mRDT, microscopy or PCR (Bousema *et al*., 2014).

3.5 Anaemia Status among Study Participants by Age Groups, Gender and Altitude

Anaemia status of the study participants were determined using Hb values. Anaemia was defined according to the WHO age-adjusted cut off for Hb, (WHO, 2011) indicated in Table 1. At the community level the prevalence of anaemia was defined according to the WHO classification of public health significance of anaemia basing on anaemia prevalence (Table 2) (WHO, 2011).

Table 1: WHO age-adjusted Hb levels to diagnose anaemia at a sea level in g/ dL

Age (years), Sex and status Of Individuals	Non-anaemic	Anaemia		
		Mild	Moderate	Severe
Children 6 -59 months of age	≥11.0	10.0-10.9	7.0-9.9	< 7.0
Children 5 - 11 years of age	≥11.5	11.0-11.4	8.0-10.9	< 8.0
Children 12 - 14 years of age	≥12.0	11.0-11.9	8.0-10.9	< 8.0
Non-pregnant women (15 years of age and above	≥12.0	11.0 -11.9	8.0-10.9	< 8.0
Pregnant women	≥11.0	11.0-10.9	7.0-9.9	< 7.0
Men (15 years of age and above)	≥13.0	11.0-12.9	8.0-10.9	< 8.0

Source: WHO (2011)

Table 2: Classification of public health significance of anaemia in populations on the basis of prevalence estimated from blood levels of Hb

Category of public health significance	Prevalence of anaemia (%)
Severe	40 or >
Moderate	20.0 - 39.9
Mild	5.0 - 19.9
Normal	4.9 or <

Source: WHO (2011)

3.6 Association between Asymptomatic Malaria and Anaemia

Association between asymptomatic malaria and anaemia was explored using logistic models, by both univariate and multivariate analysis.

3.7 Proportion of *Plasmodium* Species Submicroscopic Infection

Proportions of *Plasmodium* species submicroscopic infection from the study samples was determined by carrying out *Plasmodium* species specific PCR (Singh *et al.*, 1999). Briefly, parasites DNA were extracted from the collected DBS in respective to blood slide negative. The extraction method utilized QIAamp DNA Blood Mini (Qiagen GmbH, Hilden, Germany), as per manufacturer's instructions. Briefly, DBS were placed in 1.5 mL Eppendorf tubes, subjected to tissue lysis buffer and incubated in 85 ^{o}C for an hour.

Proteinase K was added to the mixture followed by addition of absolute ethanol. The mixture was carefully applied to the QIAamp Mini spin column followed by centrifugation and two washing steps. The trapped DNA in QIAamp mini spin column was then eluted in 150 μL of elution buffer by placing QIAamp mini spin column in 1.5 mL microcentrifuge tubes, followed by one minute incubation at room temperature and one minute centrifugation. The DNA was stored in -20^{o}C for subsequent laboratory analysis.

Aliquots of the extracted parasite genomic DNA were amplified using a Thermocycler (TProfessional, Thermocycler Biometra GmbH, Saxony, Germany) using a genus primers Plus 5 and 6 forward 5'-CCT GTT GTT GCC TTA AAT TTC-3', reverse 5'-TCA AAG ATT AAG CCA TGC AAG TGA-3'. These primers detect presence of *Plasmodium* at a genus level. Positive samples at a genus level detected by these primers (Plus 5 and 6) were subjected to species specific PCR using first round and second round of nested PCR primers for *Plasmodium* at species level detection indicated in Table 3 (Singh *et al*., 1999). First round nested PCR and second round nested species specific PCR was done using forward and reverse primers in 20 μL a reaction mixture of PCR water, primer mix 2.5 μM and Tempase (Table 4 and 5).

Table 3: Primers for *Plasmodium* species specific PCR

Parasite	Forward/ Reverse	Primer sequence (5'- 3')	Amplicons size (bp)
P. falciparum	For	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT	205
	Rev	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC	
P. malariae	For	ATA CAT AGT TGT TGT AAG AAT AAC CGC	144
	Rev	AAA ATT CCA TGC ATA AAA TTA TAC AAA	
P. ovale	For	ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA	245, 345
	Rev	GGA AAA GGA CAT TAA TTG TAT CCT AGTG	
P. vivax	For	CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC	117
	Rev	ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA	

Source: Singh *et al*. (1999)

3.8 Preparation of Controls

For *P. falciparum* strain 3D7 and sterile PCR water were used as positive and negative controls, respectively. For *P. malariae* and *P. ovale,* microscopy known positive samples and PCR water were used as positive and negative controls, respectively.

Table 4: Master mix for first round of nested PCR specific to *Plasmodium* species.

Reagents	Volume. per sample (µL)	Volume. in mix (µL)	Final conc. per sample
H_2O	8.5	H2O : 170	
First round Primer-mix PLU5&6 (conc 2.5µM)	0.5	Primers: 10	0.25 µM per. Primer
Tempase	10	PCR Buf. 200	1X

For first round of nested PCR, 19 µL master mix (Table 4) together with 1 µL DNA sample were dispensed into sample respective wells on the PCR plate, at the same time controls were added into their respective wells on the PCR Plate. First round of nested specie specific PCR were run as per thermal cycling conditions in Fig. 2.

Table 5: Master mix for second round of nested PCR specific to *Plasmodium* species

Reagents	Volume per sample (µL)	Volume in mix (µL)	Final conc. per sample
H_2O	8.0	H2O : 160	
Primermix (conc 2.5µM) Pf F&R or Pm F& R Po F&R or Pv F & R	1.0	Primers : 20	0.25 µM pr. Primer
Tempase	10	PCR Buf. 200	1X

F= forward primer, R= Reverse primer

For second round of nested PCR, 19 µL of master mix (Table 5) together with 1 µL of PCR product were dispensed into sample respective wells on the PCR plate. Second round

of nested specie specific PCR were run as per thermal cycling condition in Fig. 3. The second round of nested PCR products were subjected to gel electrophoresis. 2% agarose gel was prepared by adding 6.0 g of agarose powder into 300 mL of 1x Tris borate ethylenediaminetetraacetic acid (TBE buffer).

The solution was heated in microwave until agarose powder completely dissolved. It was allowed to cool at around 50 ^{o}C and then 10 µL of Ethidium bromide solution (10 µg/ mL) was added and the mixture poured into the casting tray. The gel was allowed to solidify for 30 minutes and thereafter transferred into the electrophoresis tank containing 1x TBE buffer.

Products were loaded with blue juice loading dye at 6x concentration. A 100 base pairs (bp) DNA ladder marker was used to identify approximate DNA bp of *Plasmodium* species as previous described (Singh *et al*., 1999). The gel was run at 107 volts for 2 hours (DNA is negatively charged molecule so it moves towards the positive electrode). The PCR products were viewed under ultraviolet light using gel documentation system BioDoc Analyzer. Picture was taken to compare positive samples against 100 bp DNA ladder marker.

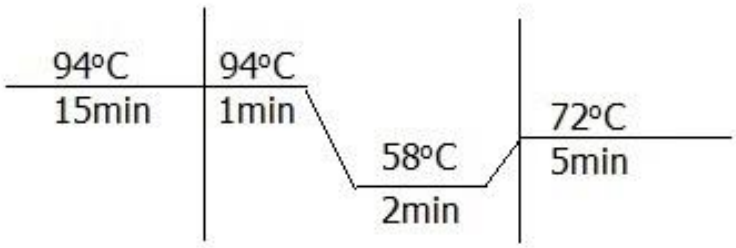

Figure 2: Thermal cycling condition for first round of nested species specific PCR

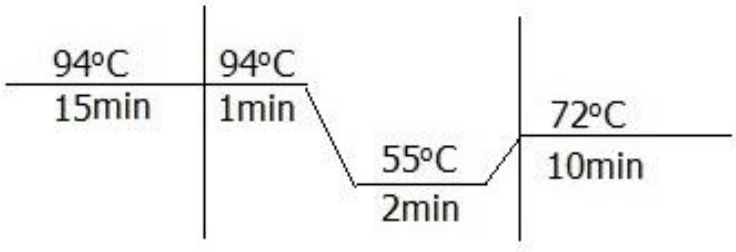

Figure 3: Thermal cycling condition for second round of nested species specific PCR

3.9 Data Analysis

Data were analyzed using STATA statistical software package version 13.1 (Stata Corp College Station, Texas USA). Proportions between groups were compared using χ^2-test. Univariate and multivariate analysis were used to estimate odds ratios (OR), adjusted odds ratios (AOR) and 95% confidence intervals (CI) for associations between malaria and other variables of interest such as malaria and Hb . Where applicable significance levels were measured at 95% CI with significant difference set at $p < 0.05$.

3.9.1 Case definitions

Asymptomatic malaria carriers were defined as individuals with axillary body temperature <37.5 ^{0}C, with no clinical signs and were malaria case positive by mRDT, microscopy or PCR (Bousema *et al*., 2014). Submicroscopic carriers were defined as those individuals with malaria infection detected by PCR but not microscopy (Singh *et al*., 1999). Anaemia was defined according to the WHO age-adjusted cut off for Hb (WHO, 2011).

3.9.2 Inclusion and exclusion criteria

The data analyzed were for individuals who were living in the study area aged 0-19 years and were not showing clinical signs of malaria. These individuals willingly consented to participate. Individuals who clinical information showed signs of malaria such as fever,

vomiting, convulsion, general body malaise, headache and loss of appetite were excluded from the study to get the true asymptomatic cases.

3.9.3 Ethical considerations

The study received ethical clearance from NIMR through the Medical Research Coordinating Committee (MRCC), certificate with approval number NIMR/HQ/R.8a/ Vol .IX/2804 (Appendix 1). The study was also approved by Sokoine University of Agriculture (reference number SUA/ADM/R.1/8/232) (Appendix 2). All ethical issues and regulation pertaining research involving human were observed as per Helsinki declaration and Belmont protocol.

CHAPTER FOUR

4.0 RESULTS

4.1 Baseline Characteristics of the Study Participants

In this study a total of 565 participants were enrolled from two villages of Korogwe District, Kwamasimba (highland village, n = 280) and Mkokola (lowland village, n = 285). Majority were aged up to 4 years (41.95%), followed by 5-9 years (25.66%) as indicated in Table 6.

Table 6: Demographic information of the study participants from Kwamasimba (highland) and Mkokola (lowland) villages, Korogwe District, north-eastern Tanzania

Variable	Number of participants	Percentage (%)
Strata		
Highland village	280	49.56
Lowland village	285	50.44
Sex		
Male	275	48.67
Female	290	51.33
Age groups		
0-4 years	237	41.95
5-9 years	145	25.66
10-14years	118	20.89
15-19years	65	11.50

4.2 Prevalence of Asymptomatic Malaria as Detected by mRDT and Microscopy

Based on mRDT 211/565 (37.34%, 95% CI: 33.99-41.49%) of the study participants had malaria infection (Fig. 4). On the other hand, only 81/565 (14.34%, 95% CI: 11.71-17.54%) were malaria positive based on microscopy (Fig. 4). Of the 565 participants, 130

(23.0%, 95% CI: 19.71-26.67%) and 33 (5.84%, 95% CI: 4.18-8.11%) had asymptomatic malaria based on mRDT and microscopy, respectively (Table 7 and 8). In under-five year's children, the proportion of asymptomatic malaria was lower over other age groups, by both mRDT and microscopy, 16.88% and 2.53%, respectively (Table 7 and 8). *P. falciparum* was the most predominant malaria parasite detected by mRDT and microscopy, 92.5% and 98.8%, respectively. Of the microscopy positive samples, none had a mixed infection. *P. falciparum* gametocytes were detected in five samples by microscopy. *P. malariae* was detected only in one sample by microscopy and none was found to be *P. ovale* based on microscopy. None of the mRDT negative samples were found to be positive by microscopy. Malaria species specific diagnostic PCR for submicroscopic infections detected three out of 206 (1.5%, 95% CI: 0.46-4.46%) and these infections were due to *P. falciparum* (Fig. 4). No *P. malariae* and *P. ovale* infection was detected in the study samples by PCR.

Table 7: Prevalence of asymptomatic malaria detected using mRDT by age groups of the study participants

Variable	mRDT results			Chi-square test
	Positive (%)	Negative (%)	Total (%)	p-value
Age Groups				
0-4 years	40 (16.88)	197 (83.12)	237 (41.95)	**<0.009**
5-9 years	34 (23.45)	111 (76.55)	145 (25.66)	
10-14 years	38 (32.20)	80 (67.80)	118 (20.89)	
15-19 years	18 (27.69)	47 (72.31)	65 (11.50)	
Strata				
Highland village	63 (22.50)	217 (77.50)	280 (49.56)	0.776
Lowland village	67 (23.51)	218 (76.49)	285 (50.44)	
Total	130 (23.01)	435 (76.99)	565 (100)	

Table 8: Prevalence of asymptomatic malaria detected using microscopy by age groups

Variable	Microscopy			Chi-square test
	Positive (%)	Negative (%)	Total (%)	p-value
Age Groups				
0-4 years	6 (2.53)	231 (97.47)	237 (41.95)	**<0.013**
5-9 years	10 (6.90)	135 (93.10)	145 (25.66)	
10-14 years	13 (11.02)	105 (88.98)	118 (20.89)	
15-19 years	4 (6.15)	61 (93.85)	65 (11.50)	
Strata				
Highland village	20 (7.14)	260 (92.86)	280 (49.56)	0.191
Lowland village	13 (4.56)	272 (95.44)	285 (50.44)	
Total	33 (5.84)	532 (94.16)	565 (100)	

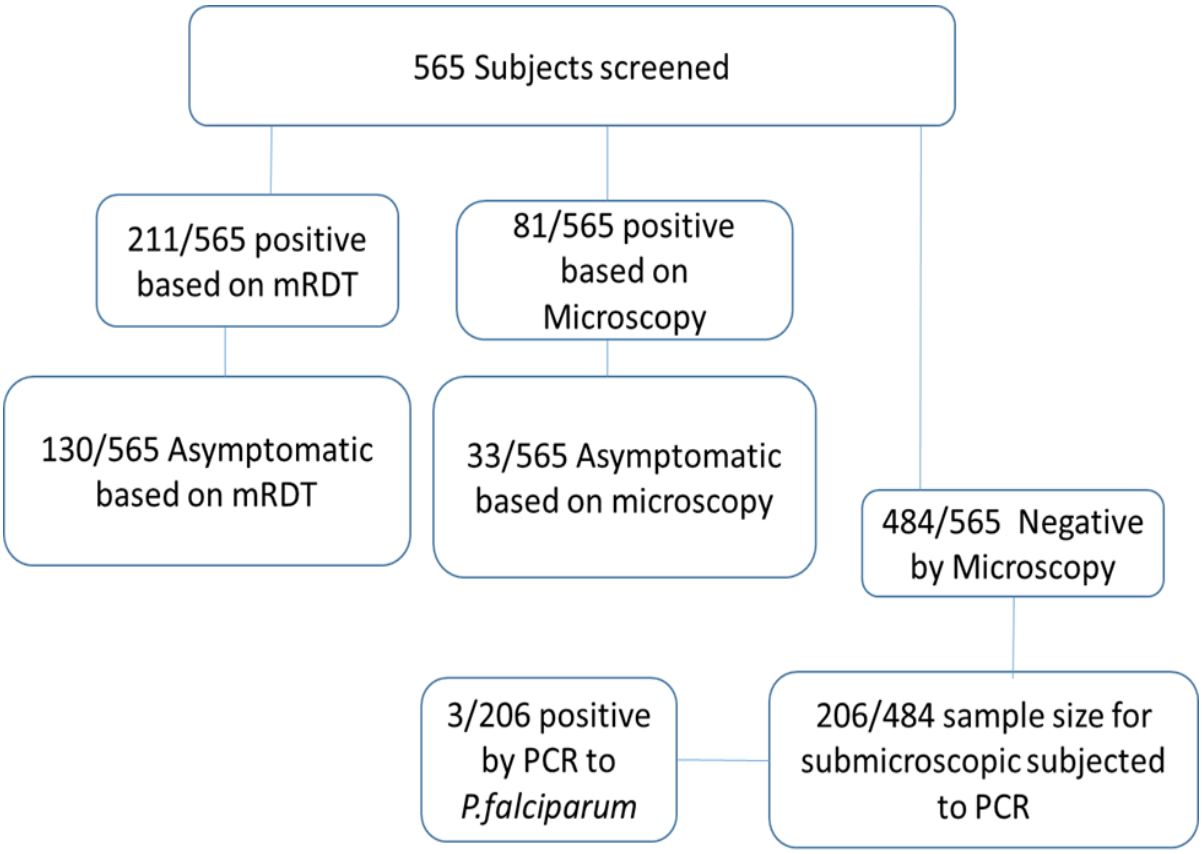

Figure 4: Study profile, showing total number of participants screened, number of asymptomatic participants and number of submicroscopic infection obtained by PCR from confirmed blood slide negative cases

4.4 Anaemia Status By Age Groups, Sex And Strata

Of the 565 participants screened, 135 (23.89%, 95% CI: 20.54-27.59%) were anaemic (Hb < 11g/ dL). Among those with anaemia, 13.8% (n = 78) had mild anaemia (Hb, 10.0-10.9 g/ dL), 8.85% (n = 50) had moderate anaemia (Hb, 7.0-9.9g/ dL), and 1.24 % (n = 7) had severe anaemia (Hb < 7.0g/ dL). There was no statistical significant difference in anaemia status between males and females participants (p = 0.248). Though a non-statistical significant difference was observed in anaemia status with age groups p = 0.221, anaemia varied with age groups, under-five years accounted for 11.15% (n = 63), 5-11 years 8.14% (n = 46), 12-14 years 2.12% (n = 12) and 15-19 years accounted 2.48% (n = 14) (Table 9).

Table 9: Anaemia status of the study participants by sex, age groups and strata

Variable		Anaemia			Total	p-value
	Non anaemic, n (%)	Mild, n (%)	Moderate n (%)	Severe n (%)		
Sex						
Male	199 (72.40)	44 (16.0)	28 (10.10)	4 (1.50)	275 (48.67)	
Female	231 (79.66)	34 (11.72)	22 (7.59)	3 (1.03)	290 (51.33)	0.248
Age groups						
0.5-4 years	174 (73.42)	43 (18.14)	17 (7.17)	3 (1.27)	237 (41.95)	
5-11 years	153 (76.88)	19 (9.55)	23 (11.56)	4 (2.01)	199 (35.22)	0.221
12-14 years	52 (81.25)	7 (10.94)	5 (7.81)	0 (0.0)	64 (11.33)	
15-19 years	51 (78.46)	9 (13.85)	5 (7.69)	0 (0.0)	65 (11.50)	
Strata						
Highland village	217 (77.50)	38 (13.57)	22 (7.86)	3 (1.07)	280 (49.56)	0.824
Lowland village	213 (74.74)	40 (14.04)	28 (9.82)	4 (1.40)	285 (50.44)	
Total	430 (76.11)	78 (13.80)	50 (8.85)	7 (1.24)	565 (100)	

4.5 Association between Asymptomatic Malaria and Anaemia

The association between asymptomatic malaria and anaemia was explored. Univariate analysis indicated that risk of anaemia was three fold among participants who were mRDT positive (OR = 3.29, 95% CI: 2.17-5.0, $p < 0.001$) whereas participants who were slide positive, the risk of anaemia was approximately two fold (OR = 1.9, 95% CI: 0.9-3.9, $p = 0.09$) as indicated in Table 10. There was a borderline significant difference in risk of anaemia between males and females participants, $p = 0.05$. Participants from lowland areas had higher risk of getting anaemia compared to those from highland village (OR = 1.34, 95% CI: 0.9-2.02, $p = 0.15$). Multivariate logistic regression analysis for predictors of anaemia showed that, malaria positives at the age group of 5-9 and 10-14 years were significantly associated with anaemia, (AOR = 0.6, 95% CI: 0.37-0.98, $p = 0.04$), (AOR = 0.32, 95% CI: 0.14-0.75, $p = 0.01$), respectively (Table 10).

Table 10: Association between asymptomatic malaria and anaemia by age groups, sex and strata

	Univariate			Multivariate		
Variable	**Crude OR**	**95% CI**	**p-value**	**AOR**	**95% CI**	**p-value**
mRDT						
Negative	Ref					
Positive	3.29	2.17-5.0	**<0.001**	3.8	2.45-5.89	**<0.001**
Microscopy						
Negative	Ref					
Positive	1.900	0.90- 3.97	0.09	2.09	0.98-4.47	0.06
Age group						
under 5 years	Ref					
5-11 years	0.82	0.53-1.3	0.42	0.60	0.37-0.98	**0.04**
12-14 years	0.46	0.21-1.03	0.06	0.32	0.14-0.75	**0.01**
15+ years	0.78	0.40-1.54	0.48	0.61	0.3-1.25	0.18
Sex						
Male	Ref					
Female	0.66	0.44-1.0	**0.05**	0.67	0.44-1.03	0.07
Strata						
Highland village	ref					
Lowland village	1.34	0.9-2.02	0.15	1.35	0.88-2.08	0.17

OR= Odds ratio, AOR Adjusted Odds ratio, CI= Confidence interval, significant p-values are in boldface

4.5 Proportion of Submicroscopic Parasitaemia

Of the negative samples by microscopy, nested species specific PCR for *Plasmodium* identified 3/206 (1.5%, 95% CI: 0.47-4.46%) were submicroscopic infection due to *P. falciparum*. The positive samples were samples with laboratory identification number 272, 130 and 552 while the rest were negative (Fig. 5). No study sample was found to be positive for *P. malariae* and *P. ovale* subspecies *P. ovale* wallikeri and *P. ovale curtisi*. The DNA band size for *P. falciparum* is approximately 205 bp, for *P. malariae* is approximately 144 bp and for *P. ovale* (*P. ovale* wallikeri = 245 bp and *P. ovale* curtisi = 345 bp).

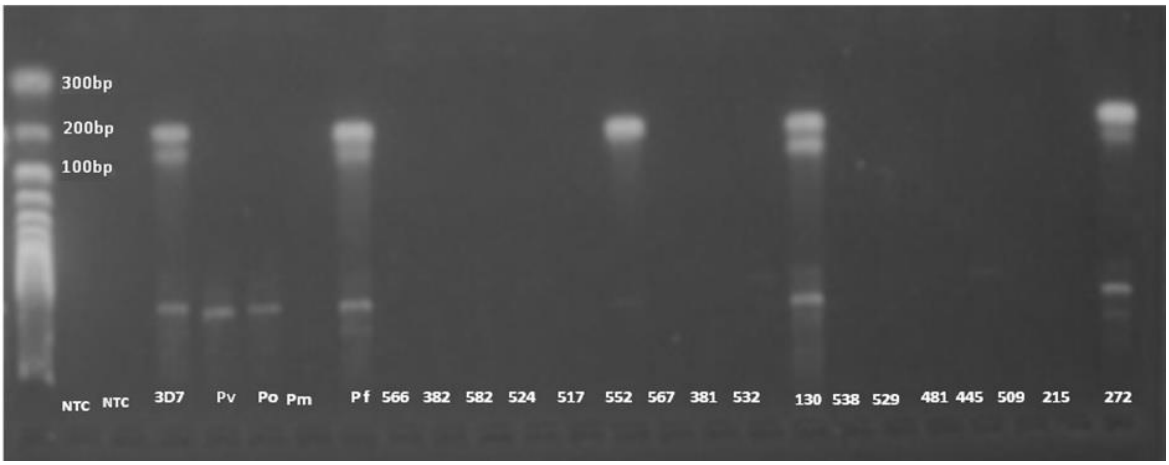

Figure 5: Detection of *P. falciparum (Pf)* by PCR in samples which were negative by microscopy. The expected band size was 205 bp using primers *Pf*F and *Pf* R. The Positive controls include 3D7, *P. ovale (Po)*, *P. vivax*, (Pv) *P. malariae (Pm)* and non-template control. The ladder used was 100bp.

CHAPTER FIVE

5.0 DISCUSSION

This study aimed to determine the prevalence of asymptomatic and submicroscopic malaria parasitaemia in Korogwe, a district in north-eastern Tanzania. Though it was previously reported that malaria had significantly decreased in communities of north-eastern Tanzania, studies aimed at malaria elimination are still recommended to be conducted (Ishengoma *et al*., 2013; Mahende *et al*., 2015; Drakeley *et al*., 2017). Large proportion of human malaria infection are asymptomatic (Gudo *et al*., 2013). In asymptomatic malaria carriers, treatment is not triggered by clinical illness and hence infected individuals continue to spread malaria parasites in the community (Lindblade *et al*., 2013). The study findings demonstrated the burden of asymptomatic malaria infection and anaemia in endemic communities of north-eastern Tanzania. In addition, the findings highlights the importance for increased effort to tackle asymptomatic infections to accelerate malaria control and elimination.

The findings revealed that asymptomatic malaria is high in participants aged above five years implying that there is an obvious epidemiological shift of malaria to school age groups. Most existing malaria control strategies are often targeting children under-five years of age, leaving school-aged children vulnerable to frequent infections. Other studies done elsewhere shows similar findings where school children are more prone to malaria (Mboera *et al*., 2015; Nzobo *et al*., 2015; Walldorf *et al*., 2015; Chacky *et al*., 2018).

The findings showed difference in the prevalence of asymptomatic malaria obtained by mRDT from that obtained by microscopy. This could be due the fact that mRDTs have been reported to show false positive results and tend to overestimate the parasite

prevalence (Grobusch *et al*., 1999; Iqbal *et al*., 2000; Lee *et al*., 2014). The mRDT shows false positive results due to the fact that it can detects HRP2 of *P. falciparum* circulating in blood for more than two weeks even after successful clearance of infected erythrocytes in the blood stream (Humar *et al*., 1997). Similar finding has been reported in Korogwe where the prevalence of malaria was high by mRDT compared to microscopy (Mahende *et al*., 2016).

In the current study a rebound of malaria cases in the study area has been observed with more cases in the highland strata. This is in contrary to the findings of the previous study (Mmbando *et al*., 2010). There was no simple explanation as to why malaria has increased in the highland village. This could be attributed to combination of factors such as an interplay of array between environmental, ecological, health services and social economic development. Previous studies done in the same study area predicted striking back of malaria in the study area if the scaled up malaria intervention will not be maintained (Mmbando *et al*., 2010).

In this study few *Plasmodium falciparum* gametocytes have been observed by microscopy. Previous studies have shown that the prevalence of gametocyte carriage is high in settings with higher burden of asymptomatic infections (Vantaux *et al*., 2018). Therefore, sensitive diagnostic tools for submicroscopic gametocytes in this setting may guide future implementation of strategies to interrupt transmission (Shekalaghe *et al.,* 2007).

The study also assessed the status of anaemia among the study participants. According to WHO public health significance of anaemia at a community level the prevalence of anaemia obtained in this study was in the category of moderate anaemia (WHO, 2011).

The finding implies that one fifth of the participants had moderate anaemia within the study area.

Univariate analysis indicated that risk of anaemia was three folds among participants who were asymptomatic malaria case positive. Similar findings have been reported elsewhere by (Pava *et al*., 2016). Participants from lowland areas had high risk of anaemia compared to highland. This finding is similar to what has been reported previously in the study area (Mmbando *et al*., 2010). Multivariate logistic regression analysis for predictors of anaemia showed that, malaria positives, age 5-14 years were significantly associated with anaemia. Similar findings has been reported elsewhere (Mboera *et al*., 2015).

The low prevalence of submicroscopic parasitaemia observed in the study samples implies that malaria transmission is still high as the level of immunity is high due to greater exposure thus maintaining asymptomatic parasite densities in individuals living in areas of high transmission settings. This was shown in previous study where high proportion of submicroscopic infections were shown to be more likely to occur in areas of low transmission compared to high transmission settings (Okell *et al*., 2012). Other similar findings have been reported elsewhere (Harris *et al*., 2010; Rodríguez *et al*., 2018).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The current study observed that asymptomatic malaria is much more prevalent in participants aged above five years. Furthermore, participants aged 5-14 years were at risk of asymptomatic malaria associated with anaemia. The findings highlight the need for targeted interventions focusing on those participants aged above five years in malaria control interventions. A low proportion of submicroscopic parasitaemia was observed in this study.

6.2 Recommendations

Further research is recommended to better understand the epidemiology of asymptomatic malaria in north-eastern Tanzania. Also implementation research is recommended to assess the progress of malaria intervention in north-eastern Tanzania. The use of high throughput molecular tool is recommended to accurately assess the magnitude of submicroscopic parasitaemia in the study area. The high prevalence of malaria observed in the age group of 10-14 years suggests that this group should be covered by different intervention apart from the existing ones. In addition, asymptomatic and submicroscopic malaria should be considered when implementing elimination strategies.

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APPENDICES

Appendix 1: Ethical clearance certificate. Approved by the National Institute for Medical Research and Health Research Review Committee in Tanzania

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
3 Barack Obama Drive
P.O. Box 9653
11101 Dar es Salaam
Tel: 255 22 2121400
Fax: 255 22 2121360
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Ministry of Health, Community Development, Gender, Elderly & Children
University of Dodoma, Faculty of Arts and Social Sciences
Building No. 11
P.O. Box 743
40478 Dodoma

NIMR/HQ/R.8a/Vol. IX/2804

22nd June 2018

Paul Martine
Sokoine University of Agriculture
P.O. Box 3019
Morogoro

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Prevalence of asymptomatic and submicroscopic malaria parasitaemia in Korogwe, North-Eastern Tanzania (Martine P *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, Community Development, Gender, Elderly & Children 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, Community Development, Gender, Elderly & Children 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 as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Site: Tanga region in Tanzania.

Approval is valid for one year: 22nd June 2018 to 21st June 2019.

Name: Prof. Yunus Daud Mgaya

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Prof. Muhammad Bakari Kambi

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: RMO of Tanga region
DMO/DED of Korogwe district

Appendix 2: Institutional Review Board Approval from Sokoine University of Agriculture

CLEARANCE PERMIT FOR CONDUCTING RESEARCH IN TANZANIA

SOKOINE UNIVERSITY OF AGRICULTURE
OFFICE OF THE VICE-CHANCELLOR
P.O. Box 3000 CHUO KIKUU, MOROGORO, TANZANIA
Phone: 255-023-2640006/7/8/9, Direct VC: 2640015; Fax: 2640021;
Email: vc@suanet.ac.tz;

Our Ref. SUA/ADM/R.1/8/232 **Date:** 3rd October, 2018

The Regional Administrative Secretary,
Tanga Region,
P. O. BOX 5095,
TANGA.

Re: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

The Sokoine University of Agriculture was established by University Act No. 7 of 2005 and SUA Charter, 2007 which became 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 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 to issue research clearance to staff, students, research associate and researchers of SUA on behalf of the Tanzania Commission for Science and Technology.

The purpose of this letter is to introduce to you **Mr. Martine Paul** a bonafide **MSc. (Parasitology)** student with registration number **MPA/D/2017/0002** of SUA. By this letter **Mr. Martine Paul** has been granted clearance to conduct research in the country. The title of the research in question is "**Prevalence of Asymptomatic and Submicroscopic Malaria Parasitaemia in Korogwe, north-eastern Tanzania**".

The period for which this permission has been granted is from **October, 2018** to **July, 2019**. The research will be conducted in **Korogwe District (Mkokola and Kwamasimba village) and NIMR – Tanga for Lab Analysis.**

Should some of these areas/institutions/offices be restricted, you are requested to kindly advice the researcher(s) on alternative areas/institutions/ 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. Peter R. Gillah
FOR: VICE-CHANCELLOR

Copy to: Student – **Mr. Martine Paul** - Researcher