MOLECULAR DETERMINATION OF *Plasmodium falciparum* PARASITES WITH HISTIDINE-RICH PROTEIN 2/3 GENE DELETIONS IN A HOLOENDEMIC AREA, SIAYA COUNTY, WESTERN KENYA

BY

SHARLEY ACHIENG' WASENA

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CELL AND MOLECULAR BIOLOGY

SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES

MASENO UNIVERSITY

DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

Signature..... Date.....

Sharley Achieng' Wasena
MSC/SC/00084/2020

Supervisors

This thesis has been submitted for examination with our approval as supervisors:

Signature..... Date.....

Dr. Patrick Onyango,
Department of Zoology,
Maseno University,
Maseno, Kenya

Signature..... Date.....

Dr. Elly. Ochieng' Munde,
Department of Clinical Medicine,
Kirinyaga University,
Kutus, Kenya

Signature..... Date.....

Dr. Evans Raballah,
Department of Medical Laboratory Sciences,
Masinde Muliro University of Science and Technology,
Kakamega, Kenya

ACKNOWLEDGEMENT

The fruition of this thesis would not have been possible without the steadfast support of my dedicated Mentors: Dr. Elly Munde, Dr. Evans Raballah, and Dr. Patrick Onyango. Their unwavering commitment to mentoring and guiding me throughout the entire process has been instrumental in the successful completion of this work.

To Prof. Douglas J. (DJ) Perkins, (University of New Mexico (UNM), USA), thank you for trusting my capabilities as a student. Prof. Perkins's tremendous support and mentorship were instrumental in bringing this project to fruition. I am deeply grateful for his guidance and expertise. My sincere gratitude goes to Prof. Collins Ouma for his consistent support and guidance during my studies. His encouragement and constructive feedback have significantly contributed to the shaping of the final outcome of my successful work. I would like to acknowledge Dr. Sammy Anyona for his invaluable assistance and support throughout my studies (Thank you for always sparing time-several hours to help me understand the data in details). I express special appreciation to Miss Anne Akinyi Ong'ondo for her indispensable administrative and moral support, playing a pivotal role in maintaining positivity amid challenges. Gratitude extends to all mentors at UNM, postdocs, students, and staff, contributing to the project's success. This work would not be a reality if not for the financial support from the National Institutes of Health: R01 AI130473 (DJP), D43 TW005884 (DJP), D43 TW010543 (DJP), and Bill and Melinda Gates INV-031511 (EOM).

I further extend my appreciation to my fellow students at UNM, who played a key role in the smooth operation of daily activities in the laboratories. My sincere thanks also go to all study participants, their parents, and guardians for their invaluable contributions. I appreciate the collaborative efforts of the UNM/Siaya County Referral Hospital medical laboratory staff during sample collection from the study participants.

Without the general support and prayers from my family—Mr. Wasena Elijah Oguta(dad), Mrs. Wasena Mary Akinyi(mom), and my five siblings—I probably would have given up along the way, "*Lol"*. Most importantly, I am grateful to God for the good health and strength that enabled me to pursue my Master's degree.

DEDICATION

I dedicate this work my parents, Mr. and Mrs. Wasena, for their support, prayers and understanding throughout this entire process

ABSTRACT

Malaria remains endemic in western Kenya despite the various control interventions. Accurate diagnosis is key to the treatment and control of malaria. As such, the World Health Organization (WHO) recommends parasite-based confirmation of malaria prior to treatment. *Plasmodium falciparum* Histidine Rich Protein 2 (*PfHRP2*) based malaria Rapid Diagnostic tests (mRDTs) kits are commonly used throughout western Kenya as an alternative to microscopy in malaria diagnosis. However, the emergence of *P. falciparum* isolates with *HRP2/3* deletions has threatened the sensitivity and performance of the mRDT kits. In western Kenya, a high transmission holoendemic area, polyclonal *P. falciparum* infection could be present but masked by the wild type gene. This may lead to both underestimation of deletion prevalence and increase in the spread of parasites with deletion. False negative mRDTs pose a public health threat towards malaria treatment and elimination progress. This is because the proportion of malaria infected patients having these gene deletions will go undetected by the *PfHRP2*-mRDTs, and therefore remain untreated. Siaya County borders western Uganda, an area where massive reports of HRP2/3 gene deletions have been reported and hence the need to conduct HRP2 surveillance in the study area. The study therefore investigated *PfHRP2/3* deletions in a paediatric cohort from Siaya county, in western Kenya. Specifically, the study determined the prevalence of *PfHRP2/3* deletion and the relative strain abundance of deleted strains in polyclonal infections, compared *P. falciparum* parasite densities estimated by qPCR and microscopy and evaluated the performance of the mRDT and microscopy techniques that routinely used for malaria diagnosis in Siaya County, western Kenya. The study being retrospective in nature, archived RBC pellets extracted from EDTA blood of children (n=219) who were previously enrolled in the study was used for DNA extraction. In order to achieve all the objectives, the study utilized one-step multiplex quantitative polymerase chain reaction (qPCR). The multiplex qPCR was designed using three differently labelled TaqMan assays detecting the *PfHRP2* (PF3D7_0831800) and *PfHRP3* (PF3D7_1372200) genes. Overall, *PfHRP2/3* deletions were detected in 12 (5.6%) parasite isolates. The *PfHRP2* monoclonal deleted strains were present in 2 isolates (1%) isolates while no parasite isolates harbored *PfHRP3* single deletion. Further, 9 (4.1%) isolates had deleted *PfHRP2* and 1 (0.5%) had *PfHRP3* deleted but were masked in polyclonal infection. The average relative abundance of *PfHRP2* deleted parasites was 9.6% while wild type was 90.4% in polyclonal infections. The multiplex qPCR demonstrated a higher ability in detecting malaria parasites compared to microscopy with median (IQR) of $8.28E^4$ ($2.75E^6$) and $6.24E^3$ ($2.45E^4$) respectively ($P \leq 0.001$). Further, there was a positive correlation between parasite detection by qPCR and microscopy
($r=0.59$, $P \leq 0.001$). On evaluation of the performance of clinical diagnostic techniques used in Siaya County Referral Hospital (SCRH), microscopy demonstrated a higher diagnostic sensitivity of 97.6% (95%CI, 89.8-105.0) and specificity of 26.0% (95%CI, 22.4-29.6) as compared to mRDT. Cohen Kappa's test revealed a fair agreement between microscopy and qPCR, ($k=0.30$, $P \leq 0.001$). The study provides evidence of *PfHRP2* deleted strains including those that are masked in polyclonal infections and their relative abundance in Siaya County. Further the study highlights microscopy as a more sensitive and specific technique in detecting malaria infection as compared to mRDT test.

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Human malaria remains a major global health threat throughout the existing years. In 2021 for instance, the World Health Organization (WHO) estimated an 247 million malaria cases in 84 malaria endemic countries, an increase of about 2 million malaria cases from 2020 (WHO, 2022). Human malaria is caused by parasites of the genus *Plasmodium*, that is; *P. ovale, P. vivax, P. knowlesi*, *P. malariae* and *P. falciparum*, with *P. falciparum* species being the most infectious and consequently accounting for the most severe cases of malaria globally (WHO, 2021). According to Kenya Malaria Indicator Survey (KIMS), malaria prevalence varies greatly depending on endemicity of the zone as it is estimated to be higher in children living in rural setup as compared to those in urban setup (Malaria Indicator Survay., 2020).

P. falciparum follows a holoendemic transmission patterns in western Kenya is holoendemic (>75% prevalence in children but low prevalence in adults) (Ratti and Wallace, 2020). In the treatment plan for malaria, the WHO recommends confirmation of symptomatic cases using microscopy and/or malaria rapid diagnostic test (mRDTs) prior to treatment administration (WHO, 2021). Although microscopy remains the gold standard, its use is limited in resource constrains areas due to factors such as lack of skilled personnel, unreliable electricity supply and lack of quality reagents (Wongsrichanalai *et al.*, 2007). In order to circumnavigate this drawback, the WHO and Division of National Malaria Control Program Kenya (DNMP) introduced and recommends use of mRDT as a good alternative test to microscopy in areas where microscopy based diagnosis may not be available or unreliable (WHO, 2021).

The first mRDT was developed in 1990 and later introduced in Kenya in 2012 in an effort to help overcome the shortcomings of microscopy (Boyce *et al.*, 2017; WHO, 2000). The mRDTs are easy to use and they provide rapid results within 20 minutes (WHO, 2010). The mRDTs recognises specific antigens (proteins) present in the blood of malaria infected individuals (WHO, 2004). The antigens targeted by commercially available mRDTs include Histidine-rich protein 2 (HRP2), lactate dehydrogenase (LDH) and aldolase.

Aldolase and LDH mRDTs exists in three forms, that is, *P. falciparum*-specific, *P. vivax*specific and genus-specific (Maltha *et al.*, 2013). Although non-PfHRP2 mRDT's can also be used for malaria diagnosis, non-HRP2 mRDTs are limited in sensitivity and unstable under room temperatures and compared to HRP2 based mRDTs (Gatton *et al.*, 2015). Therefore, *HRP2* targeting mRDTs accounts for most of the global mRDT procurements (Cunningham *et al.*, 2019). HRP2 is an abundantly produced thermostable protein which can also be retained in previously infected RBCs several days to weeks after parasite clearance (Desakorn *et al.*, 1997;
Poti *et al.*, 2019). Due to the above reasons and the high prevalence of *P. falciparum* in western Kenya, the majority of mRDTs used in Siaya County targets HRP2 protein. Moreover, the HRP2 antigen is homologous to another *P. falciparum* antigen, HRP3, and hence HRP2-based kits can still detect HRP3 antigens (Kong *et al.*, 2021).

The performance of PfHRP2-mRDTs can however vary due to a number of factors. These include antigenic variability of the target protein, its persistence in the bloodstream following elimination of parasites, and parasite density below the detection limit (Maltha *et al.*, 2013; Mayxay *et al.*, 2001; Obeagu *et al.*, 2018; Watson *et al.*, 2019). The common antigenic variability of PfHRP2/3 are due deletions and persistent antigenemia following parasite clearance (Lee *et al.*, 2006; Plucinski *et al.*, 2017).

Importantly, in high transmission areas such as western Kenya, *PfHRP2/3* deleted mutants can be present but undetected in polyclonal infections (existence of the *PfHRP2/3* wild type and mutant in the same individual) (Berhane *et al.*, 2018; Grignard *et al.*, 2020). Consequently, this not only leads to underestimation of the prevalence but also spread of the deleted mutants within a population. The *PfHRP2/3* deletions mutations have further been reported in multiple locations including Kenya (Beshir *et al.*, 2017; Grignard *et al.*, 2020), Uganda (Agaba *et al.*, 2020), Peru (Gamboa *et al.*, 2010), Nigeria (Funwei *et al.*, 2019), Equatorial Guinea (Berzosa *et al.*, 2020) among others. The prevalence of HRP2 and HRP3 gene deletions varies across different regions worldwide. For example, Eritrea has reported up to an 80% prevalence of these deletions, leading to changes in diagnostic strategies (Berhane *et al.,* 2018). However, in western Kenya, specifically in Mbita, Homabay County, the initial case of *PfHRP2/3* deletions was documented in 2017, but it did not significantly impact the use of mRDTs in the region (Beshir *et al.,* 2017).

Despite the availability of global information on deletions, there is currently a lack of comprehensive data regarding the extent of *PfHRP2/3* deletions, including strains hidden within polyclonal infections, in the high holoendemic area of Siaya County, western Kenya. Siaya County shares its border with western Uganda, an area that has reported a high prevalence of HRP2/3 gene deletions. This underscores the importance of conducting HRP2 surveillance in the study area (Agaba *et al.,* 2020).

Accurate diagnosis and prompt malaria treatment is necessary to prevent severe malaria and death. As such, low parasitemia beyond detection threshold of mRDT remains a challenge to the diagnosis of malaria especially using mRDT. Additionally, as malaria transmission decreases, the proportion of low-density infections are likely to increase, further compromising mRDT sensitivity (Martiáñez-Vendrell *et al.*, 2022). Although mRDTs are designed to detect clinically relevant

HRP2 concentration, this can be compromised at levels below the detection threshold of the (100 parasites/μL) (Plucinski *et al.*, 2017). A study conducted in 2022 showed that mRDTs are likely to miss detecting malaria cases mainly due to low density parasitemia when compared to other malaria diagnostic like light microscopy and quantitative polymerase chain reaction (qPCR) (Kaaya *et al.*, 2022). For this reason, the current study estimated and compared the parasite densities obtained from multiplex qPCR and microscopy. Further, the parasitaemia effects on mRDT results were also determined. Additionally, the effectiveness of the conventionally used clinical diagnostics in Siaya County referral hospital (light microscopy and mRDT technique) was evaluated.

1.2. Statement of the problem

The deletions of *PfHRP2/3* genes remain a challenge to the diagnosis of malaria since most of the mRDTs used currently recognize HRP2 proteins (WHO, 2020b). Importantly, the holoendemic transmission patterns in western Kenya may include *PfHRP2/3* deleted mutants that are present in polyclonal infections, which are hard to detect using the normal molecular methods. Further, this not only leads to an underestimation of deletion prevalence but increase in the spread of parasites with deletion within the region, potentially increasing the rate of false negative mRDT results (Grignard *et al.,* 2020)

False negative mRDTs pose a public health threat towards malaria treatment and elimination progress, as a high proportion of malaria positive patients may be undetected by *PfHRP2*-mRDTs, and thus no treatment will be provided. Potentially, this may lead to increased risk of malaria morbidity and mortality, and continued malaria transmission. The success of malaria control and elimination programs partly if not entirely, depends on accurate diagnosis, which partly relies on

the determination of *PfHRP2/3* deletions prevalence and the impact they generate on *PfHRP2*-mRDT kit.

Studies demonstrated that parasite densities below the detection limit could also lead a false negative *PfHRP2*-mRDTs results (Plucinski *et al.*, 2017; WHO, 2018). As a result, undetected parasitemia levels by the HRP2-mRDT further leads to a misdiagnosis of symptomatic patients and therefore no treatment may be administered. Consequently, impacting negatively on malaria control and elimination. The current study therefore determined the prevalence *PfHRP2/3* deletions and the relative abundance of deleted clones that are masked in polyclonal infections. It also compared the estimated parasite densities obtained from multiplex qPCR and microscopy relative to mRDT results. Additionally, it determined the effectiveness of the conventionally used clinical diagnostics in SCRH. The results from the study generated useful information on malaria diagnosis that is being integrated into Kenya Division of National Malaria Program (DNMP).

1.3. Objectives of the study

1.3.1. General objective

To investigate the presence of *Plasmodium falciparum* parasites with *Histidine-Rich Protein 2*/3 (*PfHRP2/3*) deletions infections in a holoendemic area, Siaya County, western Kenya.

1.3.2. Specific objectives

i. To determine the prevalence of *PfHRP2/3* deletions and the relative strain abundance of deleted genes in polyclonal infections among children with symptomatic malaria in Siaya County, western Kenya.
ii. To compare *P. falciparum* parasite densities estimated by qPCR and microscopy in children with *P. falciparum* infection in Siaya County, western Kenya.
iii. To determine the variations in performance of the conventional clinical diagnostic techniques (mRDT and microscopy) in Siaya County Referral Hospital, western Kenya compared to multiplex qPCR.

1.3.3. Research questions

i. What is the prevalence of *PfHRP2/3* deletions and the relative strain abundance of deleted clones that are present in Polyclonal infections among children with symptomatic malaria in Siaya County, western Kenya?

ii. What is the difference between *P. falciparum* parasite densities estimated by qPCR and microscopy in children with *P. falciparum* infection in Siaya County, western Kenya? iii. What is the variation in performance of the conventional clinical diagnostic techniques used in Siaya County Referral Hospital, western Kenya compared to multiplex qPCR?

1.4. Significance of the study

The findings of this study show an overview of the prevalence of *PfHRP2/3* deletions including in polyclonal infections that might affect the performance of *PfHRP2*-mRDT diagnosis. Further, the data from the study generated useful information on malaria diagnosis that is being integrated into Kenya Division of National malaria Program (DNMP). The *PfHRP2*-mRDT kit performance and lower parasite densities below the detection limit of *PfHRP2*-mRDT generated insights that will be used for evidence-based decision making by Kenya Division of National Malaria Program.

CHAPTER TWO

LITERATURE REVIEW

2.1. Malaria Epidemiology

Malaria is a vector-borne parasitic disease caused by *Plasmodium* parasite. It is endemic in different regions with nearly 40% of the globle's population remaining at risk of infections (WHO, 2017). In 2020 for instance, the estimates of malaria deaths and infections increased due to the impact of disruptions on control efforts during the COVID-19 pandemic (WHO, 2021). Despite the impressive progress towards malaria control, the situation remains worrying especially in sub-Saharan Africa where its burden remains unacceptably high (WHO, 2021). Reducing the burden of malaria relies in a number of factors, including accurate estimations of malaria transmission intensities. For instance, all age groups in regions of low transmission intensities, are susceptible to severe malaria whereas in areas of high transmission intensities, older children and adults are less affected. Additionally, in high transmission holoendemic areas, infants below the age of three years are the most affected (Snow and Marsh, 2002).

Malaria has a short life cycle with the merozoite stage destroying each red blood cell (RBC) they infect bringing about the common clinical symptoms. The symptoms usually appear days after a bite by the female anopheles mosquito, with the first symptoms being fever, headache and chills, varying from one individual to another (WHO, 2008). Due to the rapid increase in malaria infections, the WHO recommends that all cases of suspected Malaria, must be confirmed using parasite based diagnostic testing prior to the administration of treatment (WHO, 2018)

2.2. Malaria Diagnosis

The accuracy of malaria diagnosis is an important aspect since a misdiagnosis impacts negatively on the efforts geared towards controlling and preventing malaria (Iriart *et al.*, 2020; Wu *et al.*, 2015). Molecular diagnosis of malaria still remains a challenge especially in developing countries (WHO, 2016, 2017; Wu *et al.*, 2015). Malaria can be suspected mainly based on symptoms and physical finding at examination (WHO, 2004; Zimmerman and Howes, 2015). Malaria parasites can be identified by examination using the three main examination techniques described below.

2.2.1. Molecular techniques for malaria diagnosis

Nucleic acid amplification tests (NAATs) have the highest sensitivity and specificity for malaria diagnosis (Andrews *et al.*, 2005). Polymerase Chain Reaction (PCR) is the commonly used NAATs to detect parasite DNA in blood of an infected individual (Andrews *et al.*, 2005). It is an efficient technique in the identification and differentiation of malaria parasites (Kasetsirikul *et al.*, 2016). Despite their high diagnostic performance, NAATs are not used for routine malaria diagnosis because they are expensive and highly technical (Berzosa *et al.*, 2018). The multiplex qPCR has the ability to identify malaria infections and *PfHRP2/3* deletions accurately with a high performance. Additionally, it can also detect parasitaemia levels up to less than 5 parasites/μL (Schindler *et al.*, 2019).

2.2.2. Malaria Microscopy

Microscopy still remains the gold standard for laboratory confirmation of the presence of malaria parasites (WHO, 2017; Wongsrichanalai *et al.*, 2007; Wu *et al.*, 2015). The efficiency of microscopy however is dependent on a number of factors the quality of reagents used, the quality of the microscope as well as the experience of the personnel handling the technique (Wongsrichanalai, 2001). In addition, sustaining dependable microscopists where malaria

transmission has been reduced due to interventions efforts, may be challenging. Moreover, its ability to detect parasites at very low levels is limited (Otambo *et al.*, 2022). As such, microscopy is an imperfect reference standard used in the detection of malaria parasites (Ohrt *et al.*, 2002). The limitations of microscopy, further led to the emergence of mRDTs.

2.2.3. Malaria Rapid Diagnostic Test Kits

Since 2018, over 259 million mRDTs have been proqured and used in Sub-Saharan Africa annually with a number of health facilities without expert microscopists depending on mRDTs for diagnosis (Grignard *et al.*, 2020; Mukkala *et al.*, 2018; WHO, 2019). mRDTs have helped reduce malaria transmission intensity in most part Kenya especially in the island regions (Mfangano, Takawiri, Kibuogi and Ngodhe) in Lake Victoria (Idris *et al.*, 2016). *P. falciparum* specific mRDTs recognize *PfHRP2/3* antigen that codes for the *PfHRP2/3* gene. The antibodies of *PfHRP2* cross-react with *PfHRP3* antibodies (Lee *et al.*, 2006). The HRP3 gene is a member of the histidine-rich protein family . It is found in a wide range of organisms, including humans and other mammals (Thomson *et al.*, 2019). This gene encodes a protein characterized by an unusually high content of histidine residues, which makes it unique among genetic sequences(Thomson *et al.*, 2019). Histidine-rich proteins, including HRP3, have been implicated in various biological functions (Kong *et al.*, 2021). The high density and high thermal stability of *PfHRP2/3* in a malaria infected individual's blood and it's expression by *P. falciparum* in the erthrocytic stage of development, qualifies this antigen as a valuable biomarker for detecting malaria in mRDTs (Howard *et al.*, 1986). The mRDTs are based on an immuno assays (LFIA) that allows for the detection of malaria antigens i.e. HRP2/3, lactate dehydrogenase (LDH) and aldose depending on the antigen being targeted (Wongsrichanalai *et al.*, 2007). mRDTs based on LDH and Aldolase can also be used in diagnosis of malaria since they can recognize non-*falciparum* species from mixed infections, although, they

are associated with lower sensitivity and reduced stability at high temperatures (Howard *et al.*, 1986; WHO, 2017; Wu *et al.*, 2015; Zimmerman and Howes, 2015). For this reason, the study deployed use the gold standard and *PfHRP2*-mRDT instead of two mRDT combination diagnosis. The efficiency of *PfHRP2*-mRDTs has however been reduced by a number of factors. First, the emergence of *PfHRP2/3* gene deletions has threatned the effectiveness of PfHRP2mRDTs (Berhane *et al.*, 2018; Beshir *et al.*, 2017). This is because a high proportion of malaria infected patients will go untreated due to lack of detection by the *PfHRP2*-mRDTs as deletions are associated with false negative results. Consequently, most cases with *PfHRP2*-deletions remain undetected and untreated (Berhane *et al.*, 2018). Ultimately, such cases potentially lead to increased malaria infections and deaths. Deletions of *PfHRP2/3* genes remains a challenge to the diagnosis of malaria since most of the mRDTs used currently rely on these proteins for diagnosis of malaria (Gendrot *et al.*, 2019; WHO, 2017, 2020a).

Another major drawback of mRDTs is persistence of *HRP2* antigenemia, for several days to weeks after the clearance of parasites (Iqbal *et al.*, 2004; Michael *et al.*, 2021). Although antigenemia can be an indication of prior infection, it reduces the efficiency of *PfHRP2*- mRDTs use in detecting re-infection especially in a high transmission area (Iqbal *et al.*, 2004). This further makes it difficult to initiate follow-up of re-infections for instance in pregnant women (Mayxay *et al.*, 2001). According to the *PfHRP2* protocal developed by WHO the main cause of false negative mRDTs are linked to a number of factors including product quality and performance, transportation or storage conditions, operator error or parasite densities that cannot be detected by the mRDTs (WHO, 2020b). Deletions of the genes coding for *PfHRP2* threatenes the effectiveness *PfHRP2*-mRDTs leading to false negatives mRDTs (Gamboa *et al.*, 2010). It is therefore necessary to

investigate *PfHRP2* deleted mutants to include polyclonal infections especially among children who are most affected.

2.3. Plasmodium falciparum Histidine Rich Protein 2/3 gene deletions

The existence of *P. falciparum* parasites with *PfHRP2/3* deletions has threatened the efforts geared toward the control and elimination of malaria. This is because the mutant parasites could circulate widely within the population leading to infected individuals not being diagnosed by the readily available *PfHRP2*-mRDT kits. This will futher lead to the infected individulas not being treated and serving as parasite reservoirs hosts increasing transmission within the potential area. The first case of *PfHRP2* and *PfHRP3* deletions was reported in Peru in the year 2010 after discrepancies in mRDT and microscopy results were encountered (Gamboa *et al.*, 2010). Since then, the deletions surveys have been conducted in different parts of the world. In Eritrea, there was an estimated prevalence of 80% of *PfHRP2* in *P. falciparum* deletions among hospitalized malaria patients, which was a high prevalence relative to other African regions (Berhane *et al.*, 2018). Further in Eritrea, it led to the massive change in diagnostic strategy (Berhane *et al.*, 2018). The *PfHRP2* and *PfHRP3* deletion mutations have been reported in a number of countries in western Africa, which is a region associated with high malaria burdens. The countries with reports of deletions in Africa include Senegal in West Africa, (Wurtz *et al.*, 2013), Rwanda and Democratic Republic of Congo in central Africa, (Kozycki *et al.*, 2017; Parr *et al.*, 2017) and Kenya, Uganda and Tanzania east Africa, (Beshir *et al.*, 2017; Thomson *et al.*, 2019).

In Kenya, the first *PfHRP2/3* deletions case was reported in 2017 in Mbita, Homabay County with very low prevalence in monoclonal infections (Beshir *et al.*, 2017). However, there are gaps in knowledge on whether these deletions occur in polyclonal infections. Since these parasites with these deletions exist in the region of Kenya, it is important to determine their

prevalence as it will help the DNMP in their decision-making policy on whether mRDT has reached a threshold that require diagnostics change. The WHO recommends estimations of the prevalence of *PfHRP2/3* especially in previously reported countries (WHO, 2016, 2017, 2019). In addition, the WHO recommended standardized methods being used to detect the deleted parasites across all nations (WHO, 2020b). The current study therefore determined *PfHRP2/3* gene deletions in a paediatric cohort with symptomatic malaria, residing in Siaya County.

2.4. Parasitemia estimation using qPCR and microscopy

Although mRDTs were standardized for malaria diagnosis and case management, their operational limit for parasite detection (LOD) still requires a clear definition (Obeagu *et al.*, 2018). The currently available mRDTs are designed to detect clinically relevant antigen concentrations, and the estimated LODs are thought to be consistent with the *PfHRP2* concentrations (WHO, 2015,2016). Importantly, the ability of mRDTs to detect *PfHRP2* and *PfHRP3* at lower parasite concentrations can be compromised (Plucinski *et al.*, 2017). For this reason, the study quantified the parasite densities in malaria infected children using both microscopy and qPCR in Siaya County, western, Kenya in order determine whether low parasitemia could also be the cause of mRDT false negatives.

In malaria quantification, microscopic examination and PCR genotyping are the most common and reliable techniques. However, estimation of parasite counts using microscopy technique is dependent the microscopists accuracy and methods used for parasitological examination (Trape, 1985). In microscopy technique, parasitemia are estimated by multiplying number of parasite counted (trophozoites) by a standard 26.7 (8000 white cells per µL/300 number of white cells counted) (Ong'echa *et al.*, 2006b). Importantly, there has been a surge in the prevalence of sub-microscopic malaria infection which cannot be detected by microscopy technique especially in the

western Kenya region (Otambo *et al.*, 2022). Nucleic acid amplification tests (NAAT) have demonstrated a greater sensitivity in quantifying malaria parasitemia since they tend to quantify parasitemia with more accuracy compared to blood films examination, especially at low parasitemia counts (Murphy *et al.*, 2014; Murphy *et al.*, 2013).

Quantitative NAAT methods include quantitative Polymerase Chain Reaction (qPCR) and quantitative reverse transcriptase Polymerase Chain Reaction (qrtPCR). qPCR can be used for precise parasite quantification with a high sensitivity (Andrews *et al.*, 2005; Jaureguiberry *et al.*, 1990; Kamau *et al.*, 2011; Lee *et al.*, 2002). Quantification of parasite densities correctly is important for diagnosis since lower parasite densities with small number of DNA targets could lead to discrepancies in results, for instance, Microscopy positive and mRDT negative repeat thus, false conclusive results which impacts negatively on malaria control (Baidjoe *et al.*, 2016; Okell *et al.*, 2012). This study therefore determined parasitemia levels using both malaria gold standard and multiplex qPCR. The two techniques were then compared on their accuracy basis of parasite densities estimations.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area

The study was conducted at the Siaya County Referral Hospital (SCRH), located in Siaya County of western Kenya (Appendix 1). As at 2019 census, Siaya county had a total population of about 842,325 (Lumwagi *et al.*, 2019). SCRH is in rural health facility in western Kenya that has a fairly well-equipped paediatric wing and maternity services as well as general adult medical and surgical wards. One of the primary reasons of childhood morbidity and mortality in Siaya County is *P. falciparum* malaria (Kapesa *et al.*, 2018; Perkins *et al.*, 2013). SCRH is the major government referral facility in Siaya County. SCRH borders Kisumu, Busia, and Vihiga Counties. It is approximately 1520 square kilometres.

3.2. Study Design and patient population

3.2.1. Recruitment of study participants

The study utilized previously collected samples. Samples used in this investigation were selected from a longitudinal birth cohort of children (0-36 months, n=750) collected between July 2017 and August 2021. As such, it was a retrospective cohort study design. The current study used stored RBC pellets extracted from EDTA blood of children who were recruited in SCRH in a previous paediatric study. The recruitment of the study participants was done after the parent/guardian of the child consented. Sociodemographic information was collected using questionnaires whereas clinical information was recorded as per the hospital's guidelines for the management of malaria. The SCRH is the major government referral hospital in Siaya County hence it offered a better opportunity to capture malaria patients.

3.2.2. Enrolment of study participants

Enrolment of participants into the study was done as previously described (Raballah *et al.*, 2022). Briefly, the parent/guardian of the children received an explanation of what the study entails after which written informed consent were sought in languages of choice. Venous blood samples (<3.0 mL) was collected in EDTA- containing tubes at the time of enrolment, prior to provision of treatment or any supportive care.

3.3. Inclusion criteria

- Symptomatic malaria- Including individuals with symptomatic malaria ensures that the study focuses on those who are actively infected with the malaria parasite and exhibiting symptoms.
- Presence of axillary temperature ≥37.5°C - Elevated body temperature (fever) is a common symptom of malaria. It is often used as an indicator of malaria infection, as it can be associated with the body's immune response to the parasite
    - Parent/legal guardian providing informed written consent. This is because the study focuses on children below legal age.

3.4. Exclusion criteria

3.5. Sample size determination

According to the WHO protocol *PfHRP2/3* deletions and bio banking, the sample size was calculated based on a proportion obtained from simple random sampling with a sampling design effect (deft) =1.5 (to account for observations correlated within *PfHRP2/3* deletions) and probability of committing a type 1 error =95% (1.96), such that 95% CI does not overlap with the threshold of 5%. This study therefore used the following formula;

$$N \geq \text{deft}\left[\frac{Z^2\,(P)(1-P)}{2*D^2}\right]$$ (WHO, 2020a)

Where;

Z= Standard error at 95%CI (1.96) deft= design effect

P=Expected proportion in population based on previous studies-22% (Kapessa *et al.*, 2018)

D=Absolute error at 95%CI (0.04)

From the above formula above, the calculated minimum sample size is 206. An estimated assay failure rate of 6% was factored in the calculated sample size to include

6/100×206= 12.35 (Andreson *et al.*, 2008).

Therefore, the estimated sample size was 206+13=219

3.6. Collection and processing of blood samples

3.6.1. Sample collection and malaria diagnosis using Microscopy and mRDT

Thick and thin blood films were prepared for microscopic malaria diagnosis. Malaria parasite densities were also estimated by microscopy based on prior methods (Ong'echa *et al.*, 2006a). Briefly, Peripheral blood smears were prepared and stained with Giemsa reagent and examined under oil immersion for malaria parasites. Asexual malaria parasites were counted against 300 leukocytes and parasite densities estimated assuming a count of 8,000 white blood cells per microliter of blood.

Following microscopic diagnosis of malaria, about 10.0 uL of the whole blood was used for mRDT diagnosis using *PfHRP2*-mRDT (CareStart Malaria *HRP2* (Pf), Access Bio, Inc, 65

Clyde Rd. Suite A Somerset, NJ 08873 USA) as per manufacturer's instructions. The CareStart mRDT kit has a detection rate of 98% at a parasite density ≥ 100 parasites/uL (Ali *et al.*, 2021). Further, the collected blood samples were also used for performing different laboratory tests including determination of haematological parameters. Haemoglobin levels and complete blood counts were determined using the Beckman Coulter ACT diff2™ (Beckman-Counter-change this kwa power point Corporation, Miami, FL, USA). The remaining blood samples were then processed to obtain WBC, RBC and Plasma by spinning the samples at 3500 revolutions per minute (rpm) for 3 minutes. A pipette was used to aliquot the three into labelled Eppendorf tubes and stored in the -20^{o}C freezer in their respective freezer boxes. All participants were promptly treated with appropriate antimalarial treatment and required supportive therapy given as per the Kenya Ministry of Health (MoH) guidelines.

3.6.2. DNA extraction

DNA was extracted using DSP DNA mini kit with slight modifications of the manufacture's protocol (QIAGEN, Germany). The adjustments of the manufactures protocol was done with no significant difference as published by (Beshir *et al.*, 2017). Briefly, whole blood samples were placed in a deep-well plate. Buffer ATL (180.0 µL) and proteinase K (20 µL) were added to each well and mixed by thermomixer at 900 rpm at 56 °C for 15 min. The deep-well plate was then placed directly into the sample compartment of the QIAsymphony for DNA extraction. The extracted DNA samples were used for further molecular investigation

3.7. Molecular assays

Multiplex qPCR was used to perform the molecular assays. The multiplex qPCR has the ability to identify *PfHRP2/3* deletion status correctly in both polyclonal and monoclonal infection as well as the estimation of parasite densities less than 5 parasites/ µL within a single reaction (Schindler *et al.*, 2019). Briefly, the conditions for running a multiplex qPCR assay included an initial hold step at 95°C for 6 minutes, followed by 45 cycles consisting of a denaturation step at 95°C for 15 seconds, an annealing step at 54°C for 30 seconds. The threshold for data analysis was set at 35 cycle threshold (Ct), and all four genes (*HRP2, HRP3, LDH*, and *HumTuBB*) were included in the same qPCR mix for simultaneous analysis. The primer/probe chosen for genotyping targeted a region that extended across exon 1 and exon 2 in both *HRP2* and *HRP3*.

3.7.1. Detection of the *PfHRP2/3* deletions in monoclonal infection.

This was performed based on the previously published methods (Grignard *et al.*, 2020; Schindler *et al.*, 2019). Briefly, the assay was designed using four different TaqMan assays detecting the *PfHRP2* (PF3D7_0831800) and *PfHRP3* (PF3D7_1372200) genes with the single copy gene *P. falciparum* lactate dehydrogenase (*PfLDH*, PF3D7_1324900) and Human Tubulin gene (*HumTuBB*) as the internal control. The primer/probe selected for the genotyping bound to a region spanning exon 1 and exon 2 of both genes. Deletion in the *PfHRP2* and/ *PfHRP3* (Ct set at 35) was determined by failure of their amplification in samples which were positive for the internal control, *pfLDH* and *HumTuBB* (Appendix 2). The oligonucleotide sequence that was used for HRP2/3-del assay is shown in Appendix 3

3.7.2 Determination of deletions in polyclonal infections

A range of different rations were first tested in defined mixtures and a positive correlation between abundance of isolates with either *PfHRP2* or *PfHRP3* as well as an increase in ΔCt values was

observed. Further, a cut off of ΔCt values of 2.0 was chosen to identify masked *PfHRP* deletions (Schindler *et al.*, 2019). The study correlated Ct readings from amplification of *PfHRP2* or *PfHRP3* and *PfLDH* to determine deletions in polyclonal infections based on PCR efficiency and careful adjustments of the Ct values.

3.7.3. Molecular quantification of parasite density

To explore the role of low parasitemia in negative and positive genotyping results, parasitemia levels were quantified as previously described in (Schindler *et al.*, 2019). The study used *PfLDH* (PF3D7_1324900) coded by a single-copy gene on chromosome 13, as a confirmatory gene for the presence and quality of parasite DNA as well as a target for quantification. Positive samples were defined as those that crossed a pre-determined threshold of 35 and were positive for both *PfLDH* and *HumTuBB*. The accuracy of this quantification was further correlated with parasitaemia counts from the microscopic analysis.

3.8. Data analysis

The Chi-square test was used to determine differences between sexes of participants in mRDT positive and negative samples. Parasitemia estimates and hematological comparisons between the groups were analysed using the Mann-Whitney U test. Agreement between the conventionally used microscopy and mRDT tests was tested based on Cohen Kappa's statistics where 0 indicates no agreement, 0.01 to 0.40 – fair agreement, 0.41 to 0.80 –stable agreement, and 0.81–1.00 perfect agreement. Sensitivity and specificity of mRDT and microscopy diagnostic tests were determined at 95% CI (Hess *et al.*, 2012). For estimation of the relative abundance of parasites in a sample delta relative quantification method as previously published (Beshir *et al.*, 2010) was used.

Comparison of parasite density estimated by microscopy and qPCR, was performed using Pearson's correlations (*r*). For all statistical tests, values with *P*values≤0.05 were considered significant.

3.9. Ethical Considerations

In conducting this study involving children who were under the legal age, the utmost care was taken to ensure ethical standards were upheld. Prior to any data collection, informed consent was obtained from the parents or guardians of each participating child. Their voluntary agreement was sought, and they were informed about the nature and purpose of the study, as well as any potential risks and benefits involved.

To minimize any discomfort or pain experienced by the children, the study collected only a minimal amount of blood (3.0 mL) during the data collection process. It was ensured that the procedures were carried out carefully, and any potential pain associated with the collection of malaria data was explained to the parents or guardians. They were informed that any discomfort would likely be temporary in nature.

Confidentiality was rigorously maintained throughout the study to protect the privacy of the participants. To safeguard the identities of the children, their names were not included in any research-associated documents or reports. Instead, unique identifiers or codes were used to maintain anonymity.

The study received ethical approval from the School of Graduate Studies of Maseno University, and further authorization was obtained from the Maseno University Ethical Review Committee (Appendix 4). These approvals ensured that the research was conducted in accordance with established ethical guidelines and standards.

CHAPTER FOUR

RESULTS

4.1. Demographic and clinical laboratory characteristics of the study participants

The characteristics for the pediatric participants whose samples were selected for the molecular characterization are shown in **Table 1**. For the mRDT assay performed in 219 individuals, 88.5% (n=194) were positive and 11.4% (n=25) were negative. Of the mRDT (+) samples, 91.2% (n=177) were positive for microcopy and 8.8% (n=17) were negative. Furthermore, all samples that were mRDT (-) were microscopy (+) 100.0% (n=25). Across group comparison of the mRDT (+/-) and microscopy (+/-) categories did not yield a significant difference (P=0.103). Sex was comparable between mRDT (+) and (-) groups (P=0.388). The determination of the demographic characteristics including sex was necessary for proper clinical characterization of the study participants. Peripheral parasitemia was higher in the patients with mRDT (+) results [median (IQR); 7674.8 (28790.4)] relative to the mRDT (-) group [median (IQR); 907.8 (2870.3), P=5.01E^{-34}]. All hematological parameters were comparable between the groups, except for the MCHC that was higher in mRDT (+) patients (P=0.008).

Table 4. 1. Clinical, demographic and laboratory charasteristics of the study participants.

Characteristic	Clinical characteristics mRDT Positive (n=194)	mRDT Negative (n=25)	P-value
Microscopy n (%)			
Positive	177 (91.2)	25 (100)	
Negative	17 (8.8)	0 (0.0)	a 0.103
Sex n (%)			
Male	112.0 (57.8)	13.0 (52.0)	b
Female	82.0 (42.2)	12.0 (48.0)	0.388
Parasitemia (MPS/µL)	7674.8 (28790.4)	907.8(2870.3)	**5.01E-13**
Hematological Parameters			
Hgb g/dL	10.1 (2.2)	8.8 (3.2)	0.164[c]
RBC $\times 10^6$/µL	4.6 (1.0)	4.5 (1.1)	0.799 [c]
MPV fL	8.1 (1.0)	8.2 (2.0)	0.586 [c]
Plt $\times 10^3$/µL	197.0 (165.3)	195.7 (142.0)	0.929 [c]
MCV fL	66.8 (10.5)	66.0 (10.1)	0.544 [c]
MCH	21.3 (4.3)	20.8 (3.0)	0.119 [c]
MCHC g/dL	**31.8 (1.5)**	31.2 (1.8)	**0.008** [c]
Hct %	31.0 (6.7)	28.5(9.6)	0.408[c]
RDW	20.6 (4.3)	19.7 (7.6)	0.313[c]

Data are presented as the median (Interquartile range) and n (%) number of children. [a]Statistical significance was determined by Fischer's exact test. [b]Statistical significance was determined by Chi-square (χ^2) test. [c]Statistical significance was determined by Mann-Whitney U test. **Abbreviations**: mRDT-malaria Rapid Diagnostic Tests, RBC-Red blood cells, Hct-Haematocrit, MCV-Mean corpuscular volume, RDW- Red cell distribution width, MCHC- Mean corpuscular haemoglobin concentration, MPV- mean platelet volume, Pltplatelets.

4.2. The prevalence of *PfHRP2/3* deletions and the relative strain abundance of deleted strains in Polyclonal infections among children with symptomatic malaria in Siaya County, western Kenya

4.2.1 The prevalence of *PfHRP2/3* deletions among children with symptomatic malaria in Siaya County, western Kenya

The results for the multiplex qPCR assay are shown in **Table 4.2.** For these analyses, qPCR (+) refers to samples that were positive for all the genes, while qPCR (-) refers to those samples that are negative for all genes, except the internal control (*HumTuBB*). A total of 219 samples from different study participants were genotyped using the multiplex qPCR. Of the 219 samples that were genotyped, 59.8% (n=131) were qPCR (+), while 22.8% (n=50) were qPCR (-). There were 5.9% (n=13) samples negative for all genes, including the internal control. Therefore, individuals with no amplification in the HRP2 and HRP3 genes but had lower parasite densities less than 5, were excluded from further analysis. This exclusion was a precautionary measure, as the absence of gene amplification might be attributed to an insufficient parasite template rather than gene deletions, making the results inconclusive. Additionally, 5.9 (n=13) of the samples with low parasite densities (≤5 parasites/μL) were classified as indeterminate and excluded from definitive deletion calling for *PfHRP2*/3. Of the indeterminate samples, 3.2% (n=7) did not amplify for *PfHRP2* (C_t set at 35) but were positive for the other three genes, while 2.7% (n=6) were positive for *HumTuBB* and *PfLDH* but did not amplify for either *PfHRP2* or *PfHRP3*. As such, it was not clear whether the lack of amplification of *PfHRP2*/3 was due to lack of sufficient DNA template for amplification or low parasite densities that are beyond qPCR detection threshold. Based on definitive calling for the deletion patterns, *PfHRP2* monoclonal deletions were detected in 1% (n=2) of the samples, while no *PfHRP3* deletions were observed (i.e., no double deletions present).

Polyclonal deletions were detected in 4.6% (n=10) of the study participants with 4.1% (n=9) and 0.5% (n=1) had polyclonal deletion in *PfHRP2* and *PfHRP3* respectively. Overall, a total of 5.6% (n=12) of the samples had deleted clones for either *PfHRP2* or *PfHRP3* in monoclonal and polyclonal infections (see **Table 4.2**).

Table 4.2. Patterns of gene amplifications using multiplex qPCR

Clinical Samples (n=219) n (%)	***HumTuBB***	***PfLDH***	***PfHRP2***	***PfHRP3***
qPCR positive 131 (59.8)	+	+	+	+
qPCR negative 50 (22.8)	+	-	-	-
Invalid samples 13 (5.9)	-	-	-	-
< 5 Parasites/µL (Indeterminate *PfLDH* C_t Value<35)				
Isolates 7 (3.2)	+	+	-	+
Isolates 6 (2.7)	+	+	-	-
***PfHRP2* Monoclonal Deletion**				
PfHRP2 deletion 2 (1)	+	+	-	+
PfHRP3 deletion 0 (0.00)	+	+	+	+
***PfHRP2/3* Polyclonal Deletion**				
PfHRP2 polyclonal deletions 9 (4.1)	+	+	+/-	+
PfHRP3 polyclonal deletions 1 (0.5)	+	+	+	+/-
Overall deletion (*PfHRP2*-, *PfHRP2*+/-, *PfHRP3*+/-) 12 (5.6%)				

The symbol '+' indicates the gene amplification while '-' indicates no gene amplification. The '+/-' symbol shows some genes were amplified and some were not in the same sample, in the respective gene (polyclonal). n (%) represent the number of samples and their percentages. **Abbreviations:** *HumTuBB- Human tubulin*, *pfLDH- Plasmodium falciparum Lactate dehydrogenase, PfHRP2- Plasmodium falciparum Histidine rich protein 2, PfHRP3- Plasmodium falciparum Histidine rich protein 3,* Pol-polyclonal. 21

4.2.2 Relative abundance of PfHRP2/3-deleted strains in polyclonal infections

Determination of relative parasite strain abundance revealed that for all of the isolates with *PfHRP2* and *PfHRP3* polyclonal strains, the strains with deletions constituted the minority (with less than 50% abundance) while the wild type genes were the majority strains (more than 50% abundance) as shown in **Figure 4.1 (A)**. Moreover, the ΔCt values for samples that had polyclonal infections and the pure wild type were statistically different (*P*=0.007), further demonstrating the ability of the multiplex qPCR to correctly identify and distinguish mixtures in polyclonal infections.

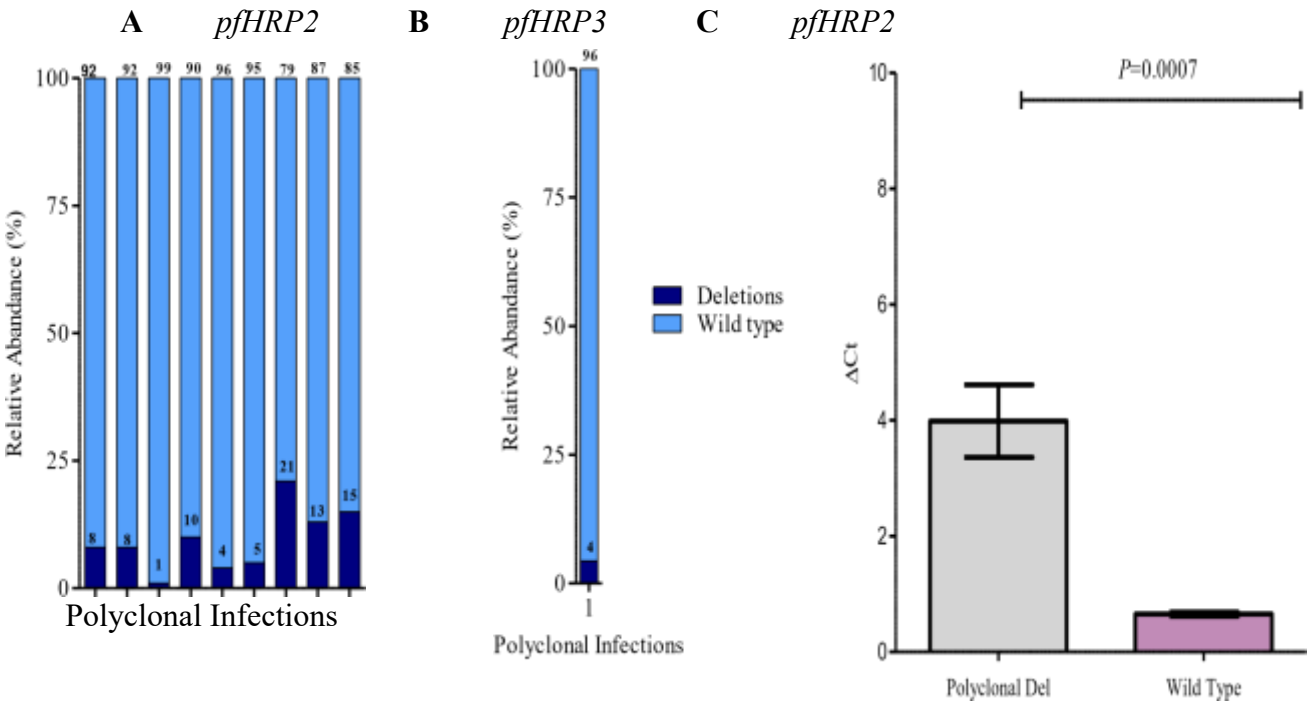

Figure 4. 1. Relative strain abundance of deleted strains in polyclonal infections.

(A) Relative Abundance of *PfHRP2* deletions in polyclonal infection. (B) Relative Abundance of *PfHRP3* deletion in polyclonal infection. (C) ΔCt values for samples with deleted strains mixed in polyclonal infections and the wild type. Statistical significance was determined using Students t-test**.**

4.3. Comparison *P. falciparum* parasite densities estimated by qPCR and microscopy and the relationship on mRDT results in children with *P. falciparum* infection in Siaya County, western Kenya.

4.3.1 Comparison *P. falciparum* parasite densities estimated by qPCR and microscopy

The study quantified and compared the median parasitemia levels for samples that were positive for qPCR and this was compared to parasitemia estimated by microscopy. The multiplex qPCR demonstrated a higher ability in detecting malaria parasites compared to microscopy with median (IQR)) of $8.28E^4$ ($2.75E^6$) and $6.24E^3$ ($2.45E^4$) respectively ($P \leq 0.001$). Further, there was a positive correlation between parasite detection by qPCR and microscopy (r=0.59, $P \leq 0.001$), **(Figure 4.2).**

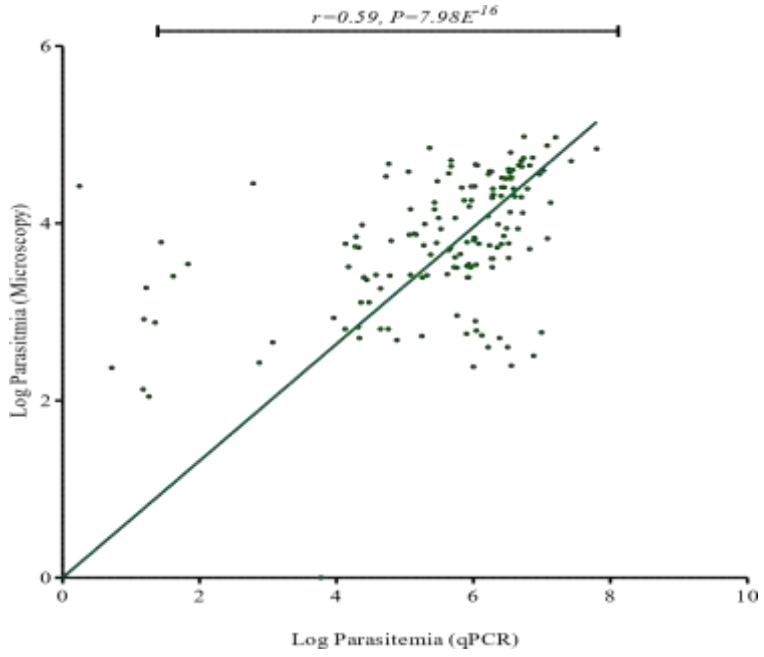

Figure 4. 2 .Correlation of parasitemia positive values obtained from diagnostic qPCR assay and microscopy.

The dots represent estimated parasitemia counts by both techniques and r is the Pearson's correlation coefficient

4.3.2. Relationship between parasitemia levels and mRDT results

To explore the possibility of the parasitemia levels and the significant effects on mRDT, samples were categorized by mRDT results. The samples that were mRDT positive had significantly higher parasite densities, median (range by qPCR), $2.6E^6$ (25180-$3.3E^6$) and

(median range by) microscopy 18427 (247-95105) as compared to mRDT negative samples

P=0.05, median (range by qPCR), 38448 (11-99476) and (median range by) microscopy 3506.31(120-7209) (*P*≤0.001), **Table 4.3.** Additionally, the 25 mRDT/microscopy discordant samples were as a results of low parasite densities that were beyond the detection threshold but not due to deletion of the target gene.

Table 4. 3. Relationship between parasitemia levels and mRDT results

Median Parasite/µL	mRDT+ (n=129)	mRDT- (n=19)	*P*-value
qPCR (Range)	6 6 2.6E (25180-3.3E)	18427 (247-95105)	**0.05**
Microscopy (Range)	38448 (11-99476)	3506.31 (120-7209)	≤0.01

Median Parasite/µL includes samples that were positive by *pfLDH* with parasitemia <5 parasites_µL. Mann-Whitney's U test was used to determine the statistical significance. mRDT= Malaria Rapid Diagnostic Test, *pfLDH*=Plasmodium falciparum Lactate Dehydrogenase. The symbol '+' represents positive, while '-'represents negative

4.3.4. Variations in performance of clinical diagnostic techniques in Siaya County Referral Hospital, western Kenya compared to multiplex qPCR

Using the multiplex qPCR as a reference, a false positivity rate of 17.9% (37) and 24.7% (50) was obtained for microscopy and mRDT test. Additionally, a false negativity rate of 1.94% (4) and 12.1% (25) was also observed by microscopy and mRDT respectively. Microscopy demonstrated a higher diagnostic sensitivity of 97.6% (95%CI, 89.8-105.0) and specificity a of 26.0 % (95%CI, 22.4-29.6) as compared to mRDT in detecting malaria parasites. Analysis using Cohen Kappa's test revealed a fair agreement between microscopy and qPCR, (k=0.30, $P \leq 0.001$). However, there was no agreement between mRDT and qPCR results, (k= 0.0, $P \leq 0.001$) **(Table 4.4)**

Table 4. 4. Performance of clinical diagnostic techniques relative to multiplex qPCR

Clinical diagnostics (n)	Multiplex qPCR (n) Positive	Negative	Sensitivity% (95%CI)	Specificity% (95%CI)	PPV%	NPV%	Kappa's Test	*P*-value
Microscopy								
Positive	152	37	97.4	26.0	80.4	76.4	0.30	≤0.001
Negative	4	13	(89.8-105.0)	(22.4-29.6)				
mRDT								
Positive	131	50	84.0	0.0	72.4	0.0	≤ 0.0	0.002
Negative	25	0	(77.3-90.0)	(0.0)				

Data are presented as n, total number of samples. *P* value is significant at values ≤ 0.05. Significant test was done using Cohen's Kappa's test. Significant values are presented in bold. PPV=Positive predictive value, NPV=Negative predictive value

CHAPTER FIVE

DISCUSSIONS

5.1. The prevalence of *PfHRP2/3* deletions and the relative strain abundance of deleted strains in Polyclonal infections among children with symptomatic malaria in Siaya County, western Kenya.

The emergence of *P. falciparum* isolates lacking the *HRP2*/3 and escaping surveillance and detection by *PfHRP2*-mRDT threatens the control and elimination strategies deployed by malaria management programs. This study reports the presence of *P. falciparum* isolates lacking *HRP2* gene including in polyclonal infections in a high transmission holoendemic area. The deletions were detected with a prevalence of 1% and 4.6% in monoclonal and polyclonal infections respectively. The *PfHRP2/3* deletion prevalence including in polyclonal infection found in this study was relatively high compared to those previously reported in Kenya (Beshir *et al.*, 2017; Grignard *et al.*, 2020; Rogier *et al.*, 2022). In other parts of the world such as southwestern Ethiopia and Brazil, there was high prevalence of HRP3 deletions to up to 73% (Vera-Arias *et al.*, 2021). These findings emphasize the variability in the prevalence of HRP2 and HRP3 deletions across different geographical regions worldwide.

This increased risk of single *PfHPR2* deletion in parasite isolates has not yet been reported in this high transmission holoendemic area. There is need for surveillance to cover a wider area to determine the extent of single *PfHPR2* mutations in the whole of western, Kenya to include detection in polyclonal infection as this brings the true picture of the parasites that might have the mutation. This will further improve on answering the question on whether the *PfHRP2*mRDT false negativity has reached a decision that require a diagnostic change (WHO, 2020a) .

Although the study was able to detect single *PfHPR2* mutations, the deletions had no significant impact on the *PfHPR2* -mRDT probably due to the present of its intact homologue high parasitemia present in monoclonal deletion and polyclonal infection respectively. Importantly, the spread of *PfHRP2* deletions is affected by transmission intensities due to the high complexity of infection. In high transmission areas for instance, *PfHRP2* deletions can be present but undetected in polyclonal infections due to technical limitations of detection (Balmer *et al.*, 2011). This is because in polyclonal infections, *PfHRP2/3*-deleted variants are masked by intact *PfHRP2/3*-positive variants and thus false-negative mRDT results can be rare, even though *PfHRP2* deletions are common. This can however can be exacerbated when transmission intensity drops due to successful control interventions, potentially challenging diagnostics as improvements in reducing transmission are made (Balmer *et al.*, 2011; Fola *et al.*, 2017). In order to overcome this potential challenge and underestimation of prevalence, especially in this high transmission holoendemic area, masked clones and their relative abundance in the population were identified. In summary, the results presented demonstrates that *P. falciparum* parasites lacking *HRP2*/3 genes are present in this holoendemic region of Kenya but with no effect on the currently used HRP2-mRDT due to the intact homologues still being present despite the deletion. As such, there is no need to change the diagnosis by HRP2-mRDT in Siaya County, western Kenya.

Although they are present, they are still detectable by the currently used *PfHPR2* -mRDT. The findings presented underscores the need for further surveillance of *PfHRP2/3* mutant to cover the entire western Kenya.

5.2 Comparison of *P. falciparum* parasite densities estimated by qPCR and microscopy and the effects on mRDT results in children with *P. falciparum* infection in Siaya County, western Kenya.

Low density malaria infections can lead to a high human morbidity as they are commonly missed by the routinely used malaria diagnostic techniques (Plucinski *et al.*, 2019). Malaria parasites in the blood of a malaria patient range from 1 parasite/µL to millions of parasites/µL of whole blood depending on the specie being quantified (Aschar *et al.*, 2022). Microscopy and qPCR are the most common methods used for quantification of malaria parasites and thus it is important that the two methods can be interpreted as giving equivalent results. This is because the concordance assures healthcare providers, researchers, and public health authorities that the results obtained from these methods are reliable, leading to better patient care, effective treatment decisions, accurate epidemiological data, and improved research outcomes in the fight against malaria. The multiplex qPCR method that was used in this study could detect up to 3 parasites/µL which can be very hard to detect using microscopy tests (Grignard *et al.*, 2020; Otambo *et al.*, 2022; Schindler *et al.*, 2019).

Although, the gold standard is limited at low levels and its accuracy is influenced by the film type (thick or thin), microscopist's expertise, and WBC count (theoretical or actual) used (O'Meara *et al.*, 2005)**.** Furthermore, microscopy its quantification at low levels is limited (Ballard *et al.*, 2019). The result of this study showed that qPCR is a more precise method in quantifying parasitemia especially when being used to detect parasitemia at very low levels, which can be hard to detect using the microscope. The current study's findings, which revealed the existence of sub-microscopic parasitemia, align with a previous study conducted in western

Kenya that also identified sub-microscopic infections. (Otambo *et al.*, 2022). These precise estimates using qPCR may be attributed to its ability of quantifying sexual life stages which may not be included in microscopy counts (Ballard *et al.*, 2019). Parasitemia values from microscopy and qPCR further demonstrated a good positive correlation, with no proportional biasness whatsoever.

5.3. Performance of clinical diagnostic techniques in Siaya County Referral Hospital, western Kenya compared to multiplex qPCR.

Accurate malaria diagnosis plays a pivotal role in enabling appropriate and effective malaria treatment. Therefore, there is a pressing need to advance the development of highly sensitive and reliable diagnostic tools, including quantitative polymerase chain reaction (qPCR). Such advanced tools have proven invaluable in research settings, allowing for the monitoring of interventions and detection of sub-microscopic infections that would otherwise remain undetected by conventional microscopic techniques (Oboh et al., 2021; Otambo et al., 2022). This study therefore evaluated the 2 routinely used clinical diagnostic techniques (*PfHRP2*mRDT and microscopy) with the multiplex qPCR as a reference. Analyses from this study revealed microscopy as a highly sensitive and specific diagnostic tool as compared to mRDT This further explains why despite emergence of more sophisticated diagnostic tools, microscopic examination continues to be considered the gold standard for laboratory confirmation of malaria (Azikiwe et al., 2012; WHO, 2010a). Microscopy has been the gold standard for decades; its use is still limited especially in resource contains areas with limited microscopists. The specificity of microscopy however can be limited in areas where sub microscopic infections occur making them to be missed by microscopists (Otambo *et al.*, 2022). Additionally, microscopist/ microscopy errors are common especially in scenarios where a reading is done by one microscopist (Ohrt *et al.*, 2002). For quality control, the

WHO recommends at least two microscopist to be present at one particular time during a routine diagnosis (WHO, 2000). As such, microscopic technique is limited for detection parasitaemia in a number of ways (Berzosa *et al.*, 2018). Due of the limitations of microscopy technique, mRDT evolved as they can be interpreted within 20 minutes (WHO, 2004). This study demonstrated a high false positivity rate with *PfHRP2*-mRDT. This is worrying as treatment may be administered to patients who may not infected by malaria in areas where the diagnosis and treatment of malaria is entirely based on this technique. This low specificity of *PfHRP2*-mRDT has however been linked to the high thermal stability of the target gene, making the *HRP2* gene to persist for up to a month after parasite clearance in the blood stream of an infected patient (Iqbal *et al.*, 2004; Toote *et al.*, 2017). As such, this persistent antigenemia makes it hard for *PfHRP2*-mRDT to distinguish between passive and active infection.

Additionally, the performance of *PfHRP2*-mRDT is subjected to a number of factors which can actively affect its sensitivity (Kozycki *et al.*, 2017). First, antigenic variations due to parasites lacking the target gene and its homologue renders the antigen undetectable by the kit (Funwei *et al.*, 2019; Iriart *et al.*, 2020). Secondly, low parasitemia levels that are beyond the detection limit of *PfHRP2*-mRDT may lead to false negative results impacting negatively on malaria control and elimination efforts (Kozycki *et al.*, 2017). Third, the ability of mRDT to identify true positive results may be limited due to operational and storage errors (Parr *et al.*, 2021). Although RDTs are used for rapid diagnosis, diagnosis by microscopy should go hand in hand because it has a high specificity (97%) as compared to mRDT test. In addition, microscopy allows the calculation of parasite densities and differentiation of species as opposed to mRDT test. Although PCR is the best diagnostic method with the highest sensitivity and specificity, it is laborious, expensive and may be limited for routine diagnostics (Berzosa *et al.*, 2018).

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

The study highlights the presence of *PfHRP2/3*-deletion infections in a holoendemic high transmission area, in Siaya County, western Kenya with a 5.6% total prevalence. Additionally, the parasites harbouring deletions had no significant effects of *PfHRP2/3* -mRDT test. The multiplex qPCR demonstrated higher performance when compared to microscopy in estimation of parasite densities

6.1. Conclusion

1. The current study reports a total prevalence of 5.6% parasites with *PfHRP2/3* deletions suggesting that *PfHRP2/3* are in circulation within western Kenya and are more likely to be detected in polyclonal infections.
2. From the study, the multiplex qPCR could detect up to 3 parasites/uL in whole blood, suggesting it is the most accurate and robust approach in detecting malaria parasites compared to microscopy.
3. The mRDT had low specificity and was in not agreement with qPCR, thus although mRDTs are used for routine rapid malaria diagnosis, diagnosis by microscopy is more sensitive and specific.

6.2 Recommendations from the current study

The study underscores the need for further surveillance in an expanded region of western Kenya.

1. Parasite densities should be estimated when carrying out studies of this nature to avoid false deletion calls
2. Multiplex qPCR should be used in determining *PfHRP2/3* deletions in polyclonal infections

6.3 Recommendations for future studies

1. The estimation of prevalence of PfHRP2/3 deletions should encompass the detection of deletions hidden within polyclonal infections. It is crucial to incorporate gene sequencing for a comprehensive overview of the regions where these deletions occur.
2. Low parasitaemia as the cause of negative PCR should be ruled out before reporting on *PfHRP2/3*deletions for accurate reporting.
3. The multiplex qPCR should be used for accurate parasitaemia estimations as it has the ability to detect parasites as low as 3 parasites/μL

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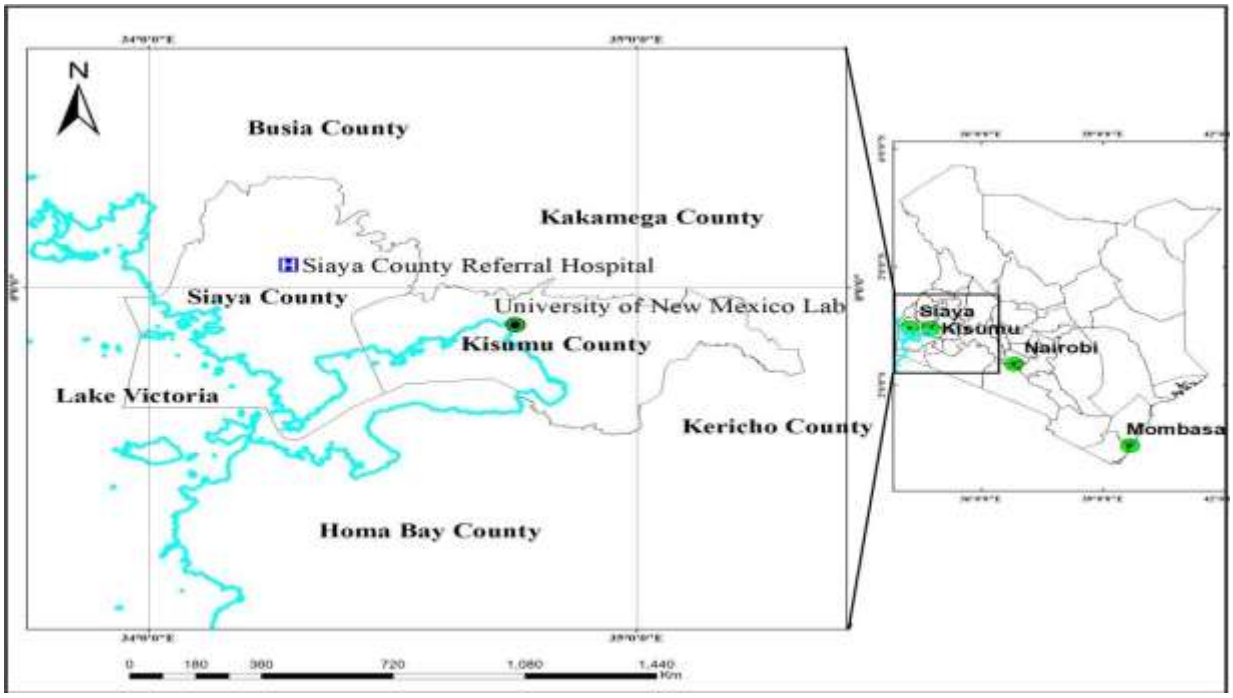
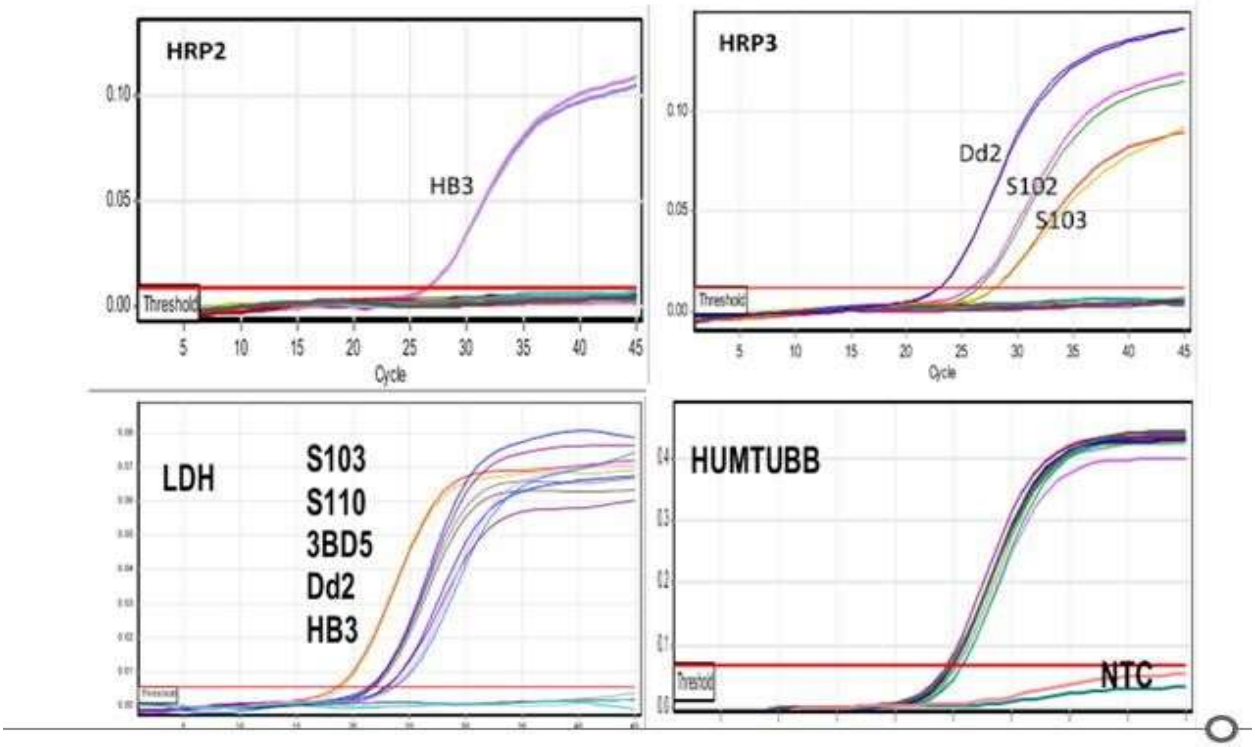
APPENDICES

Appendix 1: The map of Study area



Location of Siaya County Referral Hospital.

Appendix 2: Genotyping graphs generated using the multiplex qPCR



Quantitation Report

Experiment Information

Run Name	HRP2 QPCR plate 1 a9-a12 and plate2 a1-a4 23092022 (1)
Run Start	9/23/2022 9:23:25 AM
Run Finish	9/23/2022 11:29:16 AM
Operator	
Notes	
Run On Software Version	Rotor-Gene Q Software 2.3.1.49
Run Signature	The Run Signature is valid.
Gain Green	5.
Gain Yellow	5.
Gain Orange	5.
Gain Red	5.
Machine Serial No.	0315116

Quantitation Information

Threshold	0.09872
Left Threshold	1.000
Standard Curve Imported	No
Standard Curve (1)	N/A
Standard Curve (2)	N/A
Start normalising from cycle	1
Noise Slope Correction	No
No Template Control Threshold	% 0
Reaction Efficiency Threshold	Disabled
Normalisation Method	Dynamic Tube Normalisation
Digital Filter	Light
Sample Page	Page 1
Imported Analysis Settings	

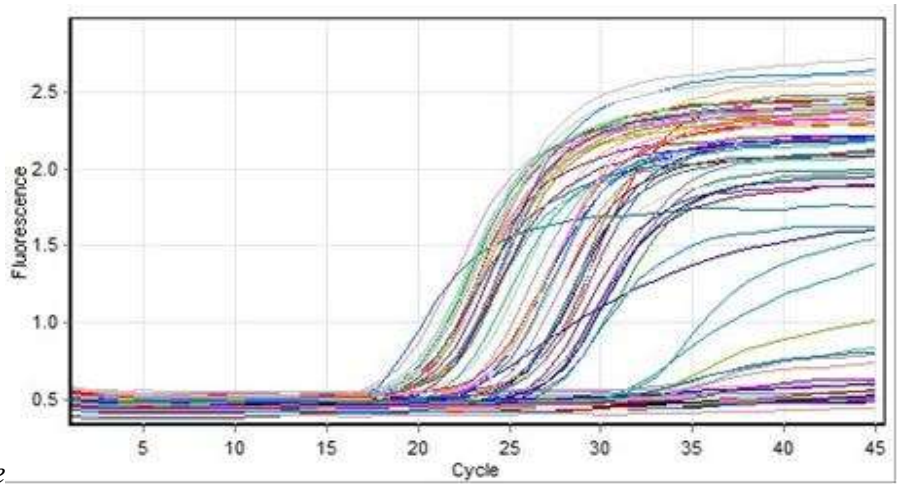
Messages

Message
Acquisition timed out, trying to resend at Cycling @ Repeat 33

Profile

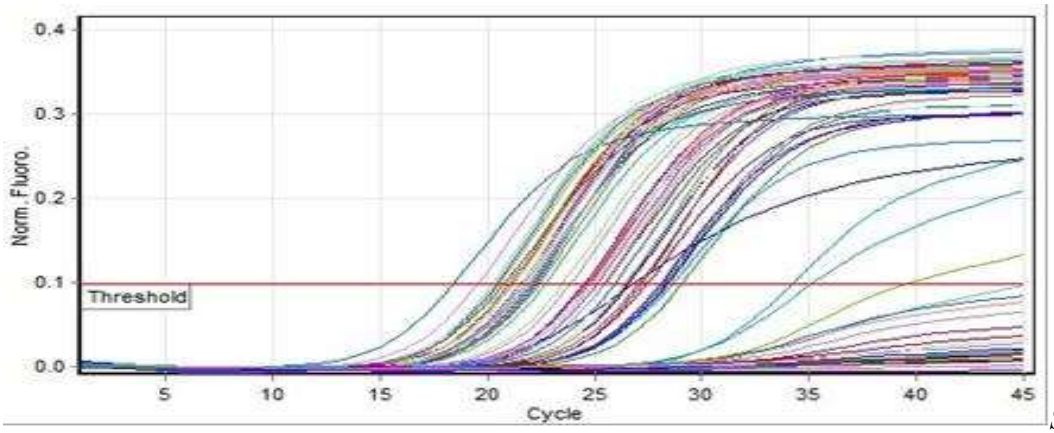
Cycle	Cycle Point
Hold	Hold @ 95°C, 6min 0s
Cycling (45 repeats)	Step 1: Hold @ 95°C, 15s
	Step 2: Hold @ 54°C, 30s, acquiring to Cycling A([Green][1][1],[Orange][3][3],[Red][4][7],[Yellow][2][2])
	Step 3: Hold @ 72°C, 30s

Raw Data For Cycling A.



A.Orange

Quantitation data for Cycling A.Orange



Standard Curve

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
A1		Unknown	Unknown	27.23			
A2		Unknown	Unknown	22.11			
A3		Unknown	Unknown	26.60			
A4		Unknown	Unknown	27.44			
A5		Unknown	Unknown				
A6		Unknown	Unknown	22.24			
A7		Unknown	Unknown	28.32			
A8		Unknown	Unknown				
B1		Unknown	Unknown	29.08			

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
B2		Unknown	Unknown				
B3		Unknown	Unknown				
B4		Unknown	Unknown	21.89			
B5		Unknown	Unknown	23.52			
B6		Unknown	Unknown	20.24			
B7		Unknown	Unknown				
B8		Unknown	Unknown	23.88			
C1		Unknown	Unknown				
C2		Unknown	Unknown	21.71			
C3		Unknown	Unknown	21.09			
C4		Unknown	Unknown	25.48			
C5		Unknown	Unknown	22.39			
C6		Unknown	Unknown				
C7		Unknown	Unknown	35.14			
C8		Unknown	Unknown				
D1		Unknown	Unknown	26.79			
D2		Unknown	Unknown				
D3		Unknown	Unknown	21.01			
D4		Unknown	Unknown	26.36			
D5		Unknown	Unknown	21.52			
D6		Unknown	Unknown				

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
D7		Unknown	Unknown	22.22			
D8		Unknown	Unknown	27.08			
E1		Unknown	Unknown	21.06			
E2		Unknown	Unknown	21.20			
E3		Unknown	Unknown	24.52			
E4		Unknown	Unknown	22.04			
E5		Unknown	Unknown	19.59			
E6		Unknown	Unknown	28.63			
E7		Unknown	Unknown	18.42			
E8		Unknown	Unknown				
F1		Unknown	Unknown	20.44			
F2		Unknown	Unknown	24.57			
F3		Unknown	Unknown				
F4		Unknown	Unknown	22.68			
F5		Unknown	Unknown	25.42			
F6		Unknown	Unknown	21.05			
F7		Unknown	Unknown	20.26			
F8		Unknown	Unknown				
G1		Unknown	Unknown				
G2		Unknown	Unknown	20.56			
G3		Unknown	Unknown	25.46			

No.	Color	Name	Type	Ct	Ct Comment	Given Conc (Copies)	Calc Conc (Copies)
G4		Unknown	Unknown				
G5		Unknown	Unknown	26.51			
G6		Unknown	Unknown	39.51			
G7		Unknown	Unknown	34.21			
G8		Unknown	Unknown	25.93			
H1		Unknown	Unknown				
H2		Unknown	Unknown	21.56			
H3		Unknown	Unknown				
H4		Unknown	Unknown				
H5		Unknown	Unknown	21.56			
H6		Unknown	Unknown	22.11			
H7		Unknown	Unknown	28.16			
H8		Unknown	Unknown	20.76			
I1		Int	Unknown	24.73			
I2		Int	Unknown	24.66			
I3		d2D	Unknown	28.40			
I4		d2D	Unknown	28.06			
I5		hb3	Unknown	24.91			
I6		hb3	Unknown	25.12			
I7		ntc	Unknown				
I8		ntc	Unknown				

Legend:

NEG (NTC) - Sample cancelled due to NTC Threshold.

NEG (R. Eff) - Sample cancelled as efficiency less than reaction efficiency threshold.

Appendix 3: Primer and probe sequences used in determination of *Pf*HRP2/3 deletions

Target gene	Size	Oligo name	Oligo sequence (5'-3')	Conc 5×PrimerMix[a]
PfHRP2	286 bp	*PfHRP2* fwd[b]	GTATTATCCGCTGCCGTTTTTGCC	1.5 µM
(PF3D7_0831800)		*PfHRP2* rev[b]	TCTACATGTGCTTGAGTTTCG	1.5 µM
		*PfHRP2*TxRd	TTCCGCATTTAATAATAACTTGTGTAGC	0.375 µM
PfHRP3	289 bp	*PfHRP3* fwd	ATATTATCCGCTGCCGTTTTTGCT	1.5 µM
(PF3D7_1372200)		*PfHRP3* rev	CCTGCATGTGCTTGACTTTCGT	1.5 µM
		PfHRP3 YY	CTCCGAATTTAACAATAACTTGTTTAGC	0.75 µM
PfLDH	287bp	*PfLDH* fwd	ATG GCA CCA AAA GCA AAA ATC GTT	0.2 µM
(PF3D7_1324900)		*PfLDH* rev	TTTG CAT TTG TTT CTC TCT TTG TTG CA	0.2 µM

Name Primer sequence

ReferencePfhrp2_F15’ TAATTSCGYATTTAATAATAACTTGTG-‘3This studyPfhrp2_F25’-

TAATTCCGCATTTAATAATAAC**G**TGTG-

3’Pfhrp2_F35’TAATTCCGCATTTAATAATAAC**G**TT**G**G-3’Pfhrp2_R15’-

CATCATCTACATGTGCTTGAG -‘3**Pfhrp2_R2**5’- CATCATCTACATGTGCT**G**GAG -

‘3Pfhrp2_R35’- CATCATCTACATGTGC**G**TGAG -‘3**Pfhrp2_probe**FAM 5’-

ATGCAAAAGGACTTAATTTAAATAAGAGATT-‘3 BHQ2Pfhrp3_F15’-

TCCGAATTTAACAATAACTTGTTTAGC-‘3Pfhrp3-R15’-

GTCAAGCACATGCAGGTGATG-‘3Pfhrp3_P1JOE 5’-

ATGCAAAAGGACTTAATTCAAATAAGAGATTA-‘3 BHQ1**Pfhrp3_F2**5’-

ACGGATTTCATTTTAACCCTTCACGA-

‘3**Pfhrp3_R2**5’TGAGAATCATCAAAACAAGCATTAGC-‘3**Pfhrp3_probe**JOE’-

ACAATTCCCATACTTTACATCATGCA-‘3 BHQ1**Pfldh_F**5’-

ACGATTTGGCTGGAGCAGAT-‘3[1]**Pfldh_R**5’-

TCTCTATTCCATTCTTTGTCACTCTTTC-‘3**Pfldh_probe**ROX 5’-

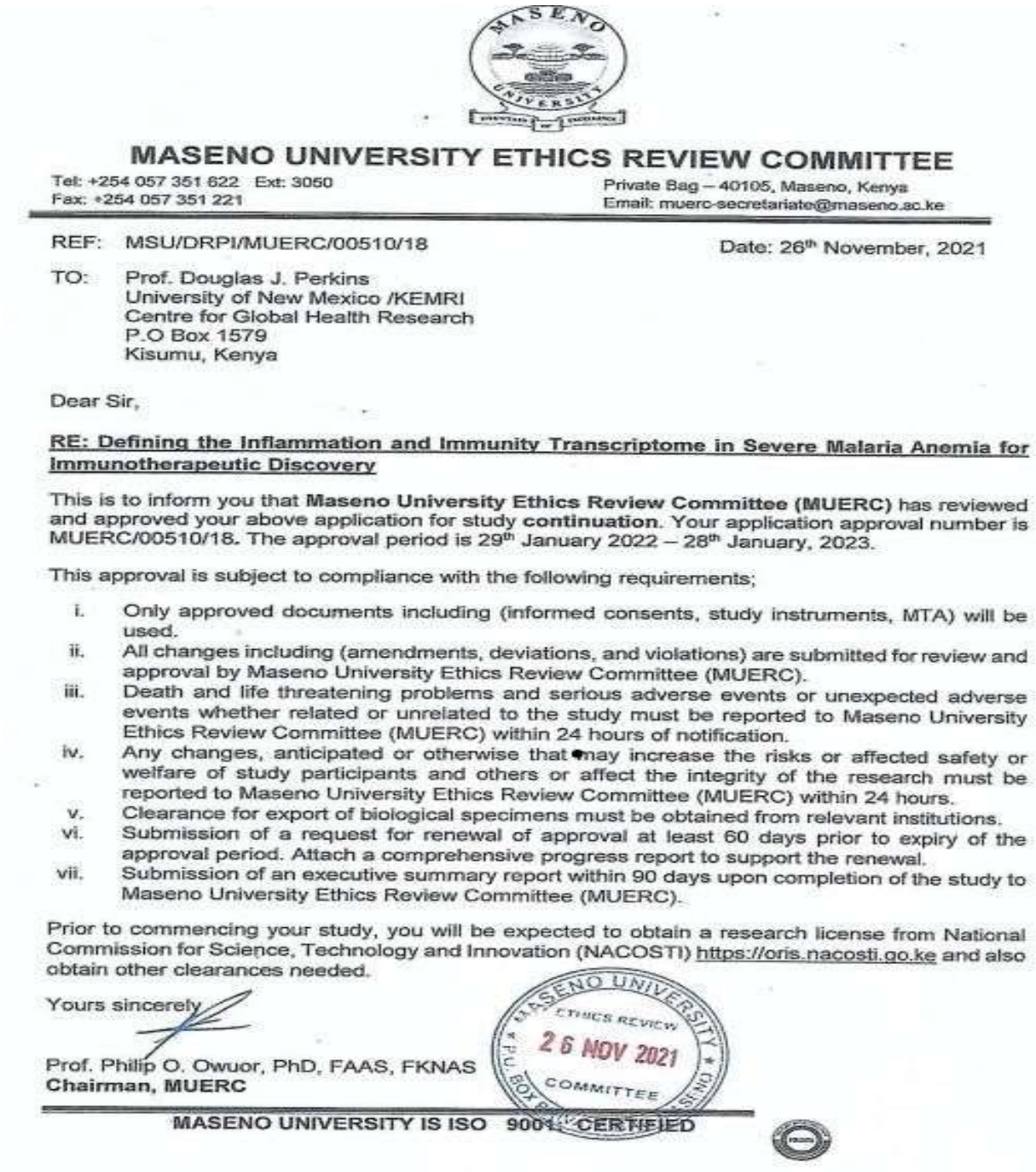
GTAATAGTAACAGCTGGATTTACCAAGGCCCCA-‘3

BHQ1**HumTuBB_F**5’AAGGAGGTCGATGAGCAGAT-‘3[2]**HumTuBB_R**5’-

GCTGTCTTGACATTGTTGGG‘3**HumanTuBB_P**CY5 5’-

TTAACGTGCAGAACAAGAACAGCAGCT-‘3 BHQ2

Appendix 4: Study Approval



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

REF: MSU/DRPI/MUERC/00510/18

Date: 26th November, 2021

TO: Prof. Douglas J. Perkins
University of New Mexico /KEMRI
Centre for Global Health Research
P.O Box 1579
Kisumu, Kenya

Dear Sir,

RE: Defining the Inflammation and Immunity Transcriptome in Severe Malaria Anemia for Immunotherapeutic Discovery

This is to inform you that **Maseno University Ethics Review Committee (MUERC)** has reviewed and approved your above application for study **continuation**. Your application approval number is MUERC/00510/18. The approval period is 29th January 2022 – 28th January, 2023.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by Maseno University Ethics Review Committee (MUERC).
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to Maseno University Ethics Review Committee (MUERC) within 24 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to Maseno University Ethics Review Committee (MUERC) within 24 hours.
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to Maseno University Ethics Review Committee (MUERC).

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely

Prof. Philip O. Owuor, PhD, FAAS, FKNAS
Chairman, MUERC

MASENO UNIVERSITY ETHICS REVIEW COMMITTEE
26 NOV 2021

MASENO UNIVERSITY IS ISO 9001: CERTIFIED

Appendix 5: Study

consent form

Study Consent Forms (English Version) Screening Form 1a: Written consent for parent/guardian for screening of child for malaria aneamia hospital-based prospective study

The Kenya Medical Research Institute (KEMRI) and the University of New Mexico are doing a research study at the Siaya County Referral Hospital (SCRH) to see how children fight malaria. Dr. Douglas Perkins of University of New Mexico would like your child to be screened to see if your child can be in the study since you live in an area where nearly all children get malaria. The title of the study is "The genetic basis of severe malaria aneamia". This research study is funded by the National Institutes of Health (NIH) in the United States. We want to learn how children fight malaria during the first three years of life. We will enroll children who are 6 to 36 months of age and follow them for 3 years for the date of enrollment. The study ends on February 2023. We might learn ways to help improve treatment or prevention of malaria. Before being joining the research study your, child must have several tests to see if he or she is in the groups of children that the research study will be done in.

Screening Procedure: Since this study will only have only children with malaria and aneamia (low blood), we will have to screen your child to see if he or she has malaria and low blood. If you choose for your child to have the screening done, we will ask you about your child's health and check for malaria and low blood. To check for malaria and low blood, we will get blood (several drops) by sticking your child's heel or finger with a small needle.

Also, we know that HIV/AIDS is a problem in this area. HIV/AIDS affects the way the body fights infections. There are blood tests that can be done to learn if your child has the virus that causes AIDS. This virus is called HIV. We would like to test your child's blood for HIV exposure. The research study will enroll children that have HIV exposure and those that do not have HIV exposure. However, it important for the research study to know who has HIV exposure since it affects the way your child fights infections. If your child has HIV exposure, this means that he or she may or may not have HIV. To see if your child has HIV exposure, we must do a rapid blood test from the several drops of blood we will get by sticking your child's heel or finger with a small needle. If the rapid blood test is positive, then we must do another test to see if your child has HIV in his or her body.

If you want your child to be tested for HIV, you will have to talk to one of our HIV counselors. The counselor will give you facts about HIV. These facts will be about the HIV blood tests and how you can keep your child from getting HIV. You will receive the results of the rapid test (for HIV exposure) the same day. If your child has a positive rapid test for HIV, we will do the additional test. The additional test will tell you for certain if your child has HIV. The HIV counselor will give you the results of the additional HIV test result within two weeks when you come back to the hospital. Whether the blood test is negative or positive, you will be the only

positive for HIV, we would like you to talk with a doctor. However, if you choose, only you will get the HIV test results of your child. You do not have to speak with a doctor. We will not tell any other person the result of the test unless you ask us to do so. Testing for HIV and talking to HIV/AIDS counselors will be free of charge. If you want, we will refer your child to the Patient Support Center (PSC) at SDH so that your child can get medicine that can help fight the HIV virus. HIV can also cause low blood in your child. Professional counselors will give you facts about the problems of low blood. They will also talk with you about why it is important to go to follow-up visits and take the vitamins and drugs for malaria and HIV.

Only a person trained to get blood will take blood from your child. If your child has no problems with bleeding, taking blood should not cause harm. There may be a small bruise or short time of discomfort when we do the finger- or heel-stick. If your child has a problem with the finger- or heel-stick, Dr. Benjamin Esiaba, of the MOH, will treat the problem at SDH.

Benefits from the screening for malaria, low blood, and HIV are access to malaria and HIV testing and counseling for your child. If your child has malaria, you will be referred to MOH doctors to provide appropriate treatment. You can also get drugs for your child from the MOH if your child has HIV. Other benefits include getting vitamins with iron for your child that may improve his or her health status.

Having the screening done is up to you. If your child is eligible for the research study, based on the screening results, you may choose to have your child enrolled in the research study. If you do not want your child to be in the research study, your child will still get the best possible medical care at the hospital. If you decide you want your child to be in the research study, you must discuss the research study with a member of the research team and sign the consent form for enrollment (participation).

If you have questions about HIV or AIDS, you are free to ask the HIV/AIDS counselors. If you have questions about the study or feel you have been harmed, you can contact Dr. Douglas Perkins (KEMRI/University of New Mexico, PO Box 1578, Kisumu, Kenya, telephone: 0733360098). You can also contact Dr. John Michael Ong'echa (KEMRI/University of New Mexico, PO Box 1578, Kisumu, Kenya, telephone: 0733447920). You can also contact Dr Benjamin Esiaba (telephone 321055/321554) at SDH, Nyanza Province, P.O. Box 144, Siaya. In the United States, Dr. Perkins address is: University of New Mexico, MSC10-5550, 1 University of New Mexico, Albuquerque, NM, 87131 (telephone 505-272-6867, e-mail dperkins@salud.unm.edu). For questions or problems about your rights as a research subject, please call or write: The National/KEMRI Ethical Review Committee, PO Box 54840, Nairobi, telephone: 02-20722541.

Parent/guardian's name: _____ **Child's name:** _____
(Please Print) (Please Print)

Date: _____ **Study #:** _____

Parent/guardian's statement:

□ The above screening process has been explained to me. The screening consent form has been read to me or I have read the screening consent form. My questions have been answered to my satisfaction. I have received a copy of this form. I was told that being in the research study is my choice. I was told that for my child to be in the research study that I must discuss the research study with a member of the study team. I was told that to be in the research study I must sign the consent form for the research study that is separate from this form. I agree for my child to be screened for taking part in the research study. By signing this form, I give my consent for my child to having screening for the research study.

Signatures:

Parent/guardian's signature: _____ **Date:** _____

Witness Signature: _____ **Date:** _____

Parent/guardian' thumbprint: _____

HIV Testing:

□ The above screening process has been explained to me. The consent form for screening has been read to me or I have read the screening consent form. My questions have been answered to my satisfaction. I have been told that HIV counseling is available to me before I decide if my child will have HIV testing. I agree that my child's blood sample can be tested for HIV.

Parent/guardian's signature:_____ **Date :**_____

Parent/guardian's thumbprint: _____

INVESTIGATOR'S CERTIFICATION

I certify that the nature and purpose, the potential benefits and possible risks associated with participation in this research study have been explained to the above individual and that any questions about this information have been answered.

Investigator's signature: _____ **Date:** _____