UNIVERSITY OF NAIROBI

FUNCTIONAL ANALYSIS OF IMMUNE RESPONSE TO *PLASMODIUM FALCIPARUM* GAMETOCYTES SURFACE ANTIGENS PFS25, PFS48/45 AND PFS230

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination or award of a degree. Where other people's work has been used, this has been properly acknowledged and referenced in accordance with the University of Nairobi's requirements.

Signature: ……

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ABSTRACT

Malaria Transmission Blocking Vaccine (MTBV) candidates capable of eliciting effective and long-lasting protection, thereby blocking or reducing community *Plasmodium falciparum* transmission rates are becoming more fashionable. Domain 1 of pre-fertilization *P. falciparum* surface protein 230 (Pfs230 D1), Pfs48/45 D3, and post-fertility protein Pfs25 are key fronted vaccine candidates. Aside from eliciting a strong antibody response, their candidacy's success will be determined by their ability to overcome obstacles such as antigenic variations that may affect their functionality, resulting in strain transcendence or allele-specific immunity, or loss of antigenicity due to conformation of antibody binding epitopes or antigen structure. This study determined polymorphisms with functional significance on the immunodominant domains of the three antigens in *P. falciparum* populations from different rural parts of western Kenya with varying transmission intensities.

Clinical blood smears, dry blood spots and patients' demographic data were collected at health facilities in Malaria-endemic Homa Bay (endemic area with ongoing indoor residual spray), Kisumu (endemic area without IRS), and Kisii (epidemic prone highland). Microscopy and real-time polymerase chain reaction were used to detect microscopic and submicroscopic infections, respectively. On parasite positive DNA samples, each target gene was genotyped and genetic computations were performed.

In epidemic-prone rural area of Kisii highland, there were 231/1115 (20.7%) positive malaria cases. Morara and Nyabikondo village had the most microscopic infections (24/57, 42%). Submicroscopic infections were uncommon (14.7%) and more common in females (19.7%) and adults (21.1%). In malaria endemic zone of Homa Bay, clinical microscopic infections were low (14.7%). The majority of positive clinical cases (36%, 112/313) were submicroscopic, resulting in an overall prevalence of 45.2%. Submicroscopic infections were more prevalent in females (35.6%, 72/202) than in males (24.2%, 40/165). Among 156

genotyped samples, the *Pfs230* D1 had low nucleotide diversity (π=0.15×10^{-2}), with variation observed per study site. Six segregating sites (1616, 1813, 1955, 1964, 1967, and 1983) were identified, each resulting in nonsynonymous mutations (I539T, G605S, T652R, E655V, T656N, and K661N) and eight haplotypes. Recombination, inbreeding, purifying and balancing selection were key drivers of these mutations. The Pfs48/45 D3 sequences of the 118 clinical isolates had a high conservation index and low nucleotide diversity (0.063×10^{-2}). Three polymorphic sites were identified: 911, 940, and 979, which resulted in nonsynonymous mutations (V304D, L314I, and C327G). C327G polymorphism occurred on one of the cysteine residues involved in structural conformation. Except for recombination, the drivers for these mutations were similar to those for Pfs230 D1. On the 177 *Pfs25* sequences, low mutation frequencies (1.3% to 7.7%) were observed, resulting in a low nucleotide diversity (0.04×10^{-2}). Ten dimorphic codons were discovered (H41H, L42M, I83I, C110C, C115W, L122L, T124T, T130T, V132I, and V143G), nine of which were novel (H41H, L42M, I83I, C110C, C115W, L122L, T124T, T130T, V132I, and V132I). Two polymorphic codons (C110C and C115W) were of functional importance and occurred on cysteine residues. There was strong purifying selection at all polymorphic loci, as well as a high level of inbreeding.

This study established that there are a high number of clinical malaria cases in epidemic-prone zone of Kisii highland, and two villages are potential sentinel sites for future public health intervention if a malaria epidemic occurs. Despite low levels of microscopic infection in malaria endemic zone (Homa Bay), a high proportion of positive (submicroscopic) malaria cases are missed in health facilities; a more sensitive diagnosis test should be used to supplement microscopy. This will help to reduce the fraction of false negative results, potentially lowering the risk of escalating or maintaining residual *P. falciparum* community transmissions. With the presence of polymorphic sites of functional significance in Pfs230 D1, Pfs48/45 D3, and Pfs25, there is a need for further immunological investigation of the effect

of these polymorphisms when designing TBVs based on the sequences of these genes and their potential impact on antibody binding epitopes.

DEDICATION

This work is dedicated to my late parents Naomi Akeyo Ochwedo and Retired Sergeant Charles Ochwedo Wang'ore, who ensured that I completed my undergraduate studies by not only paying my school fees but also encouraging me through their hard work and dedication. I would also like to appreciate my wife Beatrice Katunge Ndinda and my son Rein Nillan Ochwedo for their unwavering support during this project.

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LIST OF ABBREVIATION AND ACRONYMS

COVID-19 - Corona virus disease of 2019

P. falciparum – Plasmodium falciparum

WHO – World Health Organisation

LLIN – Long-lasting insecticidal net

IRS – Indoor residual spraying

mRDT – Malaria rapid diagnosis test

usmRDT– Ultrasensitive malaria rapid diagnosis test

TBA – Transmission-blocking activity

TRA – Transmission-reducing activity

MTBVs – Malaria transmission-blocking vaccines

Pfs230 D1 – Domain 1 of *Plasmodium falciparum* surface protein 230

Pfs48/45 D3 – Domain 3 of *Plasmodium falciparum* surface protein 48/45

Pfs25 D1 – Domain 1 of *Plasmodium falciparum* surface protein 25

Pfs25 D2 – Domain 2 of *Plasmodium falciparum* surface protein 25

Pfs25 D3 – Domain 3 of *Plasmodium falciparum* surface protein 25

Pfs25 D4 – Domain 4 of *Plasmodium falciparum* surface protein 25

AMA1 – Apical membrane antigen 1

MSP1 – Merozoite surface protein-1

MSP2 – Merozoite surface protein-2

MSP3 – Merozoite surface protein-3

PV – Parasitophorous vacuole

PEXEL/VTS – Protein export motif

NLS – Nuclear localization signal

RBCs – Red blood cells

PfsRhs – *Plasmodium falciparum* reticulocyte binding homologs

EBAs – Erythrocyte binding antigens

DNA – Deoxyribonucleic acid

AP2-G – Transcription factor

pLDH – *Plasmodium* lactate dehydrogenase

PfHRP2 – *Plasmodium* histidine rich protein 2

ACD – Active case detection

PCD – Passive case detection

HCW – Health Care Workers

KEMRI – Kenya Medical Research Institute

FMP012/AS01B – *Falciparum* malaria protein12 with adjuvant 3-O-desacyl-4′-monophosphoryl lipid A and the saponin QS-21

ITNs – Insecticide-treated nets

PFSPZ – *Plasmodium falciparum* sporozoite

CD4+ – Cluster of differentiation 4

CD8+ – Cluster of differentiation 8

3D7 – *Plasmodium falciparum* strain

GPI – Glycophosphatidylinositol

IgG – Immunoglobulin G

SNP – Single nucleotide polymorphism

kDa – Kilodalton

NF54 – *Plasmodium falciparum* strain

mAb – Monoclonal antibody

SMFA – Standard membrane-feeding assay

MoH – Ministry of health

DBS – Dry blood spots

PBS – Phosphate buffered saline

RT-PCR – Realtime polymerase chain reaction

SPSS – Statistical Package for the Social Sciences

CHAPTER ONE: INTRODUCTION

1.1 Background

Despite the existence of numerous interventions against malaria caused by *Plasmodium falciparum*, an estimated population of over 3.2 billion are still at risk of the disease. More cases and deaths from malaria were reported in 2020 as compared to 2019 (WHO, 2021a). The increased cases were partly attributed to the disruption of the malaria surveillance system, which includes vector control, diagnosis, and treatment by the Corona virus disease of 2019 (COVID-19) (WHO, 2021a). Majority (95%) of reported cases and deaths (96%) were from the Africa region with high death rates being observed among children under the age of five (WHO, 2021a). In Kenya, over 20 million people are at risk of malaria infection, and high transmission rates occur in endemic regions (consistent transmission) as well as epidemic-prone (unstable transmission) zones bordering them (Halliday *et al.,* 2014; Kamau *et al.,* 2020; MoH, 2021, 2021; Oduma *et al.,* 2021; Ochwedo *et al.,* 2021). Some of the rolled-out intervention in malaria endemic region in western Kenya to curtail the high prevalence include localized indoor residual spraying (Abong'o *et al.,* 2020), long lasting insecticidal net (LLIN) use (Santos *et al.,* 2019; Ng'ang'a *et al.,* 2021; Ochwedo *et al.,* 2021), partial introduction of RTS,S malaria vaccine (Akech *et al.,* 2020), diagnosis and treatment. Although the use of IRS, LLIN, and RTS,S in some areas, such as Homa Bay, has resulted in a slight decrease in vector populations and improved malaria surveillance, overall malaria positive cases remains slightly high (Abong'o *et al.,* 2020; Oduma *et al.,* 2021; Orondo *et al.,* 2021; Ochwedo *et al.,* 2021). High positive cases has also been observed in epidemic prone areas such as Kisii highland which is bordering Homa Bay, Migori and Kisumu all of which are malaria endemic zones (Wanjala *et al.,* 2011; Wanjala & Kweka, 2016; Zhou *et al.,* 2018).

Among the key interventions, IRS has drastically reduced primary vector populations in western Kenya (Abong'o *et al.,* 2020; Orondo *et al.,* 2021), however, without prompt diagnosis and treatment chances of residual *P. falciparum* transmission is at large. Undetected *P. falciparum* infections, that is infections that cannot be microscopically diagnosed (subclinical or submicroscopic) or are beyond detection threshold of a microscope remain key challenges to diagnosis and affect efforts towards malaria reduction and elimination. As a result, there are various attempts to developed ultrasensitive kits such as ultrasensitive malaria rapid diagnosis test kits (usmRDT) to counter this.

Other efforts aiming at reducing transmission include the designing of malaria vaccines that target various growth stages within *P. falciparum* complex life cycle (Ouattara & Laurens, 2015; Kaslow & Biernaux, 2015; Draper *et al.,* 2018). With the recent focus being on transmission reduction or blocking activities (TRA/TBA), malaria transmission-blocking vaccines (MTBVs) are of great interest in malaria reduction and elimination strategies (Griffin *et al.,* 2010). The MTBVs are expected to elicit antibodies that would continually offer long-lasting protection to the host. To achieve this, the elicited host antibodies by the vaccines are supposed to block sporogonic cycle within the vector gut thus inhibiting transmission to the next available host or conferring indirect protection to the community. Among the notable pre-fertilization and post-fertilization vaccine candidates are the domain 1 of gametocyte surface protein 230 (Pfs230 D1), domain 3 of gametocyte surface protein 45/48 (Pfs45/48 D3) and full transcript of gametocyte surface protein 25 (Pfs25) (Miura *et al.,* 2013; Jones *et al.,* 2015b; Skinner *et al.,* 2015; MacDonald *et al.,* 2016; Stone *et al.,* 2018; Healy *et al.,* 2021). The two pre-fertilization proteins (Pfs230 D1 and Pfs48/45 D3) are present on the surface of gametocytes and are exposed to host peripheral blood system thus rendering them a target for both monoclonal and polyclonal antibodies (Brooks & Williamson, 2000). Success of Pfs230 D1, Pfs48/45 D3 and Pfs25 antigens as MTBVs will rely mostly on their ability to overcome

challenges imposed by frequent polymorphisms occurring on the parasite genome and effect of evolutionary plasticity. The three are undergoing various clinical trials either as single (Clinical Trial on Malaria, 2019, 2022; Lee *et al.,* 2019; University of Oxford, 2020; de Graaf *et al.,* 2021), combined (National Institute of Allergy and Infectious Diseases (NIAID), 2019; Healy *et al.,* 2021) or fusion antigens (Singh *et al.,* 2019) vaccine candidates .

1.2 Problem statement

Despite significant progress in reducing the burden of malaria in Sub-Saharan Africa, the disease remains a major public health concern. The spread of insecticide resistance, difficulties in diagnosis and treatment, the emergence of antimalaria drug resistance strains, and the allelic specificity of first-generation malaria vaccines such as RTS,S are major impediments to progress (Neafsey *et al.,* 2015; Alout *et al.,* 2017; Githinji *et al.,* 2020; Orondo *et al.,* 2021). Prompt *P. falciparum* diagnosis is critical for disease management and reducing community transmission. However, when microscopy is used as the gold standard, asexual and sexual parasites that are beyond the detection threshold of microscopy are frequently missed. These submicroscopic infections may be common in Homa Bay or Kisumu (malaria endemic zones) and Kisii highland (malaria epidemic prone zone) and can progress to infectious gametocyte stages that infect vectors and halt the progress made by other interventions such as IRS in western Kenya (Lin *et al.,* 2016; Gonçalves *et al.,* 2017). Additionally, in the area of Marani sublocation, Kisii highland (an epidemic prone zone), malaria prevalence based on outpatient data has consistently remained high over time (Aidoo *et al.,* 2018; Kapesa *et al.,* 2018). With limited literature on the sentinel sites where infections are localised in the Marani sublocation, initiation of appropriate disease prevention, control, and management will be limited in the event of a full-blown epidemic.

Other intervention challenges have necessitated the development of new strategies and tools, one of which is the development of effective malaria transmission blocking vaccines (MTBVs). However, key MTBV candidates such as Pfs230 D1, Pfs48/45 D3, and Pfs25 sequence based must overcome challenges posed by antigenic variations, as seen with previous vaccine candidates such as *P. falciparum* Apical membrane antigen 1 (AMA1) and merozoite surface protein-1 (MSP1) or MSP2, which are expressed by asexual stage parasites (Genton *et al.,* 2002; Takala *et al.,* 2007; Ogutu *et al.,* 2009; Bergmann-Leitner *et al.,* 2012; Miura *et al.,* 2013). Polymorphisms in immunodominant sequences of *Pfs230* D1, *Pfs48/45* D3, and *Pfs25* genes can cause developed gametocyte-stage vaccines to be allele-specific or change the conformation of antibody binding epitopes, reducing their efficacy, as seen with AMA1 (Osier *et al.,* 2010; Thera *et al.,* 2011; Ouattara & Laurens, 2015), MSP1 and MSP2 (Genton *et al.,* 2002; Galamo *et al.,* 2009; Bergmann-Leitner *et al.,* 2012). As a result, the polymorphisms can act as a precursor that is acted upon by natural selection, ensuring that *P. falciparum* gametocytes are not destroyed by vaccine-induced immunity.

Several dimorphic sites in the target domains of the Pfs230, Pfs48/45, and Pfs25 antigens have been identified (Roeffen *et al.,* 1995; Kocken *et al.,* 1995; Drakeley *et al.,* 1996; Escalante *et al.,* 1998; Conway *et al.,* 2001; Jones *et al.,* 2015b; Juliano *et al.,* 2016; MacDonald *et al.,* 2016; Patel *et al.,* 2017; Kaur *et al.,* 2017; Kundu *et al.,* 2018; Sookpongthai *et al.,* 2021; Coelho *et al.,* 2021); however, there is limited knowledge on the extent of genetic diversity, signatures of selection, and other evolutionary forces that may be shaping alleles in *P. falciparum* from different focalized malaria transmission zones.

1.3 Justification

The existing literature has not hinted on the sentinel sites where the persistent high levels of infections originate in epidemic prone areas such as the Marani subcounty in Kisii highland.

This study identifies hotspots that will be used as a reference point for implementing interventions or as a target for preventing disease spread to other "non-endemic" sites, thereby preventing a full-blown epidemic in the future. Additionally, the exclusive use of a microscope in asexual and sexual parasite diagnosis remains a major concern for malaria reduction and elimination. This is because the technique may be insufficiently sensitive in detecting submicroscopic infections. Despite the significant impact of undetected and untreated *P. falciparum* infections on malaria surveillance progress, no study had established the basis of occurrence and prevalence of submicroscopic infections in malaria endemic zones of Kochia, Homa Bay County. Early detection of submicroscopic infections among patients with clinical malaria symptoms opens the door to predicting the potential consequences of these infections on ongoing interventions. As a result, calls for the development of a highly sensitive and specific diagnostic kit that can supplement or be used solely in future diagnosis within malaria endemic and epidemic prone zones have been made.

Since *P. falciparum* is under immune surveillance in a vector or host, its immunogenic genes undergo mutations to circumvent its destruction as it penetrates tissues. Key drivers for the mutations aiding the parasite to avoid being eliminated include natural selection, recombination, and gene conversion (Jiang *et al.,* 2011; Claessens *et al.,* 2014). Little is known about whether selection pressure is acting on polymorphic loci on *Pfs230* D1, Pfs*48/45* D3 and *Pfs25* or whether the spread of dimorphic sites across the parasite population is due to other forces such as inbreeding and recombination. Overlooking of the polymorphic sites in *Pfs230* D1, Pfs*48/45* D3 and *Pfs25* sequences has led to limited information on their significance and distribution across the immunodominant domains in malaria-endemic or epidemic areas. Findings on the presence of selection pressure in the *Pfs25* gene would imply that there are unknown factors within female Anophelines that drive the mutations. It is critical to conduct an in-depth analysis to determine if there are polymorphic sites with functional consequences

across sequences of the three genes in malaria-endemic and epidemic regions of western Kenya, as this would determine their impact on the functionality of developed MTBV.

1.4 Research questions

1. How does the prevalence of *Plasmodium falciparum* infections differ in malaria endemic and epidemic prone area of western Kenya?
2. Is there evidence of polymorphisms and selection signatures in the immunodominant domain 1 of Pfs230 and domain 3 of Pfs48/45 in *P. falciparum* from rural western Kenya, and how would they affect transmission reduction and blocking (TRA or TBA) functionality?
3. What are the levels of genetic diversity and selection signatures in the immunodominant domains of Pfs25 in *P. falciparum* from rural western Kenya, and how would they affect transmission reduction and blocking (TRA or TBA) functionality?

1.5 Objectives

1.5.1 Main objective

To identify polymorphisms with functional significance in the immunodominant domains of the Pfs25, Pfs230, and Pfs48/45 antigens in *P. falciparum* populations from malaria endemic and epidemic prone rural area of western Kenya.

1.5.2 Specific Objectives

1. To determine prevalence of *Plasmodium falciparum* infections in malaria endemic and epidemic prone rural area of western Kenya.

2. Evaluate the genetic polymorphism and signatures of selection on the immunodominant domains of Pfs230 and Pfs48/45 in malaria parasites from malaria endemic and epidemic prone rural area of western Kenya.
3. Examine level of genetic diversity, signatures of selection and drivers for polymorphisms on transmission-blocking vaccine candidate *Pfs25* gene in malaria parasites from malaria endemic and epidemic prone rural area of western Kenya.

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria infections and transmission settings

Malaria is still regarded as one of the most infectious diseases on a global scale (WHO, 2021c). Despite the rolled-out interventions, in 2019, 1 million more cases were reported as compared to 2018 where the total global tally was at 228 million (WHO, 2020). Mortalities, on the other hand, fell from 411, 000 to 409, 000 in 2019, with the greatest number recorded in children under the age of five. Apart from Africa, which accounted for 95% of reported cases, other regions still on the WHO risk list include the Eastern Mediterranean, South-East Asia, the Americas, and the Western Pacific, according to the WHO report (WHO, 2021a). *Plasmodium falciparum* infections accounted for the vast majority of reported cases in Africa (99.7%), the Eastern Mediterranean (71%), the Western Pacific (65%), and South-East Asia (50%) (WHO, 2020). The high biting behaviour of infective *Anopheles* is attributed to the *P. falciparum* malaria burden in Africa (Braack *et al.,* 2015; WHO, 2020; Zekar & Sharman, 2021). As a result, Sub-Saharan Africa accounted for more than half of all malaria deaths reported globally in 2019 (WHO, 2020). Kenya accounted for 1% and 3% of all *P. falciparum* malaria cases and deaths worldwide, respectively. The majority of these reported infections originate in malaria-endemic areas of the country. Whereas, 70% of the population is still at risk of infection, making the disease a public health concern (USAID, 2022).

Kenya is divided into five malaria zones based on the disease prevalence: highland epidemic, lake endemic, coastal endemic, seasonal, and low risk (Kenya Malaria Indicator Survey, 2020). The epidemic-prone, seasonal, and low risk have a positivity rate of less than 2.5% by mRDT or microscopy (USAID, 2022). In Kenya, most of the epidemic prone zones are highland (elevation >1,500 m), malaria epidemics have been reported with recent effects being on Kericho and Baringo areas (MoH, 2019a, 2022b). Malaria cases or incidence in these

areas occur sporadically and have largely been attributed to changes on rainfall patterns, rising temperature, increased human population and their activities (Alonso *et al.,* 2011; Ruiz *et al.,* 2014; Kapesa *et al.,* 2017; Krsulovic *et al.,* 2021). The infections in the western Kenya highlands are characterized by high transmission, which is slightly unstable depending on location and is dependent on favourable conditions for vector breeding and malaria parasite transmission (Hay *et al.,* 2002; Zhou *et al.,* 2004; Aidoo *et al.,* 2018; Kapesa *et al.,* 2018). Based on location, the western Kenya highlands with V-shaped valleys such as Marani (Kisii) and Fort Tenan (Kericho) have been shown to have a tendency for explosive malaria epidemics during high transmission periods, as opposed to the stable transmission seen in the U-shaped valleys of Iguhu (Kakamega) and Emutete (Vihiga) (Wanjala & Kweka, 2016). Variations in transmission levels in western Kenya's highlands have also been linked to varying levels of malaria immunity among residents (Wanjala & Kweka, 2016). Initially, *Anopheles gambiae* was found to be the most common indoor resting vector in the V-shaped valley of Marani subcounty; however, this changed over time, and *Anopheles funestus* became the most common indoor resting vector, accounting for 74% of all indoor resting vectors (Kapesa *et al.,* 2017). During this time, the area (Marani) was also marked by a 2°C increase in temperature as well as increased in asymptomatic cases among pupils.

The endemic western Kenya zone includes three counties bordering Lake Victoria (Migori, Homa Bay, and Kisumu) and three others (Vihiga, Kakamega, and Busia) (Bashir *et al.,* 2019). Mombasa, Kwale, Kilifi, and Lamu (which border the Indian Ocean) are all part of the endemic coastal zone, as is Taita Taveta (Gopal *et al.,* 2019). According to data from the 2020 Kenya Malaria Indicator Survey, children aged 6 months to 14 years from the Lake endemic zone had the highest number of reported malaria infections in Kenya (USAID, 2022). The age group's recent positivity rate by malaria rapid diagnosis test (mRDT) and microscopy was 22.8% and 18.9%, respectively. This was followed by the Coastal endemic zone, which

had an mRDT positivity rate of 4.9% and a microscopy positivity rate of 4.5%, respectively. Because of the favourable climate that supports a large population of malaria vectors that drive *P. falciparum* transmission to hosts, the Lake and Coastal endemic zones continue to have high prevalence (Gopal *et al.,* 2019; Bashir *et al.,* 2019).

In Kenya's endemic and epidemic zones, the key malaria vectors are *An. gambiae*, *An. funestus* and to some extent *An. arabiensis* (Bayoh *et al.,* 2011; Degefa *et al.,* 2017; Ondiba *et al.,* 2018; Karungu *et al.,* 2019; Zhong *et al.,* 2020; Mustapha *et al.,* 2021; Debrah *et al.,* 2021). The distribution, abundance, and vectorial capacity of these vectors vary by region; some, such as *An. arabiensis* predominate in areas such as Ahero, acting as primary *P. falciparum* transmitters (Degefa *et al.,* 2017; Wanjala & Kweka, 2018), whereas in other areas, such as Karachuonyo and Kochia in Homa Bay County, they contribute minimally to transmission despite their high population (Lefèvre *et al.,* 2013; Orondo *et al.,* 2021).

2.2 Malaria transmission cycle

Plasmodium falciparum infection and transmission by vectors are dependent on intrinsic and environmental factors such as *Plasmodium*-vector interactions, vector gut flora, its diet, infection history, and ambient temperature (Dong *et al.,* 2009; Rasgon, 2011; Lefèvre *et al.,* 2013; Herren *et al.,* 2020). The entire *P. falciparum* transmission cycle is complex, involving both asexual and sexual development phases in human hosts and Anophelines, respectively (**Fig. 2.1**). The asexual phase includes the pre-erythrocytic and erythrocytic stages (Foquet *et al.,* 2018). The arrival of vector-injected sporozoites after successful blood meal intake from hosts initiates the pre-erythrocytic or asymptomatic hepatic stage (Soulard *et al.,* 2015; Foquet *et al.,* 2018). A portion of injected motile sporozoites typically pass through host endothelial cells and enter the bloodstream, where they are transported to the liver and infect hepatocytes after a few minutes (Amino *et al.,* 2006; Foquet *et al.,* 2018). Prior to infecting host

hepatocytes, sporozoites traverse the cytosol of several host cells by breaching their plasma membranes and rapidly repairing them without forming parasitophorous vacuole (PV), which allows parasite replication (Mota *et al.,* 2001). In infected hepatocytes, however, the parasite is ensconced within its formed protective PV membrane, where it undergoes hepatocytic schizogony and maturation, resulting in schizont with a large number of first generation merozoites within 6-7 days (Mota *et al.,* 2001; Vaughan *et al.,* 2012; Nyboer *et al.,* 2018). To introduce circumsporozoite (CS) protein into the cytoplasm and enter the nucleus of hepatocytes, sporozoites use the protein-export motif (PEXEL/VTS) of the hosts' cells and the nuclear localization signal (NLS) (Singh *et al.,* 2007). In infected hepatocytes, P52 and P36, two other members of the *P. falciparum* surface protein 48/45 family, are thought to play critical roles in the formation and maintenance of the PV membrane, which is required for complete maturation and merosome formation (Ploemen *et al.,* 2012). Merozoites within merosomes are released into the host bloodstream via liver sinusoids (**Fig. 2.1**) and invade red blood cells (RBCs) during the symptomatic asexual blood stage (Amino *et al.,* 2006).

Merozoites in erythrocytes undergo erythrocytic schizogony within 24-72 hours, producing approximately 32 merozoites that are released and invade new RBCs (Venugopal *et al.,* 2020). If the asexual cycle (invasion and replication) is not interrupted, it will result in acute and eventually chronic infections. To aid in the process, the parasite may use sialic acid (SA) dependent or independent pathways to invade erythrocytes with the help of invasion antigens from the *P. falciparum* reticulocyte-binding homologs (PfRhs) and erythrocyte-binding antigens (EBAs) families (Persson *et al.,* 2008; Awandare *et al.,* 2018). During the blood stage cycle, a small percentage of merozoites (about 1% of all merozoites) commit to sexual development before schizogony (Alano, 2007). After replication, committing merozoites will produce either male or female gametocyte schizonts, but not both (**Fig. 2.1**).

However, some merozoites may commit during the early ring stage, forming gametocytes without undergoing schizogony or forming schizonts (Bancells *et al.,* 2019).

The process of gametocytogenesis is influenced by antimalarial drugs (Peatey *et al.,* 2009) or biomolecules such as DNA-binding proteins, which include the transcription factor AP2-G, which regulates the sexual commitment process (Sinha *et al.,* 2014). Others include the host's lysophosphatidylcholine, which the parasite uses for its own fatty acid and choline metabolism and whose depletion accelerates the gametocytogenesis process (Hofer, 2018). Within a 10-day period, gametocytes go through five stages of maturation (I to V). Stages I-IV are isolated from peripheral circulation, leaving only stage V available for Anopheline uptake during a bloodmeal (Gardiner & Trenholme, 2015). During bloodmeal, approximately 103 gametocytes are ingested (**Fig. 2.1**), which round up, egress from RBC, and differentiate to gametes while inside the midgut lumen (Billker *et al.,* 1997; Sinden, 1999; Smith *et al.,* 2014). Male gametes then undergo exflagellation, which is triggered by factors such as temperature and PH differences between the host and the vector, as well as xanthurenic acid production by infected vectors, and transform to haploid microgametes (Billker *et al.,* 1997, 1998; Smith *et al.,* 2014).

After detaching from exflagellation centres, the microgamete seeks out and fertilises macrogametes, producing a diploid zygote, then a tetraploid, before differentiating into ookinetes over a 16-24 hour period (Pimenta *et al.,* 2015; Bennink *et al.,* 2016). Because development takes place in the midgut, only ookinetes within the blood bolus or far enough away from the destruction by epithelium-secreted digestive enzymes within the vector midgut survive (Abraham & Jacobs-Lorena, 2004; Smith *et al.,* 2014). The motile ookinete penetrates the peritrophic matrix of the vector, travels through the vector intestinal epithelium, and settles beneath the basal lamina before differentiating into oocysts (Smith *et al.,* 2014; Pimenta *et al.,*

2015). The oocyst develops, goes through sporogony, producing thousands of sporozoites, then matures and releases the sporozoites into the vector's haemolymph, where they circulate, invading the salivary gland and ready to be injected into the available host (**Fig. 2.1**).

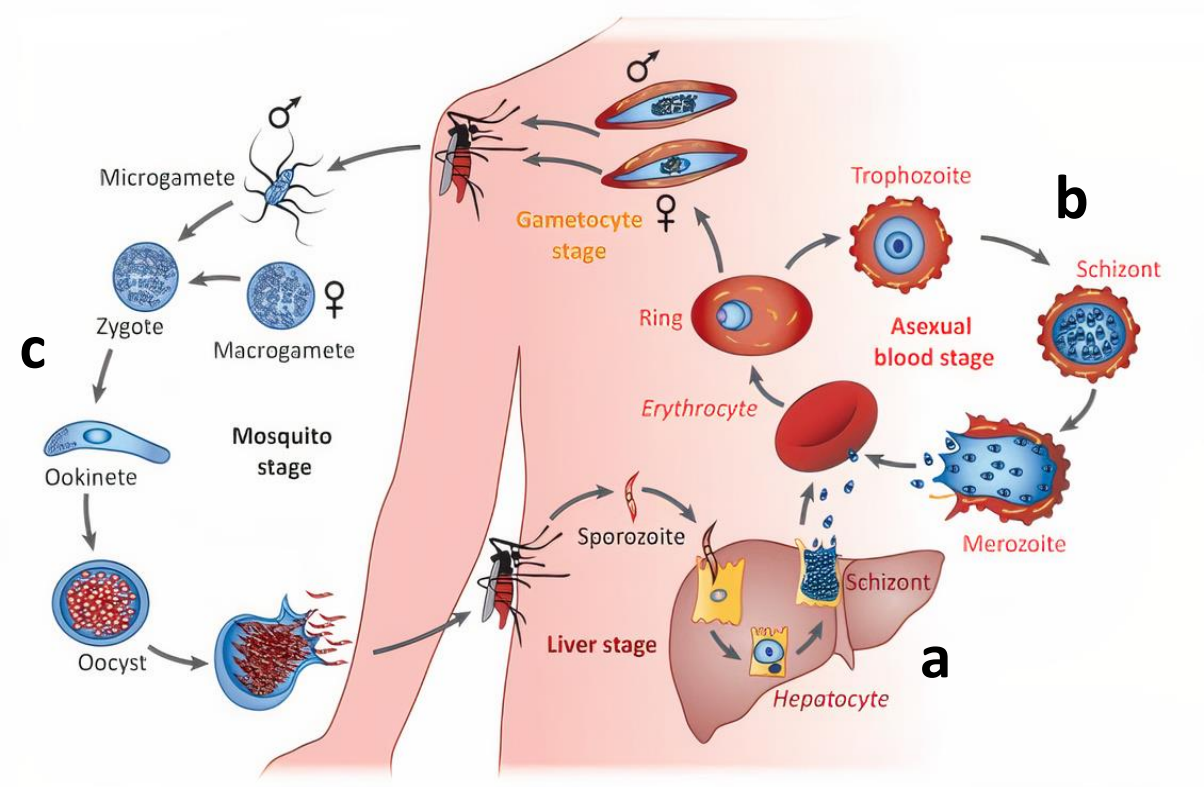

Figure 2.1 Lifecycle of *Plasmodium falciparum*. Infected Anophelines inject sporozoites into the host during a blood meal. A portion of the injected sporozoites (**a**) invade hepatocytes in the host liver. Schizonts release first-generation merozoites into the blood after successful asexual replication and maturation. The merozoite invades the erythrocyte, transforms into a ring stage, and then replicates asexually to become a blood stage schizont (**b**). After erythrocyte lysis, merozoites are released for reinvasion or commitment to gametocytes which signal the start of the sexual reproduction cycle. Anopheline consumes the formed gametocytes during bloodmeal and sexually reproduces (**c**) within the mosquito midgut (Maier *et al.*, 2019).

For vector parasite infection to be successful, various *P. falciparum* antigens must interact with the vector midgut. *P. falciparum* surface protein 230 (Pfs230) and Pfs48/45, as

well as post-fertilization protein Pfs25, are key parasite antigens involved in gametocyte fertilisation. The aforementioned antigens are candidates for malaria vaccine development due to their ability to elicit a host immune response. If developed, the vaccines are intended to supplement other existing malaria intervention programmes.

2.3 Malaria surveillance system

The implementation of malaria control strategies in malaria-endemic countries has resulted in a decrease in the number of new infections over time (WHO, 2020). Globally, parasite and vector control interventions such as active and passive case detection and treatment, indoor residual spraying (IRS), and distribution of insecticide-treated nets (ITN) are becoming more popular (Okumu & Moore, 2011; Bhatt *et al.,* 2015; Deng *et al.,* 2018; Stuck *et al.,* 2020; Abong'o *et al.,* 2020; Ochwedo *et al.,* 2021). The three mainstream malaria transmission prevention strategies mentioned above, as well as the recently approved use of the RTS,S/AS01 vaccine (MoH, 2019b; WHO, 2021b), have been implemented in endemic and epidemic-prone areas in Kenya (Ochomo *et al.,* 2017; Deng *et al.,* 2018; Abong'o *et al.,* 2020). These tools are essential components of the malaria surveillance system, which is divided into three categories: diagnosis and anti-malarial treatment, vector control, and malaria vaccine use.

2.4 Diagnosis and anti-malarial treatment

Malaria diagnosis and treatment, which are components of the malaria surveillance system, are critical in efforts to prevent the re-establishment of transmission in previously declared disease-free areas (Nasir *et al.,* 2020). The only approved diagnostic tools in malaria-endemic areas are microscopic examination of human blood smears and the use of a point-of-care malaria rapid diagnostic kit (mRDT) to detect parasite antigen (WHO, 2011; Cunningham *et al.,* 2019). Malaria RDTs are immunochromatographic lateral flow qualitative diagnostic devices that detect antigens expressed by asexual parasites or gametocytes, such as *Plasmodium* lactate

dehydrogenase (pLDH) and or histidine rich protein 2 (PfHRP2), which is expressed by asexual parasites in the blood of hosts(Moody, 2002). Only mRDTs are used in reactive or active case detection (ACD) to diagnose malaria positive cases in asymptomatic community members (Sturrock *et al.,* 2013). For symptomatic cases, microscopy and/or mRDT are used in a health facility or by a community health worker (CHW) in what is known as a passive case detection (PCD) system (Gueye *et al.,* 2013; Hsiang *et al.,* 2020). Globally, ACD and PCD have made significant contributions to the reduction of malaria transmission and cases(Nasir *et al.,* 2020).

However, the success of ACD and PCD is dependent on well-trained personnel, the efficacy of each diagnostic tool, and the efficacy of each treatment (Landier *et al.,* 2016). In eight Sub-Saharan African countries, training and supervision of health care workers (HCW) involved in malaria diagnosis improved general health facility performance or adherence to malaria case management guidelines from an average of 85% to 95% when using mRDT (Eliades *et al.,* 2019). Microscope readings from 1,037 health facilities in seven African countries yielded nearly identical results (Alombah *et al.,* 2019). Lack of skilled personnel or appropriate supervision impacts negatively on ACD and PCD and may result in under or over prescription of antimalarials (Mekonnen *et al.,* 2014; Ochwedo *et al.,* 2021; Otambo *et al.,* 2022). Under and over prescription are most likely the result of false positive or negative diagnostic tests, as well as presumptive treatment by unskilled or unsupervised personnel using a microscope or mRDT to detect the presence of malaria parasites in patients seeking treatment (Edson *et al.,* 2010; Berzosa *et al.,* 2018; Otambo *et al.,* 2022). As a result, antimalarial drug overuse has reduced their efficacy, with reports of drug-resistant parasites being recorded globally.

Overuse stresses the parasite genome, resulting in a selective sweep that favours drug-resistant or parasite-beneficial alleles (Jiang *et al.,* 2011; Cheeseman *et al.,* 2012; Takala-

Harrison *et al.,* 2013, 2015; Berzosa *et al.,* 2018). Misdiagnosis can occur not only as a result of insufficient supervision or inexperienced personnel, but also as a result of tools' inability to detect parasites due to detection threshold. Microscopy, for example, may underestimate parasite presence in hosts with low parasite density infections of less than 62 parasites per microlitre of blood (Okell *et al.,* 2009; WHO, 2014; Joanny *et al.,* 2014). Other factors, such as the availability of reagents, functional microscopes, and electricity, may also influence the quality of microscopy readings.

Malaria RDTs are not exceptional; despite being recommended by WHO for community point-of-care use due to their accuracy and speed (WHO, 2010), there have been reports of false positive and negative results leading to inappropriate treatment (Berhane *et al.,* 2017; Boyce & O'Meara, 2017; Parr *et al.,* 2021). This has been observed while using a common SD Bioline or Alere PfHRP2 RDT kit as a malaria case confirmation tool in most Sub-Saharan African health facilities (Otambo *et al.,* 2022). False negative results have been attributed to parasites that evade detection due to a lack of *Pfhrp2,* a gene that encodes an antigen detected by mRDTs (Cheng *et al.,* 2014; Thomson *et al.,* 2020). False positives, on the other hand, are caused by the extended half-life of targeted PfHRP2 antigens, despite parasite clearance from host blood (Chiodini, 2014; Mukkala *et al.,* 2018). Despite this, in a variety of transmission settings, mRDTs have demonstrated higher parasite detection accuracy and specificity than microscopy, making them an important tool in malaria reduction and elimination (Berzosa *et al.,* 2018; Otambo *et al.,* 2022).

Furthermore, efforts to develop efficient kits have resulted in the Alere^{TM} ultra-sensitive RDT (usmRDT) kit by Abbott, South Korea, which has a higher detection threshold than microscopy and SD Bioline malaria RDTs (Landier *et al.,* 2018; Acquah *et al.,* 2021). The detection threshold of the usmRDT is 3,019 *Plasmodium* parasites per microlitre of blood,

which is lower than the detection threshold of the SD Bioline RDT, which is around 11,352 parasites per ml of blood (Jimenez *et al.*, 2017; Das *et al.*, 2017; Landier *et al.*, 2018). In Kenya, the Kenya Medical Research Institute (KEMRI) recently unveiled a locally made malaria RDT that it intends to use to help scale up diagnosis and treatment, thereby reducing malaria cases and incidence (MoH, 2022a).

2.5 Malaria vaccine candidates

Malaria vaccines remain an important complement for the next era of disease prevention as malaria intervention challenges such as drug and insecticide resistance emerge. The search for an effective vaccine has been ongoing since the 1960s, with RTS,S/ASO1 (Mosquirix^{TM}) being the only vaccine to be released in 2021 (Centers for Disease Control and Prevention, 2021). The process of developing an effective malaria vaccine has encountered numerous challenges, including not only the parasite's diverse life cycle, but also coevolution of the parasite genome during parasite-host or parasite-vector interactions, which results in inherent variations. The complex parasite life cycle has limited vaccine development or vaccine candidate selection to specific life cycle stages. From these, rolled-out vaccine or candidates either aim to elicit host antibodies that are restricted to antigens expressed by *P. falciparum* in vectors (transmission blocking), at the hepatic, erythrocytic, or sporogonic stages in humans (to prevent or control disease). The vaccine or candidates may inhibit sporozoites from infecting hepatocytes or kill infected hepatocytes (in the liver) (Adepoju, 2019; Akech *et al.*, 2020), inhibit merozoite invasion of RBCs (at the blood stage), prevent clinical outcomes during pregnancy (Sirima *et al.*, 2017; Doritchamou *et al.*, 2022), and inhibit sexual development or blocking transmission (Clinical Trial on Malaria, 2019, 2022).

2.5.1 Pre-erythrocytic vaccine candidates

Among the vaccines that elicit antibodies that prevent *P. falciparum* sporozoites from infecting host hepatocytes (**Fig. 2.2**) are circumsporozoite protein-based RTS, S/ASO1 (Alonso *et al.,* 2011; Neafsey *et al.,* 2015; Adepoju, 2019; Akech *et al.,* 2020). The vaccine (RTS, S/ASO1) has a protective efficacy of less than 60% against severe and clinical malaria in children and infants infected with *P. falciparum* with genotypically matched circumsporozoite protein C-terminal as vaccine template (Alonso *et al.,* 2011; Neafsey *et al.,* 2015). For parasites with mismatched or heterogeneous alleles, efficacy drops to around 33.4%, demonstrating the importance of genetic variability in *P. falciparum* (Neafsey *et al.,* 2015).

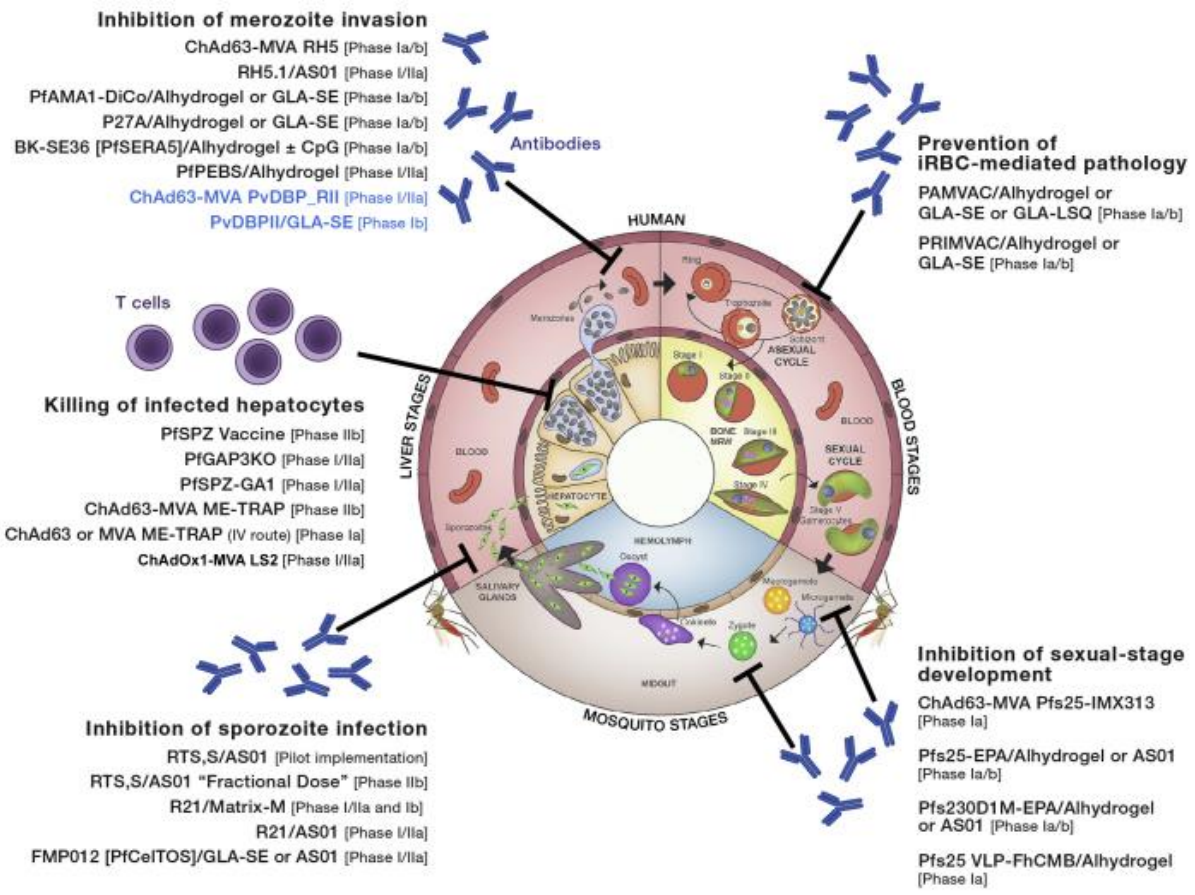

Figure 2.2 Malaria vaccine candidates and targets. The circle represents the three stages: hepatic, blood, and sporogonic. Each stage has a representative vaccine candidate, their role, mode of formulation, and clinical trial stage (Nilsson *et al.*, 2015; Draper *et al.*, 2018).

Other vaccine candidates based on circumsporozoite protein include R21 with adjuvant Matrix-M (R21/Matrix-M) and R21 with adjuvant based on liposome made of immunostimulant mycobacterial cell wall skeleton (MPL) or 3-O-desacyl-4'-monophosphoryl lipid A and saponin QS-21 or AS01 (R21/ASO1) (Didierlaurent *et al.*, 2017; Datoo *et al.*, 2021; Moorthy & Binka, 2021). The R21 vaccine is a next-generation vaccine that has been shown to be safe, highly immunogenic, and to have a high efficacy of 77% during a phase 2b clinical trial that targeted children aged 5 to 17 years (Datoo *et al.*, 2021). However, unlike RTS, S/AS01, there are no reports on allele specificity. Another sporozoite inhibitory under

consideration is *Falciparum* malaria protein 12 (FMP012)/AS01B, a cell transversal antigen-based vaccine candidate (U.S. Army Medical Research and Development Command, 2021).

In contrast to previously discussed pre-erythrocytic vaccines which act via eliciting host antibodies, radiation-attenuated *P. falciparum* sporozoite (PFSPZ) (Mwakingwe-Omari *et al.,* 2021; Oneko *et al.,* 2021), PFGAP3KO which is genetically attenuated by deletion of *P. falciparum* protein 52 (p52), p36 and sporozoite specific protein (sap1) (Kublin *et al.,* 2017), and viral vectored Chimpanzee adenovirus 63 containing modified vaccinia virus Ankara and multiple epitope thrombospondin-related adhesion protein (Chad63-MVA-ME-TRAP) (O'Hara *et al.,* 2012; Afolabi *et al.,* 2016), are vaccine candidate generation that clear infected hepatocytes through T-cell mediated immunity (Oneko *et al.,* 2021). These vaccine candidates are expected to generate high antibody titres as well as reactive malaria CD8+ and CD4+ T-lymphocytes. PFGAP3KO, unlike PFSPZ and Chad63-MVA-ME-TRAP, is administered via mosquito vector injection during a blood meal (Kublin *et al.,* 2017). All three vaccine candidates have been found to be highly tolerable and to elicit strong immune responses, particularly after booster administration in the case of PFSPZ and Chad63-MVA-ME-TRAP (O'Hara *et al.,* 2012; Afolabi *et al.,* 2016; Kublin *et al.,* 2017). These vaccine candidates are still in clinical trials, but unlike blood stage candidates, there is little information on whether they are affected by allele specificity.

2.5.2 Erythrocytic vaccine candidates

Key blood stage vaccines (**Fig. 2.2**) that are currently under clinical trials include candidates based on *P. falciparum* apical membrane antigen 1 diversity-covering (PfAMA1-Dico) (Remarque *et al.,* 2008, 2021; Spiegel *et al.,* 2017), Reticulocyte-binding protein homologue 5 (RH5) (Jin *et al.,* 2018; Minassian *et al.,* 2021), P27 a synthetic peptide designed from *P. falciparum* trophozoite exported protein 1 (TEX1) (Karch *et al.,* 2017; Steiner-Monard *et al.,*

2019), *P. falciparum* serine repeat antigen 5 (PfSERA5) (Yagi *et al.,* 2016) and *P. falciparum* pre-erythrocytic and blood-stage (PfPEBS) (Spertini & Druilhe, 2012; Salamanca *et al.,* 2019).

Several blood stage vaccine candidates failed to confer strain or variant-transcending immunity during clinical trial stages due to antigenic variations. Candidates based on merozoite surface protein 1 (MSP1), an antigen important during the parasite development stage, are among these (Child *et al.,* 2010). Immune responses elicited by recombinant proteins from the MSP142 molecule have been shown in BALB/c or ICR mice to be allele specific and to lack cross-reactive response. The immune response was specific to 3D7 vaccine regimens that targeted the protein 33 (p33) molecule (Bergmann-Leitner *et al.,* 2012). Similarly, antibodies targeting MSP1 block 2 have been reported to induce a monocyte response, resulting in allelic specific cellular inhibition (Galamo *et al.,* 2009). The allelic specificity was also observed in a clinical trial of FMP1/AS02, which is based on the MSP142 sequence, in western Kenya (Ogutu *et al.,* 2009). Other vaccine candidates that have faced antigenic specificity challenges include FMP2.1/AS02A, a vaccine candidate based on AMA1(Osier *et al.,* 2010; Ouattara *et al.,* 2013), and merozoite surface protein 3 (MSP3) (Polley *et al.,* 2007).

2.5.3 Transmission blocking vaccines candidates

In contrast to the previously discussed vaccine candidates that protect against clinical malaria, transmission blocking vaccine candidates remain exceptional due to the site of action of their respective elicited host antibodies (**Fig. 2.2**) and goal of reducing the number of infectious vectors (Blagborough *et al.,* 2013). The elicited antigen-specific antibodies function within the vector midgut, thereby inhibiting sporogonic development and reducing or blocking *P. falciparum* transmission at the community level (Wu *et al.,* 2017; Acquah *et al.,* 2017; Doumbo *et al.,* 2018; Chichester *et al.,* 2018).

When antibodies are taken in by vectors during successful bloodmeal, they may target antigens expressed on the surfaces of gametes (pre-fertilization targets), thereby blocking fertilisation and subsequent zygote formation (Wu *et al.,* 2017; Acquah *et al.,* 2017; Kundu *et al.,* 2018; Lee *et al.,* 2020; Singh *et al.,* 2020), or they may target antigen expressed on the surface of zygotes (post-fertilization target) thus blocking formation of oocyst (Kaslow *et al.,* 1988; Chichester *et al.,* 2018). These host antibodies typically remain active within the vector midgut and neutralise their target by restricting gamete movement following agglutination, minimising interaction between micro and macrogametes by coating, and gamete lysis via activation of the complement system or opsonization (Stone *et al.,* 2016). Pfs230 and Pfs48/45, both pre-fertilization proteins, and Pfs25, a post-fertilization protein, are the most likely TBV candidates (Sagara *et al.,* 2018; Takashima *et al.,* 2021).

2.5.4 *Plasmodium falciparum* surface protein 230

Plasmodium falciparum surface protein 230 was found in 1987 as a possible vaccine candidate and is among the leading sequence-based TBV candidates (Quakyi *et al.,* 1987; Singh *et al.,* 2019; Lee *et al.,* 2019; Tachibana *et al.,* 2019; Huang *et al.,* 2020; Singh *et al.,* 2020; Healy *et al.,* 2021). The ability of the *Pfs230* gene to be expressed by gametocytes in stages III-IV and spontaneously generate anti-Pfs230 antibodies in infected hosts has aroused TBV researchers' interest (Riley *et al.,* 1994; Roeffen *et al.,* 1995; Bousema *et al.,* 2010; Amoah *et al.,* 2018; Acquah *et al.,* 2020). This is in line with previous study that discovered varying amounts of IgG, IgG1, IgG3, and IgM specific to Pfs230 in children and adults from Ghana (Amoah *et al.,* 2018; Acquah *et al.,* 2020; Broni *et al.,* 2021), Tanzania (Bousema *et al.,* 2010), Gambia (Riley *et al.,* 1994; Holden *et al.,* 1996) and Cameroon (Roeffen *et al.,* 1995). Some naturally induced anti-Pfs230 antibodies, although not all, have been linked to TRA or TBA (Stone *et al.,* 2016).

The protein is expressed as a 360 kDa antigen composed of 3135 amino acid residues (**Fig. 2.3**) and is cleaved leaving 230 kDa with conserved 14 cysteine-rich domains that stay exposed on the surface of newly formed male gametes egressing erythrocytes while in the midgut of the vector (Brooks & Williamson, 2000; Miura *et al.*, 2022). It is hypothesised that the antigen forms bridges between cysteine residue pairs 1 and 2, 3 and 6, and 4 and 5, resulting in a convoluted-like shape (Gerloff *et al.*, 2005; Tachibana *et al.*, 2019). The antigen's size and abundance of cysteine residues have impeded efforts to produce it as a full-length protein (Tachibana *et al.*, 2019). However, it has been partially expressed as a recombinant protein expression system such as in plants, wheat, *Pichia pastoris*, *Escherichia coli*, and *Saccharomyces cerevisiae* (Williamson *et al.*, 1995; Vincent *et al.*, 1999; Farrance *et al.*, 2011; Tachibana *et al.*, 2011; MacDonald *et al.*, 2016). Pfs230 has an important role in attaching male gametes to the surface of erythrocytes, allowing vectors to be actively infected (Eksi *et al.*, 2006). Because it lacks a glycophosphatidylinositol (GPI) linkage, it complexes with Pfs48/45, which binds the complex to the plasma membrane, assisting in the adhesion and penetration of female gametes (Dijk *et al.*, 2001). The antigen organisation on the surface of macro and microgametes varies, and it can exist as a single antigen or in conjunction with its fusion counterpart Pfs48/45 (Singh *et al.*, 2020). Humoral immunity to the antigen (ant-Pfs230) disrupts the fusion or complex formation step, blocking the development of zygotes (Stone *et al.*, 2016).

Among the 14 cysteine-rich domains of Pfs230 antigen, domain 1 (D1), a 4-cysteine domain (**Fig. 2.3**), has been shown to be highly immunogenic and to suppress gametocyte development using anti-Pfs230 DI sera produced from mice and tested using the standard membrane feeding assay (SMFA) (Lee *et al.*, 2019; Tachibana *et al.*, 2019; Singh *et al.*, 2020; Healy *et al.*, 2021). From these assays, monoclonal antibodies such as 4F12 (isotype IgG1), subclone 4F12 (3B6), 3G2 (isotype IgG2b), 5G3 (isotype IgG1), 5H1(isotype IgG1) and 7G4

(isotype IgG1) including 4F12 IgG (rh4F12) (recombinant chimera) which recognizes Pfs230 D1 in its native or recombinant form or on surface of unfixed gametes have been identified (MacDonald *et al.,* 2016; Singh *et al.*, 2020).

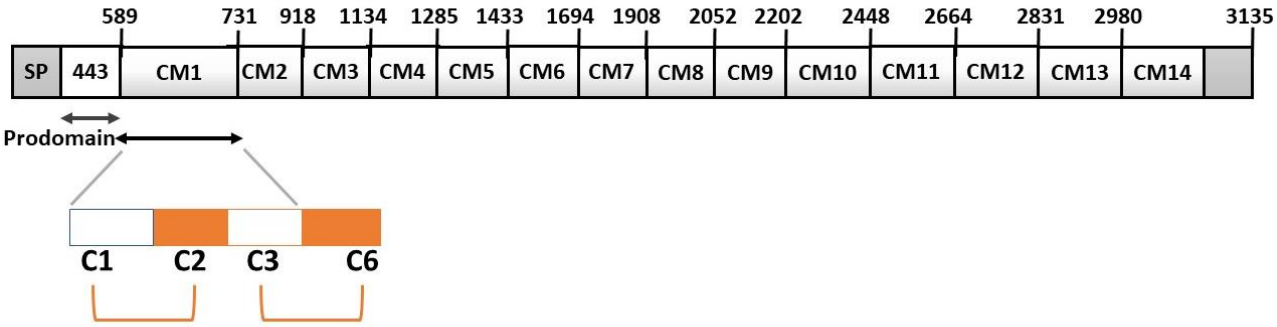

Figure 2.3 Graphical display of Pfs230 domains and cysteine linkage bridges for immunogenic domain 1. The amino acid at position 443 denotes the cleavage site, whereas CM1-CM14 symbolises the cysteine motifs or the 14 domains. The second arrow depicts Pfs230D1, with C1, C2, C3, and C6 denoting the four cysteine residues. Cysteine residue one (C1) forms bridge with C2 whereas C3 links to C6 (Tachibana *et al.,* 2019).

Only 4F12 and 5H1 show demonstrable TBA or TRA compared to the other 3G2, 5G3, and 7G4 mAbs against Pfs230 D1 (Singh *et al.*, 2020). Disulphides between 593 and 611 amino acids stabilises the epitope binding 4F12 antibodies. When binding to its corresponding epitope on Pfs230 D1, 4F12 light chain contacts roughly 98 aa and interacts with residues in different beta-strands from positions 581-586, 594, 596, 597, 598, 600, 602, 603, 604, 607, and 609 (Singh *et al.*, 2020). In contrast, the heavy chain interacts with 12 aa residues, including 601, 597, and 600. Other mAbs (5G3 and 3G2) bind to epitopes between 542 and 592 aa (Singh *et al.*, 2020). There are currently clinical trials underway to examine the safety, tolerability, and efficacy of vaccine candidates (Pfs230D1M-EPA/AS01, based on Pfs230 D1 sequences) in malaria-endemic countries such as Mali (National Institute of Allergy and Infectious Diseases

(NIAID), 2021). n other clinical trials, the vaccine candidate (Pfs230D1-EPA/Alhydrogel) demonstrated a significant TBA and TRA (Healy *et al.,* 2021).

Despite earlier assumptions that the gene was conserved, several polymorphic loci in the *Pfs230* gene from different isolates globally have been unravelled (Gerloff *et al.,* 2005; Singh *et al.,* 2020). Gerloff *et al.,* (2005) discovered 27 non-synonymous polymorphic sites, eight of which (H1159, E1152, Y1194, H1180, Q1196, N1209, K1254, and Q1250) were found to map on one side of domain IV of the Pfs230 antigen. These polymorphic loci are thought to be in the region involved in intermolecular interactions between male and female gametes (Gerloff *et al.,* 2005). Two non-synonymous SNPs, K661N and G605S, were discovered in domain 1 (Pfs230 D1) (MacDonald *et al.,* 2016). The two SNPs were thought to be unlikely to interfere with the Pfs230-based MTBV in initial phase 1 clinical trials. Aside from the two previously found frequent polymorphisms that resulted in missense K661N and G605S, fifteen single nucleotide polymorphisms (SNPs) were detected from variant call data in 2020 by Singh *et al.,* (2020). This resulted in amino acid modifications P550S, K661T, K644Q, E654K, T652R, K665Q, E655V, E656N, A699T, D713Y, D714N, D714G, N732S that required validation (Singh *et al.,* 2020). Furthermore, the drivers of these mutations were unknown, with hypothesised hypotheses pointing to a possibility of selection pressure arising from either low levels of humoral immunity or the vectors' midgut microenvironment (Singh *et al.,* 2020).

2.5.5 *Plasmodium falciparum* surface protein 48/45

In contrast to Pfs230, Pfs48/45 is expressed by both female and male gametes, is anchored on the gamete surface via the GPI moiety, and plays an important role in fertilisation (Kocken *et al.,* 1993). The gene is expressed in both humans and vectors' midguts, where it remains linked to gametes. Male gametes defective in the *Pfs48/45* gene lack bound Pfs230 and have lower fertility than female gametes with a similar deficiency (Dijk *et al.,* 2001). The hydrophobic

polypeptide is a doublet with 48 kDa and 45 kDa proteins, 16 cysteine residues, 3 domains, 448 aa (**Fig. 2.4**), a hydrophobic C-terminus, a signal sequence at the N-terminus, and seven N-glycosylation sites (Kocken *et al.,* 1993). As with Pfs230, expression of recombinant Pfs48/45 has been challenging due to the three domain structure maintained by eight pairs of disulphide bonds (Kocken *et al.,* 1993; Lee *et al.,* 2020).

Since Pfs48/45 has immunogenic domains and is expressed on the surface of sexual parasites in humans, humoral antibodies (anti-Pfs48/45) have been discovered. These have been detected in the sera of people living in malaria-endemic areas of Ghana, Tanzania, Burkina Faso, and Gabon (Bousema *et al.,* 2010; Ouédraogo *et al.,* 2011; Jones *et al.,* 2015b; Ateba-Ngoa *et al.,* 2016; Ayanful-Torgby *et al.,* 2021; Broni *et al.,* 2021). Humoral anti-Pfs48/45 has been demonstrated to have TBA and TRA, which constituted the basis for this antigen's candidacy (Bousema *et al.,* 2010; Jones *et al.,* 2015b; Stone *et al.,* 2016). Similarly to Pfs230, not all domains of Pfs48/45 antigen elicit strong antibodies with high TBA or TRA; only epitope I within domain 3 (**Fig. 2.4**) was shown to be the most immunogenic with high TBA and TRA levels (Chowdhury *et al.,* 2009; Jones *et al.,* 2015b; Kapulu *et al.,* 2015; MacDonald *et al.,* 2016; Kundu *et al.,* 2018; Singh *et al.,* 2019; Lee *et al.,* 2020).

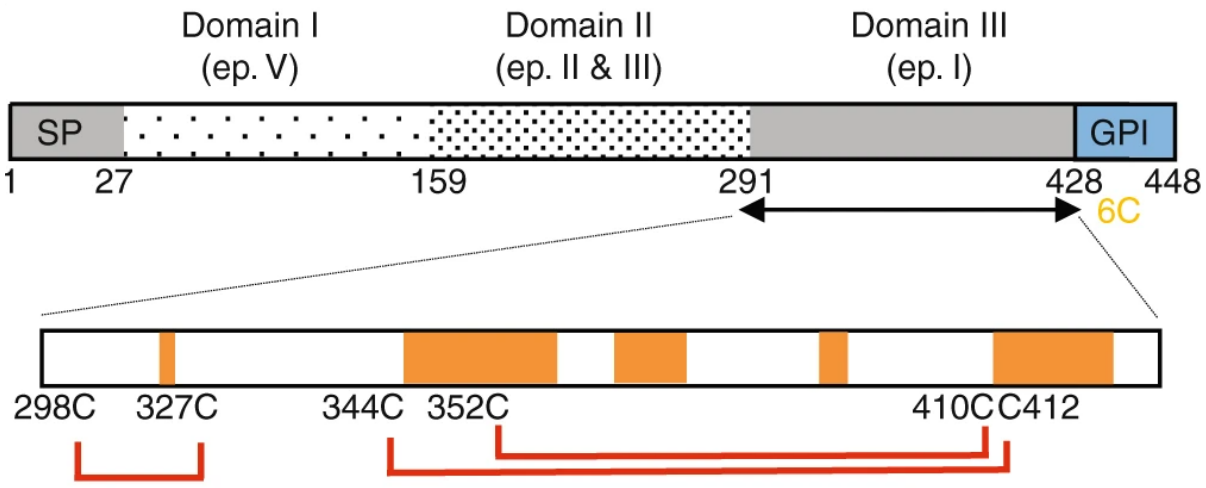

Figure 2.4 Graphical presentation of the structure of Pfs48/45 with three domains and four antibody binding epitopes. The locations of amino acid residues are represented by the numerals 1, 27, 159, 291, 428, and 448. The yellow labelled 6C represents domain 3, which is highly immunogenic with high levels of TBA and TRA and consists of six cysteine residues connected in a specific pattern (298C to 327C, 344C to C412, and 352C to 410C) (Kundu *et al.*, 2018; Lee *et al.*, 2020).

Despite possessing seven N-glycosylation potential sites, the antigen has been expressed successfully in a variety of expression platforms, including Vaccinia Virus (Milek *et al.*, 1998), *Pichia pastoris* (Milek *et al.*, 2000), *E. coli* (Outchkourov *et al.*, 2008), *Chlamydomonas reinhardtii* (Jones *et al.*, 2013), *Drosophila melanogaster* (Lennartz *et al.*, 2018), human embryonic kidney cell 293 (HEK293) cell (Kundu *et al.*, 2018), *Nicotiana benthamiana* (Mamedov *et al.*, 2019), *Lactoccus lactis* (Singh *et al.*, 2019) and baculovirus (Sf9 cells) (Lee *et al.*, 2020). Antibodies against expressed recombinant domain 3 (**Fig. 2.4**) have successfully prevented vector transmission of *P. falciparum* strains from the field and laboratory (Chowdhury *et al.*, 2009; Jones *et al.*, 2015b; Kapulu *et al.*, 2015; MacDonald *et al.*, 2016; Kundu *et al.*, 2018; Singh *et al.*, 2019; Lee *et al.*, 2020). There are currently plans to conduct

a phase 2 clinical trial of a vaccine based on D3 of Pfs48/45 (R0-PF10C) in Africa (Clinical Trial on Malaria, 2019).

Key identified monoclonal mAbs binding to Pfs48/45 epitopes include 85RF45.5 (epitope V in D1), 85RF45.2b (epitope IIb in D2), 85RF45.3 (epitope III in D2), and ones (32F3, 85RF45.113, 85RF45.1, and TB31F) binding highly immunogenic epitope I in D3 (Roeffen *et al.*, 2001; Outchkourov *et al.*, 2007; Kundu *et al.*, 2018). Other mAbs with TBA or TRA include 29F432 and 32F5, but 7F3 and 32F1 lacked TBA or TRA despite binding to Pfs48/45 epitopes (Vermeulen *et al.,* 1985). The essential player is the monoclonal antibody 85RF45.1, which interacts with amino acids at positions 347, 349, 351, 355, 365, 394, 413, 414, 415, and 416 (Kundu *et al.,* 2018). Three of these amino acids were discovered to be polymorphic (I349V, Q355L, and K414Q), and two (I349V and Q355L) were tested and shown not to give parasite protection by allowing them to escape the action of TB85RF45.1 mAbs (Kundu *et al.,* 2018). Other discovered variants on Pfs48/45 D3 from parasites circulating in India, Venezuela, and Thailand include V304D, S313G, L314I, D315G, S322N/C, E333G, V356A, A387T, K404R, and T422I (Escalante *et al.*, 2002; Anthony *et al.*, 2007; Jones *et al.*, 2015b; Kundu *et al.,* 2018). Other polymorphic sites have been discovered in Pfs48/45 D1 sequences from Tanzania and Ghana parasite isolates (at nucleotide positions 396 and 468) (Jones *et al.*, 2015b). In D2, two variants (253 and 254) have been found, with one (codon 254) thought to play a significant role in determining which mAb will attach to which epitope in D2 (Drakeley *et al.,* 1996). The antigen region where the two mutations occurred was thought to be exposed or on the outside part of the antigen that is exposed to humoral antibodies (Drakeley *et al.*, 1996).

2.5.6 *Plasmodium falciparum* surface protein 25

Plasmodium falciparum surface protein 25 (Pfs25), in contrast to pre-fertilization antigens Pfs230 and Pfs48/45, is a post-fertilization, cysteine-rich 25 kDa protein with a GPI linkage expressed on the surface of ookinetes (Vermeulen *et al.,* 1985). The antigen assists ookinetes in epithelial penetration, aggregation, and maturation to oocysts within the protease-rich vector's midgut (Tomas *et al.,* 2001; Baton & Ranford-Cartwright, 2005). The gene encoding the Pfs25 antigen is found on chromosome 10 and spans a length from 1253417bp to 1254070bp, with a single exon count that codes for a polypeptide chain of 217 amino acids (Kaslow *et al.,* 1988).

The gene has also been proven to be genetically connected to *Pfs28* by (Duffy & Kaslow, 1997) since it does not assort independently. The close genetic linkage of the two genes suggests that the locus where they are situated may allow transcription during the sexual stages. The Pfs25 gene is thought to be transcribed during the early stages of gametocytogenesis or during the trophozoite stage. In support of an early gametocytogenesis stage, Pfs25 mRNA transcripts have been detected 35 and 52 hours after invasion of the monolayer by NF54 and ItG2 strains (Dechering *et al.,* 1999; Niederwieser *et al.,* 2000). However, (Santolamazza *et al.,* 2017) recently recovered modest quantities of mRNA in the F12 strain's trophozoite stage supporting transcription at this stage. The discovery of Pfs25 mRNA transcript has supported its use as a marker for stage IV gametocyte exposure (Santolamazza *et al.,* 2017). Despite the presence of Pfs25 mRNA in host peripheral blood infected with stage IV gametocytes, the gene is thought to be expressed only in mosquito vectors at the ookinete stage (Stowers *et al.,* 2000). In humans, Pumilio/FBF family RNA-binding protein activities repress Pfs25 antigen translation (Miao *et al.,* 2013). However, the

activity of the Pfs25 promoter is induced prior to the shift of the malaria parasite from human to malaria vector (Dechering *et al.*, 1999).

The Pfs25 protein is believed to be made up of four tandem epidermal growth factor (EGF)-like domains that are anchored on the parasite's surface via the C-terminal GPI linkage (Kaslow *et al.*, 1988). The antigen's N-terminus comprises a signal sequence, which is followed by four EGF-like domains containing cysteine residues (**Fig. 2.5**). Domain 1 (D1) comprises aa residues between Alanine in position 22 (Ala22) and glutamine in position 59 (Glu59), (D2) has aa from Glu59 to Isoleucine in position 107 (Ile107), D3 is from Ile107 to Serine in position 150 (Ser150) while D4 is from Ser150 to Threonine in position 193 (Thr193) (Stowers *et al.*, 2000).

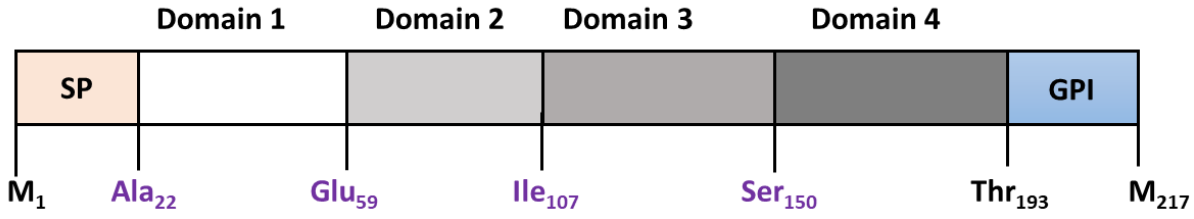

Figure 2.5 Graphical display of the four EGF like domains on the Pfs25 polypeptide chain. Domains 1-4 represent EGF-like domains and are coloured differently. The letters and subscript numbers (M_1, Ala_{22}, Glu_{59}, Ile_{107}, Ser_{150}, Thr_{193} and M_{217}) represent amino acid residues and their positions. The graphical representation was generated with reference to yeast-secreted recombinant protein (yEGF) by (Stowers *et al.*, 2000).

Domain 1 of Pfs25 has four cysteine residues (C30, C44, C46, and C58), D2 has six (C65, C74, C80, C92, C94, and C105), D3 has six (C110, C115, C120, C133, C135 and C149), and D4 has six (C157, C161, C168, C177, C179, and C192) (Stowers *et al.*, 2000; Saxena *et al.*, 2006; Lee *et al.*, 2018). The cysteine residues are linked by 11 disulphide bonds, six of which are usually correctly paired (C65-C80, C115-C133, and C161-C177), ensuring that the

four domains fold correctly and have a distinct triangle architecture (Saxena *et al.,* 2006; Scally *et al.,* 2017; Lee *et al.,* 2018). The aa residues that promote the triangle architecture are thought to be substantially conserved (Scally *et al.,* 2017). With reference to Pvs25, the triangular shape is caused by the interfacing of EGF-like domain 1 with 3 and 4 burying surfaces that are solvent accessible (Saxena *et al.,* 2006). At the boundaries of each Pfs25 domain, there is either a distinct triangle 'face up' or 'face down' architecture. The thinness of the Pfs25 prism ensures that the 'face ups' or 'face downs' architecture does not interfere with the GPI coupling to the vector's cell membrane (Saxena *et al.,* 2006). The folding is aided by the tucking in of Pfs25 D1 B loop residues with some residues in D3 and D4, leaving some residues buried from the surface (Saxena *et al.,* 2006; Scally *et al.,* 2017).

Plasmodium falciparum lacks glycosylation machinery; hence the antigen is normally expressed without glycosylation. This, however, poses a problem during full-length antigen expression due to the presence of amino acids with N-linked glycosylation patterns (Davidson & Gowda, 2001; Tsai *et al.,* 2006). Despite the challenge, glycosylated, full length Pfs25 antigen has been recombinantly expressed in a variety of expression systems, including *S. cerevisiae* (Kaslow *et al.,* 1994; Lee *et al.,* 2016), *P. pastoris* (Zou *et al.,* 2003; Tsai *et al.,* 2006; Lee *et al.,* 2016), Baculovirus (Lee *et al.,* 2016), *C. reinhardtii* (Gregory *et al.,* 2012; Patra *et al.,* 2015), and *N. benthamiana* (Farrance *et al.,* 2011; Jones *et al.,* 2013, 2015). Various expressed Pfs25 recombinant antigens have been reported to elicit antibodies that last for more than 4 months after immunisation in mice or rabbits, and when tested using SMFA, they showed TBA or TRA (Kaslow *et al.,* 1994; Zou *et al.,* 2003; Jones *et al.,* 2015; Patra *et al.,* 2015; Lee *et al.,* 2016; Parzych *et al.,* 2017).

Generated immune sera binding to epitopes in D2 following immunisation of animals or humans with Pfs25 TBV candidate have been weakly correlated to a lower number of

oocysts in SMFA as opposed to D3 (with mAb 1D2 and 4B7 binding site from aa 122–134) which has demonstrated a strong correlation (Stowers *et al.*, 2000; Scally *et al.*, 2017; McLeod *et al.*, 2019). Two mAbs (1245 and 1269) were found in transgenic mice to exhibit Oocyst reduction capabilities in SMFA, one of which (1269) bound to epitopes in D1 and D3 and overlapped epitope binding murine mAb 4B7 (Scally *et al.*, 2017). There are nine human mAbs (2530, 2534, 2544, 2578, 2586, 2587, 2595, 2598, and 2602) that have above 80% oocyst reduction capability and bind to different epitopes within Pfs25 domains (Scally *et al.*, 2017). Monoclonal antibody 2544 is the most effective of the nine and binds to an epitope in Pfs25 D3 (Scally *et al.*, 2017; McLeod *et al.*, 2019). As the most advanced TBV candidate, several clinical trials have been conducted, some of which are currently ongoing (de Graaf *et al.*, 2021). Since the *Pfs25* gene is not expressed in humans, booster vaccines will be required after the initial immunisation.

The gene (*Pfs25*) is thought to have limited sequence variation because to its inability to be expressed in humans, which means it is not subject to immune surveillance or selection pressure (Kaslow *et al.*, 1989; Lee *et al.*, 2016). Currently, 10 loci (117, 226, 333, 392, 412, 428, 433, 519, 561, and 651) have been found to be variable. These various polymorphic sites have been detected in parasite populations from Brazil, China, India, Cambodia, Laos, Thailand, and Vietnam, but none from Africa (Juliano *et al.*, 2016; Patel *et al.*, 2017; Sookpongthai *et al.*, 2021).

CHAPTER THREE: MALARIA PREVALENCE AND TRANSMISSION FOCI IN MARANI, A RURAL AREA IN A EPIDEMIC-PRONE ZONE OF KENYA

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https://pubmed.ncbi.nlm.nih.gov/35880192/

3.1 Abstract

The vulnerable population within the malaria epidemic zone remains at risk of increased burden and fatality. This is because of unpreparedness and overstretching of healthcare capacity in the event of a full-fledged epidemic. The purpose of this study was to determine the prevalence of microscopic and submicroscopic infections, as well as map specific *Plasmodium* transmission foci, within the malaria epidemic-prone zone of Kisii highland. Patients seeking malaria treatment at Eramba health facility in Marani subcounty within the epidemic-prone zone of Kisii highland were enrolled in the study. Malaria outpatient data for the entire month of May were also included in the analysis. Patients' finger prick blood smears were examined for microscopic infections, while a real-time polymerase chain reaction targeting the *Plasmodium* species 18S rRNA gene was used to detect the presence of submicroscopic infections on DNA extracted from dry blood spots. Based on outpatient data, the malaria positivity rate was 20.7% (231/1115, 95% CI, 0.18–0.23). The positivity rate varied significantly by age group (χ^2 = 75.05, df 2, $p < 0.0001$). Children under the age of five had the highest positivity rate (27.8%, 78/281), followed by children aged 5–15 years (19.4%, 69/356), and individuals aged 15 years and above (17.6%, 84/478). Out of the 102 patients recruited, the positivity rate by microscopy was 57.8% (59/102) and 72.5% (74/102) by RT-PCR. Most of the microscopic infections (40.7%, 24/59) were from Morara and Nyabikondo villages in Rioma and Kiomooncha sublocations, respectively. Males had significantly more microscopic infections than females (70.7% vs 49.2%, χ^2=4.67, df 1, p=0.0307). The submicroscopic prevalence was 14.7%

(15/102) and was observed only in patients from high-infection villages in Rioma (15.8%, 9/57) and Kiomooncha (16.2%, 6/37) sublocations. Across gender and age groups, females (19.7%, 12/61) and patients aged 15 years and above (21.1%, 8/38) had high levels of submicroscopic infections. There were two mixed infections of *P. falciparum*/*P. malariae* and *P. falciparum*/*P. ovale*, both from patients residing in Kiomooncha sublocation. *Plasmodium falciparum* infections remained relatively high in the Marani subcounty. Infections were concentrated in two villages, which could serve as a target for future public health intervention, particularly during a malaria epidemic.

Keywords: *Plasmodium infection*, epidemic-prone zone, Kisii highland, microscopic, submicroscopic.

3.2 Introduction

Malaria transmission reduction interventions have primarily targeted endemic areas in Kenya, where disease burden and mortality are high. However, epidemic-prone areas, particularly the highlands bordering malaria-endemic areas, remain vulnerable (Ndenga *et al.,* 2006; Wanjala *et al.,* 2011; Wanjala & Kweka, 2016). Because of changes in rainfall and rising temperatures, which encourage the breeding of malaria vectors, some epidemic-prone areas in Kenya have recorded prevalence ranging from 10% to 28% (Kipruto *et al.,* 2017; Aidoo *et al.,* 2018; Zhou *et al.,* 2018). As a result, nearly 20% of Kenyans living in these areas are at risk of Plasmodial infections (President's Malaria Initiative, 2011), and identifying some of the key hotspots in these areas remains critical.

Increased deaths have been reported in Rift Valley epidemics-prone counties such as Baringo, Marakwet, and West Pokot as a result of a strained healthcare system during the recent malaria outbreak (MoH, 2019a). For instance, in Baringo, 12 villages (including Riong'o, Akwichatis, and Naudo) were severely impacted in 2020, with services in Akwichatis and Riong'o dispensaries being strained. Other recently affected regions include the Bureti subcounty in Kericho (MoH, 2022b). The majority of those affected were children under the age of five and pregnant women. With projected changes in climatic conditions, increased human population, and unpredictability of host activity patterns (Alonso *et al.,* 2011; Ruiz *et al.,* 2014; Krsulovic *et al.,* 2021), malaria incidence will continue to rise, particularly in Kenya's highlands bordering endemic zones. To ensure effective planning and monitoring, the Ministry of Health (MoH) will need to map villages with high *Plasmodium falciparum* infection within epidemic zones. The purpose of this study was to determine the prevalence of microscopic and submicroscopic *P. falciparum* infections in clinical patients from the epidemic-prone Kisii highland in western Kenya, with the goal of mapping existing malaria transmission pockets.

3.3 Methods

3.3.1 Study area and design

This was a health-based survey conducted at Kisii County's Eramba health facility. The research was conducted in May 2019, when the health facility received 1115 patients seeking malaria treatment. The health facility is located on a highland adjacent to the malaria-endemic Lake Victoria basin in the Marani subcounty, which is administratively divided into locations and sublocations (**Fig. 3.1**). This study location was chosen based on previous research indicating a high risk of malaria epidemics (Aidoo *et al.*, 2018; Kapesa *et al.*, 2018).

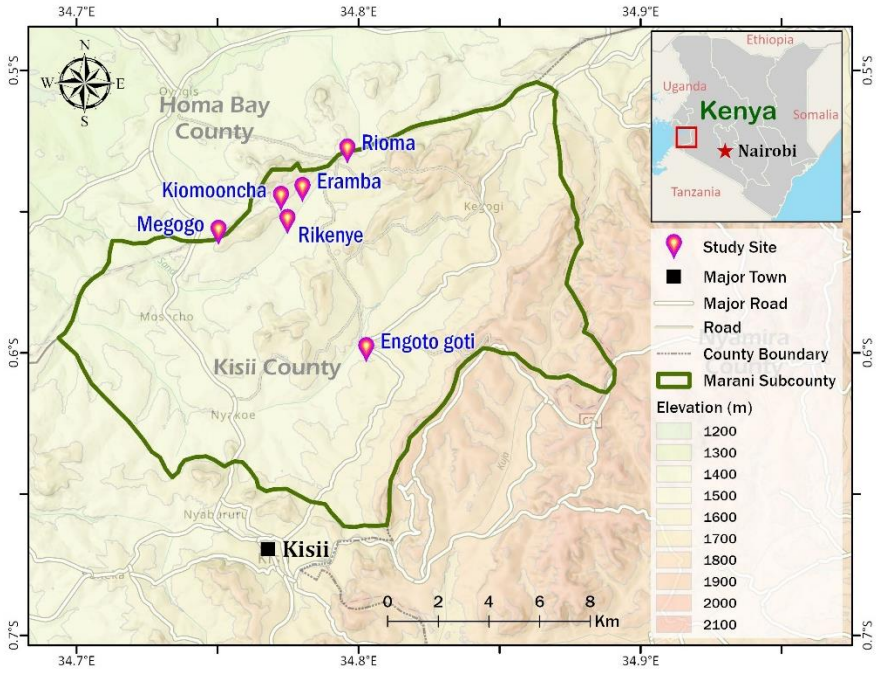

Figure 3.1 The map depicts the study area (Marani subcounty) in Kisii, as well as health facility and five sublocations from which study participants were traced. Participants in the study were residents of the Engoto Goti, Kiomooncha, Megogo, Rikenye, and Rioma sublocations, which are located at an elevation of 1501-2500 meters above sea level (Kweka *et al.*, 2017).

3.3.2 Processing of blood smears

The blood samples from finger prick were spotted on coded (with patient code, location, age, gender and collection date) Whatman™ Blood Stain Cards (GE Healthcare WB100014) and smears made on coded glass slides (Coombs & Fiscus, 2009). The blood collection was done as follows, after careful examination of the patient's middle finger and hyperextension of patients arm by the phlebotomist, the fingertip was cleaned using alcohol wipe and dried with a piece of sterile gauze. A sterile retractable lancet was firmly pressed to make a skin puncture just off the centre of the finger pad. The first drop of blood was wiped off with sterile gauze to prevent interference of the sample quality by excess tissue fluid. The subsequent full blood hanging drops were gently made to touch the filter paper at specific circled spots on the coded Whatman™ Blood Stain Card. The blood containing card was air-dried for 3-4 hours on a flat, non-absorbent surface. Once done with the collection, sterile gauze was applied to the punctured site on the patient until the bleeding stops. Each coded Whatman™ Blood Stained Card obtained was placed in individual plastic bags containing silica gel before being packed together in a labelled zip lock bag and transferred to the International Centre of Excellence for Malaria Research (ICEMR) and Tom Mboya University joint laboratories in Homa Bay for storage at -20 °C. Microscopists at the hospital prepared and read Giemsa-stained slides for the presence and density of asexual parasites. Parasite density was estimated based on visualizing microscopic fields consisting of 200 leucocytes with the assumption of a standard value of 8,000 leucocytes per μL blood. If no parasites were found after examining 200 fields at 100× magnification, the microscopy result was declared negative. All slides were subjected to a second microscopy reading for quality control at International Centre of Excellence for Malaria Research (ICEMR) and Tom Mboya University joint laboratories in Homa Bay.

3.3.3 DNA extraction

Genomic DNA was extracted from dried blood spots on filter paper following a modification of the Chelex resin (Chelex-100) saponin method (Plowe *et al.,* 1995). Briefly, A 3mm piece of blotted filter paper containing a blood sample was punched and placed into a sterile coded 1.5ml Eppendorf tube using a sterile craft store puncher. A mixture of 950µl of 1×PBS and 50µl of 10% saponin was added before incubation at 4°C overnight. The mixture was centrifuged at 12000 rpm for 10 min at room temperature before discarding the liquid content. Residual saponin was washed by the addition of 1ml PBS and spun at 12000 rpm for 5 min. The PBS was discarded and the tube content spun for 15sec before removal of the liquid component using the P200 pipette. The Eppendorf tubes containing filter paper were air-dried at room temperature for 15min. After air drying, 250µl (20%) of Chelex suspension was added and the mixture incubated in a water bath maintained at 85°C for 10min. During incubation, the mixture containing DNA was vortexed every 5min to suspend the extracted DNA. The mixture was centrifuged at 12000 rpm for 1 min and the DNA was transferred into a coded sterile 0.5ml Eppendorf tube and stored at -20°C.

3.3.4 *Plasmodium falciparum* speciation

Real-time (RT-PCR) with species-specific 18s rRNA and probes were used to identify *P. falciparum* infections as previously described (Veron *et al.,* 2009). Briefly, RT-PCR was set with a final volume of 12µl containing 2µl of sample DNA, 6µl of PerfeCTa® qPCR ToughMix™, Low ROX™ Master mix (2X), 0.5µl of the species-specific probe, 0.4µl of the species-specific forward primers (10µM), 0.4µl of the species-specific reverse primers (10µM) and 0.1µl of double-distilled water. The thermal profile used was set at 50 °C for 2 min, followed by 45 cycles of (95 °C for 2 min, 95 °C for 3 sec and 58 °C for 30 sec). Three positive samples from laboratory strain and three negative samples from blank filter paper were used as

a positive and negative control for RT-PCR respectively. The RT-PCR amplification was performed on QuantStudio 3 Real-Time PCR System (ThermoFisher, Carlsbad, CA, USA). The QuantStudio 3 Real-Time PCR System results were exported to an excel sheet, and samples with smooth curves that exceeded the predefined threshold value above background noise were declared positive.

3.3.5 Statistical analysis

Patient data were entered into Microsoft Excel v.2016, and descriptive statistics were used to summarize the study population. The Pearson chi-square test was used to compare microscopic and submicroscopic *P. falciparum* infections across gender, age groups, and sublocations. The analyses were carried out using SPSS version 25 for Windows and GraphPad Prism v.8.0.1 software. Data were considered statistically significant at $p < 0.05$.

3.3.6 Ethical approval and consenting

Maseno University's Ethical Review Committee granted ethical approval for this study (**Appendix 1**). Participants in the study provided written consent for the retrieval of patient data and the storage of their blood samples at the hospital. In the case of minor children, assent was given by their parents or guardians. To avoid ethical code violations, patients' identities were not recorded (**Appendix 2**). The Ministry of Health also granted permission for the study to be carried out (**Appendix 3**).

3.4 Results

3.4.1 Positive cases based on outpatient data

Generally, the health facility attracted 1115 patients who sought malaria treatment in the month of May 2019. Of these, blood smears of 935 were examined for the presence of asexual

parasites using microscopy whereas the remaining 180 were diagnosed using malaria rapid diagnostic kit (mRDT). Females who sought malaria treatment were 876 (78.6%) whereas males were 239 (21.4%). Across age groups, a majority 42.9% (478/1115) were adults followed by children aged 5-15 years 31.9% (356/1115) and <5 years 25.2% (281/1115). A total of 231/1115 (20.7%, 95% CI, 0.18-0.23) positive cases were recorded. Of the positive cases, 18.6% (43/231) were diagnosed by mRDT whereas the rest (188/231, 81.4%) were diagnosed by microscopy. Despite males having slightly higher number of positive cases than females, the difference was not significant (χ^2= 2.334, df 1, p=0.127). Most children under 5 years were positively diagnosed with malaria as compared to adults and children aged 5-15 years (**Table 3.1**). The variation in positive malaria cases across age group was significant (χ^2 =75.05, df 2, p<0.0001 There was a significant difference in observed cases in children aged <5 years compared to those aged 5-15 years (χ^2 =6.21, df 1, p=0.013) and also when compared to the age group 5-15 (χ^2 =10.93, df 1, p=0.001). However, there was no significant difference in observed cases between age groups 5-15 and ≥15 (χ^2 =0.45, df 1, p=0.504).

Table 3.1 Malaria positive cases across gender and age groups.

Parameter	Parameter level	N (%)	Positive cases n (%)	P-Value
Gender	Female	876 (78.6)	173 (19.7) [a]	0.127
	Male	239 (21.4)	58 (24.3) [a]	
Age group	<5	281 (25.2)	78 (27.8) [a]	<0.0001
	5-15	356 (31.9)	69 (19.4) [b]	
	≥15	478 (42.9)	84 (17.6) [bc]	

N represents the total number of individuals while n represents the cases. Different letter superscripts ([a], [b] and [c]) between parameter level estimates denotes that values differ significantly from one another within each parameter. Same letter superscripts between parameter level estimates depict non-significant differences between the estimates.

3.4.2 Characteristics of patients enrolled in the study

Of the 1115 outpatients, 102 study participants were recruited. These included participants who were diagnosed by microscopy and their blood spotted on filter paper for RT-PCR assay. Of

the recruited patients, most 57 (55.9%) were from Rioma sublocation whereas the rest were from Kiomooncha 37 (36.2%), Rikenye 6 (5.9%), Engoto Goti 1 (1%) and Megogo 1 (1%) sublocations. Sixty-one (59.8%) were female whereas 41 (40.2%) were males. Children between age group 5-15 years were the majority 41 (40.2%) followed by adults 38 (37.3%) and those <5 years 23 (22.5%). Female dominance was evident across two age groups as follows, 16 (69.6%) and 27 (71.1%) among <5 years and adults (≥15 years) respectively. More than half of recruited participants within the school-going age group (5-15 years) were males 23 (56.1%).

3.4.3 Prevalence of microscopic and submicroscopic infections among the recruited study participants

A total of 59/102 (57.8%) of screened blood smears tested positive for microscopic infections. A majority of the infected individuals were from Rioma 37 (64.9%) and Kiomooncha 16 (43.2%). In the two sublocations, two villages, Morara in Rioma and Nyabikondo in Kiomooncha each had 12 patients with microscopic infections and contributed to 40.8% (24/59) of the slide positive rate. Males had significantly more microscopic infections than females (70.7% vs 49.2%, χ^2=4.67, df 1, p=0.031). The difference was also observed across age groups (χ^2=9.47, df2, p=0.008), with children aged 5-15 years having significantly more microscopic infections than individuals in age group ≥15 (χ^2=9.18, df1, p=0.002).

There were 17.6% (15/102) of submicroscopic infections, with female patients infected more than males (**Table 3.2**). The difference in infection between the gender was however not significant (χ^2=2.98, df 1, p=0.084). Adults (≥15 years) had the most submicroscopic infections (21.1%) of any age group. The difference across the age groups was however not significant (χ^2=2.072, df 2, p= 0.355). These infections were only confirmed in patients from Rioma and Kiomooncha, with the latter having the highest rate of 16.2% (6/37) (**Table 3.2**). Two

participants from Kengambi village in the Kiomooncha sublocation had mixed infections. One participant had *P. falciparum* and *P. malariae* infections, while the other had *P. falciparum* and *P. ovale* infections. There was no statistically significant difference in submicroscopic *P. falciparum* infections between the five sublocations (χ^2 =1.500, df 4, p=0.827).

Table 3.2 The proportion of microscopic, submicroscopic and total or all *P. falciparum* infections among gender, age group and sublocations.

Parameter	Parameter level	N (%)	Microscopic infections n (%)	Submicroscopic infections n (%)	Total infections
Gender	Female	61 (59.8)	30 (49.2) [a]	12 (19.7) [a]	42 (68.9) [a]
	Male	41 (40.2)	29 (70.7) [b]	3 (7.3) [a]	32 (78) [a]
Age group	<5	23 (22.5)	12 (20.3) [ab]	3 (13) [a]	15 (65.2) [ab]
	5-15	41 (40.2)	31 (52.5) [b]	4 (9.8) [a]	35 (85.4) [b]
	≥15	38 (37.3)	16 (27.1) [a]	8 (21.1) [a]	24 (63.2) [a]
Sublocation	Engoto Goti	1 (1)	1 (100) [a]	0 [a]	1 (100) [a]
	Kiomooncha	37 (36.2)	16 (43.2) [a]	6 (16.2) [a]	22 (59.5) [a]
	Megogo	1 (1)	1 (100) [a]	0 [a]	1 (100) [a]
	Rikenye	6 (5.9)	4 (66.7) [a]	0 [a]	4 (66.7) [a]
	Rioma	57 (55.9)	37 (64.9) [a]	9 (15.8) [a]	46 (80.7) [a]

N represents the total number of individuals while n represents the cases. Different letter superscripts ([a], [b] and [c]) between parameter level estimates denotes that values differ significantly from one another within each parameter. Same letter superscripts between parameter level estimates depict non-significant differences between the estimates.

Rioma had the highest proportion of participants with total infections (microscopic and submicroscopic) (80.7%), followed by Kiomooncha (59.5%), and Rikenye (66.7%). Females had 68.9% more total infections than males (**Table 3.2**). Both infection levels were higher in children aged 5-15 years (85.4%), followed by children <5 years (65.2%) and adults (63.2%). However, the observed differences in total infections between gender, age groups, and sublocations were not statistically significant.

3.5 Discussion

In May, the malaria positivity rate among clinically ill patients was high in the epidemic-prone Marani subcounty. Males and children under the age of five were the most affected groups in this epidemic-prone zone, according to the total number of reported malaria cases in the facility. The infections were zoned to two major sublocations, Rioma and Kiomooncha, with transmission foci traced to Morara and Nyabikondo villages, respectively. The levels of submicroscopic infections, on the other hand, were low and were only confirmed on patients from the two sublocations. Females bore a disproportionate share of the submicroscopic infections compared to males, with high prevalence of total infections and parasite densities still being recorded among children aged 5-15 years.

As observed in endemic zone, more females in Kisii highland sought malaria treatment at health facility as compared to males (Ochwedo *et al.,* 2021). Furthermore, there was no difference in health seeking trends by age group in the endemic region, with more adults seeking treatment, followed by children aged 5-15 and under 5 years, respectively. The observed number of positive cases was slightly lower (20.7%) among previously reported cases by Kapesa *et al.,* (2018) (26.7%) and Aidoo *et al.,* (2018) (27.8%). With the insignificant slight variation in positive cases, the study hypothesizes that the average malaria positivity rate for the Marani subcounty could be around 21-28%. This, however, needs to be investigated further by conducting monthly clinical malaria surveillance in this region. Also, there are reports of a slight increase in malaria cases following the COVID-19 pandemic.

The majority of the microscopic infections observed were focal and could be traced back to Rioma or Kiomooncha sublocations. The two sublocations also had the highest number of patients seeking malaria treatment and are adjacent to each other while bordering an endemic region, Homa Bay County. Their proximity to endemic areas may predispose residents to

malaria infections (MoH, 1994), as well as put the sublocations at high risk of imported *P. falciparum*. Morara and Nyabikondo villages in Rioma and Kiomooncha, which have a high number of microscopic infected people, serve as a guide for possible focal parasite or vectorial intervention targets in the event of a future outbreak of *P. falciparum* infections in the Marani subcounty.

Submicroscopic infections were relatively low, with females and adult patients having the highest levels. The slightly higher prevalence of this type of infection in females and adults supports previous findings and has been linked partly to immunity (Whittaker *et al.,* 2021; Ochwedo *et al.,* 2021). However, the difference in submicroscopic infection levels across gender and age groups was not statistically significant, as observed in other health-based surveys in endemic zones (Ochwedo *et al.,* 2021). Individuals with submicroscopic infections were only found in Kiomooncha and Rioma sublocations among the five locations. The general low levels of submicroscopic and high levels of microscopic infections among patients presenting with clinical symptoms in epidemic-prone zone could be attributed to a low rate of clinical immunity build-up associated with individuals residing in malaria epidemic prone zones (Cook *et al.,* 2019).

3.6 Conclusion

This study established a high malaria positivity rates and low level of submicroscopic infections in five sublocation in the epidemic-prone region of Kisii County. The transmission was focalized to two main sublocation of which two key villages were observed as sentinel sites with the potential targeted parasite or vectorial intervention in case of malaria epidemic.

CHAPTER FOUR: PREVALENCE OF *PLASMODIUM FALCIPARUM* INFECTIONS IN KOCHIA SUB-COUNTY, A RURAL AREA OF HOMA BAY COUNTY IN WESTERN KENYA

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https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8685826

4.1 Abstract

The gold standard for diagnosing *Plasmodium falciparum* infection is microscopic examination of Giemsa-stained peripheral blood smears. The effectiveness of this procedure for malaria infection surveillance and control may be limited by a relatively high parasitaemia detection threshold. Persons with microscopically undetectable infections may go untreated, contributing to ongoing transmission to mosquito vectors. The purpose of this study was to determine the magnitude and determinants of undiagnosed submicroscopic *P. falciparum* infections in a rural area of western Kenya. To achieve this, a health facility-based survey of 367 patients seeking treatment for symptoms consistent with uncomplicated malaria in Homa Bay County was conducted. The frequency of submicroscopic *P. falciparum* infection was measured by comparing the prevalence of infection based on light microscopic inspection of thick blood smears versus real-time polymerase chain reaction (RT-PCR) targeting the *P. falciparum* 18S rRNA gene. Long-lasting insecticide-treated net use, participation in nocturnal outdoor activities, and sex were considered as potential determinants of submicroscopic infection. Microscopic inspection of blood smears was positive for asexual *P. falciparum* parasites in 14.7% (54/367) of cases. All of these samples were confirmed by RT-PCR. 36% (112/313) of blood smear negative cases were positive by RT-PCR, i.e., submicroscopic infection, resulting in an overall prevalence by RT-PCR alone of 45.2% compared to 14.7% for blood smear alone. Females had a higher prevalence of submicroscopic infections (35.6%, 72 of 202 individuals,

95% CI 28.9-42.3) compared to males (24.2%, 40 of 165 individuals, 95% CI 17.6-30.8). The risk of submicroscopic infection in LLIN users was about half that of non-LLIN users (OR = 0.59). There was no difference in the prevalence of submicroscopic infections of study participants who were active in nocturnal outdoor activities versus those who were not active (OR = 0.91). Patients who participated in nocturnal outdoor activities and used LLIN while indoors had a slightly higher risk of submicroscopic infection than those who did not use LLIN (OR=1.48). Microscopic inspection of blood smears from persons with malaria symptoms for asexual stage *P. falciparum* should be supplemented by more sensitive diagnostic tests in order to reduce ongoing transmission of *P. falciparum* parasites to local mosquito vectors.

Keywords: Submicroscopic *Plasmodium* infection, polymerase chain reaction, blood smear, Western Kenya, diagnostic tests.

4.2 Introduction

Current vector control and parasite surveillance strategies have shown remarkable progress in reducing the global malaria burden (Dhiman, 2019). In Kenya, malaria-endemic areas such as Homa Bay County have ongoing vector control intervention that include indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs) (The U.S. President's Malaria Initiative, 2018b). Malaria prevalence has decreased in these in these areas, largely as a result of these interventions (Oduma *et al.,* 2021). There is concern, however, that these gains may not be durable because the exclusive use of microscopy for passive case detection *of Plasmodium falciparum* infection may not be sufficiently sensitive to detect submicroscopic infections (Lo *et al.,* 2015). A high prevalence of undetected submicroscopic malaria cases may contribute to a parasite reservoir that is sufficient to sustain ongoing *P. falciparum* transmission in endemic communities (Lin *et al.,* 2016; Gonçalves *et al.,* 2017).

Submicroscopic infections have been observed not only in high transmission settings but also in malaria endemic areas with seasonal or low transmission (Nguyen *et al.,* 2018; Slater *et al.,* 2019). These infections also show reduced parasite genetic diversity (Branch *et al.,* 2001), fewer infective *Anopheles* (Okell *et al.,* 2012; Bousema *et al.,* 2014), lower adherence to anti-malarial drug regimens (Omer *et al.,* 2011; Bruxvoort *et al.,* 2014; Rek *et al.,* 2016), and increased asexual parasite clearance rates (Branch *et al.,* 2001; Adu *et al.,* 2020). Previous studies of submicroscopic parasitaemia have primarily been concerned with its occurrence in pregnant women (Cohee *et al.,* 2014; Elbadry *et al.,* 2017; Omer *et al.,* 2019; Unger *et al.,* 2019; Hounkonnou *et al.,* 2020), and cross-sectional community surveys of asymptomatic individuals (Pava *et al.,* 2016; Jiram *et al.,* 2019; Vareta *et al.,* 2020; Oduma *et al.,* 2021; O'Flaherty *et al.,* 2021). The objective of this study was to determine the prevalence of submicroscopic infections among patients seeking malaria treatment at a rural health centre

in western Kenya and the demographic and behavioural variables associated with these infections.

4.3 Methods

4.3.1 Study area and design

The study was conducted at the Ngegu health facility in malaria endemic zone of Homa Bay County, western Kenya. This facility had a catchment population of 6,703 persons in 2020. The sampled patient population came from the Kochia location, which is divided into smaller administrative units referred to as sub-locations. Study participants were residents of Kamenya, Kanam, Kaura, Korayo, Kothidha, and Kowili sublocations located near the shore of Lake Victoria at a latitude of 34.64190E and 0.38000S and elevation of 1143-1330 meters above sea level (**Fig. 4.1**). The mean annual temperature is 22.7°C. Rainfall is seasonal with two major peaks, March to May and October to December (The Ministry Of Agriculture & Fisheries, 2016). The study area is bordered by the Kimira-Oluch irrigation scheme, which has been demonstrated to have an impact on malaria transmission (Orondo *et al.,* 2021). Homa Bay County is predominantly malaria-endemic, with approximately 20% overall *P. falciparum* infection prevalence (The U.S. President's Malaria Initiative, 2018a). The Ministry of Health has been conducting annual IRS with formulation of microencapsulated pirimiphos methyl (Actellic®300CS) insecticide in the study area from February to March since 2018.

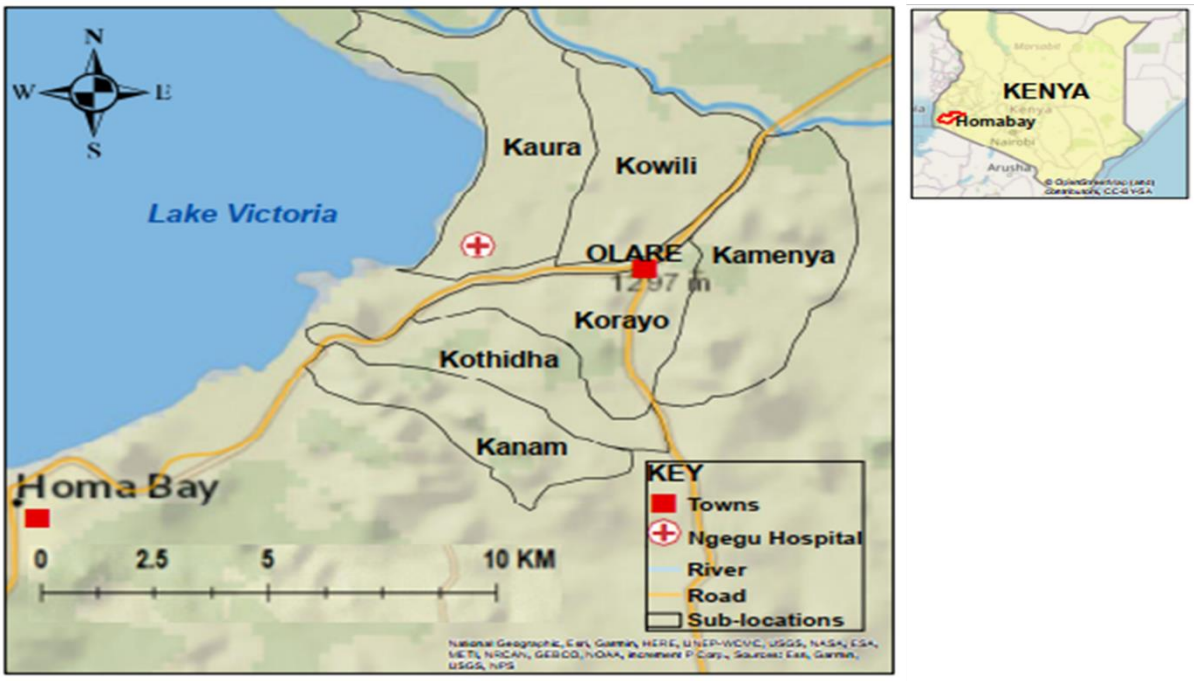

Figure 4.1 The study area map shows study sites (sub-locations in Kochia sub-county) in Homa Bay County. The circle with the red cross represents the Ngegu health facility, where patients from the six sublocations seek medical services.

A health facility-based survey in which 367 patients seeking malaria treatment from six sub-locations were enrolled was conducted. Patient information such as LLIN ownership, occupation, and participation in nocturnal outdoor activities was collected. Nocturnal outdoor activities included casting fishing nets, setting and removing fishing traps, overnight fishing, early purchase of fish by small scale traders, farming, late evening trading at open-air markets, and waiting, picking up, and dropping off clients from motorcycle taxi travel ("Bodaboda"). Patient enrolment and data collection periods occurred in July and August of 2020, which coincided with the peak of *P. falciparum* transmission. The number of samples used was determined by the number of patients who sought malaria treatment and consented or assented to the study. Blood smear microscopy slides read at the hospital laboratory were confirmed by experienced microscopists at the Sub-Saharan Africa International Centre of Excellence in

Malaria Research (ICEMR) laboratory. All samples were tested for submicroscopic infection by RT-PCR at the ICEMR laboratory at Tom Mboya University, Homa Bay.

4.3.2 Processing of blood smears

Blood samples used in this study were obtained by antecubital venepuncture and immediately pipetted on coded Whatman™ Blood Stain Cards (GE Healthcare WB100014) and glass slides. The staining of blood smears, slide reading, and estimate of asexual parasite density were all carried out as previously described (**Section 3.3.2**).

4.3.3 DNA extraction

Genomic DNA were extracted as described in **section 3.3.3**, transferred into a coded sterile 0.5ml Eppendorf tube and stored at -20°C.

4.3.4 *Plasmodium falciparum* speciation

Real-time (RT-PCR) with species-specific 18s rRNA and probes were used to identify *P. falciparum* infections as previously described (**Section 3.3.4**).

4.3.5 Patient behavior, LLIN usage and other variables associated with submicroscopic infection

To assess the relationship between submicroscopic *P. falciparum* infection and LLIN use and participation in nocturnal outdoor activities, health centre study staff interviewed and completed a questionnaire for each participant (**Appendix 4**). Information on LLIN ownership and usage, engagement in nocturnal outdoor activities, and occupation (i.e., student, non-student (children <5 years), farmer, trader, fishermen, motorcycle taxi riders, teacher, security, construction, unemployed and other) were collected. Only participants who acknowledged being outdoors from 1800hrs-2000hrs, 2000hrs-2300hrs, 2300hrs-0400hrs, and 0400hrs-

0600hrs due to occupational requirements were considered involved in nocturnal outdoor activities.

4.3.6 Statistical analysis

Patient data were entered into Microsoft Excel v.2016 for cleaning and analysis. Descriptive statistics such as sum, mean, standard deviation, standard error and 95% confidence interval were used to summarize the population under study. Before comparisons of mean value, data normality was confirmed using the Shapiro-Wilk normality test. To determine LLIN usage across sex, age and sub-location residence, multiple mean comparisons between these variables were performed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Comparisons between blood smear positive and submicroscopic (RT-PCR positive, blood smear negative) groups were performed using Pearson chi-square. Binary logistic regression models were used to determine the association between microscopic and submicroscopic infections and potential determinants such as LLIN use, engagement in nocturnal outdoor activities and sex. A multivariate analysis was used to determine the relationship between the LLIN use, nocturnal outdoor activities, microscopic and submicroscopic infections. Analyses were performed in GraphPad Prism v.8.0.1 Software and SPSS version 25 for Windows. Data were considered statistically significant at $p < 0.05$.

4.3.7 Ethical approval and consenting

Ethical approval for this study was same as in section 3.3.6.

4.4 Results

4.4.1 Participants sociodemographic and behavioural characteristics

Most of the enrolled study participants were females (55.04%) compared to males (44.96%). (**Table 4.1**). Children <5 years were the least (5.18%), followed by those aged 5-15 years (27.79%) and adults (67.03%). Most patients were from Kaura 111 (30.25%) sub-location whereas the Kothidha had the least number 23 (6.28). The number of patients from other sub-location was as follows; Kanam 78 (21.25%), Kamenya 60 (16.35), Korayo 59 (16.08) and Kowili 36 (9.81). Most were students160 (43.6%), others category 41 (11.17%) which mostly included parttime roles and small-scale traders 48 (9.54%) such as fishmongers, open air market traders as well as shopkeepers whereas the rest were non-students (<5 Children) 21 (5.72%), fishermen 19 (5.18%), motorcycle taxi riders "Bodaboda" 29 (7.9%), farmers 18 (4.9%), construction workers 6 (1.63%), teachers 2 (0.54%) and security personnel 2 (0.54%). Out of all patients, only 84 (22.88%) participated in nocturnal outdoor activities, of these, males were more likely (57/165, OR=3.42, CI: 2.01-5.76, p<0.0001) to participate in such activities than females (27/202). More females 52.48% (106/202, 95%, CI: 0.46-0.59) used LLINs compared to males 38.79% (64/165, 95%, CI: 0.31-0.46) (**Table 4.1**). The difference in LLINs use between the two groups was highly significant ($\chi^2 = 6.84$, df 1, $p = 0.009$) as females were 1.7 times (CI: 1.14-2.64, p=0.009) more likely to be users than males. Children <5 years old were the highest LLIN users compared to adults and children aged 5-15 years respectively (**Table 4.1**). The difference in LLIN use was significant (Kruskal Wallis H test, $H_{(2)} = 19.20$, df 2, $p < 0.0001$) across the three groups. However, based on Dunn's test pairwise comparison, significant difference (p<0.0001) in bed net use was between age group <5 and 5-15 as well as <5 and adults but not between 5-15 and adults (p>0.05). There was no significant difference (Kruskal Wallis H test, $H_{(2)} = 4.813$, df 5, p=0.4391) in LLIN use across the six sublocation

with Kowili sub-location having most users compared to Kothidha, Kamenya, Kanam, Korayo and Kaura respectively (**Table 4.1**). Across the occupation, motorcycle taxi riders recorded the least bed net use 6 (20.69%) with highest use being observed among non-student (<5 children) 19 (90.48%) (**Table 4.1**). Variation in number of LLIN users across the eleven occupation categories were significant (Kruskal Wallis H test, $H_{(2)}$ =, df 10, p<0.0001). There was no significant different (χ^2=2.97, df 1, p=0.085) in LLIN use between individuals who remained indoors 138 (48.76%) and those who participated in nocturnal outdoor activities 32 (38.1%).

Table 4.1 Sociodemographic attributes of study participants including usage of long-lasting insecticide-treated nets use and participation in outdoor nocturnal activities.

Parameter	Parameter Level	N (%)	LLIN use n (%)	P-Value
Gender	Female	202 (55.04)	106 (52.48) [a]	0.009
	Male	165 (44.96)	64 (38.79) [b]	
Age group	<5	19 (5.18)	18 (94.74) [a]	<0.0001
	5-15	102 (27.79)	42 (41.18) [b]	
	≥15	246 (67.03)	110 (44.72) [cb]	
Sub-location	Kamenya	60 (16.35)	29 (48.33) [a]	0.4391
	Kanam	78 (21.25)	35 (44.87) [a]	
	Kaura	111 (30.25)	48 (43.24) [a]	
	Korayo	59 (16.08)	24 (40.68) [a]	
	Kothidha	23 (6.28)	12 (52.17) [a]	
	Kowili	36 (9.81)	22 (61.11) [a]	
Occupation	Student	160 (43.60)	70 (43.75) [a]	<0.0001
	Non-Student (<5 Children)	21 (5.72)	19 (90.48) [b]	
	Farmer	18 (4.9)	10 (55.56) [bc]	
	Traders	48 (9.54)	23 (65.71) [acd]	
	Fishermen	19 (5.18)	5 (26.32) [acde]	
	Motorcycle taxi riders	29 (7.9)	6 (20.69) [acdef]	
	Teacher	2 (0.54)	2 (100) [abcdefg]	
	Security	2 (0.54)	2 (100) [abcdefgh]	
	Construction	6 (1.63)	4 (66.67) [abcdefghi]	
	Unemployed	34 (9.26)	16 (47.06) [acdefghij]	
	Others	41 (11.17)	13 (31.71) [acdefghijk]	
Nocturnal outdoor activities	Yes	84 (22.88)	32 (38.10) [a]	0.085
	No	283 (77.11)	138 (48.76) [a]	

N represents total number of individuals while n represents the proportions. Different letter superscripts ([a, b, c, d, e, f, g, h, i, j] and [k]) between parameter level estimates denotes that values differ significantly from one another within each parameter. Same letter superscripts between parameter level estimates depict non-significant differences between the estimates.

4.4.2 Prevalence of microscopic and submicroscopic or subclinical *Plasmodium falciparum* infections

A total of 54 (14.71%) microscopically screened blood smears were positive for asexual stage parasites. Male patients (28/165, 17%) had high microscopic infections compared to females (26/202, 12.9%), the difference was however not significant (χ^2=1.22, df 1, p=0.27). Across

the age groups, children <5 years had the lowest infection rates (10.53%) by microscopy whereas children between ages 5-15 recorded the highest infection rates (27.45%) followed by adults (9.76%). A similar trend was observed on assessment of parasite density per age group as children <5 years, those in age group 5-15 and adults had 218.95, 956.86 and 329.92 asexual parasites per microliter of blood respectively. Further test by RT-PCR confirmed the 54 slide positive samples to be positive of *P. falciparum* DNA. In addition to confirmed microscopic infections, 36% (112/313) of microscopy negative slides were revealed positive for parasite DNA by RT-PCR and termed as subclinical or submicroscopic infections. These infections (missed by microscopy) comprised 67.47% (112/166) of total RT-PCR confirmed Plasmodial infections and were not treated at the health facility. Compared to microscopic infections (14.71%), the levels of submicroscopic infections (30.52%) were significantly higher (χ^2=27.81, df 1, P<0.0001). Across gender, a significant difference (χ^2=4.39, df 1, p=0.036) between the two types of infection was only evident among female as opposed to male patients. Contrary to the trend in levels of microscopic infections, female patients were revealed to be harbouring more submicroscopic infections i.e., 35.64% (72/202, 95%, CI: 28.98-42.30) than males 24.24% (40/165, 95%, CI: 17.63-30.85). Most (42.11%) children <5 years had submicroscopic infections followed by adults (30.08%) and those aged 5-15 (29.41%). Only one out of eight males <5 years had submicroscopic infections as opposed to 7 that were observed among 11 sampled females in this age group.

At sublocation, Kaura had the highest proportion of both microscopic and submicroscopic infections (51.35%) whereas Kowili (33.33%) had the least. The difference in overall infections levels across the six sublocations was not significant (Kruskal Wallis H test, $H_{(2)}$ = 4.17, df 5, p=0.526). Overall infections in other sublocation were as follows; Korayo (45.76%), Kanam (44.87%), Kamenya (43.33%) and Kothidha (39.13%). There was a significant difference between microscopic and submicroscopic infections across the

sublocations (Kruskal Wallis H test, $H_{(11)} = 41.21$, $p > 0.001$). However, Dunn's test pairwise comparison revealed the significant difference between the two types infections in Korayo ($p = 0.0019$) and Kamenya ($p = 0.0320$). Patients from Korayo had the highest proportion of individual with submicroscopic infections i.e., 38.98% (23/59, 95%, CI: 26.16-51.8) whereas Kothidha had the least 26.09% (6/23, 95%, CI: 6.67-45.5). Submicroscopic infections in the rest of sublocations was as follows Kamenya 35% (21/60, 95%, CI: 22.57-47.43), Kaura 27.93% (31/111, 95%, CI: 19.45-36.41), Kowili 27.78% (10/36, 95%, CI: 12.41-43.15), Kanam 26.92% (21/78, 95%, CI: 16.86-36.99).

4.4.3 Relationship between microscopic infections, patient behaviour and LLIN use

The role of patient socioeconomic and behavioural characteristics as a predisposing factor to microscopic *P. falciparum* infections was established. Despite the slight variation in microscopic infection across gender, there was high odds (OR=1.38) of diagnosing males with these infections compared to females (**Table 4.2**). Most non LLIN users (35%) had microscopic infections with high odds (OR=1.72). This also applied to individual who did not engage in nocturnal outdoor activities whom most (44%) were diagnosed with microscopic *P. falciparum* infections (**Table 4.2**). Among the 84 individuals who participated in nocturnal outdoor activities, most infections were observed on those who did not use LLINs while indoors or not engaged in outdoor activities. The likelihood of finding microscopic infections on these individuals was 6.49 times higher than those who engaged in nocturnal outdoor activities and use LLINs while indoor.

Table 4.2 Socioeconomic and behavioural determinants of microscopic infections.

Parameter	Parameter level	N	Microscopic infections n (%)	OR (95%, CI)	P-Value
Gender	**Female**	202	26 (12.87)	1.38 (0.87-1.50)	0.270
	Male	165[ä]	28 (16.97)		
LLIN usage	**Yes**	170	19 (11.18)	1.72 (0.94-3.13)	0.078
	No	197[ä]	35 (17.77)		
[*]**Nocturnal outdoor activities**	**Yes**	84	10 (11.90)	1.36 (0.65-2.84)	0.409
	No	283[ä]	44 (15.55)		
[⁑]**Nocturnal outdoor activities**	**LLIN usage**	32	1 (3.13)	6.49 (0.78-53.89)	0.083
	No LLIN usage	52[ä]	9 (17.31)		

N represents total number of individuals while n represents the cases. The asterisk (*) represent general effect of nocturnal outdoor activities on patent and sub-patent infections while the vertical double asterisk (⁑) represents interaction effects between nocturnal outdoor activities and LLIN usage. Small letter with umlaut "ä" refers to reference category per parameter level.

4.4.4 Relationship between submicroscopic infections, patient behaviour and LLIN use

The odds of females seeking malaria treatment having submicroscopic infections was 1.73 higher than males (**Table 4.3**). Use of LLIN had a significant effect on microscopic and submicroscopic infections (F (2, 362) = 3.029, p=0.05; Wilk's = 0.984, partial $\eta 2 = 0.016$) (**Table 4.4**). However, when considered separately, the use of LLIN had a significant effect on microscopic infections (F (1, 363) = 4.499, p=0.035; partial $\eta 2 = 0.012$) but not on submicroscopic infections (**Table 4.5**). Interestingly, patients using LLINs had a significantly higher prevalence of submicroscopic infections (36.5%) (χ^2=5.29, df 1, p=0.021) than non-users (25.4%). This implies that the chance of LLINs users harbouring submicroscopic infections was about half that of non-LLINs users (OR = 0.59). Though not significant (χ^2 =0.136, df 1, p = 0.713), more patients involved in nocturnal outdoor activities had submicroscopic infections (32.1%) compared to those restricted indoors during night hours (25.3%). Participants who engaged in nocturnal outdoor activities were 0.91 times more likely to have submicroscopic infections than those who stayed indoors (**Table 4.3**). This, however,

varied when comparison was made between patients engaged in nocturnal outdoor activities but using LLINs while indoors versus non-LLIN users involved in nocturnal outdoor activities. Those LLINs users who participated in nocturnal outdoor activities were 1.48 times more likely to have submicroscopic infections and have high infections (37.50%) than non-LLINs users involved in nocturnal activities (28.85%) (χ^2=0.68, df 1, p = 0.41).

Table 4.3 Socioeconomic and behavioural determinants of submicroscopic infections.

Parameter	Parameter level	N	Submicroscopic infections n (%)	OR (95%, CI)	P-Value
Gender	**Female**	202ä	72 (35.64)	1.73 (1.10-2.74)	0.019
	Male	165	40 (24.24)		
LLIN usage	**Yes**	170	62 (36.47)	0.59 (0.38-0.93)	0.022
	No	197ä	50 (25.38)		
*__Nocturnal outdoor activities__	**Yes**	84	27 (32.14)	0.91 (0.54-1.53)	0.713
	No	283ä	85 (30.04)		
⁑__Nocturnal outdoor activities__	**LLIN usage**	32ä	12 (37.50)	1.48 (0.58-3.77)	0.411
	No LLIN usage	52	15 (28.85)		

N represents total number of individuals while n represents the cases. The asterisk (*) represent general effect of nocturnal outdoor activities on patent and sub-patent infections while the vertical double asterisk (⁑) represents interaction effects between nocturnal outdoor activities and LLIN usage. Small letter with umlaut "ä" refers to reference category per parameter level.

Table 4.4 Multivariate tests.

Effect		Value	F	Hypothesis df	Error df	P-Value	Observed Power[c]
Intercept	Pillai's Trace	0.353	98.600[b]	2	362	0.000	1.000
	Wilks' Lambda	0.647	98.600[b]	2	362	0.000	1.000
	Hotelling's Trace	0.545	98.600[b]	2	362	0.000	1.000
	Roy's Largest Root	0.545	98.600[b]	2	362	0.000	1.000
Net usage	Pillai's Trace	0.016	3.029[b]	2	362	0.050	0.585
	Wilks' Lambda	0.984	3.029[b]	2	362	0.050	0.585
	Hotelling's Trace	0.017	3.029[b]	2	362	0.050	0.585
	Roy's Largest Root	0.017	3.029[b]	2	362	0.050	0.585
Outdoor activities	Pillai's Trace	0.004	0.707[b]	2	362	0.494	0.169
	Wilks' Lambda	0.996	0.707[b]	2	362	0.494	0.169
	Hotelling's Trace	0.004	0.707[b]	2	362	0.494	0.169
	Roy's Largest Root	0.004	0.707[b]	2	362	0.494	0.169
Net usage * Outdoor activities	Pillai's Trace	0.004	0.708[b]	2	362	0.493	0.169
	Wilks' Lambda	0.996	0.708[b]	2	362	0.493	0.169
	Hotelling's Trace	0.004	0.708[b]	2	362	0.493	0.169
	Roy's Largest Root	0.004	0.708[b]	2	362	0.493	0.169

a. Design: Intercept + Net usage + Outdoor activities + Net usage * Outdoor activities

b. F-value

c. Computed using alpha ≤0.05

Table 4.5 Test of between-subject effects.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[c]
Corrected Model	Microscopy Results	0.653[a]	3	0.218	1.741	0.158	0.014	5.224	0.454
	Sub-Microscopy	1.211[b]	3	0.404	1.913	0.127	0.016	5.740	0.494
Intercept	Microscopy Results	4.089	1	4.089	32.696	0.000	0.083	32.696	1.000
	Sub-Microscopy	24.847	1	24.847	117.733	0.000	0.245	117.733	1.000
Net usage	Microscopy Results	0.563	1	0.563	4.499	0.035	0.012	4.499	0.562
	Sub-Microscopy	0.666	1	0.666	3.156	0.076	0.009	3.156	0.426
Outdoor activities	Microscopy Results	0.172	1	0.172	1.375	0.242	0.004	1.375	0.215
	Sub-Microscopy	0.055	1	0.055	0.262	0.609	0.001	0.262	0.080
Net usage * Outdoor activities	Microscopy Results	0.134	1	0.134	1.069	0.302	0.003	1.069	0.178
	Sub-Microscopy	0.018	1	0.018	0.087	0.768	0.000	0.087	0.060
Error	Microscopy Results	45.401	363	0.125					
	Sub-Microscopy	76.609	363	0.211					
Total	Microscopy Results	54.000	367						
	Sub-Microscopy	112.000	367						
Corrected Total	Microscopy Results	46.054	366						
	Sub-Microscopy	77.820	366						

a. R Squared = .014 (Adjusted R Squared = .006)
b. R Squared = .016 (Adjusted R Squared = .007)
c. Computed using alpha ≤ 0.05

4.5 Discussion

In this study age, gender and use of LLINs were factors in the occurrence of submicroscopic *P. falciparum* infections in patients with malaria symptoms seeking treatment in Homa Bay County's six sub-locations. The use of LLINs and being female was linked to a high prevalence of submicroscopic infections. Interestingly, the effect of LLIN usage on submicroscopic infection prevalence was also observed among users who participated in nocturnal outdoor activities. Furthermore, this study was unable to establish a conclusively link between the observed microscopic *P. falciparum* infections and outdoor transmission. This is due to the fact

that only one LLIN user participating in nocturnal outdoor activities had microscopic infections, compared to nine infected non-LLIN users engaged in nocturnal outdoor activities.

The study findings revealed that male patients were more likely than females to test positive for microscopic infections. This could be attributed to existing social behavioural differences, such as more males engaging in nocturnal outdoor activities, which kept them out of the intervention coverage and low LLIN use when compared to female patients. Low LLIN use and participation in activities outside of intervention coverage (Cooke *et al.*, 2015; Wamae *et al.,* 2015; Degefa *et al.,* 2017) have been demonstrated to increase exposure to biting by infected female Anophelines (Garley *et al.,* 2013; van Eijk *et al.,* 2019; Nyasa *et al.,* 2021). As a result, this study findings corroborate previous research that reported a high prevalence of *P. falciparum* slide positivity in males (Diiro *et al.,* 2016; Kassam *et al.,* 2021). The high levels of microscopic *P. falciparum* infections and parasite density in the 5–15 age group were linked to a low number of LLIN use thus predisposed to bites from infected malaria vectors (Thomsen *et al.,* 2017; Kamau *et al.,* 2017; van Eijk *et al.,* 2019). Children < 5 years were less likely to have microscopic infections because the majority used LLINs. As previously reported (Walldorf *et al.,* 2015; Rek *et al.,* 2020), the low proportion of microscopic infections among this age group, as well as the high use of LLINs, was a good indicator of strict parental care.

Patients who participated in nocturnal outdoor activities were more likely to have microscopic infections than patients who stayed indoors. However, patients who engaged in nocturnal outdoor activities and did not use LLINs while indoors were at a higher risk. These findings imply that biting by infected *Anopheles* mosquitoes occurs at the transition point from LLINs coverage to outdoor or vice versa. With these findings, the study suggests that outdoor malaria transmission is low in the six sub-locations. This finding supports previous findings in 2018 and 2019 by Ondeto *et al.,* (*pers. commun*.) on an increased population of *Anopheles*

arabiensis collected outdoors, with blood meal results indicating a feeding preference for bovines in the study area.

Despite the microscopic prevalence of 14.7%, there were a large number of clinically positive cases that were missed because they were submicroscopic infections. The high levels of undetected infections may have a significant impact on existing malaria intervention strategies, potentially resulting in a plateauing of malaria cases in the future. The high number of missed cases is attributed to declining malaria prevalence, which has resulted in the area transitioning to a low malaria transmission zone (Oduma *et al.,* 2021). Additionally, these low transmission zones have been reported to be prone to submicroscopic *P. falciparum* infections (Slater *et al.,* 2019). The reduced microscopic infections rates and increased levels of submicroscopic infections within this study site may indicate declining parasite genetics or clones (Branch *et al.,* 2001), host exposure to fewer bites by infected Anophelines due to LLIN use or IRS (Okell *et al.,* 2012; Bousema *et al.,* 2014), and a faster rate of acquired immunity acquisition due to fewer parasite clones (Clark *et al.,* 2012; Adu *et al.,* 2020). These three underlining factors have been previously linked to increased levels of submicroscopic *P. falciparum* infections.

With an odds ratio of 1:8 for detecting submicroscopic infection in males versus females, these observations were slightly higher than previously reported ratio of 1:4 in patients from low transmission zones within Belaga district of Malaysia (Jiram *et al.,* 2019). The high levels of submicroscopic infections in female patients support findings that females have a higher rate of asexual stage parasite clearance than males (Briggs *et al.,* 2020). Although few in numbers, children < 5 years had the highest percentage of submicroscopic infections than the rest of age groups. The high number of adults infected with submicroscopic infections is consistence with the findings of a systematic review on drivers of these infections, which found

age to be a significant determinant (Whittaker *et al.,* 2019). These high levels of submicroscopic infections in adults could be attributed to acquired immunity that suppresses parasite load, self-prescription, poor adherence to anti-malarial drug regimens, and a high prevalence of recently acquired infections (Omer *et al.,* 2011; Bruxvoort *et al.,* 2014). Patients living in Kaura sublocation had the highest malaria burden of any of the six sub-locations studied. This sublocation is located along the shores of Lake Victoria, which could be a confounding factor in the observed periodic prevalence. Sublocations with low LLIN use had a higher prevalence of both or total (microscopic and submicroscopic) infections than those with high LLIN use.

In contrast to the previously observed link between LLIN use and microscopic infections, patients who used LLINs were more likely to have submicroscopic infections. When the study was narrowed down to establish the link between nocturnal outdoor activities, LLIN use, and submicroscopic infections, a similar observation was made. In general, patients who participated in nocturnal outdoor activities had a higher chance of having submicroscopic infections than those who stayed indoors. This was largely influenced by the high LLIN usage among those involved in nocturnal outdoor activities. These findings demonstrate that an increase in malaria vector interventions may be directly or indirectly related to an increase in submicroscopic infections in this study site. Perhaps the interventions are limiting human-vector contact, lowering both biting and sporozoite inoculation rates, implying that low biting rates are a cause of rising submicroscopic infections (Okell *et al.,* 2012; Bousema *et al.,* 2014). Additionally, the influence of LLIN integrity cannot be overlooked as the study did not consider this. Reduced LLIN integrity confers partial protection to the host against bites by infected malaria vectors (Ochomo *et al.,* 2013).

Despite continued IRS, LLIN use, and lower periodic malaria prevalence in the six study sublocations, submicroscopic infections persisted in this rural area of western Kenya. As a result, the accumulation of undetected and untreated infections may continue to stymie efforts to achieve long-term malaria elimination. Further, female *Anopheles* have been successfully infected by submicroscopic infections (Lin *et al.,* 2016; Gonçalves *et al.,* 2017). With rapid diagnostic kits clearly playing a significant role in malaria monitoring during the COVID-19 era in most countries (WHO, 2021d), this study suggests supplementing microscopy with ultrasensitive malaria Rapid Diagnostic Tests (or PCR in areas where this is feasible) targeting patients with malaria-like symptoms.

4.6 Conclusion

In the six study sub-locations in Homa Bay County, this study found a high prevalence of submicroscopic infections, resulting in a large number of undetected and untreated patients who may serve as a reservoir for continuing transmission of *P. falciparum*. This result suggests that combined diagnosis using microscopy in conjunction ultrasensitive Rapid Diagnostic Tests or PCR is appropriate in areas with low *P. falciparum* transmission.

CHAPTER FIVE: GENETIC POLYMORPHISMS AND SIGNATURES OF SELECTION ON THE IMMUNODOMINANT DOMAINS OF Pfs230 AND Pfs48/45 IN MALARIA PARASITES FROM RURAL AREAS OF WESTERN KENYA

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5.1 Abstract

Malaria elimination and eradication efforts can be advanced by including transmission-blocking or reducing vaccines (TBVs) alongside existing interventions. Key TBV candidates, such as *Pfs230* domain 1 and *Pfs48/45* domain 3, should be genetically stable to avoid developing ineffective vaccines due to antigenic polymorphisms. This study evaluated genetic polymorphism and temporal stability of *Pfs230* domain 1 and *Pfs48/45* domain 3 in *Plasmodium falciparum* parasites from western Kenya. Dry blood spots on filter paper were collected from febrile malaria patients reporting to community health facilities in malaria endemic areas of Homa Bay and Kisumu Counties and epidemic-prone area of Kisii County in 2018 and 2019. *Plasmodium* speciation was performed using eluted DNA and real-time PCR. Amplification of the target domains of the two *Pfs* genes was performed on *P. falciparum* positive samples. Sequencing of *Pfs230* domain 1 on 156 clinical isolates and *Pfs48/45* domain 3 on 118 clinical isolates was done to infer the levels of genetic variability, signatures of selection, genetic diversity indices and perform other evolutionary analyses. *Pfs230* domain 1 had low nucleotide diversity (π=0.15×10^{-2}) with slight variation per study site. Six polymorphic sites with nonsynonymous mutations and eight haplotypes were discovered. I539T was a novel variant, whereas G605S was nearing fixation. *Pfs48/45* domain 3 had a low π (0.063×10^{-2}), high conservation index, and three segregating sites, resulting in nonsynonymous mutation and four haplotypes. Some loci of *Pfs230* D1 were in positive or negative linkage disequilibrium,

had negative or positive selection signatures, and others (1813, 1955) and (1813, 1983) had a history of recombination. Mutated loci pairs in *Pfs48/45* domain 3 had negative linkage disequilibrium, and some had negative and positive Tajima's *D* values with no history of recombination events. The two transmission blocking vaccine candidates have low nucleotide diversity, a small number of zone-specific variants, high nucleotide conservation index, and high frequency of rare alleles. With the near fixation a polymorphic site and the proximity of mutated codons to antibody binding epitopes, it will be necessary to continue monitoring sequence modifications of these domains when designing TBVs that include Pfs230 and Pfs48/45 antigens.

Keywords: *Pfs230, Pfs48/45*, transmission blocking vaccines, genetic diversity, evolutionary forces

5.2 Introduction

Genetic polymorphism of *Plasmodium falciparum* antigens has hampered efforts to develop an effective vaccine that is protective against pre-erythrocytic and asexual blood-stage parasites (Genton *et al.,* 2002; Takala *et al.,* 2007; Ogutu *et al.,* 2009; Bergmann-Leitner *et al.,* 2012; Neafsey *et al.,* 2015). Recent efforts, however, have been made to develop vaccines that reduce and block *Plasmodium falciparum* transmission at the community level. Two of the existing transmission-blocking vaccine (TBV) candidates, *P. falciparum* surface protein 230 (Pfs230) (Sabeti *et al.,* 2007; Singh *et al.,* 2019; Tachibana *et al.,* 2019; McLeod *et al.,* 2019; Lee *et al.,* 2020; Huang *et al.,* 2020; Singh *et al.,* 2020; Healy *et al.,* 2021) and *P. falciparum* surface protein 48/45 (Pfs48/45) (Singh *et al.,* 2019, 2021; Lee *et al.,* 2020), have been shown to elicit antibody responses in mice and people that block *P. falciparum* gametocyte fertilization in the mid-gut of the *Anopheles* vector.

Pfs230 is a cysteine-rich 230 kDa protein expressed by both male and female gametocytes (Rener *et al.,* 1983; MacDonald *et al.,* 2016). The antigen is thought to play a role in gamete fusion in the mosquito blood meal after forming a complex with another cysteine-rich protein, Pfs48/45 (Eksi *et al.,* 2006). In comparison to antibodies elicited by immunization with other Pfs230 domains, Domain 1 (D1) has been shown to elicit transmission-blocking monoclonal antibodies with strong inhibitory activity against oocyst development in standard membrane feeding assays (Singh *et al.,* 2019; Lee *et al.,* 2019; Tachibana *et al.,* 2019; Huang *et al.,* 2020; Singh *et al.,* 2020; Healy *et al.,* 2021). Like Pfs230 D1, fusion with its counterpart Pfs48/45 D3 has good potential as a component of a TBV. The latter fused doublet antigen consists of three domains linked by disulphide bonds and contains 16 cysteine residues (Kocken *et al.,* 1993). Unlike Pfs230, Pfs48/45 is anchored on the gamete surface membrane by glycophosphatidylinositol (Kocken *et al.,* 1993; Dijk *et al.,* 2001; Gilson *et al.,* 2006) and

is essential for male gamete fertility. Domain 3 has been shown to elicit antibodies in the host (Graves *et al.,* 1988; Roeffen *et al.,* 1994; Dijk *et al.,* 2001; Bousema *et al.,* 2010; S. Jones *et al.,* 2015a; Acquah *et al.,* 2017). Pfs48/45 D3 is located at the C-terminus of the protein and contains binding sites for non-inhibitory and inhibitory human and mouse mAbs that reduce *P. falciparum* infection in mosquitoes (Vermeulen *et al.,* 1986; Graves *et al.,* 1988; Outchkourov *et al.,* 2007; Chowdhury *et al.,* 2009; Singh *et al.,* 2017, 2019; Lennartz *et al.,* 2018; Kundu *et al.,* 2018; Lee *et al.,* 2020).

Antigenic polymorphism of Pfs230 D1 and Pf48/45 D3 should be assessed in malaria endemic areas on a regular basis to support the successful development of these TBV candidates due to the fact that, if the targeted regions are genetically unstable, polymorphisms may cause critical codon changes within immunogenic epitopes, thereby reducing TBV efficacy. Several dimorphic sites on Pfs230 D1 and Pfs48/45 D3 have previously been identified (Kocken *et al.,* 1995; Drakeley *et al.,* 1996; Escalante *et al.,* 1998; Conway *et al.,* 2001; Jones *et al.,* 2015a; MacDonald *et al.,* 2016; Kundu *et al.,* 2018; Singh *et al.,* 2020; Coelho *et al.*, 2021); however, there is limited knowledge of the extent of genetic diversity, signatures of selection, and other evolutionary forces that may be shaping alleles in *P. falciparum* from different malaria transmission zones. This study performed an in-depth genetic analysis of *Pfs230* D1 and *Pfs48/45* D3 in parasites isolated from patients with uncomplicated *falciparum* malaria from three different areas in western Kenya.

5.3 Materials and Methods

5.3.1 Study site and sampling

Dry blood spots (DBS) were collected on filter paper from febrile malaria patients at health clinics in malaria endemic sites with ongoing IRS intervention (Kochia in Homa Bay

County), endemic site with no IRS intervention (Chulaimbo in Kisumu County) and an epidemic prone zone (Eramba in Kisii County) in 2019 and 2020 (**Fig 5.1**).

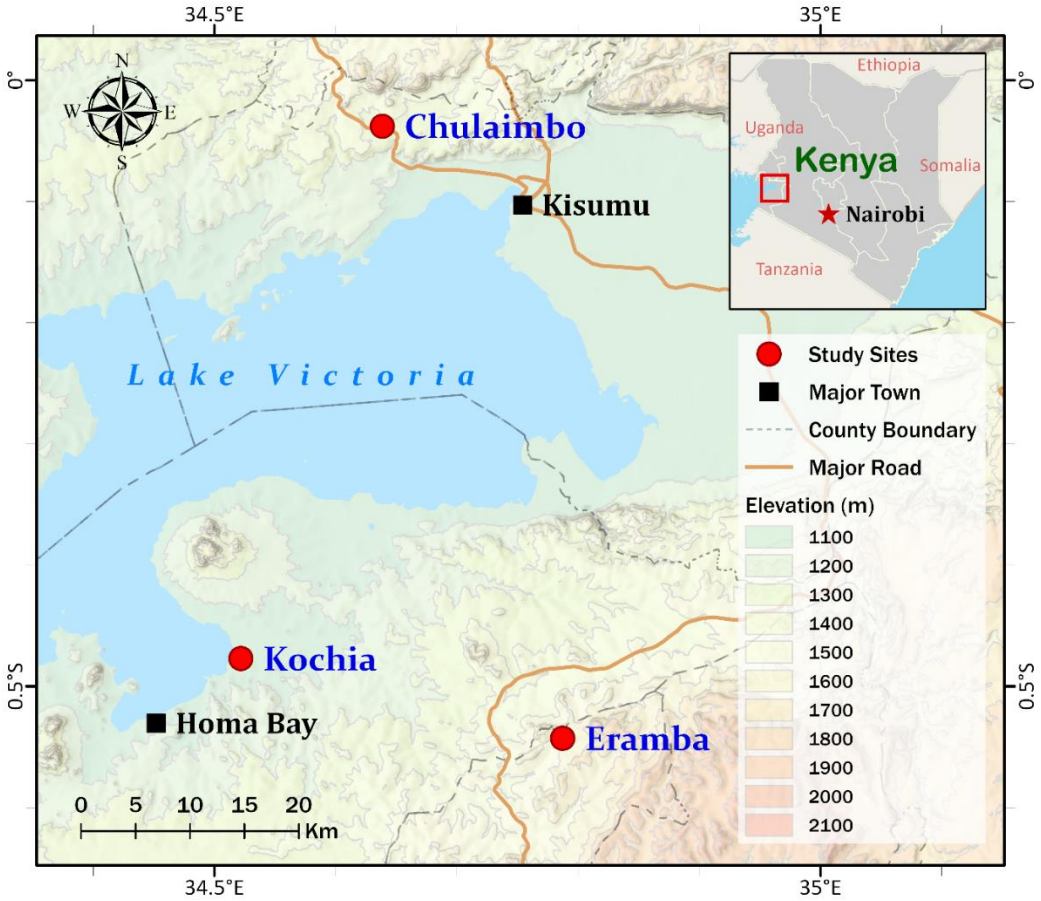

Figure 5.1 Map showing Kochia (Homa Bay County), Chulaimbo (Kisumu County) and Eramba (Kisii County) study sites in western Kenya. The sampling points are represented by the red circles on the map. This figure was prepared with ESRI ArcGIS Pro 2.8 with field survey results and publicly available datasets. The map contains information from OpenStreetMap and OpenStreetMap 115 Foundation, which is made available under the Open Database License.

The study site in Homa Bay is characterized by perennial transmission. Vector control consists of universal distribution of long-lasting insecticidal bed nets with annual indoor residual spraying of insecticides. The study site in Kisumu County also has perennial

transmission. Vector control consists of long-lasting insecticidal bed nets alone. The site in Kisii County is malaria epidemic-prone with low transmission and residents use LLINs (Kapesa *et al.,* 2018). Health facilities selected for sample collection in malaria endemic zone of Homa Bay lie at 34.64190°E-0.38000°S and 1134-1330 metres above the sea level (asl), in Kisumu at 00.03572°S-034.62196°E and 1328–1458 m asl and in Kisii at 34°48′E, 00°35′S; 1540–1740 m asl. Patients presenting with fever and symptoms of uncomplicated malaria, e.g., myalgia, fatigue, non-localizing symptoms, were recruited to donate a finger prick blood sample. In brief, four drops of approximately 25µL of blood from each patient were spotted on Whatman™ Blood Stain Cards (GE Healthcare WB100014) as previously described in section 3.3.2. 150 of the 372 DBS collected came from endemic rural areas in Homa Bay; 120 and 102 were from Kisumu and Kisii, respectively.

5.3.2 Ethical approval and consenting

Ethical approval, consenting and assenting was done as stated in section 3.3.2.

5.3.3 *Plasmodium falciparum* genomic DNA extraction

Genomic DNA were extracted as described in section 3.3.3, transferred into a coded sterile 0.5ml Eppendorf tube and stored at -20°C.

5.3.4 Amplification and sequencing of *Pfs230* domain 1 and *Pfs48/45* domain 3

Plasmodium species-specific real-time PCR targeting 18S ribosomal RNA gene was used to confirm *P. falciparum* positive DNA samples as described in section 3.3.4. before amplification of specific target fragments of each gene using designed domain specific primer sets. Primer sets were designed using Primer3 version 0.4.0 for *Pfs230* D1 and *Pfs48/45* D3 and in silico validation of each set was performed using the Sequence Manipulation Suite to confirm targeted base pairs (Stothard, 2000). Among the 372 samples, 332 (89.3%) tested

positive for *P. falciparum* DNA (n=150, 120 and 62 from malaria endemic zone of Homa Bay, Kisumu and epidemic prone zone of Kisii highland, respectively) and were used to amplify *Pfs230* D1 and *Pfs48/45* D3 in a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Prior to amplification of targeted region of the two genes in samples that tested positive for parasite DNA, gradient PCR was conducted using NF54 (positive control DNA) to determine the annealing temperature of each primer set. For *Pfs230* D1, the annealing temperature tested ranged from 57°C to 60°C whereas for *Pfs48/45* D3 ranged from 55°C to 57°C. Upon determining the annealing temperature, amplification of each gene was done as follows. Briefly, 3 µL of sample DNA was added to a mixture of 11.5 µL of DreamTaq Green PCR Master Mix (2X), 0.5 µL of *Pfs230* D1 forward (5'-TGGTGAAGCTGTCGAAGATG-3') and reverse primers (5'-GTGTACCACAGGGGGAAGAG-3') targeting 514 base pairs and 7.5 µL of double-distilled water. The thermal profile was set as follows 95°C for 3 min, 34 cycles (94°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec) and final extension at 72°C for 6 min. For *Pfs48/45* D3, similar reaction volume was prepared using forward (5'-TTTTCAAGAAGGAAAAGAAAAAGC-3') and reverse primers (5'-GCCAAAAATCCATAATATGCTGA-3') targeting 600bp. The PCR conditions were set as follows 95°C for 3 min, 34 cycles (94°C for 30 sec, 55°C for 30 sec and extension at 72°C for 45 sec) final extension at 72°C for 6 min. All the amplicons were assessed by gel electrophoresis in 1.5% w/v agarose gel before sequencing. For *Pfs230* D1, 82 and 35 samples from malaria endemic zone of Homa Bay and epidemic prone zone of Kisii, respectively, were amplified (**Appendix 5**). For *Pfs48/45* D3, 36, 44, and 38 samples from Homa Bay, Kisumu, and Kisii, zones respectively, were amplified (**Appendix 7**). All the PCR amplicons, together with positive control were purified by the addition of Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-IT) and incubated at 37°C for 15 min. The ExoSAP-IT in purified PCR products was inactivated by heating at 80°C for 15 min. The cleaned PCR products with

quantity above 25ng/µl were selected for sequencing after testing 1µl of each using NanoDrop™ Lite Spectrophotometer (Thermo Scientific™). Each purified amplicon was bi-directionally sequenced using 3730 BigDye® Terminator v3.1 Sequencing Standard kit on ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

5.3.5 Data analysis

All sequences were assembled using Geneious version 11.1.5 software, and multiple sequence alignment was performed using ClustalW. Polymorphic locus and codons were inferred after comparing each sequence to the respective sequence of positive control (NF54) as well as 3D7 (PF3D7_0209000 for *Pfs230* and PF3D7_1346700 for *Pfs48/45*). DnaSP Version 6.12.03 (Rozas *et al.,* 2017) and Arlequin version 3.5.2 (Excoffier & Lischer, 2010) were used to compute genetic diversity indices such as nucleotide diversity (π), haplotype diversity (*Hd*), number of haplotypes (h), number of segregating sites (S) and mean number of pairwise difference (k). Population Analysis with Reticulate Trees (Popart) version 1.7 software (Clement *et al.,* 2000) was used to infer haplotype networks. Neutrality tests; Tajima's *D*, Fu and Li's D, Fu and Li's F and Fu's *Fs* statistics and test for the presence of Recombination events (Rm) and linkage disequilibrium (LD) were computed in DnaSP Version 6.12.03 and Arlequin version 3.5.2. Generated Tajima's *D* values (values that differ from a standard neutral model in which observed mutations are assumed to be neutral and have no effect on organism fitness) were plotted using GraphPad version 8.3.0 (Tajima, 1989). Both antigen structural delineation was done using Protein Homology/analogY Recognition Engine (PHYRE2) version 2.0 and generated models visualized and edited in UCSF Chimera version 1.15 (Pettersen *et al.,* 2004).

5.4 Results

5.4.1 Analysis of mutations detected in Pfs230 D1 in *P. falciparum* population from western Kenya

For *Pfs230* D1, 82 and 35 consensus sequences from malaria endemic zone of Homa Bay and epidemic-prone zone of Kisii were retrieved from respective contigs. These were compared to 39 sequences from Kisumu. Generally, a total of six loci (1616, 1813, 1955, 1964, 1967and 1983) in *Pfs230* D1 were found to be polymorphic, resulting in nonsynonymous mutations in parasites from western Kenya (**Table 5.1; Appendix 6**). The mutations were skewed toward transversion, with a transversion to transition ratio (Tv: Ts) greater than 0.5. Two polymorphic sites were singletons (1964 and 1967), whereas four dimorphic sites (1616, 1813, 1955, and 1983) were parsimony informative. These polymorphisms resulted in I539T, G605S, T652R, E655V, T656N, and K661N codon changes.

Table 5.1 Polymorphic sites on Pfs230 domain 1 from Homa Bay, Kisumu and Kisii region in western Kenya.

Pfs230 **(N=156)**								
Segregating sites	**Domains**	**Allelic frequency**			**Substituted bases**	**Type of Substitution**	**Codon change**	**Type of mutation**
		Homa Bay n (%)	**Kisumu n (%)**	**Kisii n (%)**				
1616	D1	-	2 (5.1)	-	*T/C	Transition	I539T	Nsyn
1813	D1	81 (98.8)	37 (94.9)	35 (100)	*G/A	Transition	G605S	Nsyn
1955	D1	2 (2.4)	1 (2.6)	2 (5.7)	*C/G	Transversion	T652R	Nsyn
1964	D1	-	-	1 (2.9)	*A/T	Transversion	E655V	Nsyn
1967	D1	-	1 (2.6)	-	*C/A	Transversion	T656N	Nsyn
1983	D1	35 (42.7)	19 (48.7)	16 (45.7)	*A/C	Transversion	K661N	Nsyn

n: number of sequences harbouring mutations; ∗: reference (3D7and NF54) allele only; D: Domain; Nsyn: Non-synonymous mutation; Syn: Synonymous mutation; A: Adenine; C: Cytosine; T: Thymine; G: Guanine.

Nonsynonymous alterations T652R and K661N were on separate beta (β) pleated sheets connected by a loop containing mutated codons E655V and T656N (**Fig. 5.2**). G605S was also

on the loop connecting two different β pleated sheets. In general, western Kenya parasites had a high allelic frequency of G605S (98.08%), followed by progressively lower frequencies of K661N, T652R, and I539T, E655R and T656N were each observed at a frequency of <1%.

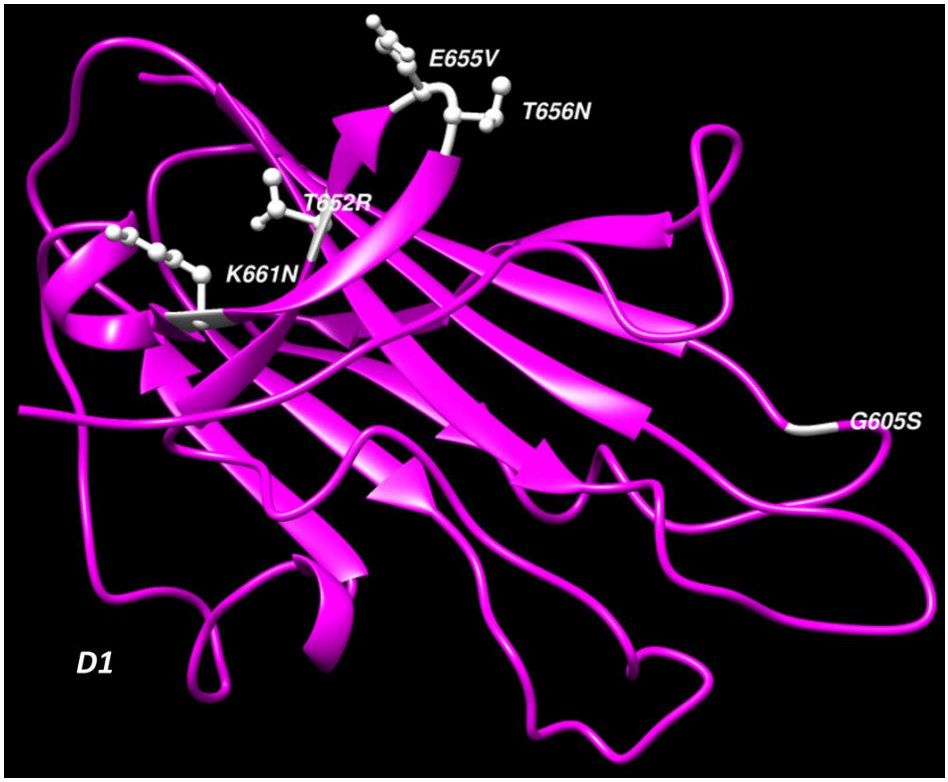

Figure 5.2 Computed structure with mutated codons on domain 1 of Pfs230 antigen. The antigen structures are presented in the form of Beta strands (β) and loops in magenta colour. The white balls and sticks represent codons with mutations (Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311).

The prevalence of various alleles was almost similar across the various study sites. For example, as shown in **Table 5.1**, G605S was the most common codon change in the three study sites. Only two *P. falciparum* isolates from malaria endemic zone of Kisumu and one from Homa Bay County lacked this mutation.

5.4.2 Analysis of mutations detected in Pfs48/45

In contrast to the six nonsynonymous mutated sites observed in *Pfs230* D1, *Pfs48/45* D3 had three segregating sites (**Table 5.2; Appendix 8**). Singleton sites were found at loci 911 and 979 in parasites isolated from patients residing in malaria endemic zone of Homa Bay County and epidemic-prone zone of Kisii County, respectively. A low frequency polymorphism at locus 940 was observed across parasite populations in all three counties, and was parsimony-informative. These transition bias mutations at loci 911, 940, and 979 resulted in nonsynonymous mutations V304D, L314I, and C327G, respectively.

Table 5.2 Polymorphic sites on Pfs48/45 domain 3 from Homa Bay, Kisumu and Kisii region in western Kenya.

Pfs48/45 **(N=118)**								
Segregating sites	**Domains**	**Allelic frequency**			**Substituted bases**	**Type of Substitution**	**Codon change**	**Type of mutation**
		Homa Bay n (%)	**Kisumu n (%)**	**Kisii n (%)**				
753	D2	-	-	1 (2.6)	*T/C	Transition	Y251Y	Syn
757	D2	-	4 (9.1)	3 (7.9)	*A/G	Transition	K253E	Nsyn
762	D2	1 (2.8)	3 (6.8)	3 (7.9)	*C/G	Transversion	N254K	Nsyn
911	D3	1 (2.8)	-	-	*T/A	Transition	V304D	Nsyn
940	D3	3 (8.3)	3 (6.8)	9 (23.7)	*T/A	Transition	L314I	Nsyn
979	D3	-	-	1 (2.6)	*T/G	Transversion	C327G	Nsyn

n: number of sequences harbouring mutations; *: reference (3D7and NF54) allele only; D: Domain; Nsyn: Non-synonymous mutation; Syn: Synonymous mutation; A: Adenine; C: Cytosine; T: Thymine; G: Guanine.

The variants were in the Pfs48/45 D3 antigen loop connecting different β pleated sheets (**Fig. 5.3**). Codon change C327G in D3 was found in only one sequence in parasites isolated from a patient in Kisii County (**Table 5.2**). The ability of the designed primer set to cover *Pfs48/45* D3 also allowed for the discovery of a singleton site 753 (Y251Y) and parsimony-informative sites 757 and 762 (K253E and N254K) (**Table 5.2**; **Fig. 5.3**).

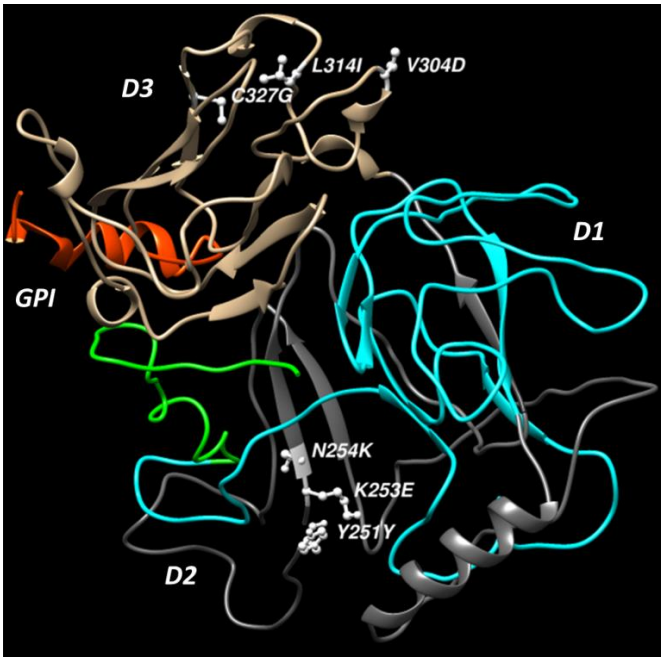

Figure 5.3 Computed structure with mutated codons on domain 2 and 3 of Pfs48/45 antigen. The Glycosylphosphatidylinositol (GPI) is represented in orange red colour, D1, D2 and D3 are represented in cyan, light slate grey and tan colours respectively. The antigen structures are presented in the form of Beta strands (β) and loops. The white balls and sticks represent codons with mutations (Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311).

Except for G605S, which was near dimorphic codons on Pfs48/45 domain 2, the superimposed structure revealed dimorphic codons of Pfs48/45 D3 antigen close to those of Pfs230 D1 (**Fig. 5.4**).

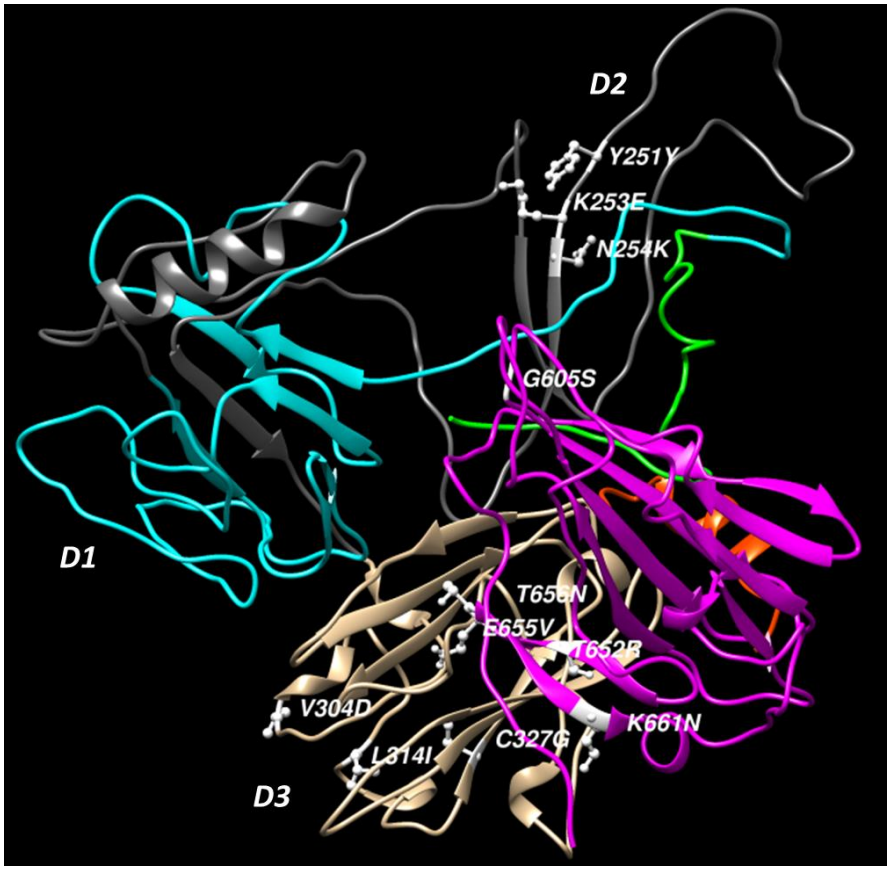

Figure 5.4 Superimpose structure with mutated codons on Pfs230 D1 and Pfs48/45 antigen. The antigen structures are presented in the form of Beta strands (β) and loops. The magenta ribbon represents Pfs230 D1 structure whereas respective domains of Pfs48/45 are labelled as D1, D2 and D3 a. The white balls and sticks represent codons with mutations (Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311).

5.4.3 Genetic diversity of *Pfs230* and *Pfs48/45* genes in western Kenya

Domain 1 of *Pfs230* from the three sites had π of 0.15×10^{-2}, k; 0.68 and haplotype diversity (*Hd*) of 0.57 (**Table 5.3**). The domain had a nucleotide conservation index of 98.7% with a total of 8 haplotypes circulating in western Kenya (**Fig. 5.5**). Malaria endemic zone of Kisumu had the highest π (0.18×10^{-2}) followed by epidemic-prone zone of Kisii (0.15×10^{-2}) and Homa

Bay (0.12×10^{-2}). The site also had the most haplotypes (6) and the highest *Hd* (0.63) when compared to Kisii and Homa Bay zones, which had four haplotypes each and *Hd* of 0.60 and 0.52, respectively (**Table 5.3)**.

Table 5.3 Summary of genetic diversity indices for Pfs230 domain 1 and Pfs48/45 domain 3 from parasites in western Kenya.

***Pfs230* D1 Region**	**N**	**C (%)**	**S**	**π ($\times10^{-2}$)**	**h**	***Hd***	**Tajima's *D***	**Fu's Fs**	**FLD***	**FLF***
Homa Bay	82	99.40	3	0.12	4	0.52	-0.11	-0.21	-0.54	-0.45
Kisumu	39	98.90	5	0.18	6	0.63	-0.80	-1.94	-0.73	-0.82
Kisii	35	99.40	3	0.15	4	0.60	-0.15	-0.39	-0.31	-0.29
W. Kenya	156	98.70	6	0.14	8	0.56	-0.82	-3.33	-0.94	-1.03
***Pfs48/45* D3**										
Homa Bay	36	99.50	2	0.05	3	0.21	-1.09	-1.42	-0.80	-0.95
Kisumu	44	99.80	1	0.03	2	0.13	-0.60	-0.30	0.55	0.244
Kisii	38	99.50	2	0.10	3	0.41	-0.21	-0.12	-0.81	-0.74
W. Kenya	118	99.30	3	0.06	4	0.25	-0.94	-1.87	-2.06	-2.00

N: Sample size; C: Conservation index; S: Segregating sites; π: nucleotide diversity; Vars: Variants; *Hd*: Haplotype diversity.

Haplotype 2 (Hap_2) with the mutated codon G605S was the most common in western Kenya and at each study site. This was followed by Hap_4 (mutated codons G605S and K661N), Hap_7 (G605S, T652R, and K661N), and Hap_8 (T652R and K661N) (**Table 5.4**). The remaining haplotypes (Hap_1, Hap_3, Hap_6, and Hap_5) were observed at a lower frequency. Only one sequence (from Homa Bay rural areas) and two from Kisumu in western Kenya lacked a mutated site and had 100% sequence identity to the laboratory strain PF3D7_0209000 or NF54 sequence used as a positive control (**Fig 5.5)**.

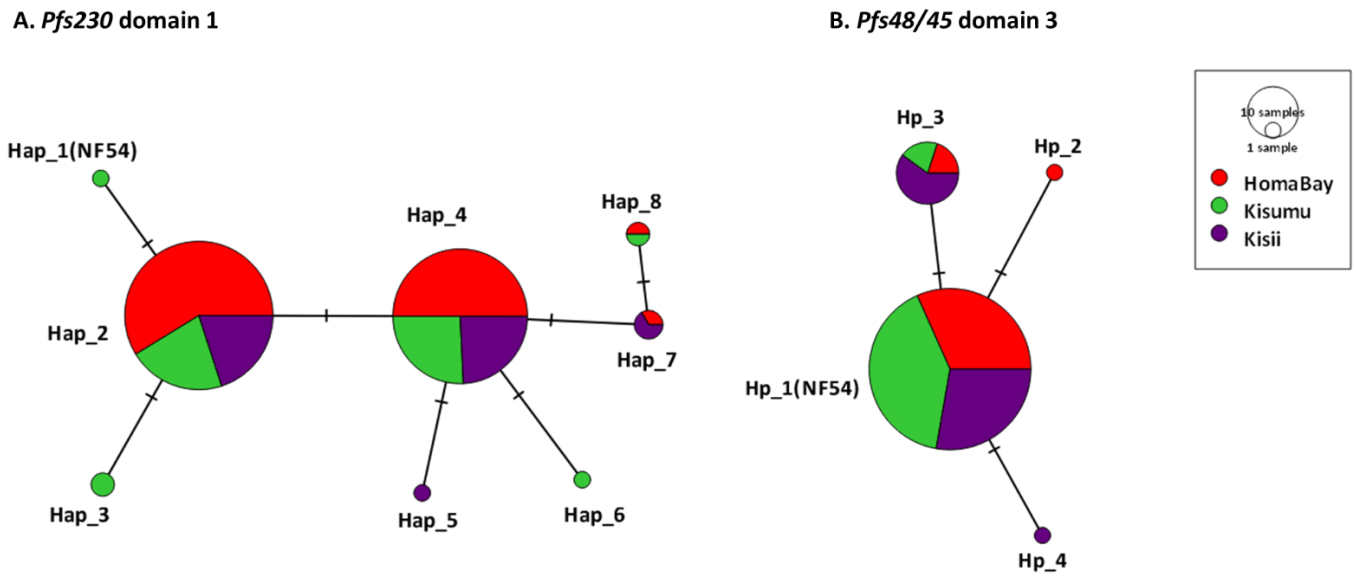

Figure 5.5 TCS-network analysis of the relationship of haplotypes based on the of Pfs230 domain 1 and Pfs48/45 domain 3 from parasites in malaria-endemic and epidemic-prone region of western Kenya. A. represents haplotypes based on observed sequence variation in Pfs230 D1 whereas B represents haplotypes in Pfs48/45 D3. The network shows the distribution of haplotype within malaria-endemic zones of Homa Bay (red) and Kisumu (Chulaimbo) (green) as well as epidemic prone zone of Kisii highland (purple). The hatch marks represent the number of mutations resulting in a specific haplotype whereas the size of the circle equates to the frequency of the observed haplotypes. Haplotypes labelled Hap_1(NF54) for Pfs230 D1 and Hp-1 (NF54) for Pfs48/45 D3 lacked mutated locus and have 100% sequence identity to laboratory strain NF54 or 3D7. Nucleotide sequences of all haplotypes were submitted to GenBank (accession: MW624857- MW625101).

The *Pfs48/45* D3 from western Kenya had low π (0.06×10^{-2}) and *Hd* (0.25) (**Table 5.3**). The domain had a conservation index of 99.3%, with four haplotypes circulating in the study area (**Fig. 5.5**). Parasites from rural areas of Kisii had the highest π (0.10×10^{-2}) followed by Homa Bay (0.05×10^{-2}) and Kisumu (0.03×10^{-2}) (**Table 5.3**). Kisii and Homa Bay study sites each had three haplotypes in circulation, while Kisumu had four (**Fig. 5.5**). The majority of

haplotypes lacked a mutation (Hp_1) or had 100% sequence identity to the laboratory strain PF3D7_1346700 or the NF54 sequence used as a positive control (85.6%). This was followed by Hp_3 (mutated codon L314I), which had an overall frequency of 12.7%, while the rest (Hp_2 and Hp_4) had a frequency <1% (**Table 5.5**).

5.4.4 Signatures of selection, linkage disequilibrium and recombination events

Pfs230 D1 from *P. falciparum* isolates deviated from a standard neutral model. Tajima's *D* (-0.8), FLD* (-0.9) and FLF* (-1.0) tests were all negative and non-significant (p>0.05) (**Table 5.3**). However, Fu's F_S result (-3.3), was significant (p=0.023). Tajima's *D* test results were also non-significant (p>0.05) in each site (**Table 5.3**). Despite the overall negative Tajima's D results, there was a slight variation among individual mutated loci on *Pfs230* D1. Locus 1983 (codon change K661N) had a significant (p<0.05) positive (1.9) Tajima's *D* result, whereas the rest had negative results (**Fig 5.6**).

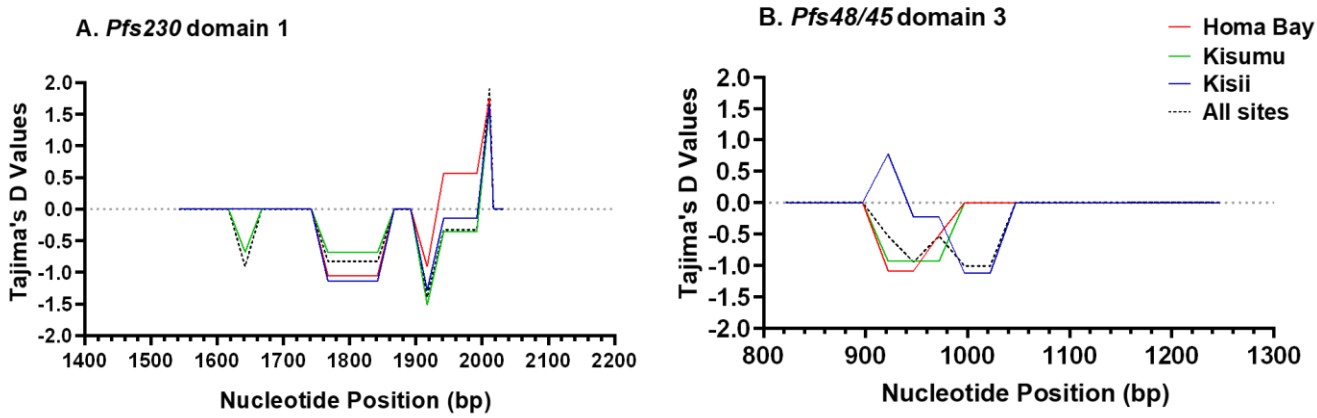

Figure 5.6 Sliding window plot of Tajima's *D* values for Pfs230 domain 1 and Pfs48/45 domain 3 in western Kenya. The X-axis displays the nucleotide position (Window midpoint) whereas the Tajima's *D* values are represented on the Y-axis. A. is a representation of Pfs230 domain 1 whereas B. represents of Pfs48/45 domain 3. The blue curve represents computed Tajima's *D* plot for sequences of *P. falciparum* circulating in epidemic-prone zone of Kisii, the red colour is for Homa Bay, the green colour is for Kisumu whereas the black dotted colour represents the population from the three study sites. The middle horizontal dotted line (intersecting the Y-axis at 0.0) represents a standard neutral model where the Tajima's *D* value is equal to zero. Positive deviation from the grey dotted line signifies balancing selection whereas negative deviation represents purifying selection.

Apart from deviating from a standard neutral model, some loci pairs (1813, 1955) had positive LD (D') results with highly significant ($p<0.001$) χ^2 values of 19.7 and 12.7 among Homa Bay and Kisumu sequences, respectively (**Table 5.4**). Among Kisii sequences, loci pairs (1813 and 1983) had positive D' results, despite the fact that the χ^2 values was non-significant and the r^2 value was low (0.03). Other loci with positive D' results, low r^2 values and non-significant ($p>0.05$) χ^2 values in Homa Bay and Kisumu parasites include loci pairs (1813,1983), (1955,1983) and (1967,1983). Some loci pairs on Kisumu and Kisii sequences had negative D' results (**Table 5.4)**. On *Pfs230* D1 from parasites in rural areas of Homa Bay

and Kisumu, recombination events were detected across loci pairs (1813, 1955) and (1813, 1983) (**Table 5.4**).

Table 5.4 Haplotype frequencies and defining codon changes on the domain I of Pfs230 across Homa Bay, Kisumu and Kisii *Plasmodium falciparum* population.

Homa Bay (N=82)						
Haplotype	**Mutated codon**	**Haplotype Frequency n (%)**	**LD (Linked loci) χ 2**	**D'**	**r^2**	**Rm**
Hap_1 (NF54)	0	0.00	(1813, 1955) 19.738*** [B], (1813,1983) 0.052 [ns], (1955,1983) 2.811 [ns]	0.49 0.14 1.00	0.31 0.06×10^{-2} 0.03	(1813, 1955)
Hap_2	G605S	47 (57.32)				
Hap_4	G605S/K661N	33 (40.24)				
Hap_7	G605S/T652R/K661N	1(1.22)				
Hap_8	T652R/K661N	1(1.22)				
Kisumu (N=39)						
Hap_1 (NF54)	0	1(2.56)	(1616, 1813) 0.171 [ns], (1616, 1955) 0.054 [ns], (1616, 1967) 0.054 [ns], (1616, 1983) 1.905 [ns], (1813, 1955) 12.650*** [B], (1813, 1967) 0.083 [ns], (1813, 1983) 0.261 [ns], (1955, 1967) 0.026 [ns], (1955,1983) 1.134 [ns], (1967,1983) 1.134 [ns]	-1.00 -1.00 -1.00 -1.00 1.00 -1.00 -0.30 -1.00 1.00 1.00	0.38×10^{-2} 0.14×10^{-2} 0.14×10^{-2} 0.05 0.32 0.21×10^{-2} 0.66×10^{-2} 0.07×10^{-2} 0.03 0.03	(1813,1983)
Hap_2	G605S	17(43.59)				
Hap_3	I539T/G605S	2(5.13)				
Hap_4	G605S/K661N	17(43.59)				
Hap_6	G605S/T656N/K661N	1(2.56)				
Hap_8	T652R/K661N	1(2.56)				
Kisii (N=35)						
Hap_1 (NF54)	0	0.00	(1813, 1955) 0.061 [ns], (1813, 1964) 0.029 [ns], (1813, 1983) 1.150 [ns], (1955, 1964) 0.061 [ns], (1955, 1983) 1.895 [ns], (1964, 1983) 0.920 [ns]	-1.00 -1.00 1.00 -1.00 -1.00 -1.00	0.17×10^{-2} 0.08×10^{-2} 0.03 0.17×10^{-2} 0.05 0.03	-
Hap_2	G605S	16(45.71)				
Hap_4	G605S/K661N	16(45.71)				
Hap_5	G605S/E655V/K661N	1(2.86)				
Hap_7	G605S/T652R/K661N	2(5.71)				

N: Sample size; Capital letters before numerical denotes amino acid change at respective codon; n: Proportion of sequences under respective haplotypes; LD: Linkage Disequilibrium; χ^2: Chi-square values; [***B]: Highly significant by the Bonferroni procedure; [ns]: Non-significant; D': Parameter for estimating LD; r^2: Parameter for estimating LD; Rm: Recombination events.

All of the *Pfs48/45* D3 sequences had non-significant (p>0.05) negative Tajima's *D* -1.9, FLD*: -2.1 and FLF*: -2.0 results. The Fu's F_S (-1.9) result was, however, significant (p=0.096). Most mutated loci on *Pfs48/45* D3 were found to be under negative selection. Locus 940 (L314I) among Kisii sequences had a significant (p>0.05) positive (Tajima's *D* = 0.8) result (**Fig 5.6**). There was no evidence of Rm or positive D' results at any of the dimorphic loci within *Pfs48/45* D3. However, some loci pairs from Homa Bay and Kisii had negative D' results (**Table 5.5**).

Table 5.5 Haplotype frequencies and defining codon changes on domain III of Pfs48/45 across Homa Bay, Kisumu and Kisii *Plasmodium falciparum* population.

Homa Bay (N=36)					
Haplotype	**Mutated codon**	**Haplotype frequency n, (%)**	**LD (Linked loci) χ 2**	**D'**	**r^2**
Hp_1(NF54)	0	32 (88.89)			
Hp_2	V304D	1 (2.78)	(911, 940) 0.091^{ns}	-1.00	0.25×10^{-2}
Hp_3	L314I	3 (8.33)			
Kisumu (N=44)					
Hp_1(NF54)	0	41 (93.18)	-	-	-
HP_3	L314I	3 (6.82)			
Kisii (N=38)					
Hp_1(NF54)	0	28 (73.68)			
Hp_3	L314I	9 (23.68)	(940, 979) 0.308^{ns}	-1.00	0.79×10^{-2}
Hp_4	C327G	1 (2.63)			

N: Sample size; Capital letters before numerical denotes amino acid change at respective codon; n: Proportion of sequences under respective haplotypes; LD: Linkage Disequilibrium; χ^2: Chi-square values; ^{***B}: Highly significant by the Bonferroni procedure; ^{ns}: Non-significant; D': Parameter for estimating LD; r^2: Parameter for estimating LD.

5.5 Discussion

The Pfs230 D1 and Pfs48/45 D3 antigens are important candidate antigens in the development of an effective TBV. Despite having a high frequency of rare alleles in western Kenya, both targets had low nucleotide diversity. Two variants, each on Pfs230 D1 and Pfs48/45 D3, were novel and private to western Kenya. The study validated five previously described polymorphic

sites on *Pfs230* D1 (Singh *et al.,* 2020). In this study, G605S, one of the five mutated codons, was fixed in some study areas but not in others. *Pfs230* D1 had the most mutations, while the Pfs48/45 D3 was the most conserved. Mutated loci from both domains were either under purifying or balancing selections. Other genetic forces revealed to have shaped alleles on the two genes included inbreeding and genetic drift with recombination being discovered only *Pfs230* D1.

Domain 1 of *Pfs230* from western Kenya had low nucleotide diversity, with significant Fu's F_S results indicating a high frequency of rare alleles. In addition to the previously reported 15 polymorphisms on *Pfs230* D1 from parasites in Asian (Bangladesh, Cambodia, Laos, Myanmar, Thailand, and Vietnam) and African (Democratic Republic of the Congo, Ghana, Guinea, Malawi, Mali, Nigeria, Senegal, and Gambia) countries (MacDonald *et al.,* 2016; Singh *et al.,* 2020), this study discovered one additional mutation (I539T). This novel variant was identified only at the Kisumu study site along with five other polymorphisms (G605S, T652R, E655V, T656N, and K661N). These findings validate five previously described polymorphisms reported by Singh *et al.,* (2020). We speculate that the relatively higher nucleotide diversity index and number of haplotypes in Kisumu compared to other sites in western Kenya is related to the region's slightly higher malaria transmission and absence of IRS activities (Oduma *et al.,* 2021).

Missense mutation G605S was found in parasites at a slightly higher allelic frequency (AF=0.98) than in other geographical regions, as described by Singh *et al.,* (2020) (AF=0.94), MacDonald *et al.,* (2016), (AF= 0.11), and Coelho *et al.,* (2021) (AF=0.91). With only three clinical isolates in western Kenya lacking this mutation, G605S is almost completely fixed. This indicates the presence of selection pressure from either host antibodies, vector immune response or genetic drift (decreased variation and increasing homozygosity), may be stronger

on *P. falciparum* populations from epidemic-prone zone of Kisii (low parasite population size) or Homa Bay (endemic site with declining parasite population size) compared to Kisumu (endemic zone with no ongoing IRS) (Hancock & Rienzo, 2008; Honnay, 2013; Oduma *et al.,* 2021). In contrast, the second most common polymorphism, K661N, was found in malaria endemic zone of Kisumu at a higher frequency than in rural areas of Kisii (epidemic) and Homa Bay (endemic). This reversal in the observed G605S and K661N frequencies could be attributed to factors such as recombination events (Rm), which are known to interfere with linked loci and could be effective on linked dimorphic loci pair 1813 and 1983 (responsible for G605S and K661N mutations respectively), thus increasing diversity in Kisumu (Chulaimbo) (Mejia, 2012) as opposed to Kisii and Homa Bay parasites, which also lack Rm between the two sites. Since immunogenic epitope binding light chain of transmission-blocking 4F12 monoclonal antibodies (TB 4F12 mAb) is close to the dimorphic codon G605, selection pressure on the epitope may be affecting the surrounding codons (MacDonald *et al.,* 2016; Singh *et al.,* 2020). This codon is located within a disulphide loop (from 593–611 aa) that is thought to be stabilizing the epitope binding of TB 4F12 mAb (Singh *et al.,* 2020). With near-complete fixation, the mutation may be beneficial to parasites but have a negative effect on the epitope binding affinity of TB 4F12 mAb. This needs to be looked into further by immunoassays of haplotypes with this polymorphism.

Polymorphism I539T was found near codons 542–592 that contain 3G2 and 5G3 mAb binding epitopes which were previously shown to have no detectable oocyst reduction activity (Singh *et al.,* 2020). Other polymorphisms, T652R, K661N on different β pleated sheets, and E655V, T656N on disulphide loops linking the two loops, were distally located from the epitope that binds TB 4F12 mAb (Singh *et al.,* 2020). When the two fusion proteins were superimposed, these four polymorphic codons were closer to mutated codon V304D, L314I, and C327G on Pfs48/45 D3, supporting the hypothesis that antibodies could be sterically

interfering with protein-protein interaction (Singh *et al.,* 2020). *Plasmodium falciparum* may induce these mutations in response to antibody-induced pressure in order to circumvent the blockade of fusion between Pfs230 D1 and Pfs48/45 D3, resulting in an uninterrupted gametocyte fertilization process.

The novel missense polymorphism C327G on Pfs48/45 D3 has the potential to be very important because it can interfere with one of the six cysteine residue pairings (pairing between codon C298 and C327) on the 85RF45.1 mAb epitope (Kundu *et al.,* 2018). Other polymorphisms (Y251Y, K253E, N254K in Pfs48/45 D2 and V304D, L314I in Pfs48/45 D3) have been observed in *P. falciparum* populations in other malaria endemic regions (Conway *et al.,* 2001; S. Jones *et al.,* 2015b; Kundu *et al.,* 2018). However, none of these polymorphisms had been previously reported by a study conducted in the Asembo Bay area of western Kenya (Escalante *et al.,* 1998). Though not the focus of this study, polymorphisms on codon 254 is thought to influence the type of host antibody that binds at the epitope bearing this mutation on Pfs48/45 antigen (Kocken *et al.,* 1995). The three polymorphic codons Y251Y, K253E, and N254K on Pfs48/45 D2, are close to the disulphide loop that stabilizes the epitope binding TB 4F12 mAb on Pfs230 D1, thus suggesting steric interference from the antibodies. *Pfs48/45* domain 3 is highly conserved, with low nucleotide and haplotype diversity when compared to *Pfs230* D1. The key polymorphism based on this domain was L314I, which has a higher allelic frequency in epidemic prone zone of Kisii highlands than in endemic zone of Homa Bay and Kisumu. Despite the presence of a high frequency of rare alleles, the majority of parasites lacked polymorphic loci on *Pfs48/45* D3.

Inbreeding, recombination, and natural selection were identified as major drivers of the observed mutations in *Pfs230* D1 and *Pfs48/45* D3. The presence of linkage disequilibrium confirmed the history of selection pressure and inbreeding across various loci in *Pfs230* D1 and *Pfs48/45* D3 (Larrañaga *et al.,* 2013). Some polymorphisms were considered intermediary

because they had negative linkage disequilibrium (D') values (Silvela *et al.*, 1999). The negative D' values also confirmed a history of random drift, which is decreasing the number of variants while increasing homozygosity that may play a role in the parasite's loss of favourable mutations if it persists (Barton, 2010).

The presence of natural selection was confirmed by the Tajima's *D* values. Overall negative Tajima's *D* results revealed that purifying selection was affecting the majority of loci within *Pfs230* D1 and *Pfs48/45* D3, reducing genetic diversity (Cvijović *et al.*, 2018). The aforementioned selection was, however, weak because the computed negative Tajima's *D* values in both antigens were not significant. Individual Tajima's *D* results for each codon revealed all other dimorphic codons to be under purifying selection, with the exception of K661N on Pfs230 D1 from all study sites and V304D on Pfs48/45 D3 from Kisii, which are under strong and weak balancing selection, respectively. The two mutated loci under balancing selection may play an important role within the Pfs230 D1 and Pfs48/45 D3 fusion proteins, which may explain why they are maintained in the *P. falciparum* population from western Kenya (Escalante *et al.*, 1998). These findings support previous postulation (Jones *et al.*, 2015b) that selection pressure is acting on immunogenic domains of Pfs48/45.

The presence of weak purifying selection acting on dimorphic sites may impact not only host mAb binding and functional activity but also be affected by selective pressure in the mosquito vector (Lombardo & Christophides, 2016). This pressure could be exerted on individual antigens before or after complex formation. Findings in the present study support future investigations that examine functional antibody responses such as the ability of PfS230 and Pfs48/45 antibodies that activate human plasma complement and reduce mosquito infectivity in membrane feeding assays.

5.6 Conclusion

The *Pfs230* D1 and *Pfs48/45* D3 in *P. falciparum* from western Kenya have low nucleotide diversity and a high conservation index with high frequency of rare alleles. Among the observed polymorphisms in *Pfs230* D1, G605S is nearly fixed in the population. Natural selection, inbreeding, and, to some extent, recombination are important driving forces in shaping these alleles in the two antigens. With the discovery of novel polymorphic sites, the two domains of the *Pfs230* and *Pfs48/45* from different malaria-prone regions, including areas where clinical trials have been conducted, should be monitored indefinitely. This will help track the genetic stability of the two TBV candidates.

CHAPTER SIX: SIGNATURES OF SELECTION AND DRIVERS FOR NOVEL MUTATION ON TRANSMISSION-BLOCKING VACCINE CANDIDATE *Pfs25* GENE IN WESTERN KENYA

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https://pubmed.ncbi.nlm.nih.gov/35390042/

6.1 Abstract

Leading transmission-blocking vaccine candidates such as *Plasmodium falciparum* surface protein 25 (*Pfs25* gene) may undergo antigenic alterations which may render them ineffective or allele-specific. This study examines the level of genetic diversity, signature of selection and drivers of *Pfs25* polymorphisms of parasites population in regions of western Kenya with varying malaria transmission intensities. Dry blood spots (DBS) were collected in 2018 and 2019 from febrile outpatients with malaria at health facilities in malaria-endemic rural areas of Homa Bay, Kisumu and the epidemic-prone highland area of Kisii. Parasites DNA were extracted from DBS using Chelex method. Species identification was performed using real-time PCR. The 460 base pairs (domains 1-4) of the *Pfs25* were amplified and sequenced for a total of 180 *P. falciparum*-infected blood samples. Nine of ten polymorphic sites were identified for the first time. Overall, *Pfs25* exhibited low nucleotide diversity (0.04×10^{-2}) and low mutation frequencies (1.3% to 7.7%). Malaria endemic zone of Kisumu had the highest frequency (15.4%) of mutated sites followed by epidemic-prone zone of Kisii (6.7%), and Homa Bay (5.1%). Neutrality tests of *Pfs25* variations showed significant negative values of Tajima's *D* (-2.15, $p<0.01$) and Fu's F_S (-10.91, $p<0.001$) statistics tests. Three loci pairs (123, 372), (364, 428) and (390, 394) were detected to be under linkage disequilibrium and none had history of recombination. These results suggested that purifying selection and inbreeding might be the drivers of the observed variation in *Pfs25*. Given the low level of nucleotide diversity, it

is unlikely that a Pfs25 antigen-based vaccine would be affected by antigenic variations. However, continued monitoring of Pfs25 immunogenic domain 3 for possible variants that might impact vaccine antibody binding is warranted.

Keywords: *Plasmodium falciparum*, Transmission blocking vaccines, Pfs25, genetic diversity, signature of selection

6.2 Introduction

Over the past decade remarkable progress has been made in reducing the global malaria burden by coordinated public health interventions targeting both vectors and parasites (WHO, 2019). However, progress in malaria control has been stalled partly due to the spread of insecticide and antimalarial drug resistance (Alout *et al.,* 2017; WHO, 2019). One of the interventions needed to reduce the malaria burden is an effective *P. falciparum* vaccine (Ouattara & Laurens, 2015; Kaslow & Biernaux, 2015; Draper *et al.,* 2018). Barriers to advances in vaccine development include allelic variation of target antigens due to mutations in their immunodominant protein domains (Genton *et al.,* 2002; Takala *et al.,* 2007; Ogutu *et al.,* 2009; Bergmann-Leitner *et al.,* 2012; Miura *et al.,* 2013). Currently, there are attempts towards developing transmission-blocking vaccines (TBVs) that target *P. falciparum* sexual stage parasites (Ouattara & Laurens, 2015; Kengne-Ouafo *et al.,* 2019). TBVs elicit host antibody responses that block the sporogonic cycle of the parasite in the mosquito vector, thereby thus reducing and, ultimately, stopping ongoing transmission in local communities.

Among the various TBVs under development a vaccine based on the Pfs25 protein sequence is perhaps the most advanced (Scally *et al.,* 2017; Brod *et al.,* 2018; Chichester *et al.,* 2018; University of Oxford, 2020). The cysteine-rich 25 kilo-Dalton molecule is expressed in the protease-rich mosquito vector midgut post-fertilization, where it facilitates ookinete epithelium penetration, aggregation, and maturation to oocysts (Tomas *et al.,* 2001; Baton & Ranford-Cartwright, 2005). Protein expression of the Pfs25 protein is translationally repressed in the human host by the Pumilio/FBF family RNA-binding protein (Stowers *et al.,* 2000; Miao *et al.,* 2013). The Pfs25 protein consists of four tandem epidermal growth factor (EGF) domains with 22 cysteine residues that is anchored on the parasite surface by a C-terminal glycosylphosphatidylinositol (GPI) (Kaslow *et al.,* 1988). Recombinant Pfs25 antigen in combination with other TBV antigens (Brod *et al.,* 2018; Huang *et al.,* 2020) or alone have

been shown to elicit anti-Pfs25 antibodies (Kumar *et al.,* 2014; Lee *et al.,* 2016; Li *et al.,* 2016; Talaat *et al.,* 2016; McGuire *et al.,* 2017; Chichester *et al.,* 2018; McLeod *et al.,* 2019) that block oocyst development in standard membrane feeding assays. Notably, translational repression of Pfs25 protein expression in the human host tempers enthusiasm for field deployment of a TBV based on this antigen alone due to the absence of natural boosting in malaria endemic communities (Talaat *et al.,* 2016; McGuire *et al.,* 2017).

The *Pfs25* exon is considered to be highly conserved despite the existence of various sites with mutations (Escalante *et al.,* 1998; Patel *et al.,* 2017; Kaur *et al.,* 2017). Documented mutations include locus 392 in *P. falciparum* isolates from China and India and locus 428 of isolates from Cambodia and India (Juliano *et al.,* 2016; Patel *et al.,* 2017; Kaur *et al.,* 2017). The aim of this study was to determine the distribution of polymorphic sites, level of genetic diversity and identify possible signatures of selection in the four domains of the Pfs25 protein from *P. falciparum* parasites circulating in malaria-endemic and epidemic-prone regions of western Kenya.

6.3 Materials and Methods

6.3.1 Study site and sampling

This was a cross-sectional study based on clinical DBS collected in 2018 and 2019, as described in section 5.3. 1.

6.3.2 DNA extraction and *Plasmodium* speciation

Genomic DNA was extracted as described in section 3.3.3, transferred into a coded sterile 0.5ml Eppendorf tube and stored at -20°C.

6.3.3 Detection of *Plasmodium falciparum* infections

Plasmodium speciation was done as described in section 5.3.4.

6.3.4 *Pfs25* gene amplification and sequencing

A total of 180 *P. falciparum* infected samples (78, 40 and 62 from malaria endemic zones in Homa Bay, Kisumu, and malaria epidemic prone zone in Kisii, respectively) confirmed positive by RT-PCR were used to amplify the target *Pfs25* gene. Amplification of the 460bp exon was performed through nested PCR in T100™ Thermal Cycler (Bio-Rad) as described previously (Mlambo *et al.,* 2008). Briefly, for Nest I PCR, a final reaction volume of 13µL was prepared by addition of 3µL of parasite DNA to a mixture containing 5.75µL of DreamTaq Green PCR Master Mix (2X), 0.5µL of Nest I forward primer (10µM), 0.5µL of Nest I reverse primer (10µM) and 3.25µL of double-distilled water. For Nest II, a final reaction volume of 23µL was prepared by addition of 3µL of Nest I amplicon to a mixture containing 11.5µl of DreamTaq Green PCR Master Mix (2X), 0.5µL of Nest I forward primer (10µM), 0.5µL of Nest I reverse primer (10µM) and 7.5µL of double-distilled water. Nested I and II PCR conditions were set as follows: initial denaturation at 94°C for 3 minutes, 34 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, primer extension at 68°C for 1 min and final extension at 72°C for 6 min. PCR amplification was performed on T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The quality of the 460bp amplicons (**Appendix 9**) from Nested II PCR were assessed by performing gel electrophoresis in 1.5% w/v agarose gel after amplification and before sequencing. Each of the 180 PCR products was purified by the addition of Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-IT) and incubated at 37°C for 15 min. The ExoSAP-IT in purified PCR products was inactivated by heating at 80°C for 15 min. The cleaned PCR products with quantity above 25ng/µl were selected for sequencing after testing 1µl of each using NanoDrop™ Lite Spectrophotometer

(Thermo Scientific™). Bi-directionally sequencing was done using BigDye® Terminator v3.1 Sequencing Standard kit on ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Repeat sequencing was done on samples with poor reads to confirm mutated sites. Three amplicons were excluded from further analysis: one from the DNA template of a *P. falciparum* isolate from malaria endemic zone of Kisumu and two from epidemic-prone zone of Kisii.

6.3.5 Ethics approval

Ethical approval was obtained as described in section 3.3.6.

6.3.6 Data analysis

The sequence assembly was done using Geneious version 11.1.5 software. Multiple sequence alignment was done using ClustalW, a matrix-based algorithm that is in build in Mega X software (Kumar *et al.,* 2018). Segregating sites, mutated codons, and type of mutation were determined by inputting sequences from each region and reference sequence NF54 (Accession number X07802.1) in CodonCode Aligner version 9.0.1 (CodonCode Corporation, www.codoncode.com). The PHASE function of DnaSP Version 6.12.03 was used to infer the haplotypes of mixed infections. The Pfs25 antigen structural delineation was done by inputting full-length sequence (X07802.1) on Protein Homology/analogY Recognition Engine (PHYRE2) version 2.0. The generated Pfs25 model by Phyre2 was visualized, modified and mutated sites marked in UCSF Chimera version 1.15 (Pettersen *et al.,* 2004). The genetic diversity indices; nucleotide diversity (π) (Nei, 1997), the mean number of pairwise difference (k) (Tajima, 1989), number of segregating sites (S), number of haplotypes (h), haplotype diversity (*Hd*) were computed per region from the aligned sequences using DnaSP software (Rozas *et al.,* 2017), and results confirmed using Arlequin version 3.5.2 (Excoffier & Lischer, 2010). Haplotype data generated from DnaSP in nexus format with slight modification was

used to draw a haplotype network using Population Analysis with Reticulate Trees (Popart) version 1.7 software (Clement *et al.,* 2000). The ratio of nonsynonymous to synonymous mutations (d_N/d_S) was computed to give a hint on selection pressure acting on Pfs25 antigen from this region (Yang & Nielsen, 1998; Suzuki & Gojobori, 1999; Koonin & Rogozin, 2003; Yang *et al.,* 2005). Presence and type of natural selection or deviation from the standard neutral model were determined through computation of neutrality tests such Tajima's *D* (Tajima, 1989), Fu and Li's *D*, Fu and Li's *F* and Fu's *Fs* statistics (Fu & Li, 1993). The output of Tajima's *D* values was exported to GraphPad version 8.3.0 and used to generate the Tajima's *D* curve for both regions. The negative values from these tests correspond to a purifying natural selection whereas positive values are a representation of positive selection. Other factors or drivers sustaining or spreading the observed mutations in parasite population such as recombination and inbreeding were confirmed by detecting the presence or absence of Recombination events (Rm) and Linkage disequilibrium (LD) were confirmed using DnaSP software.

6.4 Results

6.4.1 Distribution of polymorphisms in four domains of Pfs25 antigen

A total of 10 segregating or polymorphic sites (123, 124, 249, 330, 345, 364, 372, 390, 394 and 428) were identified across the 460bp of *Pfs25* gene from *P. falciparum* isolates that were in circulation in western Kenya (**Appendix 10**). Malaria endemic zone of Homa Bay had 3/78 (3.8%) mutated sequences, Kisumu had 3/39 (7.7%), and Kisii had 3/60 (5%). The polymorphic sites were observed at a frequency of 7.9% (14/177) among sampled parasites (**Table 6.1**). The distribution of these polymorphic sites per study area was as follows: Homa Bay 5.1% (4/78), Kisumu 15.4% (6/39) and epidemic-prone zone of Kisii 6.7% (4/60). Most (80%) of the polymorphic sites (loci 123, 124, 249, 330, 345, 364, 372 and 428) were singletons

or single nucleotide polymorphs (SNPs) whereas others (loci 390 and 394) were parsimony informative as they were observed in more than one sample. Transition and transversion equally contributed to the observed mutations across the 10 segregating sites (**Table 6.1**). The base substitution resulted in more (60%) synonymous amino acid or codon changes compared to nonsynonymous. Nonsynonymous changes in *Pfs25* sequences from Homa Bay (endemic zone) resulted in codon change L42M (Leucine to Methionine), whereas synonymous changes included H41H (Histidine to Histidine), C110C (Cysteine to Cysteine), and T142T (Threonine to Threonine). Each of these changes occurred at a 1.3% frequency, with two (H41H and T124T) occurring on the same sequence. On polymorphic sequences in malaria endemic zone of Kisumu, synonymous T130T (Threonine to Threonine) and nonsynonymous mutation V132I (Valine to Isoleucine) occurred as a pair at a frequency of 7.7% each. Kisii parasites had codon changes I83I (Isoleucine to Isoleucine), C115W (Cysteine to Tryptophan), L122L (Leucine to Leucine), and V143G (Valine to Glycine), which were observed at a frequency of 1.61% each (**Table 6.1**). Polymorphic codons L122L and V143G occurred on a single sequence.

Table 6.1 Polymorphic sites in Pfs25 sequences from Homa Bay, Kisumu and Kisii region in western Kenya.

Segregating sites	Domain	Allelic frequency			Substituted bases	Type of Substitution	Codon change	Type of mutation
		Homa Bay n (%)	Kisumu n (%)	Kisii n (%)				
123	D1	1 (1.3)	-	-	T/C	Transition	H41H	Syn
124	D1	1 (1.3)	-	-	T/A	Transversion	L42M	Nsyn
249	D2	-	-	1(1.7)	A/T	Transversion	I83I	Syn
330	D3	1 (1.3)	-	-	T/C	Transition	C110C	Syn
345	D3	-	-	1(1.7)	T/G	Transversion	C115W	Nsyn
364	D3			1(1.7)	T/C	Transition	L122L	Syn
372	D3	1 (1.3)			A/G	Transition	T124T	Syn
390	D3	-	3 (7.7)	-	T/A	Transversion	T130T	Syn
394	D3	-	3 (7.7)	-	G/A	Transition	V132I	Nsyn
428	D3	-	-	1(1.7)	T/G	Transversion	V143G	Nsyn

n represents the proportion of sequences per study site with polymorphic sites.

Out of the mutated 10 codons in Pfs25 antigen, 7 (330, 345, 364, 372, 390, 394 and 428) were present in D3 (**Fig. 6.1**). Domain 2 had 1 (I83) mutated codon whereas D1 had two mutated codons (H41 and L42) which were close to D3 codons. In D3, mutated codon C110, L122, T130 and V132 were within structural proximity.

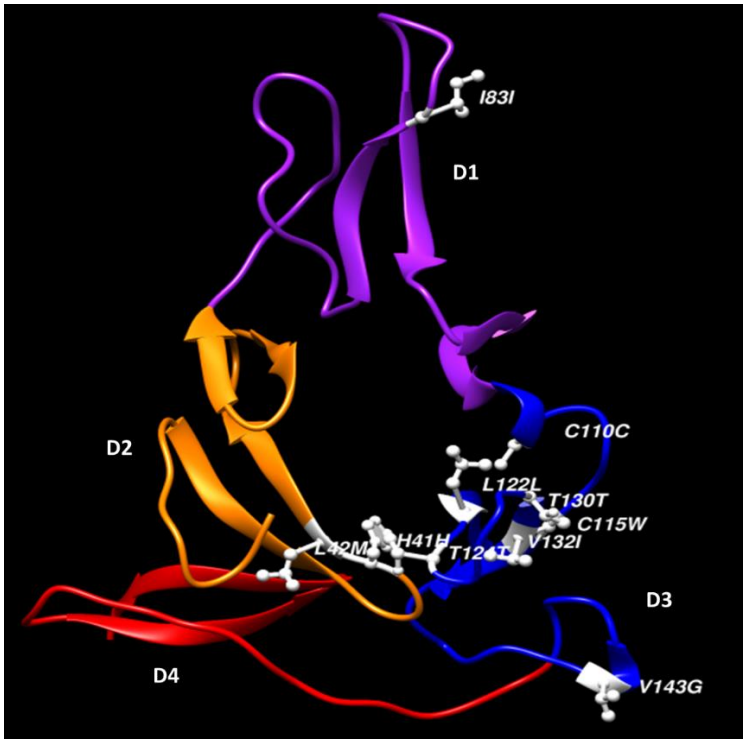

Figure 6.1 Predicted structure of Pfs25 antigen and distribution of polymorphic codons on its domain 1, 2 and 3. Beta strands (β) and loops in orange represent D1; D2 is in purple β-strands and loops; D3 is in blue β-strands and loops whereas D4 is represented by red β-strands and loops. The white balls and sticks represent codons with mutations in the Pfs25 antigen (Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311).

6.4.2 Genetic diversity indices of *Pfs25* gene

Relatively low nucleotide diversity (π) was observed among the sequences analysed. The 177 Pfs25 sequences had π of 0.04×10^{-2}, k of 0.16 and a nucleotide conservation index of 97.7% that slightly varied per study site (**Table 6.2**). Kisumu isolates had the highest observed π (0.07×10^{-2}) and *Hd* (0.15) followed by Kisii (π=0.03 and *Hd*=0.10) then Homa Bay (π=0.02 and *Hd*=0.08). Parasite from malaria endemic zone of Homa Bay, Kisumu and epidemic prone

zone of Kisii had a Fst value of 0.03 and number of migrants (Nm) of 8. There was no significant difference (p=0.400) when the population pairwise FSTs were computed for Homa Bay and Kisii sequences (FST=0.00). Similar results (p=0.160) were observed for population differences between Kisumu and Kisii (FST=0.037). No significant difference (p=0.110) in observed allelic variation between Homa Bay and Kisumu sequences (FST=0.039). The highest diversity index in malaria endemic zone of Kisumu was due to a high frequency of T130T and V132I codon changes (**Table 6.3**). Homa Bay zone and Kisii had the highest number of Pfs25 haplotypes (four each) compared to Kisumu (**Fig. 6.2**). Among analysed samples from Kisumu, blood samples of patients from the same household were found to harbour different haplotypes. Six blood samples, 3 from each different household had either Hap1 with no mutated sites or Hap5 with both mutated codon T130T and V132I. This was however not observed in sequences from malaria endemic zone of Homa Bay and epidemic-prone zone of Kisii.

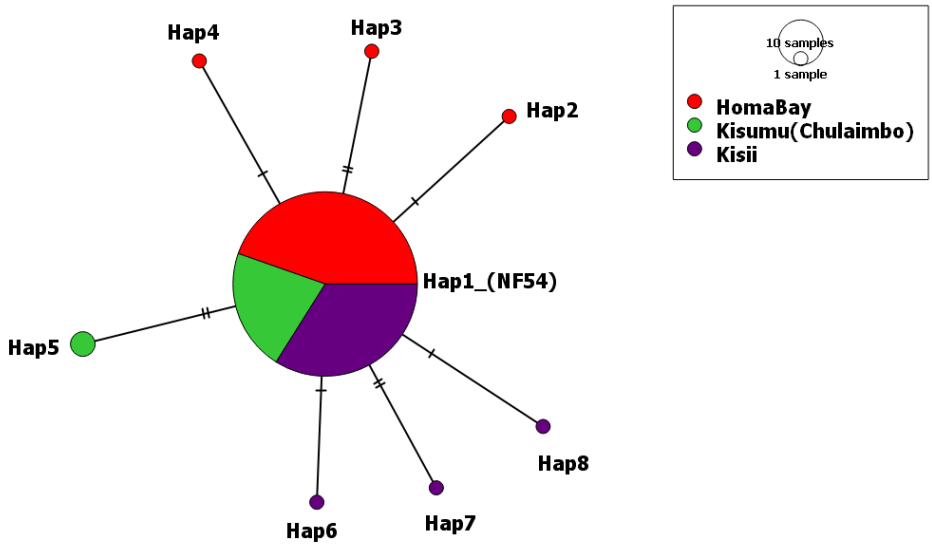

Figure 6.2 TCS-network analysis of the relationship of Pfs25 haplotypes in malaria-endemic and epidemic-prone regions of western Kenya. The network shows the distribution of haplotype within malaria-endemic lowlands of Homa Bay (red) and Kisumu (Chulaimbo) (green) as well as Kisii highlands (purple). The hatch marks represent the number of mutations (double hatch marks equate to two polymorphic sites) whereas the size of the circle equate to the relative frequency of haplotypes. Hap 1 (lacked mutated site, identical with Pfs25 reference sequence of NF54 strain or 3D7 strain), Hap2 (had codon change L42M), Hap3 (H41H and T124T), Hap4 (C110C), Hap5 (T130T and V132I), Hap6 (I83I), Hap7 (C115W) and Hap8 (L122L, V143G).

Table 6.2 Summary of genetic diversity indices of Pfs25 gene in western Kenya.

Study site	N	C (%)	S	π ($\times 10^{-2}$)	H	*Hd*	Ks or d_S ($\times 10^{-2}$)	Ka or d_N ($\times 10^{-2}$)	d_N/d_S	Tajima's *D*	Fu's F_S
Homa Bay	78	99.10	4	0.02	4	0.08	0.09	0.01	12.29	-1.81*	-4.42 *
Kisumu	39	99.50	2	0.07	2	0.15	0.16	0.04	3.93	-0.73	0.80
Kisii	60	99.10	4	0.03	4	0.10	0.07	0.02	3.89	-1.84*	-3.90*
All sites	177	97.70	10	0.04	8	0.10	0.10	0.02	5.37	-2.15*	-10.91*

N: Sample size; C: Conservation index; S: Segregating sites; π: nucleotide diversity; H: haplotype; *Hd*: Haplotype diversity; Ks or d_s: The number of synonymous (or silent) substitutions per synonymous (or silent) site by Jukes and Cantor; Ka or d_N: The number of nonsynonymous substitutions per nonsynonymous site by Jukes and Cantor; d_N/d_S: Ratio of nonsynonymous and synonymous substitutions, *: Significance ($p<0.05$).

6.4.3 Signature of selection and other mutation drivers on *Pfs25* gene

The computed ratio of nonsynonymous and synonymous substitutions (d_N/d_S) for all Pfs25 sequences from western Kenya was 5.37 (**Table 6.2**). The d_N/d_S ratio was not only greater than 1 across all sequences but also in each of the three study sites. Neutrality test results (Tajima's *D*) for all the Pfs25 sequences were negative, thus signifying the presence of natural selection. The Tajima's *D* value was -2.12 ($p<0.05$), Fu and Li's *D** test statistic, FLD*: -4.78 ($p<0.02$), Fu and Li's *F** test statistic, FLF*: -4.58 ($p<0.02$) and Fu's F_S statistic: -10.91 ($p<0.001$). A similar trend was observed on Tajima's *D*: -1.81 ($p<0.05$)), and Fu's F_S: -4.42 ($p=0.011$) values of Pfs25 sequences from Homa Bay. Other test results FLD*: -3.72 and FLF*: -3.50 were also significant ($p<0.05$). The selection pressure exacted its effect on loci existing between 98-148bp and 323-373bp in Pfs25 sequences from malaria endemic zone of Homa Bay (**Fig 6.3**). These loci harbour the observed segregating sites in Homa Bay sequences. Among sequences from parasites in malaria endemic zone of Kisumu, Tajima's *D* and Fu's F_S were -0.73 and 0.8, respectively. All these test results were not significant ($p>0.05$), with FLD* and FLF* values of 0.77 and 0.37 respectively. Deviation from the standard neutral model among the Kisumu

samples was observed on loci existing between 348-423bp where segregating sites were observed (**Fig. 6.3**). Sequences from Kisii had significant (p<0.05) Tajima's *D* (-1.84) and Fu's F_S (-3.9) values. The FLD* and FLF* results were -3.54 (p<0.02) and -3.52 (p<0.02) respectively. Purging selection on loci blocks ranging from 223-469bp was observed for these sequences (**Fig. 6.3**).

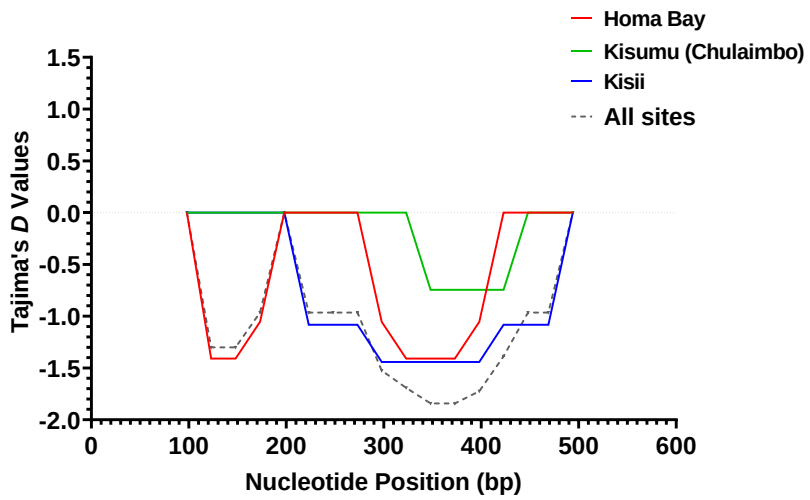

Figure 6.3 Sliding window plot of Tajima's *D* values for *Pfs25* gene in western Kenya. The X-axis displays the nucleotide position (Window midpoint) whereas the Tajima's *D* values are represented on the Y-axis. The red curve represents computed Tajima's *D* value for Pfs25 sequences from *P. falciparum* circulating in malaria endemic zone of Homa Bay, the green colour is for Kisumu zone, the blue colour is for Kisii zone whereas the black dotted colour represents the population from the three study sites. The middle horizontal dotted line (intersecting the Y-axis at 0.0) represents a standard neutral model where the Tajima's *D* value is equal to zero. Positive deviation from the grey dotted line signifies balancing selection whereas negative deviation represents purifying selection.

To further assess the presence or history of drivers shaping allelic patterns within the four domains of the Pfs25 antigen, we assessed a pair of loci that were under LD as well as

having a history of recombination events (Rm). Three pairs of loci (123, 372), (364, 428) and (390, 394) within polymorphic Pfs25 sequences were observed to be under LD (**Table 6.3**). The three loci pairs had highly significant (P<0.001) LD values. The presence of significant LD values is an indicator of inbreeding as a driver for the observed sequence changes in each study site. None of mutated Pfs25 sequences from western Kenya had a history of recombination.

Table 6.3 Pfs25 haplotypes frequencies across Homa Bay, Kisumu and Kisii regions.

Distribution of Pfs25 haplotypes					
Homa Bay					
Haplotype	**Codon Change**	**Number of Sequences**	**Haplotype Frequencies (%)**	**LD χ^2 (Loci)**	**r^2**
Hap1	0	75	96.15		
Hap2	L42M	1	1.28	79.00^{***B}	0.02×10^{-2}
Hap3	H41H, T124T	1	1.28	(Locus 123 and 372)	
Hap4	C110C	1	1.28		
Kisumu					
Hap1	0	36	92.31	40.00^{***B}	1
Hap5	T130T, V132I	3	7.69	(Locus 390, 394)	
Kisii					
Hap1	0	57	95.00		
Hap6	I83I	1	1.67	61.00^{***B}	1
Hap7	C115W	1	1.67	(Locus 364 and 428)	
Hap8	L122L, V143G	1	1.67		

LD: Linkage disequilibrium; χ^2: chi-square test; ^{*B}: Significant by the Bonferroni procedure; Rm: Minimum number of recombination events.

6.5 Discussion

This study revealed novel polymorphic sites across the four immuno-dominant domains of the Pfs25 antigen. Most of these sites were dimorphic sites and involved Pfs25 D3. They resulted in 9 haplotypes that were unique ("private") not only to malaria endemic zone of Homa Bay, Kisumu or epidemic prone zone of Kisii highlands but also to western Kenya. The *Pfs25* gene

exhibited low nucleotide diversity and a high conservation index. Natural selection was identified to be the leading cause of these mutations with purifying selection purging on specific loci blocks. Additionally, inbreeding was established as an alternative driver for linked polymorphic loci in analysed sequences from each study site. There was high level of interbreeding in *P. falciparum* population from Homa Bay, Kisumu and Kisii zone.

Generally, low nucleotide diversity was observed in Pfs25 sequences from parasites population in western Kenya compared to Asia (0.11×10^{-2}), Thailand (0.09×10^{-2}), Cambodia (0.12×10^{-2}), India (0.31×10^{-2}), Brazil (0.18×10^{-2}) and Vietnam (0.26×10^{-2}) (Sookpongthai *et al.,* 2021). Kisumu parasites sequences had high nucleotide variation compared to sequences from rural areas of Homa Bay and Kisii. The observed variation between the two malaria-endemic sites and a close similarity between Homa Bay and Kisii (epidemic) implies that ongoing indoor residual spraying targeting malaria vectors within Homa Bay (President's Malaria Initiative, 2021) may be directly or indirectly reducing the numbers of parasite clones or genetic diversity. Despite having high number of haplotypes across western Kenya, their observed number was within the set limit of the total number of segregating plus one (S+1), thus implying the population is under selective sweep or growth (Fu & Li, 1993; Depaulis & Veuille, 1998).

Apart from the dimorphic site 428 (codon V143) which had previously been reported in Pfs25 sequences from Cambodia (Juliano *et al.,* 2016; Sookpongthai *et al.,* 2021), India (Juliano *et al.,* 2016; Patel *et al.,* 2017; Sookpongthai *et al.,* 2021) and now in Kisii highland, the remaining nine sites were novel. The amino acid change observed in this codon (V143G), however, did not correspond to documented V143A mutation following differences in base substitution at locus 428 (T/G as opposed to T/C) of the gene in one of the sequences from the Kisii area. None of the sequences from western Kenya had G131A mutations that had been

previously observed in the parasite population from India and Cambodia (Juliano *et al.*, 2016). However, three sequences from Kisumu study sites had mutations at a nearby codon V132I, and was observed at a frequency of 7.7%. Compared to its analogue in *Plasmodium vivax*, two mutated codons T130 and V132 identified in Pfs25 had been described in Pv25 in Asia (Feng *et al.*, 2011; Lê *et al.*, 2019).

The majority of the polymorphic sites we discovered were in the *Pfs25* D3 region. This domain had previously been described to be highly immunogenic compared to others, and is also known for harbouring binding sites of 1D2 and 4B7 human monoclonal antibodies used in standard membrane feeding assay studies of *P. falciparum* mosquito infectivity (Scally *et al.*, 2017; McGuire *et al.*, 2017; Patel & Tolia, 2021). Among mutated codons in D3, codon change C110C and C115W targeted two of the 21 cysteine residues that are known to be highly conserved and function in maintaining the folding pattern of the Pfs25 polypeptide (Duffy & Kaslow, 1997; Juliano *et al.*, 2016; Scally *et al.*, 2017). The presence of such silent and nonsynonymous mutation points to the possibility of a weak or strong selection pressure acting on nearby codons within the Pfs25 antigen (Lawrie *et al.*, 2013). If pronounced, such mutation may interfere with the folding pattern of the Pfs25 antigen, and thereby impact interaction between host antibodies elicited by Pfs25 TBV and modify interactions with other B-cell epitopes as observed with other proteins and cysteine residues in the D2 region of Pfs47 (Canepa *et al.*, 2018).

As opposed to previous reports describing the absence of selective pressure acting on the *Pfs25* gene (Escalante *et al.*, 1998), our studies suggest that natural selection may play a role in shaping allelic diversity of the *Pfs25* in western Kenya. Specifically, purifying selection was suggested based on computation of the ratio of nonsynonymous and synonymous substitutions (Yang & Nielsen, 1998; Suzuki & Gojobori, 1999; Koonin & Rogozin, 2003;

Yang *et al.,* 2005). This was evident across all mutated codon since they displayed a significant Tajima's *D* value. The loci pairs under LD not only reaffirmed the history of selection but also confirmed inbreeding as another factor in the spread and sustenance of such mutations (Larrañaga *et al.,* 2013). We speculate that selection pressure may be arising from unknown factor in protease-rich midgut of female Anopheline vectors.

6.6 Conclusion

The *Pfs25* gene from the malaria-endemic and epidemic-prone region of western Kenya revealed varying levels of genetic diversity and novel haplotypes. Purifying selection and inbreeding were predicted as the cause and drivers for the observed variations. The low level of nucleotide diversity is an indicator that TBV based on Pfs25 sequences is less likely to be affected by antigenic variations. However, this study recommends continued monitoring of the *Pfs25* gene from different malaria-prone regions including areas where clinical trials have been conducted. This will not only aid in unravelling new polymorphic sites that could have an effect on antibody binding especially on immunogenic D3 but also monitor the durability of the *Pfs25* gene as a potential TBV.

CHAPTER SEVEN: GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 General conclusion

The goal of this study was to look for polymorphisms with functional significance on the immunodominant domains of Pfs25, Pfs230, and Pfs48/45 antigens in *P. falciparum* populations from different rural parts of western Kenya with varying transmission intensities. In addition, the study sought to determine the prevalence of clinical microscopic and submicroscopic infections in a selected (malaria endemic and epidemic prone zone) study area. The study found that in epidemic-prone areas, females sought treatment at health facilities more than males. Males and children under the age of five had the highest positivity rates. The transmission pattern in this area was found to be local and focalized in Morara and Nyabikondo villages in Rioma and Kiomooncha sublocations, respectively. Due to their proximity to Homa Bay County, the study hypothesizes that infections in these two sublocations may be influenced by the endemic zone. As a result, the two sublocations could serve as a reference point for future vectorial intervention or parasite elimination strategies. Submicroscopic infections were low in comparison to endemic areas with declining *P. falciparum* infection cases. This type of infection was mostly seen in adults and female patients, and it could only be traced back to the previously mentioned two sublocations with a high number of malaria cases. Kiomooncha sublocation had a diverse parasite composition, with mixed infections of *P. falciparum*/*P. malariae* and *P. falciparum*/*P. ovale*.

Similarly, to the observation made in Marani subcounty (epidemic prone zone), females were more likely to seek malaria treatment, whereas males were more likely to be diagnosed with microscopic infections at Ngegu health facility in Kochia location (endemic). Microscopic

P. falciparum infections and parasite densities were highest in children aged 5-15 years. The observed high prevalence in males and children aged 5-15 years was linked to the two groups' low use of LLIN. Furthermore, participation in nocturnal outdoor activities was thought to be a risk factor for the observed high prevalence of microscopic infections in males, the majority of whom were found engaging in such activities. However, this was debunked after it was discovered that staying indoors had a slightly higher chance of getting microscopic infections than participating in nocturnal outdoor activities. Furthermore, with low levels of microscopic infections among LLIN users participating in nocturnal outdoor activities, the study demonstrated that transmission in Kochia was primarily indoor and may have occurred at the point of transition to an area covered by LLINs. Outdoor transmission is low in the Kochia location if it exists.

A large number of clinically ill patients with submicroscopic infections were misdiagnosed and went untreated. This is expected to have a significant impact on malaria control programs currently underway in the study area. Individuals with undiagnosed and untreated *P. falciparum* infections may act as reservoirs of *P. falciparum* gametocytes in the future, sustaining transmission in the community. As seen in Marani (epidemic), female patients had a higher chance of having submicroscopic infections than males. This was also seen in adults across all three age groups. Furthermore, LLIN use was a risk factor for submicroscopic infections, with blood samples from patients who used LLINs being more likely to test positive for this type of infection. As a result, increased malaria intervention has been shown to increase submicroscopic infections by reducing human-vector contact, which influences further infection dynamics. This was supported by the high likelihood of detecting these types of infections in blood samples from patients who engage in nocturnal outdoor activities and use LLINs indoors. The majority of infected patients were traced to Kaura, which

is located near the shore of Lake Victoria. The proximity to the lake was thought to be a potential confounding factor for infections.

Due to declining *P. falciparum* infections in malaria endemic zone of Homa Bay, genetic analysis of immunodominant D1 of Pfs230, a gametocyte pre-fertilization antigen, revealed that parasites from this region had the lowest nucleotide diversity compared to Kisii (epidemic) and Kisumu (endemic with no implementation of IRS). In general, the domain was highly conserved among western Kenya parasites, with six dimorphic codons discovered (I539T, G605S, T652R, E655V, T656N, and K661N). Among them, I539T dimorphism was unique to the Kisumu study site, which also had the highest genetic diversity index, which was attributed to high malaria transmission. Among the five polymorphic codons, G605S was close to or fixed in the Kisii (Marani) region. This indicated the presence of selection pressure or genetic drift, which reduces variation and thus increases homozygosity. In contrast to the observed similarity in occurrence pattern on dimorphic codon G605 in the three regions, the high frequency of dimorphic codon K661 in Kisumu was linked to a recombination event that was detected only in this zone and is known to increase diversity.

According to this study, the polymorphic codon G605 is of great immunological functional importance and is thought to be under selection pressure that is targeting the immunogenic epitope-binding light chain of transmission-blocking 4F12 monoclonal antibodies (TB 4F12 mAb). Because it is part of the disulphide loop, this codon plays a stabilizing role on the epitope. The selection pressure surrounding this epitope is thought to be the driving force behind the dimorphic codons T652R and K661N (on pleated sheets) as well as E655V and T656N (on disulphide loops). Other mutated codons discovered near 3G2 and 5G3 mAb binding epitopes include the novel dimorphic codon I539T. The aggregation of polymorphic codons on Pfs230 D1 and Pfs48/45 D3 (codons V304D, L314I, and C327G) at

the same location within a superimposed structure (fusion protein) supported the hypothesis of protein-protein steric interference by antibodies. The parasite was thought to play a role in inducing these mutations in order to circumvent gametocyte fertilization process.

Despite having low nucleotide diversity and high conservation index when compared to *Pfs230* D1, the novel missense C327G on Pfs48/45 D3 interfered with one of the structurally important cysteine residues. The residue stabilizes the epitope binding 85RF45.1 mAb. Furthermore, three observed mutated codons (Y251Y, K253E, and N254K) on Pfs48/45 D2 were found to be structurally close to the disulphide loop that stabilizes the TB 4F12 mAb binding epitope on Pfs230 D1 upon fusion. A total of 8 and 4 haplotypes based on Pfs230 D1 and Pfs48/45 D3 were confirmed to be circulating in western Kenya, respectively, based on these polymorphic sites. These mutations were primarily driven by inbreeding, recombination, and natural selection, with the forces acting selectively per transmission region. Purifying selection was identified as the primary selective force acting on Pfs230 D1 and Pfs48/45 D3. Notably, some dimorphic codons (K661N on Pfs230 D1 and V304D on Pfs48/45 D3) were under balancing selection, implying their importance and the reason they are maintained by parasites in western Kenya. The selection pressure mentioned herein could be from both host antibodies and the *Anopheles* immune system, and it could be acting prior to or after the fusion of the Pfs230 and Pfs48/45 antigens.

Post-fertility (Pfs25) antigens also had low nucleotide diversity and a high conservation index. Nine of the ten observed polymorphic sites were novel, with the majority occurring in Pfs25 antigen D3, indicating that the domain is highly immunogenic and under selection pressure. In Western Kenya, eight haplotypes were discovered, with the majority lacking polymorphic sites. Polymorphisms on codons C110C and C115W, which are known for maintaining the antigen's folding pattern, were hypothesized to interfere with interactions

between host antibodies and their respective epitopes due to antigen conformation changes. Purifying selection shaped alleles in Pfs25 and the pressure was suggested to be arising in the protease-rich midgut of female Anopheline vectors because the gene is not expressed in infected humans. Inbreeding spread the mutations throughout the population. Based on the sequences of the three genes (Pfs230 D1, Pfs48/45 D3, and Pfs25), there was no distinct population structure among the three study sites, indicating that there was a high level of geneflow among the population, as evidenced by a high number of parasite migrants across sites.

7.2 Recommendations

1. Given the high number of clinically positive cases in Kisii's Marani subcounty (epidemic prone region), the study recommends biannual community malaria surveillance, particularly in the five sublocations. This will not only reveal the key predisposing or determining factors for *P. falciparum* infections in these areas, but will also guide public health policy to aid in the prevention of a future epidemic.
2. To avoid plateauing and stalling of morbidity gains from available malaria intervention tools such as IRS, the high level of undetected and untreated submicroscopic infections should be reduced. As a result, the study recommends a public health intervention that will include ultrasensitive diagnostic techniques in the routine diagnosis of patient blood samples. If at all possible, the technique should be used to supplement microscopy.
3. With the near fixation of some of the mutations on immunodominant Pfs230 D3, an immunological assay targeting gametocytes harbouring these mutations is required for further MTBV assessment based on antigen sequences. Following that, a standard membrane feeding assay should be performed in which reared female *Anopheles* are

infected with gametocytes containing the three main polymorphisms together with specific human monoclonal antibodies targeting Pfs230 D1 epitopes.

4. Furthermore, polymorphisms on Pfs230 D1, Pfs48/45 D3, and Pfs25 should be monitored in malaria-prone areas as well as areas where clinical trials using Pfs230 D1, Pfs48/45 D3, and Pfs25 TBV candidates have been conducted.

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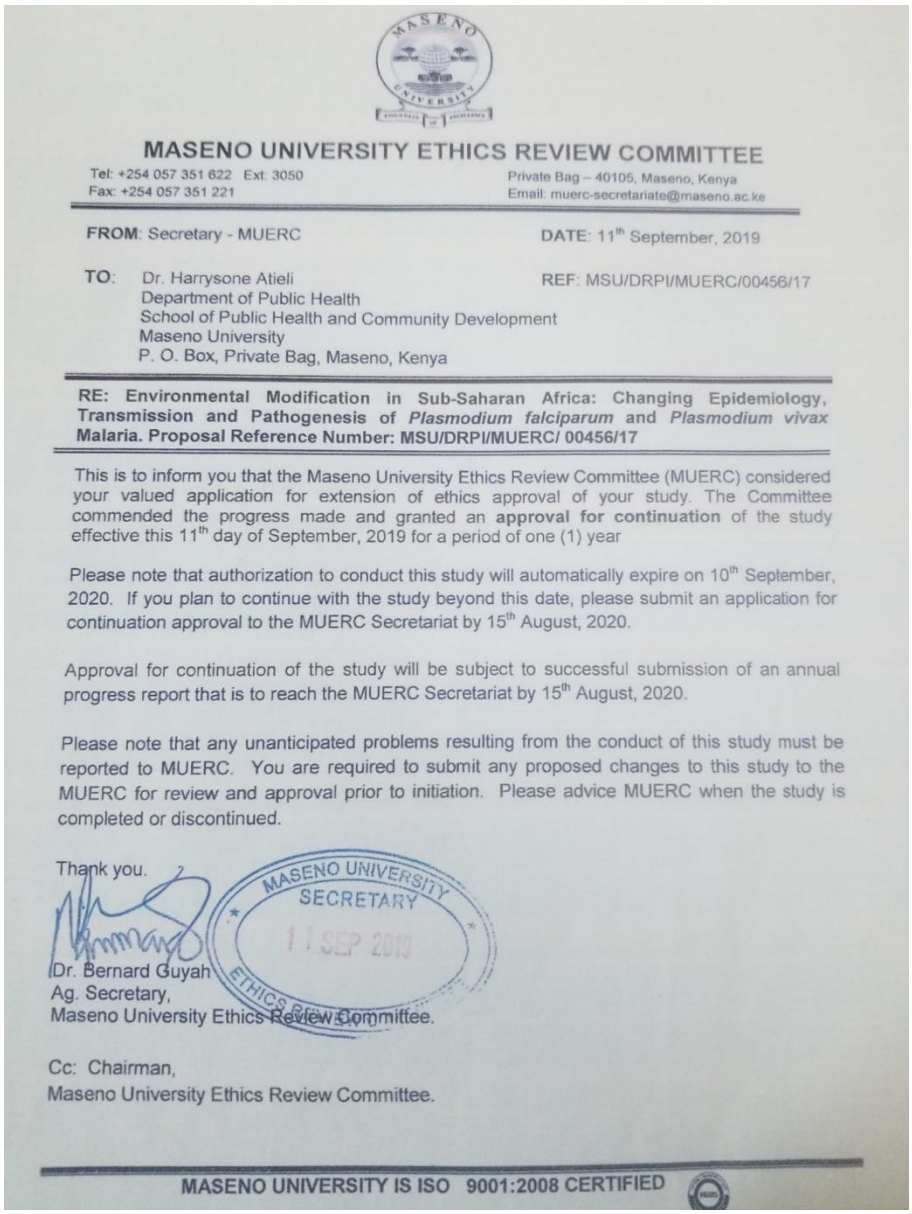
APPENDIX 1: ETHICAL APPROVAL LETTER



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 11th September, 2019

TO: Dr. Harrysone Atieli
Department of Public Health
School of Public Health and Community Development
Maseno University
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00456/17

RE: Environmental Modification in Sub-Saharan Africa: Changing Epidemiology, Transmission and Pathogenesis of *Plasmodium falciparum* and *Plasmodium vivax* Malaria. Proposal Reference Number: MSU/DRPI/MUERC/ 00456/17

This is to inform you that the Maseno University Ethics Review Committee (MUERC) considered your valued application for extension of ethics approval of your study. The Committee commended the progress made and granted an **approval for continuation** of the study effective this 11th day of September, 2019 for a period of one (1) year

Please note that authorization to conduct this study will automatically expire on 10th September, 2020. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 15th August, 2020.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 15th August, 2020.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to the MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

MASENO UNIVERSITY SECRETARY 11 SEP 2019 ETHICS REVIEW COMMITTEE

Dr. Bernard Guyah
Ag. Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

APPENDIX 2: CONSENT FORM

Functional Analysis Of Immune Response to *Plasmodium falciparum* Gametocytes Surface Antigens Pfs25, Pfs48/45 And Pfs230

This consent form will be explained and signed by each study participant

Name of Volunteer:_____

Age of Volunteer:_____

Principal Investigator: Kevin Omondi Ochwedo. **Supervisors:** Prof. Wolfgang R. Mukabana and Dr. Anthony C. Otieno. P.O. Box 30197-00100. Tel: +254714388914

The purpose of study
The purpose of this study determined polymorphisms with functional significance on the immunodominant domains of Pfs25, Pfs230, and Pfs48/45 antigens in *P. falciparum* populations from different rural parts of western Kenya with varying transmission intensities.

Procedures involved
Approximately 250ul will be collected by finger prick in heparin microtainer. 100ul of the withdrawn blood from participants will be used to prepare dried blood spots and 150ul will be used to prepare thick and thin smears/Antecubital venepuncture will be used to draw approximately 3.5 ml of blood from the participant's vein into a 5-millilitre red top vacutainer tube. 2ml will be used in serum replacement and whole blood for indirect mosquito membrane feeding assays to asses *Plasmodium* parasites infectivity to mosquitoes and presence of transmission blocking activities. All participants will have a unique identifier that links them to their laboratory results, demography and location. Samples will be analysed by microscopy and qPCR at the international centre of excellence for malaria research (ICEMR). unauthorised tests for the study will not be carried out on the blood samples.

Discomforts and risks
The antecubital venepuncture/finger prick procedure is slightly uncomfortable. For each individual, sterile needle and a syringe (followed by sterile ethanol) will be utilised. The operations will represent very minimal risk of infection by other infections.

Benefit to participants
Because enrolment is voluntary, you will receive no financial benefit from your participation.

Confidentiality
To the degree permitted by law, information about you will be kept strictly confidential. Your identity will be coded but not linked to any published results. Your code number and identity will be retained in the Principal Investigator's locked file.

Freedom to withdraw
Your participation in this study is voluntary and you may discontinue your participation at any time without prejudice and without affecting future health care.

Who to contact?
If you have questions about the study or your participation in this study, you may contact the investigators on the contact given above. For any questions pertaining to rights as a research

participant, contact person is: **The Secretary, Maseno University Ethics Review Committee, Private Bag, Maseno; Telephone numbers: 057-51622, 0722203411, 0721543976, 0733230878; Email address:** muerc-secretariate@maseno.ac.ke; muerc-secretariate@gmail.com

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I have read and understand this consent form, and I am willing to participate in the study.

Participant's Name	Participant's Signature (consent)	Date
Parent/Guardian's Name	Parent/Guardian's Signature (Assent)	Date
Investigator's Name (type or print)	Investigator's Signature	Date

APPENDIX 3: ETHICAL CLEARANCE FROM MINISTRY OF HEALTH

MINISTRY OF HEALTH

Telegrams: "MOH" Homa Bay
Telephone: 21039
When replying please quote

Homabaychc@gmail.com

Ref: MOH/CTY/GEN/VOL.III/302

MINISTRY OF HEALTH,
HOMA-BAY COUNTY
P.O. BOX 52
HOMABAY

9th January, 2018

To:

Dr. Harrysone Atieli, PhD
Project Manager, ICEMR,
Dear Sir/Madam,

RE: AUTHORITY TO CONDUCT MALARIA RESEARCH IN HOMABAY COUNTY

Following your request to conduct malaria research in Homa bay county for a study entitled '*Environmental Modifications in sub-Saharan Africa: Changing Epidemiology, Transmission and Pathogenesis of Plasmodium falciparum and P. vivax Malaria* ,' you are hereby authorized to proceed with the exercise for the duration and under the conditions permitted by the University of California, Irvine Institutional Review Board (UCI IRB) dated March 15, 2017 and the Maseno University Ethical Review Committee dated 11th September, 2017 ref, MSU/DRPI/MUERC/00456/17

You will be required to adhere to the hospitals norms and regulations during the data collection period. You are also expected to communicate your findings to the Directors' office at the end of the research.

Wish you all the best as you plan the study and am looking forward for future collaborations.

MINISTRY OF HEALTH
DIRECTOR OF MEDICAL SERVICES
09 JAN 2018
Sign.....
P.O. Box 52-40300 HOMA-BAY

Dr Gordon Okomo
County Director of Health
Homabay County

Cc: SC MOH – Homa Bay Township, Rangwe, Rachuonyo North,

APPENDIX 4: STUDY QUESTIONNAIRE

Malaria Research Questionnaire

Questionnaire on details and health status of study participants

Date……. Study Code…….

Place of resident (Village/Location) ……...……Occupation…….

Level of education…….....

Participant name…….……Sex…….……

Age…….…….

Participant's vitals

1. Body temperature…… Weight (Kg)……

a) Symptoms?

Fever (…....) Chills (……) Headache (……...) Vomiting (…....) Joint pains (……...) Running/stuffy nose (……...)

Other……

2. Do you have treated bed net in your house? **Yes** (…….) **No** (…….....)

a) Last night did you sleep under bed net? **Yes** (.....) **No** (.....)

b) How often do you sleep under bed net at night? **Every night** (.....) **Some nights** (.....) **Rarely** (.....)

3. Do you engage in activity outside your house past 1800 hours? **Yes** (.....) **No** (.....)

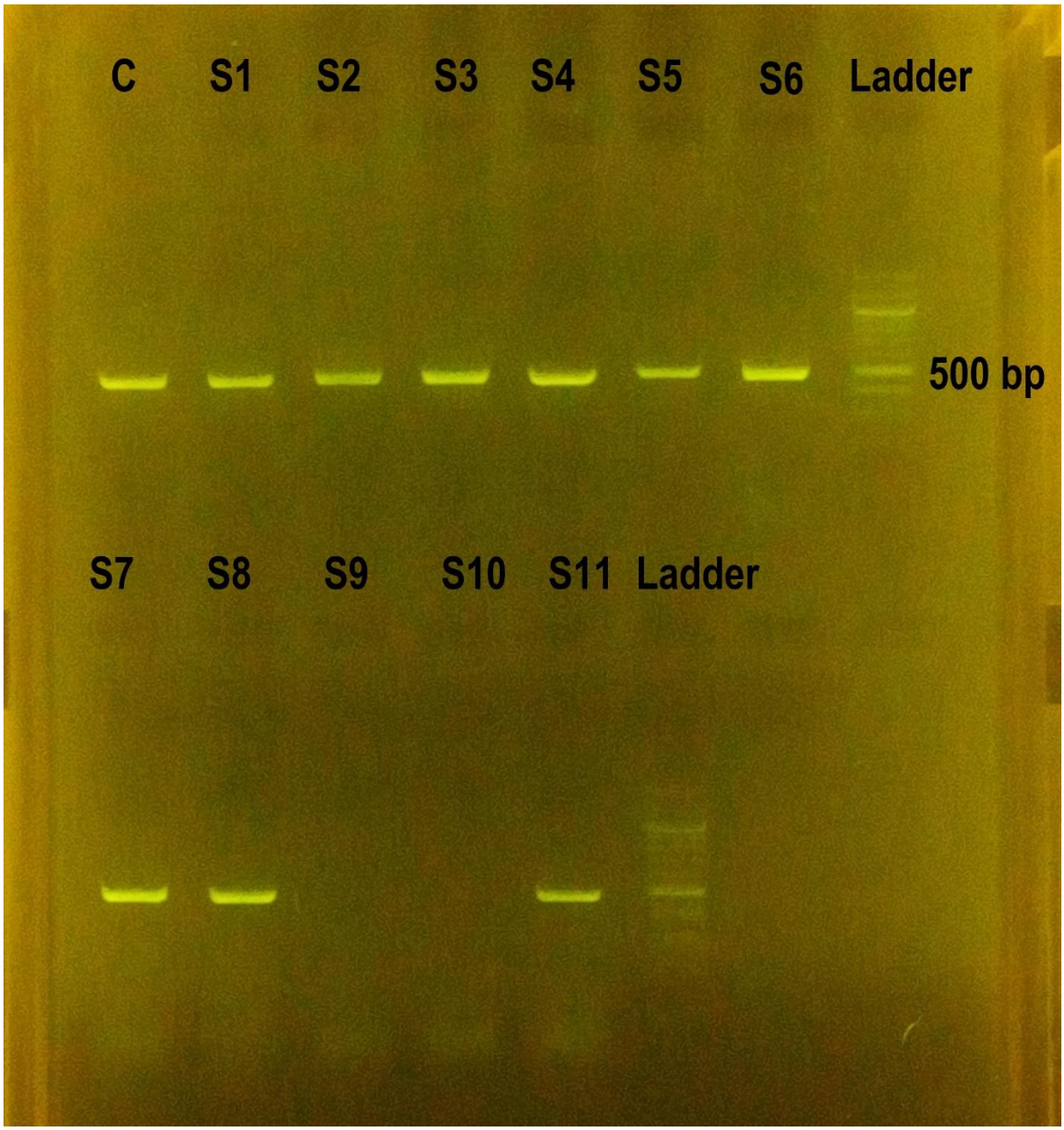
a) If yes at what time frame? **1800 h–2000 h** (.....) **2000 h–2300 h** (.....) **2300 h–0400 h** (.....)

0400 h–0600 h (.....)

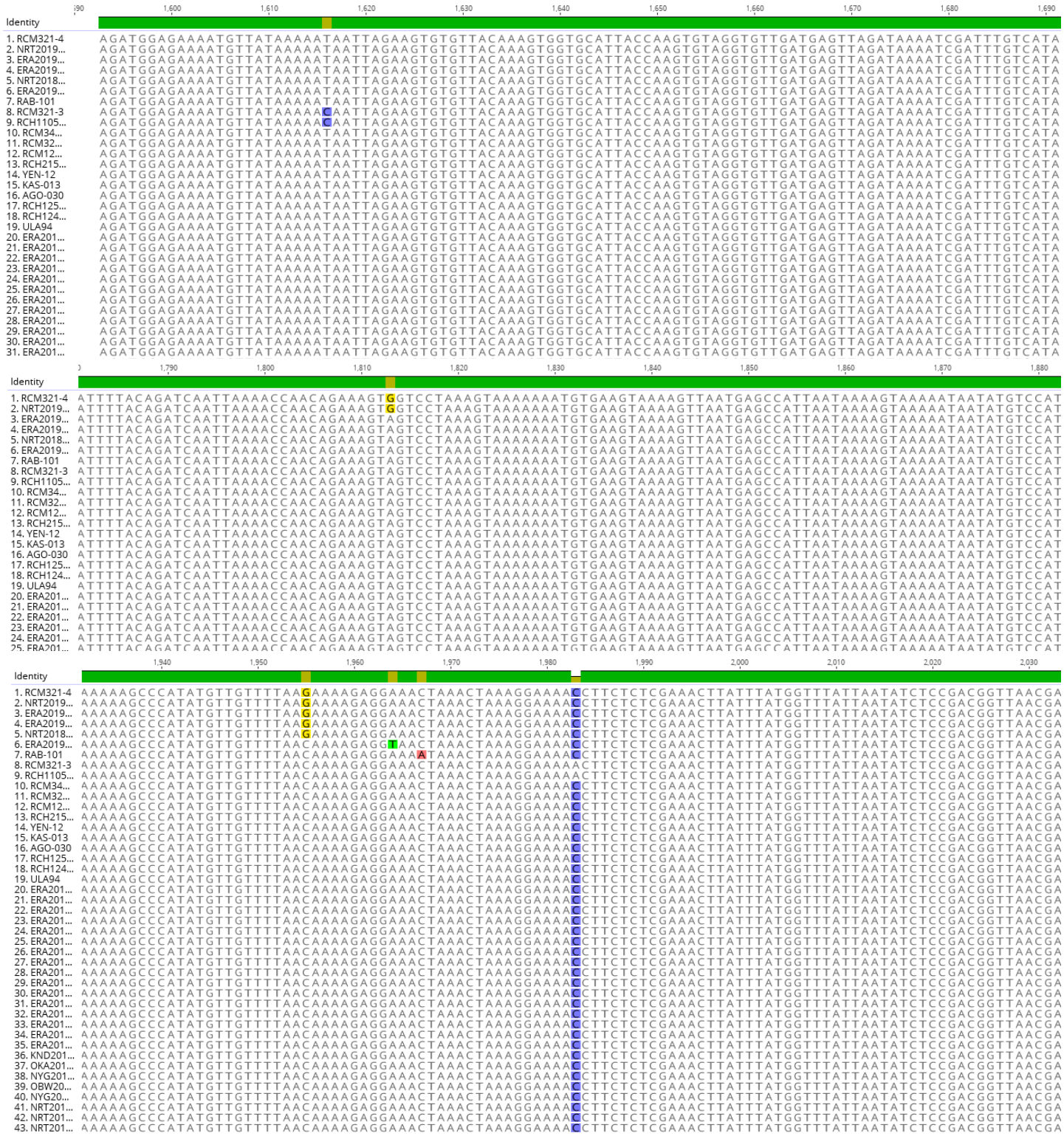
3. Microscopy results (Parasite counts) …… Gametocytes Counts……

APPENDIX 5: GEL IMAGE OF AMPLIFIED PFS230 DOMAIN 1 REGION

Letter C denotes positive control whereas letter S represents samples.

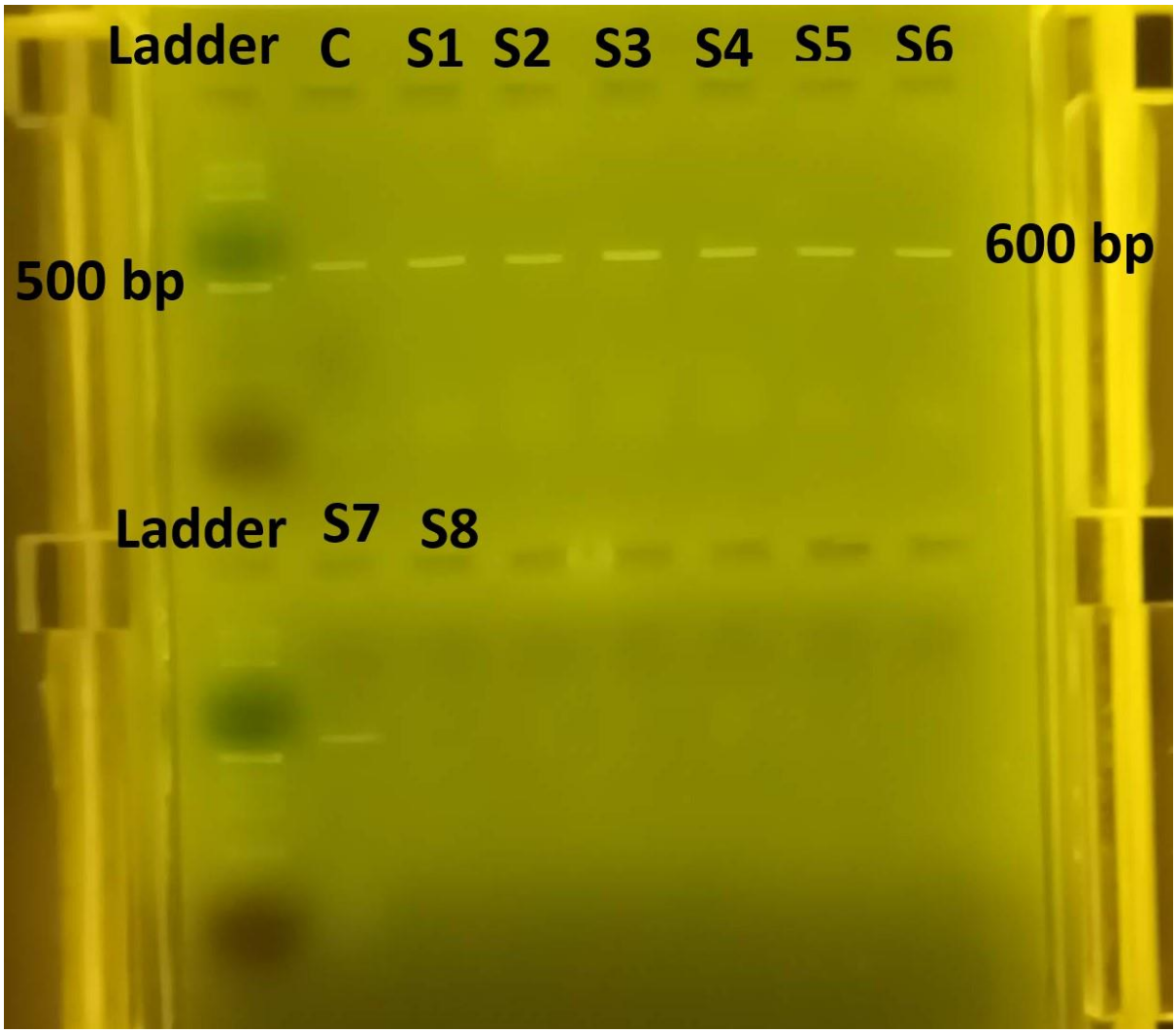


APPENDIX 6: SEQUENCE WITH MUTATED LOCI WITHIN PFS230 D1 GENE FROM *PLASMODIUM FALCIPARUM* IN WESTERN KENYA.

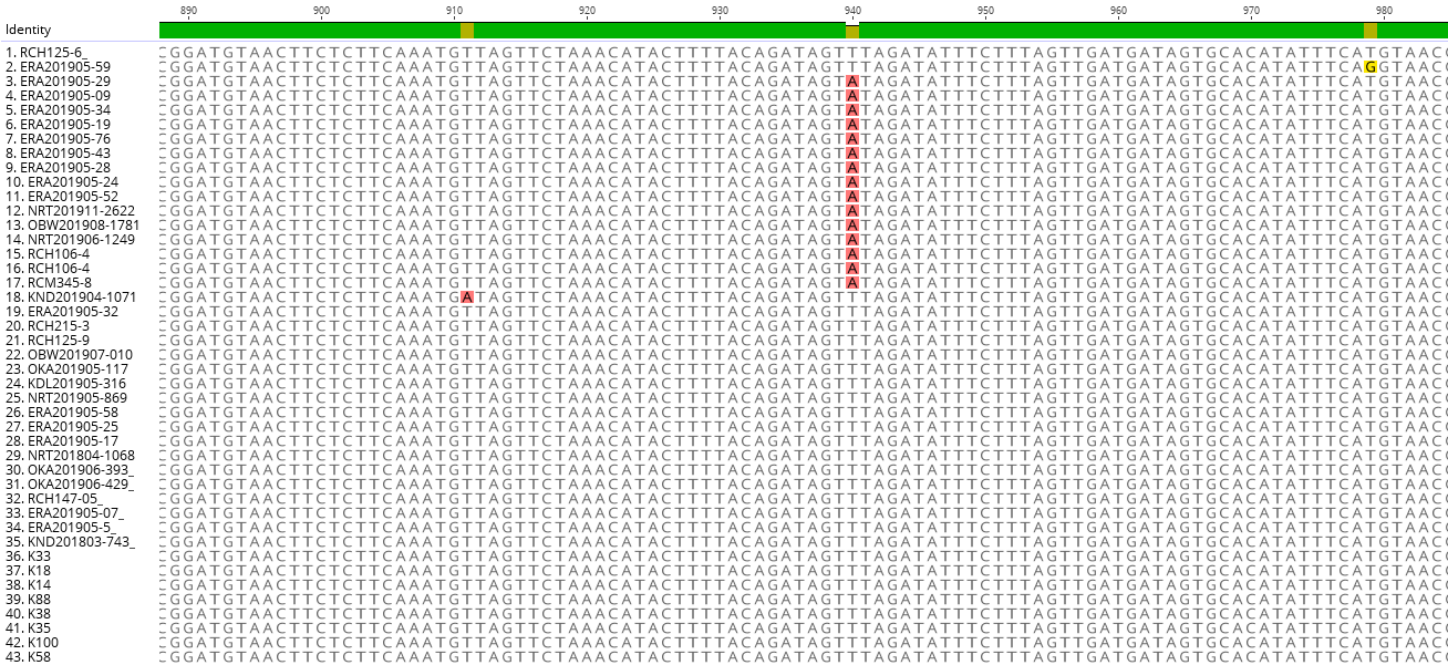


APPENDIX 7: GEL IMAGE OF AMPLIFIED PFS48/45 DOMAIN 3 REGION

Letter C denotes positive control whereas letter S represents samples.

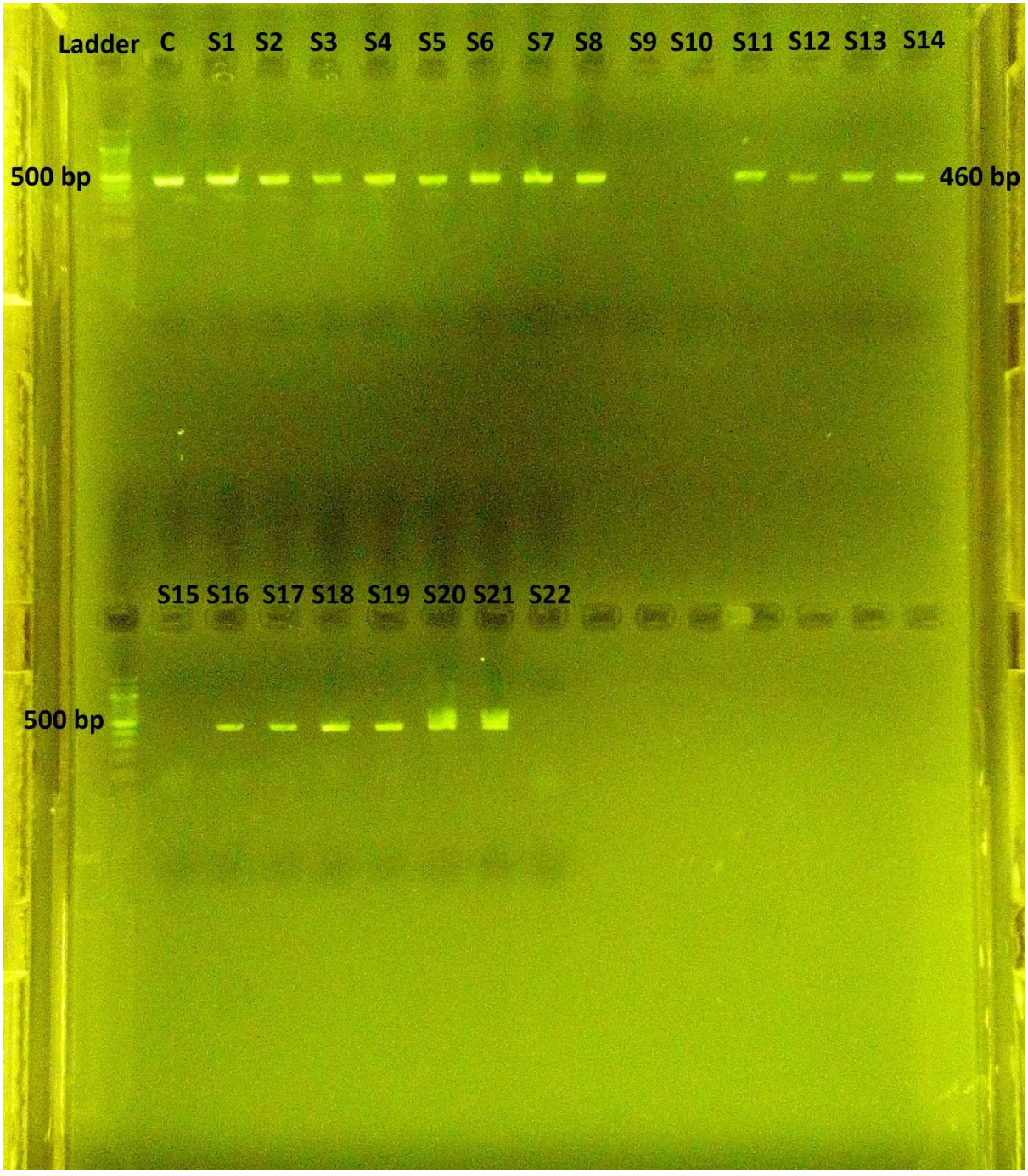


APPENDIX 8: SEQUENCE WITH MUTATED LOCI WITHIN PFS48/45 D3 GENE FROM *PLASMODIUM FALCIPARUM* IN WESTERN KENYA.



APPENDIX 9: GEL IMAGE OF AMPLIFIED PFS25 REGION

Letter C denotes positive control whereas letter S represents samples.



APPENDIX 10: SEQUENCE WITH MUTATED LOCI WITHIN PFS25 GENE FROM *PLASMODIUM FALCIPARUM* IN WESTERN KENYA.