Evaluation of *Plasmodium falciparum* histidine rich protein 2 and 3 genes deletion prevalence in Kenya and it implication to RDTs use

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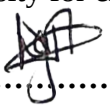
Declaration

This thesis/research project is my original work and has not been presented for a degree in any other university for examination

Signature ……  **……** **Date …**27 October 2020**……**

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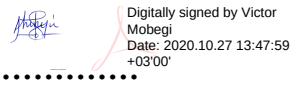
Signature …… 

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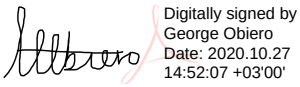
Signature …… 

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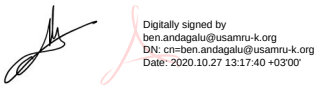
Signature …….. 

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

AA	Amino Acid
Ab	Antibody
ACT	Artemisinin Combination Therapy
Acyl- CoA	Acetyl Coenzyme A
Ag	Antigen
AL	Artemether Lumefantrine
CDC	Centre for Disease Control and Prevention
CI	Confidence interval
CT	Cycle Threshold
DdNTPs	Dideoxynucleoside triphosphates
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
ExoSAP	Exonuclease-Shrimp Alkaline Phosphatase
GTS	Global Technical strategy
ICS	Immunochromatography
IMCI	Integrated Management of Children Illness
KEMRI	Kenya Medical Research Institute
KEMRI SSC	Kenya Medical Research Institute Scientific Steering Committee

MDR	Malaria Drug Resistance lab
MSA	Multiple Sequence Alignment
PCR	Polymerase Chain Reaction
***Pfhrp*1**	*Plasmodium falciparum* histidine rich protein 1
***Pfhrp*2**	*Plasmodium falciparum* Histidine rich protein 2
***Pfhrp*3**	*Plasmodium falciparum* Histidine rich protein 3
pH	Potential of Hydrogen
PHISTb	*Plasmodium* helical interspersed subtelomeric domain b
PLDH	*Plasmodium* Lactate Dehydrogenase enzyme
RBCs	Red Blood Cells
RDT	Rapid Diagnostic test
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
SNPs	Single Nucleotide Polymorphisms
TAE	Tris Acetate EDTA
UN	United Nations
UNICEF	United Nations International Children's Emergency Fund
USAMRD-A	US Army Medical Research Directorate - Africa
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research

Abstract

Globally, Rapid Diagnostic Tests (RDTs) diagnoses about 75% of all suspected malaria cases. The accuracy of the most frequently used *Plasmodium falciparum* histidine rich protein 2 (*Pfhrp*2) based RDTs can be compromised by deletion in *Pfhrp*2 gene or cross-reaction with *Pfhrp*3 antigens. The criteria of World Health Organisation (WHO) necessitate accuracy above 95 % as the threshold for selection or withdrawal of RDTs advocating for vigorous *Pfhrp*2 gene deletions mapping. As part of continuing study on epidemiology of malaria drug resistance, the objective of this study was to determine occurrence and trends of *Pfhrp*2 and/or *Pfhrp*3 genes deletion as well as their polymorphisms in three of five malaria transmission zones in Kenya.

This study used samples from the epidemiology of malaria drug resistance in Kenya study that started in 1992. This specific study used 350 samples comprising 255 samples collected between 2013 and 2017 (Post-RDTs) in Kombewa, Kericho, and Malindi and 95 samples collected between 2003 and 2005 (Pre-RDTs) in Kericho, Malindi, Alupe and Kisumu. The samples were diagnosed for malaria by microscopy, *Pfhrp*2/pLDH RDTs and real time PCR using primers targeting 18S ribosomal RNA. RDTs were not conducted on these samples, as only DNA samples were available. To identify genes deletions and amino acid repeat types, primers targeting *Pfhrp*2 and *Pfhrp*3 genes were used to amplify and sequence all *P. falciparum* PCR positive samples.

Out of 350 samples, 327 (93.4%) were *P. falciparum* positive by PCR, comprising 93 (28.4%) pre-RDTs and 234 (71.6%) post RDTs. The prevalence of single *Pfhrp*2 gene deletion was 0.3% while that of *Pfhrp*3 was 2.1% of the 327 samples genotyped. No sample had double deletion of both *Pfhrp*2 and *Pfhrp*3 genes. All pre RDTs samples had intact *Pfhrp*2 and *Pfhrp*3 genes. For both *Pfhrp*2 and *Pfhrp*3, deletion of the gene was not associated with deletion of any of the flanking genes. Twenty one (10.8%) of the 195 samples positive by *P. falciparum* PCR (with 30 parasites per microliter) and pLDH RDTs were negative by *Pfhrp*2 based RDTs. This appeared to be false negative *Pfhrp*2 based RDTs results, but genotype analysis showed that all the 21 samples had *Pfhrp*2 and only one sample (4.8%) had deletion of *Pfhrp*3 gene.

Out of 327 samples sequenced, most had poor coverage at the intron and at the start of exon 2. However, 69 clean *Pfhrp*3 sequences were obtained. Thirty-one of the 69 sequences had no deletion while the other 38 sequences had partial deletion. Amino acid position 94 to 100 had the highest prevalence of deletion occurring in 27 (71.1%) of all 38 sequences. Amino acid repeat type 4 and type 16 were the most frequent, occurring 4 to 14 times in all 69 sequences.

This study findings shows that *Pfhrp*2 and *Pfhrp*3 genes deletion are indeed present in Kenya among the symptomatic individuals enrolled in this study. However, these genes deletion could not be associated to false negative RDTs results.

This finding heralds the need for investigating additional mechanisms of false-RDTs negativity. Further work needs to be done using concurrent cohort and include asymptomatic cases as the method used for screening in the positive could present a bias. In addition, Whole genome sequencing approach may be used rather than Sanger sequencing since it is more informative. Research on new targets of RDTs need to continue in order to increase chances of having better and more reliable RDTs.

Chapter 1.0

Introduction

1.1 Background

Malaria disease is caused by a protozoan parasite of genus *Plasmodium*. There are five *Plasmodium* species that have been known to cause malaria in humans, this include; *P. falciparum, P. vivax, P. malariae, P. ovale* (*P. ovale curtisi and P. ovale wallikeri*) *and P. knowlesi* (WHO 2015). Recently, two other zoonotic species *P. cynomolgi* and *P. simium* have been reported to infect human (Brasil et al. 2017; Hartmeyer et al. 2019; Raja et al. 2020). In sub-Saharan Africa, *P. falciparum* is most prevalent and is responsible for most of the morbidity and mortality due to malaria (WHO 2018; Snow et al. 2018). About 99.7% of all estimated malaria cases in sub-Saharan Africa are due to *P. falciparum* (WHO 2017, 2018). Malaria transmission has declined in many regions of the world but it remains a key public health threat in many countries especially in Africa. In 2018 approximately, 213 million cases and 380 000 deaths were recorded in Africa, compared to 2010, malaria cases have declined from 239 million cases. Similarly, malaria mortality has been declining from 607 000 in 2010 to 380 000 in 2018 (WHO 2019).

Malaria does not present with distinct physical signs that would allow clinical diagnosis, its signs and symptoms are like those of other febrile infections. In individuals lacking immunity to malaria, the disease symptoms include fever, sometimes with headache, sweat, chills, or other symptoms that may be similar to other diseases. The symptoms and severity of the malaria depend on several factors including parasite species and density as well as an individual's acquired immunity to malaria. WHO recommends prompt malaria diagnosis of suspected malaria cases using microscopy or RDTs before initiation of treatment (WHO 2010). This measure has contributed to improved malaria management and it may lower the occurrence and spread of malaria parasites that are resistant to drugs. Indeed, following this directive, malaria burden is reducing on the behest of effective antimalarial with 75% of the treatment decisions made based on rapid diagnostic tests (RDTs) (WHO 2010, 2017, 2018, 2019).

Malaria diagnosis can be done using microscopy, RDTs or PCR. Microscopy is the gold standard diagnostic tool and can detect up to 100 parasites per microliter. However, microscopy requires highly trained personnel; results take long and highly rely on electricity limiting its use to only regions where there is electricity. Conversely, PCR is a highly sensitive tool with the ability to detect up to one parasite per microliter. However, PCR is very expensive from purchase of the equipment to reagents needed to run the assay. This limits PCR use to only research institutes and high-level private hospitals. On this account, RDT that requires neither specialized training nor power supply remains the most relied upon diagnosis method, central for malaria management, and surveillance. The introduction of RDTs has increased number of suspected malaria cases that are undergoing diagnosis from 36% in 2010 to over 85% in 2018 (WHO 2017, 2019). RDTs accounted for approximately, 75% of all suspected malaria diagnosis in 2017, an increase from 40% in 2010. Despite documented unreliability in diagnosing low parasitaemia samples (WHO. Malaria Policy Advisory Committee Meeting 2013), the field experience of this method still argues for its deployment in regions that are in the process of transitioning to malaria elimination phase. Malaria RDTs are capable of detecting up to 30 parasites per microliter.

Malaria RDTs are designed to target various parasite's antigens in human blood. These antigens include *Plasmodium* genus-specific aldolase enzyme, *Plasmodium* Lactate Dehydrogenase enzymes (pLDH) which are found in all malaria parasite species or *Plasmodium falciparum* histidine rich protein 2 (*Pfhrp*2) (United Nations 2013). Owing to robustness, high sensitivity, low cross-reactivity rates, high specificity and high abundance of *Pfhrp*2 antigens most of the RDTs used to diagnose *P. falciparum* are based on identification of the *Pfhrp*2 antigens, which is expressed by *Pfhrp*2 gene (WHO: World Malaria report 2012 fact sheet 2012; United Nations 2013). Over 90% of malaria RDTs used in Sub-Saharan Africa are *Pfhrp*2 based due to the dominance of *Plasmodium falciparum* in this region (WHO 2017, 2018).

Several *Pfhrp*2 detecting RDTs cross-react with *Pfhrp*3, due to the presence of similar antigenic epitope among the two proteins (Gamboa et al. 2010; Baker et al. 2010; CDC 2012; WHO. Malaria Policy Advisory Committee Meeting 2016). *Pfhrp2* gene is found on

chromosome 8 whereas *Pfhrp3* gene is found on chromosome 13 of the parasite genome (Wellems et al. 1987).

As interventions continue to bring down the malaria burden, highly sensitive and specific diagnostic tools are needed for accurate case detection and management. The inconsistency in RDTs reactivity can be explained by several factors including the quantity of parasite antigens present in the blood of the patient, parasite density, storage conditions, and the interpretation of the test results. Additionally, the accuracy may be influenced by parasite factors including a lack of detectable antigen especially in *Pfhrp*2 based RDTs where the absence of *Pfhrp*2 gene or its homologue *Pfhrp*3 could affect the results. Finally, the repeats type found in either *Pfhrp*2 or *Pfhrp*3 genes can influence results (Baker et al. 2005, 2010).

1.2 Problem Statement

Reports of impaired *Pfhrp*2 and *Pfhrp*3 genes may threaten *Pfhrp*2 based RDTs accuracy in diagnosis (Gamboa et al. 2010; Berhane et al. 2018). Field parasite with partial or full deletion of *Pfhrp*2 and/or *Pfhrp*3 gene are prevalent, and these can cause false-negative RDTs results (WHO. Malaria Policy Advisory Committee Meeting 2016) which may lead to high rates of delayed treatment and consequently continued malaria transmission (Berhane et al. 2018). Although some parasites lacking *Pfhrp*2 have *Pfhrp*3 which can be detected by *Pfhrp*2 based RDTs, *Pfhrp*3 is less abundant and often undetectable at low levels of parasitaemia (Xin-zhuan and Wellems 1996) additionally some parasite may have deletion of both *Pfhrp*2 and *Pfhrp*3 genes. Though it is possible to have mixed infections of malaria parasites lacking *Pfhrp*2 gene with parasites having *Pfhrp*2, in low transmission regions mixed infection are rare.

1.3 Justification

Currently, studies done from various parts of the world have shown varying prevalence of parasites lacking *Pfhrp*2 and *Pfhrp*3 genes. Presence of field isolates with deleted *Pfhrp*2 and *Pfhrp*3 genes was first reported in Peru (Gamboa et al. 2010). In Kenya, a study carried out by Beshir et al. (2017) working with asymptomatic samples from Mbita and genome data from Kilifi found 10% *Pfhrp*2 gene deletion and 0.7% *Pfhrp*3 genes deletion prevalence.

Fortunately, none of the samples had double deletion of both *Pfhrp*2 and *Pfhrp*3 genes (Beshir et al. 2017).

Although few studies were conducted on *Pfhrp*2 and *Pfhrp*3 gene deletions in Kenya, little is known on these gene deletions, their characteristics and the amino acid repeats type present in most part of the country during the pre and post-RDT introduction. WHO guidelines on the choice of malaria diagnostic method recommends a change to non- *Pfhrp*2 detecting RDTs if the *Pfhrp*2 and *Pfhrp*3 gene deletion causing false-negative RDTs results, exceed 5% prevalence (WHO Global malaria programme 2017a).

Recent studies found that *Pfhrp*2 could be under the selection of low malaria transmission and malaria treatment based on diagnosis with *Pfhrp2* based RDTs (Watson et al. 2017). This means that under low malaria transmission, parasite clones lacking the genes are likely to become most prevalent. This transition is threat to public health since it stands in the way of prompt malaria case management. As regions continue to emphasize on proper case management in order to transition to pre-eradication stage, there is a critical need to map the distribution of parasites with deleted *Pfhrp*2 and/or *Pfhrp*3 across transmission regions in order to guide on the effective diagnostic kit to use at this crucial step.

As per the WHO recommendations, this study aimed to map *Pfhrp*2 and *Pfhrp*3 genes deletion in order to guide on regions where *Pfhrp*2 based RDT can be ineffective. The findings from this study will contribute to determining the implication of these genes deletion to *Pfhrp*2 based RDTs use policy.

The aim of this study was to evaluate the *Pfhrp*2 and *Pfhrp*3 genes deletion prevalence and polymorphisms in *Plasmodium falciparum* field isolates collected from Kombewa, Kericho, Malindi, Kisumu and Alupe regions in Kenya between 2003 and 2005 and between 2013 and 2017.

1.4 Objectives

1.4.1 Broad Objective

To assess the prevalence and distribution of *Pfhrp*2 and *Pfhrp*3 genes deletion in three of the five malaria transmission regions of Kenya and its implication to RDTs use

1.4.2 Specific Objectives

1. To determine the prevalence of *Pfhrp*2, *Pfhrp*3 and flanking genes deletions in the study population
2. To compare *Pfhrp*2 based RDTs reactivity to *Pfhrp*2 and/or *Pfhrp*3 deletion status
3. To characterize deletions and identify the repeat types in *Pfhrp*2 and *Pfhrp*3 genes

Chapter 2.0

Literature review

2.1 Overview

Malaria disease occur when a protozoan parasite of genus *Plasmodium* infect Red blood cells (RBCs) and the parasite are injected into human blood by female *Anopheles* mosquito during feeding (WHO 2015). Five species of *Plasmodium* infect humans; *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* (has two sub species *ovale wallikeri* and *ovale Curtis*) and *Plasmodium knowlesi* (WHO 2019). Recently, two other zoonotic species *P. cynomolgi* and *P. simium* have been reported to infect human (Brasil et al. 2017; Hartmeyer et al. 2019; Raja et al. 2020). Since 2010, malaria cases have been declining with 251 million cases and 228 million cases reported in 2010 and 2018 respectively and 231 million cases in 2017 worldwide (WHO 2019). Most cases of malaria take place in WHO African area, this region accounted for 93% (213 million) malaria cases in 2018 (WHO 2018, 2019).

2.2 Malaria symptoms

Malaria symptoms occur during the blood phase of the *Plasmodium* lifecycle. At first symptoms are not distinct and are similar to minor systemic viral infections. They include fever, headache, lethargy, sweating, chills, abdominal discomfort, loss of appetite, vomiting, muscle and joint pain. In severe cases, malaria may cause respiratory distress, anaemia, lactic acidosis, comma and death (Marsh et al. 1995; Miller et al. 2002; Chen et al. 2016). Malaria in children may present with cough, lethargy and poor feeding. If malaria is treated early during initial disease progression stage without organ dysfunction, full quick recovery is expected. If treatment is not done promptly, especially in *P. falciparum*, parasite density increases continuously and the infection may progress to complicated malaria.

The nature of malaria infection depends mostly on patients acquired immunity to malaria level and this is majorly influenced by patterns and transmission intensity of malaria in the region where the individual resides (WHO 2015). In regions where there is stable malaria transmission, most individuals acquire partial immunity to symptomatic infection and lower risk of acquiring severe malaria. Malaria infection can be classified into an asymptomatic and

symptomatic infection. Occurrence of malaria parasites in human blood in absence of any malaria symptoms is known as asymptomatic malaria (WHO 2016). While symptomatic malaria is parasite occurrence in the human blood accompanied by malaria symptoms.

Symptomatic malaria infection can further be classified into uncomplicated and severe malaria. Uncomplicated malaria is whereby patient present with malaria symptoms and positive parasite test either by microscopy or by malaria RDTs without any feature of severe malaria (WHO 2015, 2016). On the other hand, severe malaria definition depends on *Plasmodium* species. In *Plasmodium falciparum*, severe malaria is the presence of asexual stage parasite accompanied by any of these symptoms with no other identifiable cause. Unconsciousness, multiple seizures, prostration, acidosis (respiratory distress), renal impairment, severe malarial anaemia, hypoglycaemia, jaundice (bilirubin level greater than 50 µM with a parasitaemia greater than 100 000/ µL), significant bleeding (nose, gum or venepuncture sites), pulmonary oedema, shock or hyperparasitaemia (parasitaemia >10%). Severe *Plasmodium vivax* is defined similar to severe *falciparum* nonetheless with no parasite density thresholds. Conversely, in *Plasmodium knowlesi* severe malaria is defined similar to severe *falciparum* but differ in the fact that severe *Plasmodium knowlesi* hyperparasitaemia is parasite density greater than 100 000/µL and jaundice is bilirubin > 50 µM with a parasite density of > 20 000/µL (WHO 2015).

2.3 Malaria life cycle

In humans, malaria disease starts when female *Anopheles* mosquitoes while feeding inject sporozoites into the human bloodstream. The injected sporozoites move to the liver then attack hepatocytes and then replicate asexually leading to merozoites formation, which are then released into the bloodstream. Merozoites rapidly attack red blood cells then develop through ring, trophozoite and schizont stages. Throughout this development process, *Pfhrp*2, aldolase and pLDH antigens are increasingly being produced into the bloodstream.

Schizonts rapture to release newly formed daughter merozoites leading to further increase in parasitaemia and increase the production of antigens. The released merozoites proceed to invade new erythrocytes and proceed through another asexual replication cycle. This process

of asexual division takes about 48 hrs for *P. falciparum* and occurs multiple times, leading to increased parasitaemia within the infected host (Smith et al. 2003; Bousema et al. 2014).

Alternatively, some of the merozoites may undergo sexual development to form male and female gametocytes through a process called gametogenesis. Immature gametocytes produce histidine-rich protein 2, while mature gametocytes produce pLDH and aldolase (Figure 2.1). When taken up by a feeding mosquito, the male gametocytes differentiate into microgametes while female gametocytes differentiate into macrogametes. Subsequently, fertilization occurs to form zygotes, which then develop through ookinete, oocyst and sporozoite stages. The sporozoites are transported to the salivary glands of the mosquito where they are inserted into human while the mosquito is feeding, thus perpetuating the cycle as shown in Figure 2.1. The antigens of *Pfhrp*2 can persist in blood for several weeks once viable malaria parasites have been eliminated from the blood, but clearance of both aldolase and pLDH take place within 5 to 6 days (Global and Programme 2011).

Malaria symptoms occur during the asexual blood stage of infection, when merozoites invade, egress and re-invade the red blood cell (Baum et al. 2005). This is one of the few times of the parasite life cycle when the parasite is exposed directly to the host immune system and the merozoite has surface antigens that induce an immune response (Smith *et al.* 2003; Cowman *et al.* 2012; Wright *et al.* 2014).

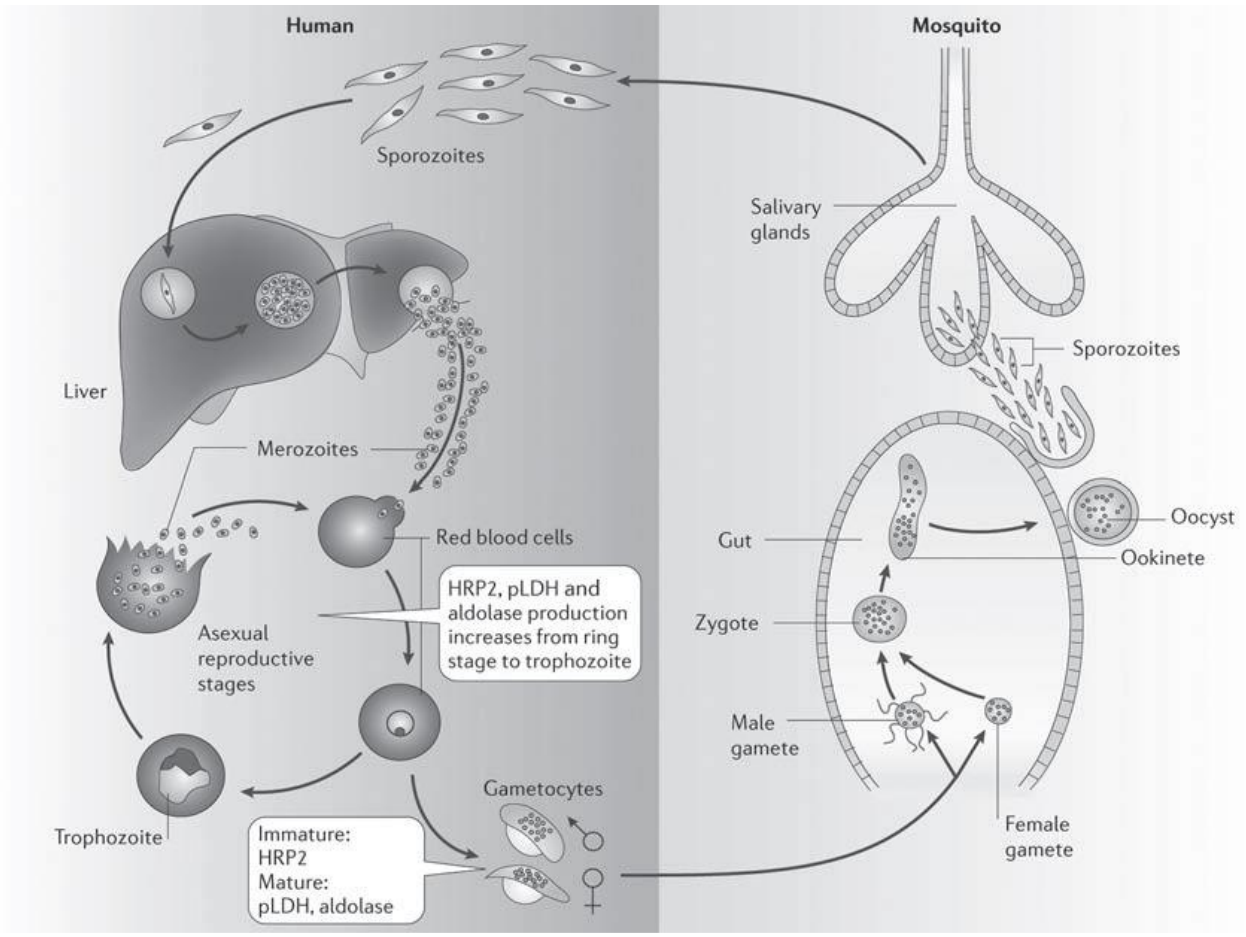

Figure 2.1: Malaria parasite life cycle demonstrating various lifecycle stages where different RDT antigens are produced (Adapted from Macmillan publishers ltd: Nature Reviews Microbiology, s7-s20 (September 2006), © 2006 nature publishing Group 68)

2.4 Malaria diagnosis

Diagnosis of malaria can be done through either parasite based diagnosis or clinical diagnosis. Clinical diagnosis of malaria involves the medical practitioner prescribing the drugs depending on the patient's signs and symptoms. Clinical diagnosis is the cheapest malaria diagnosis technique. However, it is quite challenging, as malaria does not present with distinct physical signs. Malaria signs and symptoms are like those of other bacterial, viral or other fever causing infections. In individuals with low malaria immunity, the disease symptoms include fever, headache, sometimes with chills and sweating or other symptoms that are similar to other diseases. Therefore, clinical diagnosis has very low specificity and often leads

to unselective antimalarial drugs usage which could compromise treatment of non-malarial fevers (Reyburn et al. 2004; Mwangi et al. 2005; Meredith et al. 2015; WHO 2015). An algorithm developed to diagnose common childhood illness by healthcare providers that have little training and with inadequate diagnosis equipment, produced very low specificity when used to diagnose malaria (Perkins et al. 1998; Weber et al. 1998; Tarimo et al. 2001).

On the other hand, parasite-based diagnosis of malaria involve detecting malaria parasites or antigens (parasites proteins) in patients' blood which can be done through either microscopy, RDT or by PCR (WHO 2010; United Nations 2013; WHO 2015). WHO recommends a prompt diagnosis of cases suspected to be malaria by either RDTs or microscopy before antimalarial treatment (WHO 2010, 2015). Conducting parasite-based diagnosis instead of treating patients presumably may aid in further examination of other possible causes of febrile diseases if malaria test result is negative. This is because most of the febrile illnesses particularly in most malaria-endemic regions where successful control measures of malaria have been established are not due to malaria (United Nations 2013; WHO: Malaria microscopy Quality Assurance Manual 2015). Additionally, it may reduce the unnecessary use of antimalarial drugs especially Artemether Lumefantrine (ACT) as they are quite expensive in comparison to the drugs that have previously been used (e.g. chloroquine) (Arrow, Panosian, and Gelband 2004; Connell et al. 2011). This also helps avoid ACT associated side effects. Moreover, prompt diagnosis of malaria before treatment may help reduce the rapid occurrence and spread of parasites that are resistant to antimalarial drugs (WHO 2011).

2.5 Microscopy

Microscopy involves visualisation of the Giemsa-stained parasite in patients' blood using a microscope. It allows visualisation of various malaria parasites species, their different development phases as well as determining of parasitaemia to monitor treatment response. Species determination involves visualisation of different morphology of the four human malaria parasite species and the infected red blood cells. Malaria diagnosis using microscopy requires preparation of two slides, thin and thick smear. Thick smear preparation involves placing 1 to 3 drops of blood on clean, grease and scratch free slide and spreading evenly with wood or corner of another slide, air drying, staining with 2% Giemsa, and rinsing then

allowing it to dry completely. On the other hand a thin smear is prepared by placing one tiny blood drop at the edge of clean, grease and scratch free slide and spread using another slide to have a featherlike end, air drying, then fix using methanol, stained with 2% Giemsa, then rinsed and allow to dry completely. Unlike thin smear, the thick smear should not fixed with methanol in order to allow haemolysis. The parasites are identified in the thick smear and species present confirmed in a thin smear.

The choice between microscopy and rapid diagnostic tools depend on circumstances such as number of patients, epidemiology of malaria, available skills and possibility of using microscopy to diagnose other diseases. Microscopy is advantageous because it is cost-effective, allows speciation, quantification of the parasite, evaluation of antimalarial treatment response, identification of erythrocytic stages present in patient's blood and the investigation of other diseases or factors that could be the cause of fever. However, the accuracy of the microscopy highly depends on the availability of experienced personnel, equipment that are well-maintained, steady supply of reagents, electricity and clean water. In addition, microscopy results take longer to be produced as the process of staining and interpreting is labour intensive and time-consuming. Additionally, the reliance of microscopy on light limits its utilization to health facilities with electricity supply. This makes its use not feasible in the field and in the remote rural setting such as small health care facilities lacking electricity and limited health facility resources (Erdman and Kain 2008). Another limitation of microscopy is that it has comparatively little sensitivity, mainly when parasite density is low. Even though an expert microscopist is able to detect up to 5 parasites per microliter, average microscopist is capable detecting parasite density of at least 50-100 parasites/ μl (Payne 1988). This can cause underestimation of infection rates.

2.6 Malaria RDTs

RDTs are devices that have a lateral flow containing antibodies to detect specific parasites antigens in blood samples by immunochromatography. RDTs work through a method of lateral flow Immunochromatography Strip (ICS) and indicate presence parasite antigens through changing porous nitrocellulose strip colour. Malaria RDTs consist of capture antibodies imbedded on a nitrocellulose strip that form a labelled antibody-antigen complex

with parasite antigens in the patient's blood. This antibody-antigen form a visible stripe signifying a positive malaria test result (Moody 2002; WHO/Find GMP 2014). The tagged antibody-antigen network move through the nitrocellulose strip together with product reagent through flushing with clearing buffer and via capillary action. RDTs kits for detecting antimalarial antibodies are also present, however, they are not used for case management only in some cases such as in blood bank to screen donated blood to avoid malaria transmission through blood transfusion (UNICEF 2007).

Various formats of RDTs are available commercially, including dipsticks, cassette and cards. Dipstick (test strip) RDTs require to be placed on wells containing the patient's blood or buffer. The cassettes or card format consist of cassette in which the nitrocellulose strip is placed , hence making it more costly but easier to use (UNICEF 2007; Global and Programme 2011). Over 40 malaria RDTs for detecting different *Plasmodium spp*. are available commercially. Some RDTs detects only one *Plasmodium* species (either *Plasmodium falciparum* or *Plasmodium vivax),* while others detect *Plasmodium falciparum* together with one or more of the other three *Plasmodium* species that infect human (*P. malariae, P. vivax and P. ovale).*

2.7 Diagnostic targets for RDT

Commercially available RDTs can detect mainly three antigen groups. This includes *Pfhrp*2, aldolase and pLDH antigens, generation of these antigens occur during the parasite developmental stages in the human host (Rock et al. 1987; WHO/USAID 2000; Moody 2002; UNICEF 2007; Global and Programme 2011; United Nations 2013; Obeagu et al. 2018).

The *Pfhrp*2 is produced specifically by *Plasmodium falciparum* throughout the asexual blood phase of malaria parasite development cycle and by immature gametocytes (Rock et al. 1987). *Pfhrp*2 is a soluble antigen with high thermal stability that is found abundantly in the cytoplasm and membrane of infected red blood cell (UNICEF 2007). On this basis *Pfhrp*2 detecting RDTs are the most commonly used RDTs in detection of *Plasmodium falciparum*, as they can withstand high temperature found especially in the field (WHO. Malaria Policy Advisory Committee Meeting 2016). Additionally, they are abundant and highly sensitive than pLDH, and aldolase in the detection of *Plasmodium falciparum* (Global and Programme

2011). Owing to this WHO recommends the use of *Pfhrp*2 based and pLDH specific for *Plasmodium falciparum* RDTs in sub-Saharan Africa, since *P. falciparum* malaria infections are most prevalent in this region (Global and Programme 2011). *Pfhrp*2 persists 5 to 6 days after clearance of viable parasite (WHO/USAID 2000; Moody 2002; Plucinski et al. 2019). Although this persistence may lead to a false positive RDTs result, it has the advantage of helping detect the parasite in case of ineffective treatment and poor malaria dosage.

PLDH based RDTs are available in various sub-types; pan-specific, *P. falciparum*-specific and *Plasmodium vivax*-specific pLDH antibodies (Makler et al. 1998). Although, other subtypes detect specific species, pan-specific detect all *Plasmodium* parasites as it contains pan lactate dehydrogenase found in all *Plasmodium* species (*P. malariae, P. ovale, P. vivax*) (UNICEF 2007; Global and Programme 2011). On the other hand, aldolase enzyme is found in all *Plasmodium* species. Both aldolase and pLDH are produced during the asexual blood phase of parasite development cycle and by mature gametocytes. They are key malaria parasites glycolytic pathway enzymes (Meier et al. 1992; Makler et al. 1998). They are soluble, abundant and highly conserved in the parasite (UNICEF 2007).

Combination or 'combo' tests are malaria RDTs that are designed to detect both *falciparum*-specific and non-*falciparum* or pan-specific targets antigens together. Pan-specific RDTs identifies four of the five types of *Plasmodium* species that infect humans. Test detecting *falciparum* specific and *vivax* specific pLDH are available commercially, however, they are more expensive than forms of the same tests using pan-specific pLDH (UNICEF 2007). Selection of appropriate malaria RDTs for a certain use, require consideration of defined field performance of RDTs and diagnostic needs of health care personnel. Criteria used depends on targeted species and antigens of the parasite and the RDTs performance (i.e. panel detection score, false-positive rate, the invalid rate, ease of use, thermal stability of the RDT on storage at 35 °C and 45 °C and 75% humidity for two months). It depends on recommended and national treatment guidelines, experience in use of RDTs and availability and cost of the RDT (Global and Programme 2011).

2. 8 RDT in various epidemiological settings

The suitability of various type of RDTs i.e. pan-specific, *falciparum* or non-*falciparum* RDTs depends on parasite species prevalent in the intended area of use. The WHO has categorized areas into three zones (WHO Global malaria Programme 2011):

Zone 1. This zone includes most regions of sub-Saharan Africa and lowland Papua New Guinea. In this region, *Plasmodium falciparum* parasites are predominantly prevalent and non-*falciparum* infections are rare. Mostly occurrence of non-falciparum infections is as mixed *Plasmodium falciparum* infections and rarely as single species. In this zone, RDTs detecting *Plasmodium falciparum* are recommended especially those targeting *Pfhrp*2 or those targeting *Plasmodium falciparum*-specific lactate dehydrogenase (pLDH)

Zone 2. This zone include few regions in the Horn of Africa and endemic regions of Americas and Asia where prevalent malaria infections are *falciparum* and non-*falciparum.* In this zone, both *Plasmodium falciparum* and non-*falciparum* infections are present and occur as single species. Combination RDTs detecting all *Plasmodium* species and that differentiate *P. falciparum* from non-falciparum infections are recommended. This because *Plasmodium falciparum* first-line treatment and that of non-*falciparum* cases in these zones are different, therefore, insisting on the need to distinguish different species

Zone 3. Mainly this zone includes regions of South America, East and central Asia and some highland parts where only *Plasmodium vivax* malaria are prevalent. Additionally, regions where only non-*falciparum* malaria infections are the prevalent parasites, mostly *Plasmodium vivax* malaria infections only. The recommended RDTs are non-*falciparum* detecting RDTs alone (pan or *P. vivax*-specific if *P. vivax* is the only species present). In this zone *falciparum* infections are absent and first-line treatment for all non-*falciparum* cases is similar (WHO Global malaria Programme 2011).

Unlike microscopy, RDTs do not require laboratory facilities, water nor electricity making them easy to use in remote rural areas (WHO 2011). More so, conducting RDTs and results interpretation does not involve much training, therefore can be done even in the absence of well-trained laboratory technician. Additionally, RDTs facilitates widespread availability of

malaria diagnostics, which otherwise could be infeasible by microscopy alone. In fact, with the introduction of malaria RDTs a tremendous increase in proportion of suspected malaria cases receiving diagnosis has been recorded. In 2010 when RDTs were being introduced, the number of suspected malaria cases receiving diagnosis was at 36%, in 2015 they were at 76%, and increased to 87% in 2016 (WHO 2017). This increase is majorly due to increase in use of malaria RDTs, which account for over 75% of all malaria diagnosis. It is advisable that availability of RDTs be used to better the availability and quality of microscopy where needed rather than replacing microscopy (WHO 2011).

However, the malaria RDTs performance highly depends on numerous factors, including the blood flow rate through the nitrocellulose strip, capture antibody (Ab) adherence to the strip, antibody antigen binding ability, and antibody-dye conjugate signal integrity. These influences are all prone to degradation during hostile conditions when transporting and during storage, and the degradation rate and their impact on outcomes can differ among products. Additionally, there is variation in connection between parasite density and antigen concentration depending on the level of parasites sequestration, antigen persistence after reduction and the parasite growth stage (WHO/Find GMP 2014). According to most manufacturers, RDTs should be stored at temperature of 2-30°C. Conversely, use of RDTs in remote regions involves storing in conditions that may be beyond the designed RDTs limits. The best RDTs for these zones ought to be capable of tolerating temperatures of at least 40°C to 50°C, over 2 years storage (WHO/USAID 2000). Currently, limited information is available on the stability of many RDTs in such conditions, and during transport, more extreme conditions might temporary occur. Since there could be variation in product stability and sensitivity among lots, there is need to reduce exposing RDTs to extreme temperatures, and performance of each lot should be monitored.

2.9 *P. falciparum* Histidine rich proteins

Plasmodium falciparum blood-stage produces three histidine-rich proteins, *Pfhrp*1, *Pfhrp*2 and *Pfhrp*3, in order of their discovery. *Pfhrp*1 is involved in the generation of knob-like projection on diseased erythrocyte membrane (Kilejian 1979; Rock et al. 1987), however, *Pfhrp*2 is produced both in Red Blood Cells (RBCs) having the knob and those that lack the

knob (Leech et al. 1984). *Pfhrp*2 consist of histidine, alanine and aspartate in 35, 40 and 12 per cent respectively (Wellems and Howard 1986). Conversely, *Pfhrp*3 contains 30% histidine and 29% Alanine (Stahl et al. 1985). Synthesis of *Pfhrp*2 begin at the ring stage and proceed through trophozoite stage (Wellems and Howard 1986).

The *Pfhrp*2 gene (*Pf*3D7_0831800) is 1064 base pair (bp) long, it is made up of two exons and one intron and it found in subtelomeric region of chromosome 8. *Pfhrp*2 gene is flanked immediately by *Plasmodium* exported (PHIST) protein pseudogene 1002 bp long on 5' end and by a heat shock protein 70 (*Pf*3D7_0831700) on 3' end (Figure 2.2). However, *Pfhrp*3 gene (*Pf*3D7_1372200) is 977 base pair long; it is found in subtelomeric area of chromosome 13. *Plasmodium* exported protein (PHISTb) (*Pf*3D7_1372100) flanks *Pfhrp*3 on the 3' end while in 5' end an acyl-CoA synthetase coding gene (*Pf*3D7_1372400) is found about 9.1kb (Figure 2.3).

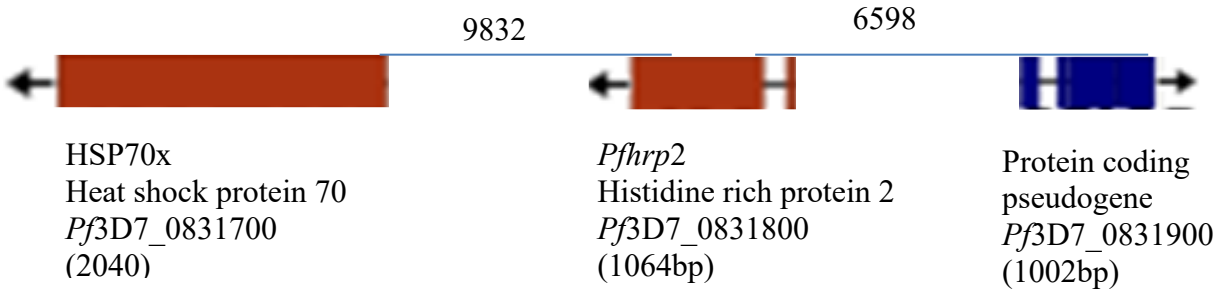

Figure 2. 2 : Structure of *Pfhrp*2 gene and its 3' and 5' ends flanking genes arrows indicating 5' to 3' of the gene. Adapted from Plasmodb release 46 (http://plasmodb.org/plasmo/).

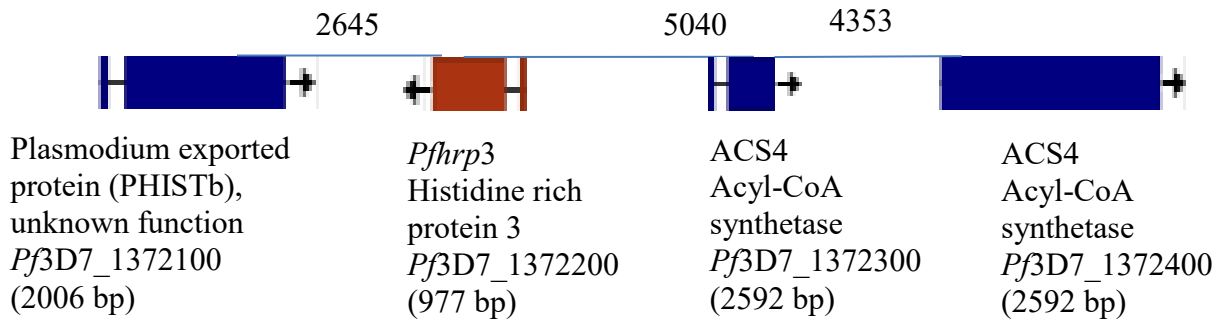

Figure 2. 3: Structure of *Pfhrp*3 genes and their 3' and 5' ends flanking genes with arrows indicating 5' to 3' of the gene. Adapted from Plasmodb release 46 (http://plasmodb.org/plasmo/).

2.10 Studies on *Pfhrp*2 and *Pfhrp*3 gene deletion

Parasites not expressing *Pfhrp*2 and/or *Pfhrp*3 genes have been reported in the laboratory parasites and as well as in field isolates. However, there is no report on parasite not expressing pLDH or aldolase targets, probably due to the fact that these enzymes are essential for parasite metabolism and survival (Meier et al. 1992; Makler et al. 1998).

Parasite lacking *Pfhrp*2 or *Pfhrp*3 genes in clinical cases were first reported in Peru (41% *Pfhrp*2 gene deletion, 70% *Pfhrp*3 gene deletion and 21.6% deletion of both *Pfhrp*2 and *Pfhrp*3 genes) (Gamboa et al. 2010). In Colombia, Solano (2015) found 18% deletion in *Pfhrp*2 gene and 52% *Pfhrp*3 gene deletion (Solano et al. 2015) while Dorado (2016) found 38.5% *Pfhrp*2 gene deletion prevalence and *Pfhrp*3 gene deletion prevalence of 43% (Dorado et al. 2016). In Suriname, 14% of the samples tested were negative for *Pfhrp*2 gene, 4% were *Pfhrp*3 negative, however, in Guyana all samples (n=97) were positive for both *Pfhrp*2 and *Pfhrp*3 genes (Okoth et al. 2015). In Brazil, the Amazon region had 31.2% *Pfhrp*2 gene deletion, the Rondonia region had 3.3% *Pfhrp*2 gene deletion prevalence while in the Para state all parasites were positive for the *Pfhrp*2 gene. On the other hand, *Pfhrp*3 gene deletion ranged from 18.3% to 50.9% in all the three states (Maria et al. 2017). In Bolivia 4% of the 25 samples tested had deleted *Pfhrp*2 gene and 68% had deleted *Pfhrp*3 gene (Maria et al. 2017). Fontecha *et al*. (2018) found 25% and 91.4% prevalence of parasites lacking the region between exon 1 and exon 2 of *Pfhrp*2 and *Pfhrp*3 genes respectively (Fontecha et al. 2018).

Outside South America lower incidence of *Pfhrp*2 and/or *Pfhrp*3 gene deletion has been reported, in India (2.4% and 1.8% respectively) (Bharti et al. 2016). In China-Myanmar border 4.1% of the 97 samples tested had a deletion of *Pfhrp*2 gene, double deletion of both *Pfhrp*2 and *Pfhrp*3 genes was in 3 of the 97 samples (Li et al. 2016). However, Pati *et al.* (2018) found a *Pfhrp*2 gene deletion prevalence of 65.5%, *Pfhrp*3 gene deletion prevalence of 41.4% and double deletion of both *Pfhrp*2 and *Pfhrp*3 genes was 29.3% in RDT negative samples (Pati et al. 2018).

In Africa *Pfhrp*2 and/or *Pfhrp*3 gene deletion has been evaluated in Mali (2.1% *Pfhrp*2 deletion) (Ousmane et al. 2012). In Senegal (2.2% and 12.8% for *Pfhrp*2 and *Pfhrp*3 genes deletion, respectively) (Deme et al. 2014). In the Democratic Republic of Congo (*Pfhrp*2 6.4%, *Pfhrp*3 3.4%) (Jonathan et al. 2017). A study in Ghana found *Pfhrp*2 gene deletion of 33.3% (Amoah *et al.* 2016). In Mozambique, 1.4% *Pfhrp2* gene deletion (Gupta et al. 2017). In Kenya, Beshir *et al.* (2017) working with 274 samples from Mbita and 61 genomic data from Kilifi, found 10% *Pfhrp*2 gene deletion in 131 *P. falciparum* PCR and microscopy positive samples. One PCR confirmed sample lacked *Pfhrp*3 gene, one genomic sample lacked *Pfhrp*2 gene and another one sample lacked *Pfhrp*3 gene. Fortunately none of the samples had deletion of both *Pfhrp*2 and *Pfhrp*3 genes (Beshir et al. 2017). Lastly Eritrea experienced the highest prevalence of *Pfhrp*2 and *Pfhrp*3 genes deletion in Africa (62% *Pfhrp*2 deletion, 41% *Pfhrp*3 deletion and 31% double *Pfhrp*2 and *Pfhrp*3 gene deleted samples) (Berhane et al. 2018). WHO has recommended the use of non *Pfhrp*2 based RDT in regions where the deletion of both *Pfhrp*2 and *Pfhrp*3 have exceeded 5% prevalence (WHO. Malaria Policy Advisory Committee Meeting 2016).

Chapter 3.0

Materials and Methods

3.1 Study setting

The study was done at KEMRI, USAMRD-A, Kenya, Malaria drug resistance laboratory (MDR) Kisumu. It used a subset of archived whole blood samples from on-going approved (KEMRI SSC# 1330, WRAIR# 1384 Protocols) epidemiology of malaria drug resistance patterns in Kenya study that started in 1992. This epidemiology study collects samples from public health facilities in Kisumu, Kombewa, Kericho, Malindi, Marigat, Kisii and Alupe district hospitals. The study participants are persons aged 6 months and above, including those that had been treated for malaria within the preceding 14 days. For the samples collected between 2013 and 2017, each participant had to present with positive *P. falciparum* or Pan RDT, depicted by both bands positive (*P. falciparum*) or pan band only (other species) or symptomatic RDT both bands negative. On the other hand, for samples collected between 2003 and 2005 participant had to present with a microscopy slide positive or symptomatic with microscopy slide negative.

3.2 Study Population

This specific study utilized 350 samples, 95 samples collected between 2003 and 2005 (pre RDTs) and 255 samples between 2013 and 2017 (post RDT). These samples were collected from Kombewa, Kericho, Malindi, Alupe, and Kisumu. Kombewa, Kisumu and Alupe are holoendemic regions with stable malaria transmission throughout the year and peaks during short and long rain seasons; Kericho is a highland with mostly imported malaria while Malindi is an area with declining malaria endemicity.

3.3 Sample size

WHO protocol to determine samples size to estimate whether false-negative *Pfhrp*2 based RDT results caused by *Pfhrp2/3* gene deletions exceeds 5% prevalence was used (Table 3.1) (WHO Global malaria programme 2017b). There is no existing data on the prevalence of *Pfhrp2/3* gene deletions causing false negative *Pfhrp2* based RDTs results in this study sites,

therefore determination of sample size for this study was based on existing data from Mbita (Beshir et al. 2017). Beshir (2017) found that *Pfhrp*2/3 genes deletion causing false negative *Pfhrp*2 based RDTs was not present. Therefore a sample size for less than 1% *Pfhrp*2/3 gene deletion causing false negative RDTs results was considered (N=150) (Table 3.1). Since this study involved both pre and post RDT samples a target sample of 150 for each study period was used totalling to 300 samples. Additional 50 samples were selected to increase the power and cater for samples that would turn negative for *P. falciparum* malaria.

Table 3. 1: WHO samples sizes for determining whether observed *Pfhrp*2 exceeds 5%

Estimated proportion of hrp2 deletion (outcome 1: Total *Pfhrp*2- & Pan-pLDH+ /Total LDH+	**Number of individuals needed with *P. falciparum* infections at province level needed to conclude 90% CI interval does not include 5%**	**Number of individuals with *P. falciparum* infection per clinic (n=10 clinics per domain)**
<1%	150	15
1%	150	15
2%	150	15
3%	350	20
4%	1550	155
5%	2280	228
6%	2280	228
7%	660	66
8%	330	33
9%	210	21
>9%	150	15

3.4 Laboratory procedures

3.4.1 *Pfhrp*2 based RDT Detection

Whole blood samples were retrieved from -80 ºC freezer and incubated at +4 ºC fridge to allow samples to thaw. Subsequently all the samples were pulse vortexed to allow mixing. Blood samples were tested using Parascreen™ (Zephyr Biomedicals, Goa India) *Pfhrp*2/ Pan

RDT as per the manufacturer's instructions (Zephyr Biomedicals, n.d.). This is a RDT inform of cassette that detects three antigens. It contains two spots, the first spot is for adding buffer while the next spot is for adding the blood sample, and the other region is for reading results in form of bands. The first band detects *Pfhrp*2 antigen, the middle band detects *Plasmodium* lactate dehydrogenase enzyme (*P*LDH) (pan) while the third band is a control band containing anti-rabbit antibody. Briefly, five microliter of the sample was poured on the RDT sample spot. Subsequently, two drops of the buffer was poured on buffer spot and incubated at room temperature for 20 min. Results were interpreted depending on bands produced. A single pink-purple line (band) on the control region depict that the sample is negative for malaria, one band (pink-purple) in the middle region together with control line (band) depict that the sample is positive for *non-falciparum* parasite, one pink-purple band together with previous two bands indicate that the sample is positive for *falciparum* parasite. If the control line does not appear the test was termed invalid and was repeated (Appendix7 2) (Zephyr Biomedicals, n.d.).

3.4.2 Extraction of DNA and *Plasmodium* typing PCR

Qiagen DNA Mini extraction spin protocol (Qiagen, Valencia, CA) was used to extract DNA from whole blood as per manufacturer's guidelines (Qiagen 2016). Briefly, 20 µl of proteinase K was added to 1.5 ml micro centrifuge tube, then addition of 200 µl of whole blood sample. Subsequently, 200 µl of Lysis buffer (AL) was added and the content pulse-vortexed to mix. The mixture was then incubated at 56 ºC for 10 minutes. Maximum yield of DNA is attained after incubation for 10 minutes, however, longer incubation do not affect yield or quality of DNA. The tubes were then centrifuged briefly. Thereafter 200 µl of absolute (96-100%) ethanol was added and mixed by pulse-vortexing, and then centrifuged briefly to remove drops from inside the lid. Absolute ethanol enables precipitation of the nucleic acid. All the mixture was applied to the QIAamp Mini spin column carefully avoiding wetting the rim then centrifuged for 1 minute at 8000 rpm. The QIAamp Mini spin column was then placed in a clean 2 ml collection tube and the filtrate discarded. The spin-column contains silica membrane, while the lysate buffer contains conditions that facilitate optimal binding of DNA to the membrane. The lysate is maintained at a certain salt and pH conditions to ensure that proteins and other contaminants that may impede PCR reactions pass through the membrane

without being retained. Five hundred microliters of AW1 buffer was used to wash the spin column that contained the bound DNA and spun down at 8000 rpm for 1 minute. Then washed with 500 µl of AW2 buffer and spun down at 14000 rpm for 3 minutes, followed by a dry spin of 14000 rpm for 1 minute. The purified DNA was then eluted by applying 200 µl of elution buffer and at room temperature for 5 minutes, then spun down for 1 minute at 8000 rpm (appendix 1) (Qiagen 2016).

Subsequently, *P. falciparum* confirmatory PCR was conducted using the primer set shown in Table 3.2 and conditions defined elsewhere (Kamau et al. 2011). This process was carried out in two-step assay, the first was to detect the *Plasmodium* genus with the primers F1 and R1 shown in the Table 3.2, the probe was labelled with FAM (6-carboxyfluorescein) in 5 prime and at the 3 prime TAMRA (6-carboxytetramethyl-rhodamine) (Kamau et al. 2011). Consequently *P. falciparum* species-specific assay was done using primers and probes described somewhere else (Veron et al. 2009) with FAM reporter. Real-time PCR assay was then carried out in Applied Bio system QuantStudio 6 flex (QuantStudio^{Tm} real-time PCR applied Biosystems by Thermos Fisher Scientific) with the following conditions; 40 cycles of 95 ºC for 10 minutes followed by 95 ºC for 15 minutes, and then 60 ºC for 1 minute as done by Kamau *et al.* (2011) (Kamau et al. 2011). This study settled for 18S rRNA target over other PCR targets for *Plasmodium* speciation such as cytochrome oxidase III gene (Isozumi et al. 2014; Echeverry et al. 2016) since parasite quantification was required and a well optimized quantitative PCR protocol for this target was already available. The results were exported into excel worksheet showing the cycle threshold (CT) value for each sample.

Only samples positive by *P. falciparum* confirmatory PCR with a cycle threshold value of at least 32 and below (assumed to be equivalent to at least 30 parasites per microliter (Kamau et al. 2013) were considered for *Pfhrp*2 and *Pfhrp*3 gene deletion analysis, samples positive but with a lower cycle threshold value were used as controls.

Table 3. 2: Genus and species-specific primers

	Primer/probe name	Primer sequence	References
Plasmodium primers	F1	5'-GCTCTTTCTTGATTTCTTGGATG-3'	(Kamau et al. 2011)
	R1	5'-AGCAGGTTAAGATCTCGTTCG-3'	
	Probe(FAM-TAMRA)	5'-ATGGCCGTTTTTAGTTCGTG-3',	
Plasmodium falciparum	*Pf*-1	5'ATTGCTTTTGAGAGGTTTTGTTACTTT3'	(Veron et al. 2009)
	Pf-2	5'GCTGTAGTATTCAAACACAATGAACTCAA3'	
	Probe(FAM-MGB)	5'CATAACAGACGGGTAGTCAT3'	

3.4.3 *Pfhrp*2 and *Pfhrp*3 and their flanking genes detection

A nested PCR was conducted to confirm presence or absence of *Pfhrp*2, *Pfhrp*3 and the flanking genes spanning between *Pf*3D7_0831900 and *Pf*3D7_0831700 upstream and downstream of *Pfhrp*2 respectively, as well as *Pf*3D7_1372100 and *Pf*3D7_1372400 upstream and downstream of *Pfhrp*3 respectively, using primer and conditions described elsewhere (Appendix 3) (Abdallah et al. 2015). Each gene had two primer sets, the first primer set was for primary PCR and the second primer set was secondary (nested PCR). In Nested PCR, the primary PCR amplifies a longer region of the gene, followed by the second primer set which amplifies a shorter gene region within the first amplification. All *Pfhrp*2, *Pfhrp*3 and flanking genes PCR amplifications were done in 20µl total volume consisting of quantifast (Qiagen,

catalogue no. 204257) master mix, 10 µM forward and reverse primers and 34.0 ng/µl DNA template. PCR conditions for all the genes amplification assays were set at 95 ºC for 5 minutes for activating the enzymes, 95 ºC for 30 seconds to allow denaturation of the template DNA. Annealing temperature specific for each primer set for thirty cycles as shown in appendix 3 Table 1, next 68 ºC for 30 seconds, final elongation at 68 ºC for 5 minutes as done by Abdallah *et al.* (2015) with quantifast master mix (Qiagen) in place of Taq polymerase (Roche). However, for *Pfhrp*3 gene, the primary amplification annealing temperature was increased to 60 °C and the number of cycles increased from 30 to 35 cycles. *Plasmodium falciparum* 3D7 strain was utilized as a positive control for all the assays. For *Pfhrp*2 and its flanking regions PCR amplification, the laboratory strain Dd2 (5 ng/µl) served as a negative control as this parasite lacks *Pfhrp*2 and its flanking genes (Wellems, Walkerijonah, and Panton 1991). Additionally, Dd2 served as a positive control for *Pfhrp*3 and flanking genes PCR amplification. Moreover, laboratory strain HB3 (5 ng/µl) was utilized as a negative control for *Pfhrp*3 and its flanking regions (Wellems et al. 1987) and as positive control for *Pfhrp*2 and flanking genes amplification.

3.4.4 Gel electrophoresis

Following PCR, gel electrophoresis was conducted to identify samples that amplified successfully for each gene (*Pfhrp*2 and *Pfhrp*3 and their flanking genes). 1.5% agarose gel was prepared using 1 x Tris Acetate EDTA (TAE) buffer. 2.25 g of agarose powder (Applied genetic technology corporations) was weighed, then added to 150 ml of 1 x TAE buffer and heated to boiling point in a microwave for three minutes to allow mixing. The mixed solution was then cooled under running tap water until it was about 55 ºC, then stained with 15 µl (10 000x) of gel red and poured into a gel tray fitted with combs. The gel was then incubated for 30 to 60 minutes at room temperature to allow it to set. For each well, four microliter of PCR product, as well as negative and positive control were individually mixed with four-microliter of six times blue orange loading dye (Promega) and loaded into wells on the gel. Additionally, four µl each of 100 base pairs hyper ladder (Bioline) was loaded into the first and final wells on the gel. After loading all the samples, running buffer (10% TAE) was then added and the gel was run at 230 volts for 50 minutes. The gel was then read on UVIsave HD5 - Gel documentation (Uvitec Limited) and the resulting picture printed and saved in a flash drive.

Results were interpreted as done previously (Abdallah et al. 2015), the amplification that resulted in a detectable band of suitable length was termed as a positive confirming expression of the corresponding gene. Successful amplification was recorded as the final. However, for samples that fail to amplify, PCR amplification was repeated. If the repeated amplification resulted in an agreement with the first result, it was recorded as an ultimate finding. Conversely, if the first and second results were disagreeing, a third assay was carried out and the two identical results of the three were recorded as the final result.

3.4.5 Mapping Primers to reference sequence

P. falciparum 3D7 *Pfhrp*2 and *Pfhrp*3 full genes length +- 100 base pairs sequences were retrieved from NCBI and imported into Sequence Manipulation Suite (SMS). All Primers were mapped to 3D7 reference sequence using online Sequence Manipulation Suite (SMS) (http://www.bioinformatics.org/sms2), primer map in order to determine the region, which the primer would amplify. *Pfhrp*2 primers from Abdallah 2015 study, Baker 2005 and Me´lanie 2013 were mapped onto *Pfhrp*2 3D7 reference sequence. Similarly, *Pfhrp*3 primers from the same studies were mapped on *Pfhrp*3 3D7 reference sequence. Both genes primers were matched and selected based on the expected fragment length to be amplified. The primers were then ordered from LGC Bio search Technologies.

3.4.6 DNA sequencing

*Pfhrp*2 gene was amplified using primers and conditions described elsewhere (Me´lanie et al. 2013). This method involved two separate PCR amplification involving two sets of primers. The primer set one was amplifying exon one and intron one of the gene while primer set two was amplifying exon two of *Pfhrp*2 gene as shown in appendix 4 (Me´lanie et al. 2013). The PCR amplification conditions for both primer sets were set as done in Me´lanie 2013 paper. Briefly, activation at 94 ºC for 15 minutes, 35 cycles of denaturation at 94 ºC for 30 seconds, followed by annealing at 55 ºC for 30 seconds and elongation at 72 ºC for one minute and final elongation at 72 ºC for 10 minutes.

On the other hand, *Pfhrp*3 gene was amplified using a combination of primers from several publications (Baker et al. 2005; Me´lanie et al. 2013; Abdallah et al. 2015). Like *Pfhrp*2, it

involved two separate PCR amplification using two sets of primers. Primer set one involved forward primer 3E12 F1: 5′ GGT TTC CTT CTC AAA AAA TAA AA 3′ (Abdallah et al. 2015) and reverse primer *Pfhrp*3-R1: 5' -TGG TGT AAG TGA TGC GTA GT-3' (Baker et al. 2005). For primer set one PCR conditions were set at activation at 94 ºC for 15 minutes, 35 cycles of denaturation at 94 ºC for 30 seconds annealing at 56 ºC for 30 seconds and elongation at 72 ºC for 1 minute. Followed by a final elongation at 72 ºC for 10 minutes.

Primer set two involved forward primer P*fhrp3*-F2: 5'AAA TAA GAG ATT ATT ACA CGA AAG-3' and reverse primer A261R: 5'-TAATCTTCGATTAAATGGATT-3' (Baker et al. 2005; Me´lanie et al. 2013). The primer set two PCR conditions were set similar to primer set one conditions with annealing temperature adjusted from 56 ºC for 30 seconds to 60 ºC for 50 seconds as shown in appendix 4. All *Pfhrp*2 and *Pfhrp*3 PCR amplifications were done in 20-µl total volume consisting of quantifast master mix, 10 µM forward and reverse primers and 34.0 ng/µl DNA template.

Amplification products were cleaned using ExoSAP-IT (Affymetrix). This method involves an enzymatic clean-up procedure and consists of two enzymes; exonuclease enzyme, which cleaves any remaining primers and Shrimp Alkaline Phosphatase (SAP) enzyme, which cleaves the phosphate, group from unincorporated dNTPs. Two microliters of ExoSAP-IT enzyme was mixed with eight microliters of PCR product. Then was incubated at 37 ºC for 20 minutes to allow the two enzymes to work then further 20 minutes at 80 ºC to inactivate the ExoSAP-IT enzymes prior to sequencing.

Big dye termination PCR was carried out using primary PCR primers with concentration lowered from 10 µM to four µM. The amplification conditions were set at 95 ºC for 5 minutes, 30 cycles of 95 ºC for 15 seconds, 55 ºC for 30 seconds and 68 ºC 2 minutes and 30 seconds followed by a final elongation at 68 ºC for 3 minutes. Big dye terminator sequencing is a modification of Sanger sequencing where dideoxynucleotides (DdNTPs) labelled with a specific fluorescent dye corresponding to each nucleotide base are added to the reaction. During sequencing, dNTPs and DdNTPs are added to the elongating strand. However, when a DdNTP is added to the strand, elongation is terminated since the DdNTP lacks the hydroxyl group at the three prime end to allow continued synthesis.

The sequencing products were cleaned using Sephadex. Ten microliter of HiDi form amide was added into the cleaned sequence products. The plates were then sealed and heated at 96 ºC for 3 minutes to denature the DNA and then analysed using capillary electrophoresis ABI 3130/3500xL Genetic analyser.

3.5 Data Analysis

3.5.1 Calculation of prevalence of *Pfhrp*2 and *Pfhrp*3 genes deletions

This was calculated by dividing *Pfhrp*2 and/or *Pfhrp*3 genes deleted samples with the total number of samples positive for *P. falciparum* by 18S ribosomal RNA PCR

3.5.2 Correlation of false negative *Pfhrp*2 based RDT to *Pfhrp*2 and *Pfhrp*3 deletion status

The inclusion criteria for this analysis:

i. Positive for *P. falciparum* PCR targeting 18S rRNA with at least 30 parasites per microliter. This is because the limit of detection of malaria RDT is 30 parasites per microliter
ii. Positive for *Plasmodium* genus by pan based RDTs

*Pfhrp*2 based RDTs sensitivity was calculated using R, version 3.6.1. A spreadsheet containing RDTs, *P. falciparum* specific PCR, *Pfhrp*2, and *Pfhrp*3 genotyping result was converted into comma-delimited file (CSV) and loaded into R. Software. Calculation of sensitivity was done using epi tool (https://cran.r-project.org/web/packages/epi/index.html). Samples that qualified for this inclusion criteria but turned negative for *Pfhrp*2 based RDT were termed as false negative. *Pfhrp*2 and *Pfhrp*3 deletion patterns for both *Pfhrp*2 based RDTs false negative and *Pfhrp*2 based RDTs positive samples were analysed in excel 2016.

3.5.3 Analysis of sequence data

Sequencing forward and reverse primer electropherograms data from sequencer were then imported into CLC Main Workbench version 8.1 for cleaning and analysis (https://www.qiagenbioinformatics.com/products/clc-main-workbench/) (Qiagen Bioinformatics, n.d.). Each sample electropherogram for forward and reverse primers were

mapped along 3D7 reference sequence to develop a consensus. Corrections to base calling were done independent of the reference sequence. Multiple sequence alignment (MSA) was carried out using Muscle in Mega software (version 7.0) (Kumar et al. 2016) to identify the insertions and deletions and assess the nature of deletion whether partial or full gene deletion.

3.5.4 Analysis of *Pfhrp*2 and *Pfhrp*3 amino acid repeats

The Multiple Sequence Alignment (MSA) was then imported to BioEdit software (Version 5.2) (Senthil Gandi, n.d.). The sequences were then translated into the amino acid sequences. Repeat sequences were searched using radar online software (https://www.ebi.ac.uk/Tools/pfa/radar/) and recorded.

Chapter 4.0

Results

4.1 *P. falciparum* PCR

In total, 350 samples were analysed, of these 255 were collected between 2013 and 2017 while 95 were collected between 2003 and 2005 (Figure 4.1). A total of 327 (93.4%) of the total samples analysed were *P. falciparum* positive by PCR this comprised 234 post RDTs and 93 pre RDTs samples. However, 23 (6.6%) samples from the Total analysed were negative for *P. falciparum* by PCR and were excluded from further analysis (Figure 4.1).

Of the 327 *P. falciparum* PCR positive samples, 317 (96.9%) samples had at least 30 parasites per microliter. They were considered for analysis of RDTs reactivity, *Pfhrp*2 and *Pfhrp*3 encoding genes genotyping. The 317 samples comprised of 93 (29.3%) pre RDTs samples and 224 (70.7%) post RDTs samples (Figure 4.1). Ten samples with less than 30 parasites per microliter were used as control.

4.2 RDT results

We managed to run Para screen RDT for the 255 post RDT samples, for the 95 pre-RDTs samples RDT was not done as only DNA was available. Out of 255 samples, 234 were positive for *P. falciparum* by PCR, while 21 were negative by both *P. falciparum* by PCR and RDT. Of 234 PCR positive samples, 224 samples had at least 30 parasites per microliter while 10 had lesser parasitaemia.

In total, 195 (87.1%) of 224 samples were positive by pan based RDT whereas 29 (12.9%) samples were negative, this depict negative for *Plasmodium* by RDT hence were excluded from analysis of *Pfhrp*2 based RDT reactivity. Twenty-one (10.8%) samples of 195 pan RDT positive samples were negative by *Pfhrp*2 based RDT suggesting false negative RDTs result. False-negative RDT results were higher in samples with less than 30 parasites per microliter (50.0%) than in samples having at least 30 parasites per microliter (10.8%) (Figure 4.1). The sensitivity of *Pfhrp*2 based RDT was 89.2% (174/195) in reference to pan positive in *P. falciparum* PCR positive samples (Figure 4.1).

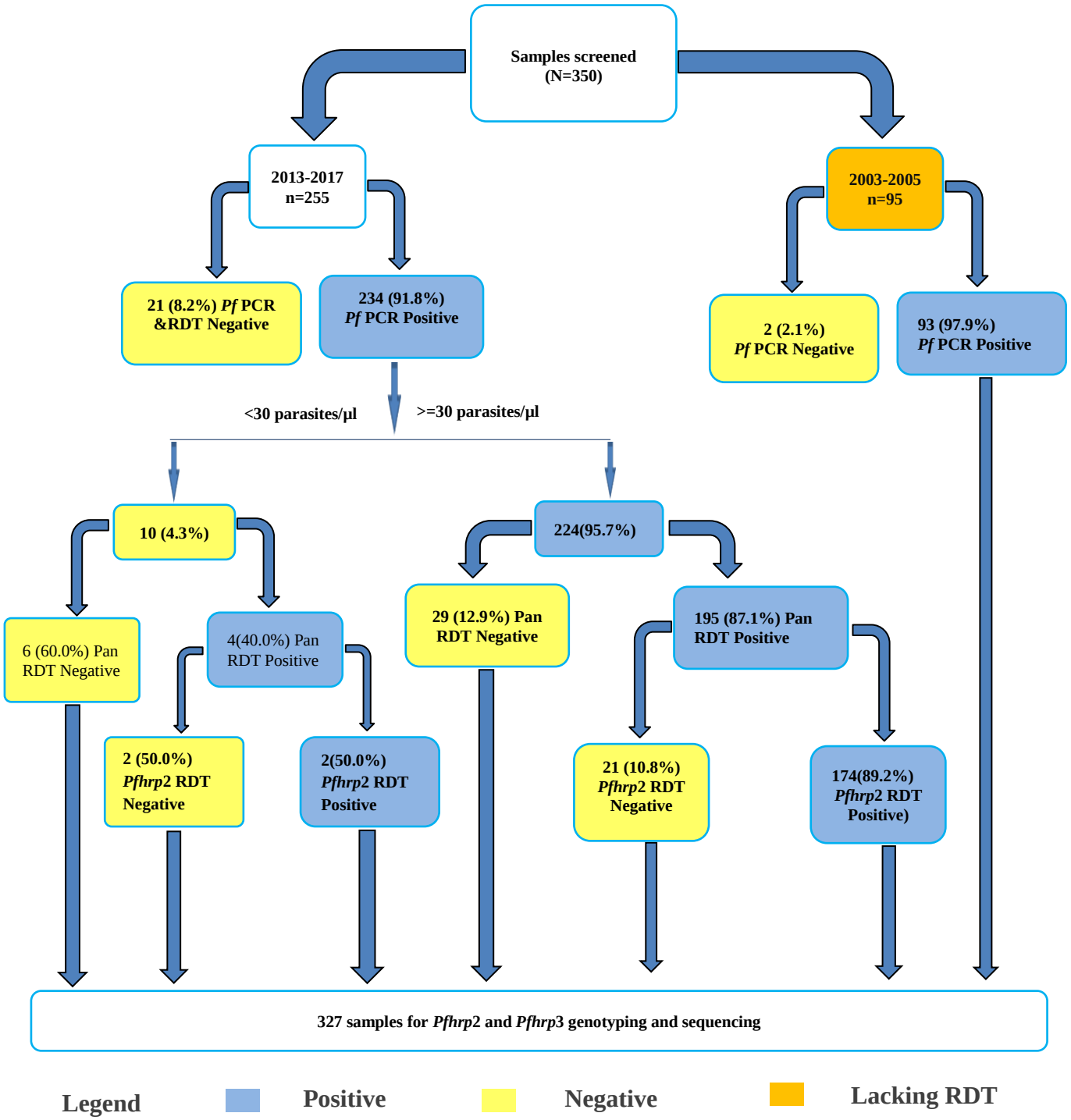

Figure 4. 1: Flow chart showing the *Plasmodium falciparum* PCR and RDT results. Twenty-one of 195 samples positive for pan RDT and *P. falciparum* PCR (with 30 parasites per microliter) were negative by *Pfhrp*2 based RDTs indicating possible false negative *Pfhrp*2 based RDTs results. Colour blue represents samples positive for the test, yellow represent negative for the test while colour orange represents samples not tested.

Analysis of *Pfhrp*2 based RDT band and pan RDT band in 224 samples shows that, 174 (89.2%) were both Pan and *Pfhrp*2 band positive, 21(9.4%) had pan band only and 22 (9.8%) had *Pfhrp*2 band only. Conversely, seven (3.1%) of 224 samples were negative for both pan and *Pfhrp*2 band. Conversely, two (20%) of 10 samples with less than 30 parasites per microliter were positive for both *Pfhrp*2 based RDT band and pan RDT band (Table 4.1).

Table 4.1: Illustration of *Pfhrp*2 and Pan bands RDT results in relation to parasitaemia in *P. falciparum* PCR positive samples (n= 234).

***P. falciparum* parasitaemia >= 30 parasites per µl (n=224)**				***P. falciparum* parasitaemia <30 parasites per µl (n=10)**			
***Pfhrp2*-band**	**Pan-band**	**n**	**Prevalence**	***Pfhrp2*-band**	**Pan-band**	**n**	**Prevalence**
Positive	Positive	174	77.7%	Positive	Positive	2	20%
Negative	Positive	21	9.4%	Negative	Positive	2	20%
Positive	Negative	22	9.8%	Positive	Negative	3	30%
Negative	Negative	7	3.1%	Negative	Negative	3	30%

4.3 Temporal and spatial trends of false RDTs negative results

In 2013, Malindi site had 28.5% prevalence of false RDT negative samples, followed by Kericho site 25.0%. Kombewa site had the least prevalence of false negative RDT result (3.6%). There was no clear temporal or spatial trend of *Pfhrp*2 based RDT negativity while in Kericho the prevalence was decreasing from 2013 to 2016 in Kombewa the prevalence was increasing. Malindi site did not have samples in 2014 and 2015. In 2017, very few numbers of samples were recorded across all sites (Table 4.2). Overall, Malindi site with 31.6% (6/19) had the highest prevalence of false-negative RDTs results, while Kericho (n=73) and Kombewa (n=103) sites had 13.7% and 4.9% respectively.

Table 4.2: Prevalence of *Pfhrp*2 based RDT Negativity in *P. falciparum* PCR and Pan RDT positive samples per year across three study sites (n=195)

	2013	**2014**	**2015**	**2016**	**2017**
Malindi	4 (28.5%) (n=14)	-	-	2(50%) (n=4)	0 (n=1)
Kombewa	1(3.6%) (n=28)	0 (n=25)	3(0.1%) (n=30)	1(5.6%) (n=18)	0 (n=2)
Kericho	5(25.0%) (n=20)	2(11.8%) (n=17)	2(0.1%) (n=20)	1(6.7%) (n=15)	0 (n=1)

4.4: *Pfhrp*2 and *Pfhrp*3 genotyping

*Pfhrp*2 and *Pfhrp*3 and their flanking genes genotyping PCR amplification results were interpreted as done previously (Abdallah et al. 2015), the amplification that resulted in a detectable band of suitable length was termed as a positive confirming expression of the corresponding gene. However, for samples that fail to amplify, PCR amplification was repeated. If the repeated amplification resulted in an agreement with the first result, it was recorded as an ultimate finding. Conversely, if the first and second results were disagreeing, a third assay was carried out and the two identical results of the three were recorded as the final result (Figure 4.2 and Figure 4.3).

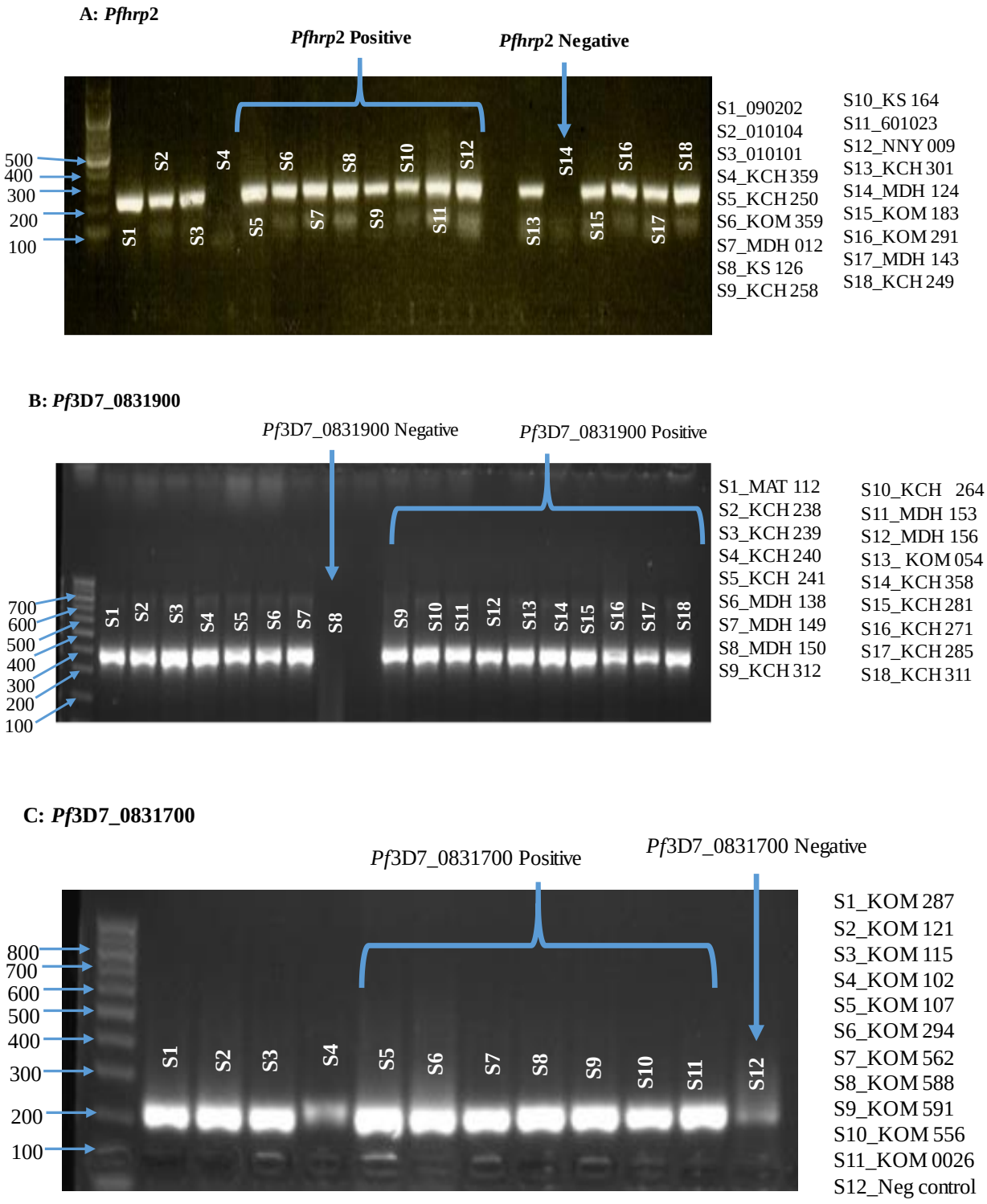

Figure 4.2: Gel electrophoresis images, A represents *Pfhrp2* gene (228bp), B represents *Pfhrp2*, 5' flanking gene (301bp) while C represent *Pfhrp2*, 3' flanking gene (198bp). A clear band represent positive and lack of band represent negative result**.**

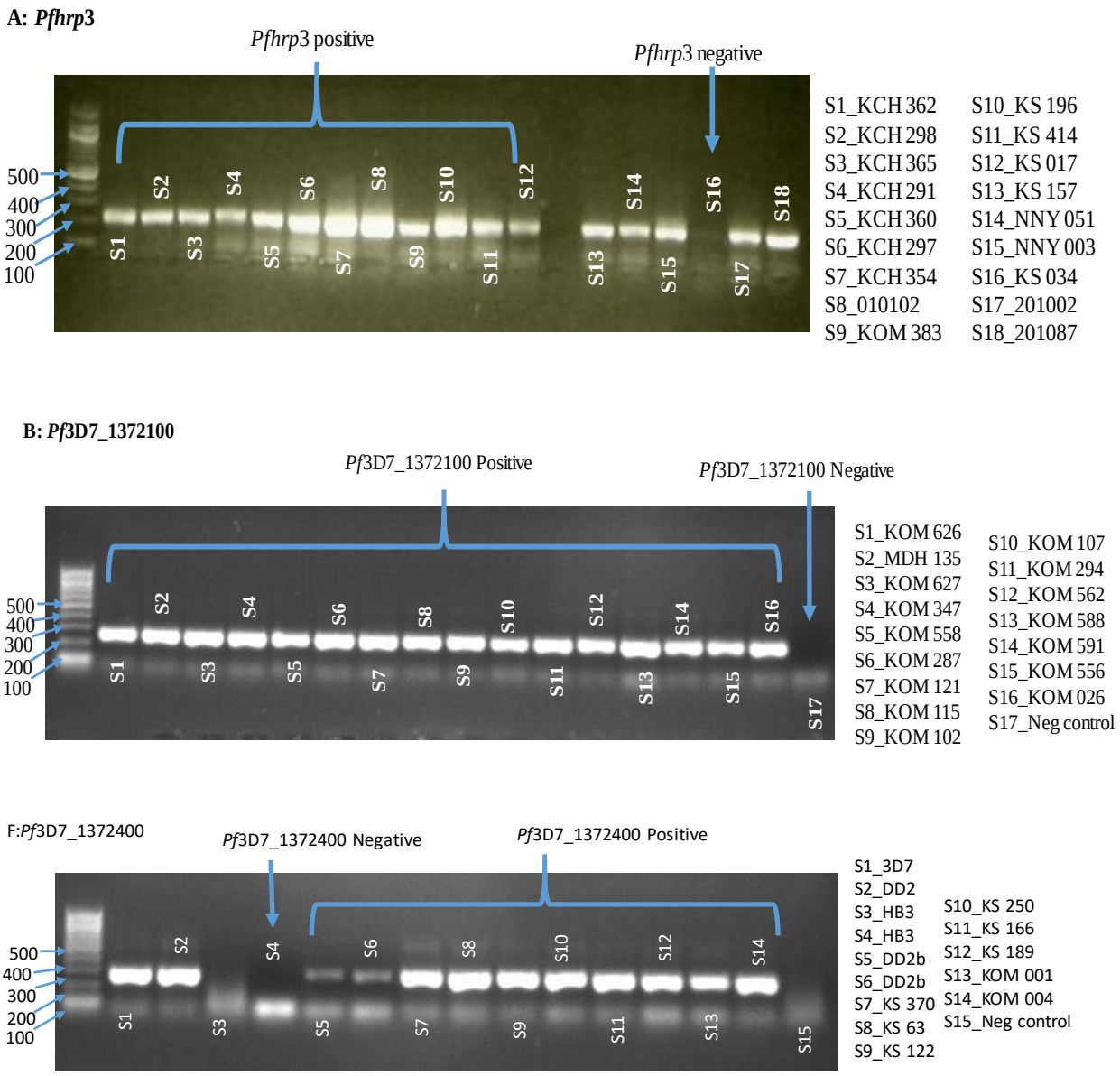

Figure 4.3: Gel electrophoresis image A represent *Pfhrp*3 gene (225 bp), B *Pfhrp*3 5' flanking gene (241 bp) and C *Pfhrp*3 3' flanking gene (225 bp) PCR Result. A clear band represent positive and lack of band represent negative result

Overall, 327 *P. falciparum* PCR positive samples proceeded to *Pfhrp*2 and *Pfhrp*3 genotyping. This comprised of 317 (93 and 224) samples that had at least 30 parasites per microliter in pre and post RDT samples and 10 samples with less than 30 parasites per microliter. The 317 samples were used to analyse the prevalence of *Pfhrp*2 and *Pfhrp*3 gene deletions, while the 10 samples with less than 30 parasites per microliter were used as control.

Analysis of *Pfhrp*2 genotyping, 311 (98.1%) of 317 were positive for *Pfhrp*2, one (0.3%) sample was negative while the remaining five samples *Pfhrp*2 assay was not done. Conversely, all the 10 samples with less than 30 parasites per microliter were positive for *Pfhrp*2.

On the other hand, 310 of 317 samples tested for *Pfhrp*3 were positive for the gene, four (1.3%) samples were negative while three samples of 317 were depleted hence *Pfhrp*3 genotyping was not done. Analysis of the 10 samples with less than 30 parasites per microliter shown that four (40%) samples were negative for *Pfhrp*3 whereas six (60%) were positive.

In total, 306 samples of 317 were positive for both *Pfhrp*2 and *Pfhrp*3 genes, four were negative for *Pfhrp*3 only, one sample was negative for *Pfhrp*2 only and no sample was negative for both genes. The remaining six samples were not analysed, as the assay of either one gene or both were not done (Table 4.3).

Table 4. 3: The patterns of *Pfhrp*2 and *Pfhrp*3 deletions in *P. falciparum* positive samples with at least 30 parasites per microliter (n=317)

*Pfhrp*2 genotyping	*Pfhrp*3 genotyping	n	Prevalence
Positive	Positive	306	96.5%
Positive	Negative	4	1.3%
Negative	Positive	1	0.3%
Negative	Negative	0	_
*	*	2	0.6%
*	Positive	3	0.9%
Positive	*	1	0.3%

Positive, represent presence of the gene; Negative, represent absence of the gene; * represents missing data

Kericho site had the highest prevalence of *Pfhrp*3 negative results 2.2% followed by Malindi site, however, the prevalence of *Pfhrp*3 in all sites was significantly low. Only one sample was negative for *Pfhrp*2 (Table 4.4).

Table 4.4: *Pfhrp*2 and *Pfhrp*3 deletions prevalence across the study sites

Site	Sample Size	*Pfhrp*2	*Pfhrp*3	*Pfhrp*2 and *Pfhrp*3 deleted
Kombewa	117	1 (0.9%)	1 (0.9%)	0
Malindi	53	0	1(1.9%)	0
Kisumu	49	0	0	0
Kericho	93	0	2(2.2%)	0
Alupe	5	0	0	0

There was no clear trend of *Pfhrp*2 or *Pfhrp*3 gene deletion across years as the prevalence of genes deletion were too low (Table 4.5). However, *Pfhrp*3 gene deletions were highest in 2013 (Table 4.5). There was no double deletion of both *Pfhrp*2 and *Pfhrp*3 genes across all the years (Table 4.5). One sample of 224 post-RDT samples had a deletion of *Pfhrp*2 only and four samples of 224 samples had a deletion of *Pfhrp*3 gene only. On the other hand, no sample of 93 pre-RDT samples had a deletion of any of genes. The deletion of *Pfhrp*2 or *Pfhrp*3 gene deletion was highest in post RDT samples than in pre-RDT samples (Table 4.5).

Table 4.5: Temporal trends of *Pfhrp*2 and *Pfhrp*3 deletions

Year	Sample Size	*Pfhrp*2 single deletion	*Pfhrp*3 single deletion	*Pfhrp*2 and *Pfhrp*3 deleted
2003 & 2005	93	0	0	0
2013	76	0	3 (3.9%)	0
2014	46	0	0	0
2015	58	0	1(1.7%)	0
2016	39	1 (2.6%)	0	0
2017	5	0	0	0

Occurrence of *Pfhrp*2 and its two flanking genes together was the most prevalent (83.6%), followed by deletion of 5' *Pf*3D7_0831900 gene with intact *Pfhrp*2 and 3' flanking gene *Pf*3D7_0831700. No sample had double deletion of *Pfhrp*2 and any of its flanking gene (*Pf*3D7_0831900 flanking on 5' or *Pf*3D7_0831700 flanking on 3' end). Similarly, no sample had deletion of all the three genes together (triple deletion) (Table 4.6).

Table 4. 6: Deletion patterns of *Pfhrp*2 and its flanking genes (n= 317)

***Pf*3D7_0831900 (Mal7pI.230)**	**Pf3D7_0831800 (*Pfhrp*2)**	***Pf*3D7_0831700 (Mal7pI.228)**	**n**	**Prevalence**
Positive	Positive	Positive	265	83.6%
Negative	Positive	Positive	41	12.9%
Positive	Negative	Positive	1	0.3%
Positive	Positive	Negative	2	0.6%
Negative	Negative	Positive	0	-
Positive	Negative	Negative	0	-
Negative	Positive	Negative	2	0.6%
Negative	Negative	Negative	0	-
6 samples had missing data				

Positive, represent presence of the gene; Negative, represent absence of the gene

Similarly, occurrence of *Pfhrp*3 and its flanking genes together was the most prevalent (88.3%). Prevalence of double deletion of *Pfhrp*3 and any of its flanking gene (*Pf*3D7_1372100 flanking on 5' or *Pf*3D7_1372400 flanking on 3' end) was very low (0% and 0.3% respectively). Similar to *Pfhrp*2, no sample had deletion of all the three genes together (triple deletion) (Table 4.7).

Table 4. 7: Deletion patterns of *Pfhrp*3 and its flanking genes (n= 317)

***Pf*3D7_1372100 (Mal13pI.475)**	**Pf3D7_0831800 (*Pfhrp*3)**	***Pf*3D7_1372400 (Mal13pI.485)**	**n**	**Prevalence**
Positive	Positive	Positive	280	88.3%
Negative	Positive	Positive	10	3.2%
Positive	Negative	Positive	3	1.0%
Positive	Positive	Negative	16	5.0%
Negative	Negative	Positive	0	-
Positive	Negative	Negative	1	0.3%
Negative	Positive	Negative	2	0.6%
Negative	Negative	Negative	0	-
	5 samples had missing data			1.6%

Positive, represent presence of the gene; Negative, represent absence of the gene

4.5 Correlation of RDT reactivity to *Pfhrp*2 and *Pfhrp*3 gene deletion status

Samples having at least 30 parasites per microliter by *Plasmodium falciparum* PCR and positive by pan based RDTs were analysed to assess relationship between RDTs reaction with *Pfhrp2* and *Pfhrp3* deletions (n=195). Of the 21 samples negative by *Pfhrp*2 based RDT, 20 (95.2%) samples were positive for both *Pfhrp*2 and *Pfhrp*3, one (4.8%) sample was negative for *Pfhrp*3 only, and none of the 21 samples lacked *Pfhrp*2 gene (Table 4.9).

Prevalence of both *Pfhrp*2 and *Pfhrp*3 being present together was not significantly different in both *Pfhrp*2 based RDT positive samples and in RDT negative samples (97.1% and 95.2% respectively). No samples had *Pfhrp*2 gene deletion in false *Pfhrp*2 based RDT negative

samples, however, 0.6% of all *Pfhrp*2 based RDT positive samples were negative for *Pfhrp*2 gene. On the other hand, single *Pfhrp*3 deletion with intact *Pfhrp*2 was 4.8% and 1.7% in false *Pfhrp*2 based RDT negative and *Pfhrp*2 based RDT positive samples respectively. One sample in *Pfhrp*2 based RDT positive samples was negative for both *Pfhrp*2 and *Pfhrp*3 while in false *Pfhrp*2 based RDT negative samples none had deletion of both genes (Table 4.8 and Table 4.9).

Table 4.8: *Pfhrp*2 and *Pfhrp*3 deletions patterns in *Pfhrp*2 based RDT positive samples with at least 30 parasites per µl

***Pfhrp*2 based RDT Positive(n=174)**			
***Pf*hrp2 (*Pf*3D7_0831800)**	***Pfhrp*3 *Pf*3D7_1372200**	**n**	**Prevalence**
Positive	Positive	169	97.1%
Positive	Negative	3	1.7%%
Negative	Positive	1	0.6%
Negative	Negative	0	-

Positive, represent presence of the gene; Negative, represent absence of the gene

Table 4.9: *Pfhrp*2 and *Pfhrp*3 deletions patterns in *Pfhrp*2 based RDT negative samples with at least 30 parasites per µl

***Pfhrp*2 based RDT Negative (n=21)**			
***Pfhrp*2 (Pf3D7_0831800)**	***Pfhrp*3 Pf3D7_1372200**	**n**	**Prevalence**
Positive	Positive	20	95.2%
Positive	Negative	1	4.8%
Negative	Positive	0	-
Negative	Negative	0	-

Positive, represent presence of the gene; Negative, represent absence of the gene

4.6 Sequence analysis

Sequencing was done for 317 samples; however, only 69 *Pfhrp*3 clean sequences were obtained (Figure 4.4). There was high failure rate in *Pfhrp*3 gene sequencing whereby most samples had poor or no primer coverage at the intron and at the start of the exon 2. Thirty-one (44.9%) samples of 69 had no deletion while 38 (55.1%) samples had partial deletions no sample had full gene deletion. The most frequent deletion was from amino acid position 94 to position 100, while deletion at position 121 to 127 and 154 to 160 amino acid position had the least frequency (Table 4.10).

Table 4.10: Frequency of *Pfhrp*3 gene deletion by amino acid position (n= 69)

Amino acid position	n	Frequency
87-90 aa and 94 -100aa	5	13.2%
87-90 aa and 96-99	1	2.6%
87-90 aa, 94 -100aa and 164-169 aa	7	18.4%
94-100aa	11	28.9%
94-100aa and 112-118aa	2	5.3%
94-100aa and 118- 124aa	2	5.3%
96-99aa and 164-169aa	2	5.3%
121-127aa	1	2.6%
130-136aa	2	5.3%
154-160aa	1	2.6%
164-169aa	4	10.5%

aa, represents amino acid

Amino acid repeat AAH and AHHAAN were the most prevalent appearing in frequency of around 4- 10 repeats in all of the 69 samples (Table 4.11). Correlation of repeat type to RDT reactivity was not done due to failure in *Pfhrp*2 sequencing.

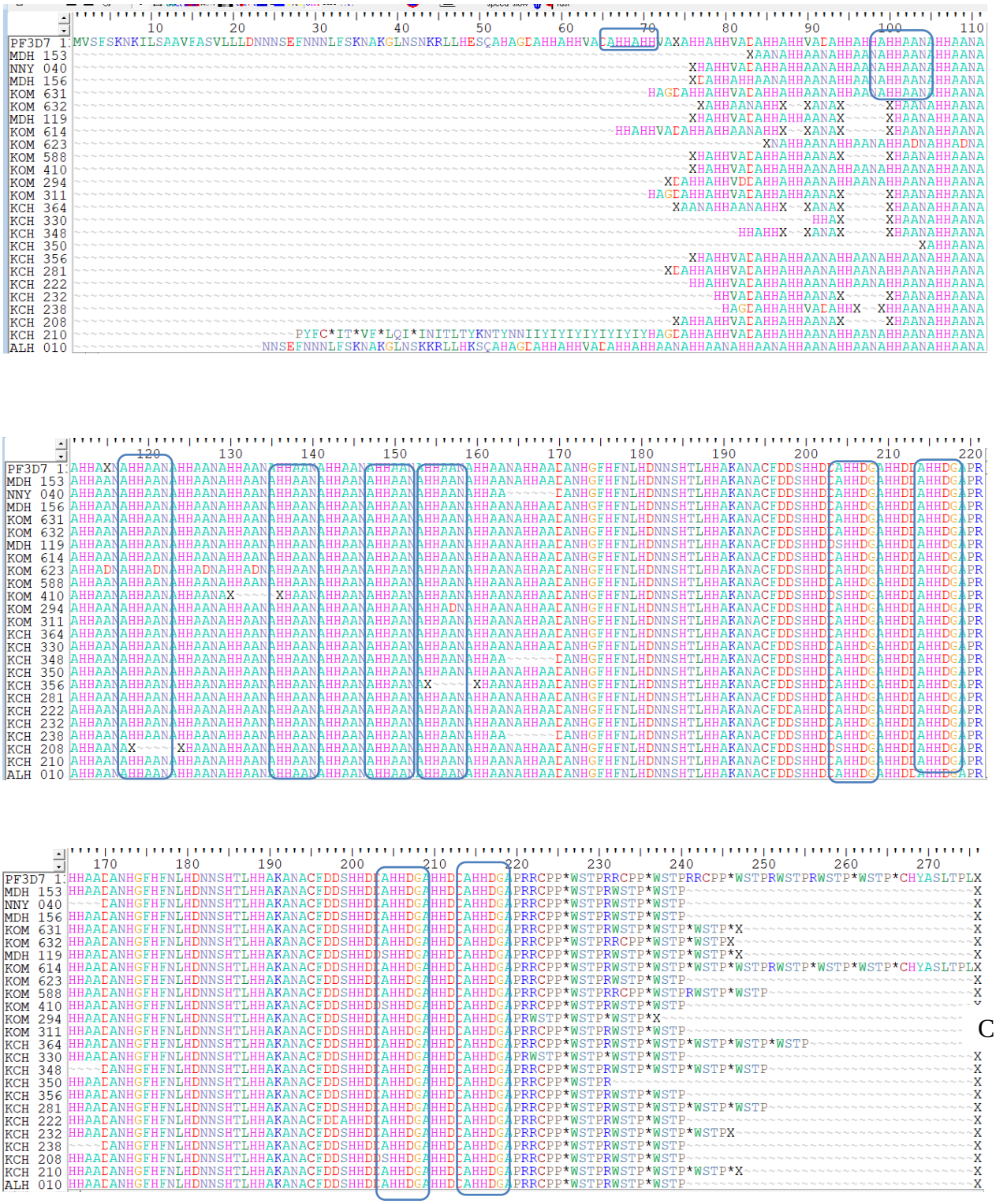

Figure 4.4: Multiple sequence alignment of the amino acid sequences, A is from 1aa to 111aa, B is 112aa to 220 aa while C is from 170aa to 275aa. For most samples the sequence start from position 90aa to 240 amino acids. The highlighted regions show various repeats types.

Table 4.11: Illustration of amino acid repeat type present in *Pfhrp*3 amino acid sequence in 69 samples

Repeat type	Repeat	No. of Repeats	n	Repeat prevalence
Type 4	AHH	4-14	69	100%
Type 16	AHHAAN	4-10	69	100%
Type 7	AHHAAD	1	16	23.2%
Type 17	AHHDG	1-2	22	31.9%
Type 18	AHHDD	1	7	10.1%

On the other hand, for *Pfhrp*2, full consensus of all the 327 sequences was not obtained. Similar to *Pfhrp*3, *Pfhrp*2 sequences had poor or no primer coverage at the intron and the start of exon two. Additionally, *Pfhrp*2 sequences had no primer coverage from 600 base pairs to 1064 base since the second primer set forward primer A118F and reverse primer A264 supposed to cover from 290 bp to 1164 bp produced very short sequence. A second primer sets were designed to sequence the exon 1 and exon 2 separately however, we were not able to attempt this approach due to lack of time and limited resources.

Chapter 5.0

5.1 Discussion

Malaria RDTs were introduced in 2010 in most parts of the world but in Kenya, they were introduced in 2012. Introduction of malaria RDTs has increased the detection level in suspected malaria cases, with RDT accounting for over 75% diagnosis of all suspected malaria cases (WHO 2017). Prompt and accurate diagnosis improves case management and may lower the occurrence and increase in strains resistant to antimalarial drugs. However, the accuracy of *Pfhrp*2 based RDTs which is the most commonly used malaria RDT could be affected by deletions in the gene (Berhane et al. 2018), or repeat type present in their amino acid sequence (Baker et al. 2005, 2010). The criteria of World Health Organisation (WHO) require accuracy above 95% as the threshold for selection or withdrawal of RDTs advocating for vigorous *Pfhrp*2 gene deletions mapping (WHO Global malaria programme 2017b).

This study analysed 350 archived samples from the epidemiology of malaria drug resistance study. These comprised of 255 samples collected between 2013 and 2017 and 95 collected between 2003 and 2005. Malaria diagnosis was done using microscopy; *Pfhrp*2/pan based RDT, and *Plasmodium falciparum* specific 18S ribosomal RNA PCR. For the 95 samples collected between 2003 and 2005, RDT was not done since only DNA samples were available.

The prevalence of *Pfhrp*2 gene deletion in all samples positive for *Plasmodium falciparum* with at least 30 parasites per microliter was 0.3%. This finding shows lower prevalence than from Beshir *et al* (2017) study where *Pfhrp*2 gene deletion prevalence was 10%. On the other hand, the prevalence of *Pfhrp*3 gene deletion was 1.3% which is slightly higher than previous finding (Beshir et al. 2017). The *Pfhrp*2 gene deletion was only in post RDT samples (0.4%), pre-RDT samples were all positive for *Pfhrp*2 gene. This finding may be owing to selection pressure brought about by utilisation of *Pfhrp*2 based RDT, but, the number of *Pfhrp*2 gene deletion was very low that the trend of deletion could not be interpreted (Watson et al. 2017). Similar to *Pfhrp*2, *Pfhrp*3 deletion were only in post RDT samples (1.8%).

Kombewa site was the only site with deletion of *Pfhrp*2 gene (0.9%) prevalence. Conversely, Kericho site had the highest prevalence of *Pfhrp*3 gene deletion (2.2%) followed by Malindi

with 1.9% and Kombewa had the lowest prevalence with 0.9% *Pfhrp*3 gene deletion. However, interpretation of these deletions should be done with caution since *Pfhrp*3 deletion prevalence was very low across all sites. Nevertheless, it is clear that Kombewa (0.9%) produced the lowest prevalence of *Pfhrp*3 gene deletion, despite it having a larger sample size than Kericho and Malindi.

In both *Pfhrp*2 and *Pfhrp*3 gene, deletion of the gene was not associated with the deletion of the flanking genes (5' and 3' flanking genes). This finding shows that the deletion of these two genes is not due to chromosomal deletion as each was occurring independently. This finding is in contrast to studies done in South America (Gamboa et al. 2010; Abdallah et al. 2015; Okoth et al. 2015; Dorado et al. 2016). Abdallah (2015) working with 68 samples collected from Honduras in 2008 and 2009 found that all *Pfhrp*2 and its two flanking genes were all present. 26% of the isolate had double deletion of *Pfhrp*3 and the 5' prime flanking gene (*Pf*3D7_1372400**)** and 15% of the isolated had deletion of all the three gene**s (***Pfhrp*3 and its two flanking genes) (Abdallah et al. 2015). Dorado et al (2016) found that all *Pfhrp*3 negative samples were also negative for 3' prime end gene *Pf*3D7_1372400 and 90% of all samples negative for *Pfhrp*3, 5' prime flanking gene (*Pf*3D7_1372100) was also negative (Dorado et al. 2016). Gamboa (2010) found that out of nine WHO FIND malaria RDTs evaluation program samples collected around Iquitos (Peruvian Amazon area) in 2007, eight were negative for *Pfhrp*2. All the eight *Pfhrp*2 negative samples were negative for *Pfhrp*2 5' flanking gene but positive for the *Pfhrp*2 3' flanking gene. However, the one sample positive for *Pfhrp*2 gene was also positive for both *Pfhrp*2 flanking genes (Gamboa et al. 2010). All four samples for the exon 2 of *Pfhrp*3 were also negative for the 5' *Pfhrp*3 flanking gene while three were negative for the 3' flanking.

*Pfhrp*2 based RDT had a sensitivity of 89.2% (174/195) in reference to pan positive in *P. falciparum* PCR positive samples. This is quite below the WHO recommendation of at least 95% sensitivity in samples with at least 100 parasites per microliter (Who- Regional office for Western Parcific/TDR 2006; WHO Global malaria programme 2017b). However, analysis of the trend of false RDT negative result from 2013 to 2016 samples in Kombewa and Kericho did not produce a clear trend. The prevalence of false negative RDT results in Kombewa was increasing from 2013 to 2015 then decreased in 2016, while that of Kericho site show

decreasing trend from 2013 to 2016. This finding confirms that although this study used archived samples, the storage did not affect negatively the RDT reactivity. However, lack of clear trend of RDT reactivity hamper evaluation of the impact of evolution on RDT reactivity.

In total 10.8% (21/195) of 195 samples positive for *P. falciparum* were negative by *Pfhrp*2 based RDT, this appear to be false negative RDT result. However, *Pfhrp*2 and *Pfhrp*3 genotype analysis shows that these samples had at least *Pfhrp*2 or *Pfhrp*3 genes. Although *Pfhrp*2 based RDTs are intended to detect *Pfhrp*2, studies have shown that they cross-react with *Pfhrp*3 due to presence of similar antigenic epitopes within the two genes (Baker et al. 2010; Gamboa et al. 2010; WHO. Malaria Policy Advisory Committee Meeting 2016; CDC 2012).

Approximately 95.2% of false negative *Pfhrp*2 based RDT samples had both *Pfhrp*2 and *Pfhrp*3 genes present, 4.8% had deletion of *Pfhrp*3 gene only, and none of the samples had deletion *Pfhrp*2 gene. This finding indicates that the false negative RDT result was not associated to either *Pfhrp*2 or *Pfhrp*3 gene deletions. Additionally, in both *Pfhrp*2 based RDT positive and false RDT negative samples the prevalence of both *Pfhrp*2 and *Pfhrp*3 being present together was not significantly different (97.1% and 95.2% respectively). The prevalence of *Pfhrp*2 gene deletion was higher in RDT positive samples than in the false RDT negative suggesting that other mechanism other than *Pfhrp2* gene deletion could be affecting the sensitivity of *Pfhrp*2 based RDT. Although, the prevalence of *Pfhrp*3 gene deletion with intact *Pfhrp*2 was higher in false negative *Pfhrp*2 based RDT samples than in positive samples (4.8% and 1.7%) *Pfhrp*2 is the main target of RDT and *Pfhrp3* is only detected in high parasite density as it is less abundant (Xin-Zhuan *et al.* 1996).

This finding is similar to Li *et al.* (2016) where two samples positive for *P. falciparum* by microscopy and PCR but negative by two *Pfhrp*2 based RDTs had intact *Pfhrp*2 gene (Li et al. 2016). In total eight (0.6%) samples of 174 *Pfhrp*2 based RDT positive samples, were negative for the *Pfhrp*2 gene with an intact *Pfhrp*3 gene, this positive RDT result could be due to signal produced by the *Pfhrp*3 gene. This finding is similar to Beshir *et al* (2017) where parasites that were *Pfhrp*2 deleted with undeleted *Pfhrp*3 turned positive for the *Pfhrp*2 based RDT but, it is in contrast to some studies (Baker et al. 2005; Baldeviano et al. 2015).

The sequencing process of both *Pfhrp*2 and *Pfhrp*3 had a very high failure rate with only 69 *Pfhrp*3 clean sequences to analyse and no *Pfhrp*2 sequences to analyse. For the high prevalence of poor primer coverage at the intron and the start of exon2 could be due presence of polyclonal parasites in the same sample having different nucleotide which may confuse the polymerase enzyme leading to production of several picks at the same location.

To better the sequencing of both *Pfhrp*2 and *Pfhrp*3 a protocol need to be optimised in order to improve sequencing at the intron, which is further affecting the start of the exon 2. Alternatively, separate primer set for exon 1 and exon 2 should be designed and leaving intron since the intron is making sequencing hectic yet it is less informative.

Limitations of the Study

Samples collected between 2003 and 2005 lacked RDTs data because they were collected prior to introduction of RDTs. This hampered comparison of the performance of RDT in pre RDT introduction to that of post RDT introduction. Nevertheless, we were able to obtain *Pfhrp*2 and *Pfhrp*3 genotyping and sequence data, which gave a rough estimate of effect of introduction of RDTs on *Pfhrp*2 and *Pfhrp*3 genes.

Varying burden of malaria across enrolling sites as well as period of site inclusion to the original study dictated the numbers of samples per site across the study period impeding spatial and temporal analysis

Additionally this study was limited by the fact that post RDTs samples (samples collected between 2013 to 2017) were recruited majorly based on pan/ *Pf* RDTs. This could have introduced some bias on the real picture of RDT reactivity and the prevalence of *Pfhrp*2 and *Pfhrp3* gene deletions. However, the original study included all symptomatic individuals even if they had negative pan/*Pf* band implying that any symptomatic malaria that failed to be detected by RDTs may have also been included.

High AT-rich intron found in both *Pfhrp*2 and *Pfhrp*3 gene affected sequencing process. Sequencing data obtained was very little due to challenges in sequencing both *Pfhrp*2 and *Pfhrp*3 genes. This made it difficult to draw a conclusion on the sequencing data, however, it gives an insight on types of amino acid repeats present in *Pfhrp*3 sequence and the nature of

deletion. To better sequence *Pfhrp*2 and *Pfhrp*3 genes, a protocol needs to be optimized, that will enable sequencing of AT-rich region in the sequence. Alternatively, the two exons of *Pfhrp*2 and *Pfhrp*3 genes should be sequenced separately to avoid effect of highly AT-rich intron.

5.2 Conclusion

This study shows that *Pfhrp*2 and *Pfhrp*3 genes deletion are indeed present in Kenya among the symptomatic individuals enrolled. However, these genes deletion could not be associated to false negative RDTs results. In addition, it confirms previous reports that double deletion of both hrp2 and hrp3 gene has not been established in Kenya. Amino acid repeat type 4 and 16 are the most prevalent at least for the 69 samples analysed.

5.3 Recommendation

This finding heralds the need for investigating additional mechanisms of false-RDTs negativity. Further work needs to be done using concurrent cohort and include asymptomatic cases as the method used for screening in the positive could present a bias. In addition, Whole genome sequencing approach may be used rather than Sanger sequencing since it is more informative. Research on new targets of RDTs need to continue in order to increase chances of having better and more reliable RDTs.

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Appendices

Appendix 1

DNA isolation from Body Fluids using Spin column method

This method is used in the extraction of entire (mitochondrial, viral as well as genomic) DNA out of body fluids such as whole blood, serum, buffy coat or plasma using a micro centrifuge.

NB:

- All steps of centrifugation are done at room temperature.
- In case the sample has less than 10,000 genome equivalents use of carrier DNA is recommended
  - Use of carrier DNA involves adding one microliter of aqueous solution comprising of 5–10 µg of carrier DNA such as poly dT, poly dA or poly dA:dT into 200 µl of Buffer AL. Ethanol addition in step six should be increased from 200 µl to 230 µl to guarantee optimal binding conditions. The DNA should be eluted in 60 µl of Buffer AE.
- The DNA yields from 200 µl whole blood is 3–12 µg. Supposing that one require greater DNA yields it is recommended to prepare buffy coat.
  - Buffy coat is white blood cells enriched whole blood portion. Its preparation increases DNA yields by about 5–10 times compared to that of same whole blood volume. Its preparation involve centrifugation of whole blood for 10 minutes at 2500-x *g* at room temperature. Three dissimilar portions are identifiable once centrifuged: The top most layer consist of plasma, the layer in the middle contains buffy coat with concentrated white blood cells while the bottommost is red blood cells enriched.

Prior to procedure initiation:

- Heating of water bath should be done until 56°C in preparation for incubation stage.
- Samples ought to be at room temperature.
- Elution buffer ought to be at room temperature in preparation of elution step.

- Confirm that AW1 buffer and Buffer AW2 have been added suitable absolute ethanol (96-100%) volume as shown on the container and the QIAGEN protease has been reconstituted appropriately.
    - To reconstitute lyophilised QIAamp protease for 50 samples DNA blood mini kit, 1.2 ml of protease solvent* should be dispensed to the QIAGEN protease vial, according to the label. For 250 samples kit, 5.5 ml of protease solvent* should be dispensed to the QIAGEN protease vial, as labelled.
    - Note that in case one is using QIAamp Mini Elute Virus Kits, reconstitution of these kit's QIAamp protease is in buffer AVE (protease resuspension buffer) which is incompatible with the QIAamp DNA Blood Mini Kit. Following reconstitution of QIAGEN protease, the vial containing the reconstituted QIAGEN Protease should be labelled to specify the buffer used to reconstitute.
- Stability of dissolved QIAGEN Protease is until 12 months at 2–8°C storage.
- Check whether the AL buffer has precipitate, then dissolve by incubation at 56°C.

Method

0. First 20 µl of proteinase K (or QIAGEN Protease) is dispensed into 1.5 ml micro centrifuge tube using a micropipette.
1. Subsequently, 200-µl whole blood or other body fluids are added into the micro centrifuge tube.

   In case the volume of the sample is below 200 µl, suitable PBS volume should be added.

QIAamp spin columns cleanse both DNA and RNA if the sample contain both. Except PCR, RNA can hinder other enzymatic downstream reactions.

If one require genomic DNA that is free from RNA; prior to addition of Buffer AL, addition of four microliter stock solution RNase A (100 mg/ml) ought to be done.

Note: Addition of proteinase K to samples that are already in the micro centrifuge can also be done. However, proper mixing after adding proteinase K is important.

2. Following, addition of 200 µl of Buffer AL to the micro centrifuge tube containing the sample is done then. Then pulse-vortex for 15 s to mix.

Thorough mixing of sample and buffer AL into a solution that is homogeneous is vital to guarantee effective lysis.

Proportional increase of proteinase K and Buffer AL in case one is using larger than 200 µl of sample should be done. E.g., 400 µl of Buffer AL and 40 µl of Proteinase K are needed to extract 400 µl of sample. To process lager than 400 µl of sample, it is recommended to use either Midi or Maxi QIAamp DNA Blood Kits, which can run a maximum of 2 ml or 10 ml of sample respectively.

Buffer AL require storage at room temperature, in which its stability can last 1 year. Thorough mixing before use is required.

Note: Avoid direct addition of proteinase K to Buffer AL.

3. The micro centrifuge containing extraction mixture above should be incubated for 10 minutes at a temperature of 56°C.

Once lysed at a temperature of 56°C for 10 minutes, maximum DNA production is achieved. Though, incubating for longer period does not affect extracted DNA yield nor quality.

4. Centrifuge the micro centrifuge tube shortly to get rid of droplets in the lid.
5. Consequently, 200 µl of absolute ethanol is added into the micro centrifuge having the extraction mixture, then pulse-vortexed for 15 seconds to mix. Followed by centrifuging shortly to get rid of drops in the lid.

Proportional increase of ethanol when using more than 200 µl is required. Such as 400 µl of ethanol when extracting 400 µl of sample.

6. The step six's mixture above is put into QIAamp Mini spin column (fitted into a collecting tube of two millilitres) cautiously to avoid wetting the rim. Followed by centrifugation for one minute at 8000 revolution per minute (6000 x g) with micro

centrifuge tube's cap closed. Thereafter, the spin column is inserted into the new two millilitre collecting tube, then dispose of filtrate holding tube.

During centrifugation, ensure all spin column's cap are closed to prevent formation of aerosol.

The reason of centrifuging process is carried out at 8000 rpm is so that the noise produced during the process can be less. Both pureness and output of DNA are not affected by centrifuging at full speed. The centrifugation should be repeated at a greater speed, in case some content of the extraction mixture have remained in the column, until all the content has passed through the column.

NB: Extraction of DNA using lymphocytes or buffy coat, it is recommended to centrifuge at full to prevent blockage.

7. Next, 500 µl of AW1 buffer is put into the spin column carefully not to wet the rim. Then centrifuged for one minute at 8000 revolution per minute. The collecting tube having the filtrate is disposed and the spin column inserted into a new two millilitre collecting tube.

Increasing Buffer AW1 volume is not necessary even if the sample volume used was larger than 200 µl.

* Sample preparation Flow-through waste comprises guanidine hydrochloride from Buffer AW1 or Buffer AL that may result in formation of very reactive compound on coming into contact with the bleach therefore do not add beach or any other acidic solution to waste from sample extraction directly.

When storing buffer AW1, it should be kept at room temperature.

At room temperature, Buffer AW1 remains stable for one year.

8. Subsequently, 500 µl of AW2 buffer is put into the spin column carefully not to wet the rim. Then centrifuged for 3 minutes at full speed (14,000 revolution per minute; 20,000 x *g*).

When storing buffer AW2, it should be kept at room temperature.

9. It is recommended to carry out a dry spin by placing the spin column into a fresh two millilitre collecting tube then the tube holding filter out is disposed and centrifuge for one minute at maximum speed.

This stage assist in eliminating possibility of Buffer AW2 residue.

10. Transfer the spin column into a new 1.5 ml micro centrifuge tube then dispose the filter out holding tube. Followed by addition of 200 µl of distilled water or Buffer AE and incubated for one minute at room temperature, followed by centrifugation for one minute at 8000 rpm.

Incubation of the QIAamp Mini spin column filled with water or Buffer AE at room temperature for five minutes prior to centrifugation normally increases yield of the DNA.

Carrying out an additional elution step using more 200-µl Buffer AE increase DNA production by a maximum of 15%.

Elution using more than 200 µl of elution buffer ought not to be done using 1.5 ml micro centrifuge tube since the spin column would encounter the eluate, which may cause formation of aerosol during centrifugation.

Eluting DNA in lesser than 200 µl, significantly more concentrated final DNA, nevertheless total yield of DNA is reduced a little. In samples with below one microgram of DNA, it is recommended to elute using 50 µl rather than 200 µl. Carrying out two elution using 100 µl instead of one elution using 200 µl does not improve the efficacy of elution.

If DNA is to be stored for long period, it is recommended to elute using buffer AE then store at a temperature of between -30 and -15°C. This is because long storage of DNA in water make it susceptible to hydrolysis (Qiagen 2016).

Appendix 2

Parascreen™ (Zephyr Biomedical, Goa India).

Intended use of the Parascreen

Parascreen® is a qualitative two-spot sandwich rapid immunoassay used to detect *Pfhrp*2 and Pan malaria specific pLDH in the whole blood. Additionally, it may be involved in distinguishing *Plasmodium falciparum* infections from non-*falciparum* infection (*Plasmodium* species) as well as monitoring success of antimalarial treatment using whole blood samples.

The test kit is for use in clinical and point of care places by health professionals to test suspected malaria infection cases.

Ability to detect and differentiate *Plasmodium* species is very important mostly because of cerebral malaria and drug resistance incidences related to *P. falciparum* malaria as well as morbidity associated with other forms of malaria.

Parascreen® identifies the manifestation of Pan malaria specific pLDH produced by parasite infected erythrocytes, to detect all malarial parasites. Conversely, to detect *Plasmodium falciparum* malaria, parascreen® uses the detection of *P. falciparum* specific histidine rich protein-2 (*Pfhrp*2), produced by infected individual's parasitized red blood cells.

If *Pfhrp*2 is absent and the Pan malaria specific band is present, indicate that non falciparum malaria species are presence. Speciation results interpreted depending on the most prevalent malarial species in the particular region.

The pan band may be utilised to determine whether treatment of malaria has been successful since pLDH is produce by viable parasites only.

PRINCIPLE

Parascreen® involves the agglutination principle between antibodies (antisera) and their complementary antigens in immuno-chromatography layout together with utilisation of nano gold particles as agent to reveal agglutination. On adding the clearing buffer, the specimen

moves along the device's assembled membrane, meanwhile agglutinating sera for *Pfhrp*2 coloured colloidal gold conjugates / colloidal gold conjugate for Agglutinating sera for Pan malaria specific pLDH binds on antigens in the sample. This combination travels further along the membrane of the device then get bound at the test area by *Pfhrp*2 Agglutinating sera / Pan malaria specific pLDH Agglutinating sera applied on the membrane resulting in pink-purple band/s formation which shows that the test result is positive. In *P. falciparum* both bands (Pan malaria specific pLDH and *Pfhrp*2) will appear, while, in non- *falciparum* malaria positive samples, only one band will appear. If no pink-purple band form at the test area, the test is negative.

The conjugate of rabbit globulin and colloidal gold together with unreacted conjugate and any unbound complex travel further along the membrane to the control area where it is attached by agglutinating rabbit globulin sera coated on the membrane, resulting in pink-purple band formation. The formation of control band depends on agglutinating sera system of rabbit globulin and fully independent of detection system of the analyte. Therefore, steady control band formation is facilitated regardless of the concentration of the analyte. This control signal helps in validating the test performance, if the test does not form control band the RDT is termed invalid.

Materials and reagents provided in the Parascreen® test kit

1. Distinct bags, each having:

   a. The test appliance, which consist of assembled membrane on which rabbit globulin-colloidal gold conjugate, pan malaria specific pLDH, Agglutinating sera colloidal gold conjugate agglutinating sera of *Pfhrp*2 and that of for *Pfhrp*2, Agglutinating sera for Pan malaria specific pLDH and Agglutinating sera for rabbit globulin at the respective regions.

   b. Desiccant sachet.

   c. Plastic sample Applicator that is disposable.

2. Dropper bottle containing clearing buffer.

3. Package leaflet.

4. Pictorial representation.

Optional: Calibrated micropipette for dispensing 5ul sample accurately.

Storage and stability

The kit's components and the sealed pouches can be saved between 1°C to 40°C until the period of its expiry date.

Never freeze the clearing buffer. Once opened, it can be kept between 1°C to 40°C until the period of its self-life.

NOTES

Carefully read the instructions prior to carrying out the test.

This test should not be used as therapeutic, it is to be utilised only in in vitro diagnosing of samples as well professional utilisation.

The aim of this test is not to be utilised in testing asymptomatic population.

Never use the kit after its expiry date.

Avoid mixing components from different lots.

The device & sample applicator are to be used only once.

Minimise contacting desiccant pouch contents as it contain cobalt chloride amid other substances. If inhaled or swallowed, it may cause harm.

All samples should be handled as potential infectious agent.

Biosafety regulation standards for managing and disposing infectious agent should be adhered to.

Avoid skin contact with Clearing buffer as contain Sodium Azide (0.1). Additionally, flush with huge amount of water to avoid building up of Azide in in the plumbing as it can lead to reaction with copper and lead in the plumbing system forming metal oxides that's are extremely explosive.

Collection and preparation of specimen

Test sample should be fresh anti coagulated whole blood. Suitable anticoagulant such as EDTA, Heparin, CPDA, Tri-sodium Citrate or Oxalate may be used. Collect the specimen in a sterile plastic or glass vessel. In case it unable to run the test immediately, the sample can be kept at 2°C to 8°C for maximum of 72 hours before testing. Avoid performing the test using Clotted or contaminated blood samples. One can utilise finger puncture fresh blood too.

Testing procedure and result interpretation

1. Before running the components of **parascreen®** kit ought to be taken to room temperature.

2. Remove the sample applicator, testing device and desiccant bag from the pouch. The colour of the desiccant should be blue. It should be assessed and in case it has become pink or colourless, the device should be discarded. Utilise the device immediately once opened.

3. The test device should then be labelled with patient's identity.

4. The testing device is then placed on a horizontal surface that is flat.

5. To pierce the nozzle of the buffer bottle, Squeeze the buffer bottle cap in the clockwise direction.

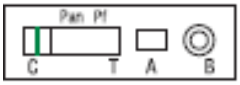
6. Swirl the anti-coagulated blood sample gently to evenly mix. The sample applicator is then dipped into the sample or in case of using finger prick blood; the sample applicator is placed to touch the finger puncture blood. Making sure that when removing the applicator is filled with blood, the blood is blotted in the sample port "A".

   (In case of finger prick blood, ensure blood has not coagulated and the blood is transferred to the sample application port immediately)

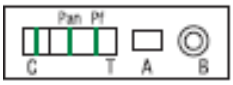
   On the other hand, micropipette can be used to transfer five microliters of the finger prick or anti-coagulated specimen to sample port 'A'.

NOTE: confirm that the entire blood sample is removed absolutely from the sample applicator to the devices sample port.

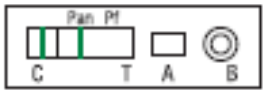
7. Immediately after blotting the sample from applicator into sample port 'A', two drops of buffer should be poured into buffer port "B".
8. Incubate at room temperature then interpret the result after 20 minutes as shown below:



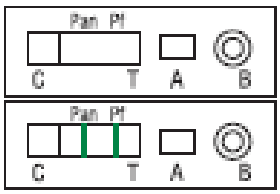
Indicate negative malaria sample: single band forms at the control region only.



Indicate that the sample is *P. falciparum* POSITIVE or has mixed infection: Two pink-purple bands forms in the test window T at "Pan" and "*Pf*" area in besides the control band.



Indicate Pan POSITIVE for non-*falciparum* species: One pink-purple band form only at "Pan" area at the test window besides the control band



Indicate INVALID RESULT: If no band form on the device or only test bands (Pan and/or *Pf*) form, the test is considered invalid. It should be repeated using new devise and adhering to the procedure carefully.

Characteristics of parascreen® RDT performance

A panel of 251 microscopically confirmed samples were tested with parascreen®. Obtained results shown below:

Sample	parascreen®		Total No. of samples tested	Specificity (%)	Sensitivity (%)
	Positive	**Negative**			
P. falciparum Positive	16	0	16	-	100
P. vivax Positive	25	0	25	-	100
Malaria Negative	0	210	210	100	-

LIMITATIONS OF THE TEST

1. The result of the test should always correspond to the clinical results.
2. The test findings interpretation should be within the context of therapeutic, clinical and epidemiological. If it appear out of this context, reference parasite-based diagnostic method such as thick and thin microscopy blood smear ought to be considered.
3. Utilisation of other reagents or adjusting the protocol above in whatever manner will make the procedure of the test to be invalid.

4. This test cannot distinguish single *P. falciparum* infection from mixed infection of *P. falciparum* and other species of malaria because in this kind of infection both, *Pf* and pan malaria bands will be positive in case of mixed infection.

5. Various studies have reported that occurrence of antibodies induced by external antigens in the blood of the patient may interfere with the test and cause immunoassay analyte detection error. However, Parascreen® utilises Heterophilic Blocking Reagent (HBR) to prevent most of these interferences.

6. During therapy monitoring with the "Pan" band, if the test reaction positivity persist with the equal concentration 5-10 days post treatment, resistant malaria strain possibility should be considered.

7. After successful antimalarial drug treatment, 'Pan' band usually turns negative. However, the test should be repeated 5-10 days after the start of therapy since clearance of parasite depends on duration of the treatment and the medication used.

8. During gametogony stage in *P. falciparum*, secretion of *Pfhrp*2 does not occur. Therefore, *Pfhrp*2 band may be absent in "Carriers".

9. *Pfhrp*2 antigens levels, persist until 15 days post treatment, hence, treatment success in *P. falciparum* may be monitored using 'Pan' band.

10. Occurrence of *Pfhrp*2 band positive with 'Pan' band negative result in few cases may indicate a case of post therapy infection. Nevertheless, such pattern of response can be seen in rare untreated malaria cases also. Therefore it is advised to retest after 2 days.

11. Never interpret test results read past 30 minutes of incubation (http://www.tulipgroup.com/Zephyr_New/html/pack_inserts/Parascreen.pdf)

Appendix 3

Table 1: *Pfhrp*2 and *Pfhrp*3 genes as well as their corresponding flanking genes genotyping primer and PCR conditions

Gene	Sequence & name of the primer	Amplification stage	Annealing Temp (°C)	Nested Amplicon size(bp) anticipated
PF3D7_0831900 (MAL7P1.230)	230 F1: 5′ GATATCATTAG AAAACAAGAGC TTAG 3′	Primary	63	301
	230 R: 5′ TATCCAATC CTTCCTTTGCAA CAC C 3′			
	230 F: 5′ TATGAACGCAA TTTAAGTGAGG CAG 3′	Secondary	65	
	230 R: 5′ TATCCA ATCCTTCCTTTG CAACACC 3′			
Pfhrp2 Exon 1–2, PF3D7_0831800	2E12 F1: 5′ GGTTTCCTTCTC AAAAAATAAAG 3′	Primary	55	228

	2E12 R1: 5′ TCTACATGTGCTTGAGTTTCG 3′			
	2E12 F: 5′ GTATTATCCGCTGCCGTTTTTGCC 3′	Secondary	62	
	2E12 R: 5′ CTACACAAGTTATTATTAAATGCGGAA 3′			
PF3D7_0831700 (MAL7P1.228)	228 F: 5′ AGACAAGCTACCAAAGATGCAGGTG 3′	Primary	60	198
	228 R: 5′ TAAATGTGTATCTCCTGAGGTAGC 3′			
	228 F1: 5′ CCATTGCTGGTTTAAATGTTTTAAG 3′	Secondary	63	
	228 R: 5′ TAAATGTGTATCTCCTGAGGTAGC 3′			

PF3D7_1372100, (MAL13P1.485)	485 F: 5′ TTGAGTGCAATGATGAGTGGAG 3′	Primary	60	241
	485 R: 5′ AAATCATTTCCTTTTACACTAGTGC 3′			
	485 F1: 5′ GTTACTACATTAGTGATGCATTC 3′	Secondary	59	
	485 R: 5′ AAATCATTTCCTTTTACACTAGTGC 3′			
Pfhrp3 Exon 1–2, PF3D7_1372200	3E12 F1: 5′ GGTTTCCTTCTCAAAAAATAAAA 3′	Primary	53	225
	3E12 R1: 5′ CCTGCATGTGCTTGACTTTA 3′			
	3E12 F: 5′ ATATTATCGCTGCCGTTTTTGCT 3′	Secondary	62	

	3E12 R: 5′ CTAAACAAGTTATTGTTAAATTCGGAG 3′			
PF3D7_1372400 (MAL13P1.475	475 F: 5′ TTCATGAGTAGATGTCCTAGGAG 3′	Primary	55	212
	475 R: 5′ TCGTACAATTCATCATACTCACC 3′			
	475 F: 5′ TTCATGAGTAGATGTCCTAGGAG 3′	Secondary	61	
	475 R1: 5′ GGATGTTTCGACATTTTCGTCG 3′			

Appendix 4

Table 1: Primer and condition for sequencing primary PCR.

Gene name	Primer name	Primer sequence	Conditions	cycles	Product size
***Pfhrp*2 exon1/intron1**	A262	5'-TTTAATAAAAATGGTTTCCTTC-3'	94 ºC 15min 94 ºC 30sec 55 ºC 30sec 72 ºC 1min 72 ºC 10min	35	315 bp
	A263	5'-GCTTGAGTTTCGTGTAATAATCT-3'			
***Pfhrp*2 exon2**	A118	5'-TTCCGCATTTAATAATAACTTGTG-3'	94 ºC 15min 94 ºC 30sec 55 ºC 30sec 72 ºC 1min 72 ºC 10min	35	1166bp
	A264	5'-AAAATCGCTATCCCATAAATTACA-3'			
***Pfhrp3* primer set 1**	3E12F1	5′ GGTTTCCTTCTCAAAAAATAAAA 3′	94 ºC 10 min, 94 ºC 50 sec, 60 ºC 50 sec, 72 ºC 1 min, 72 ºC 10 min,	35	
	*Pfhrp*3-R1	5' -TGGTGTAAGTGATGCGTAGT-3'			
***Pfhrp*3**	*Pfhrp*3-	5'AAATAAGAG	94 ºC 15min	35	

Primer set 2	F2	ATTATTACACG AAAG-3’	94 ºC 30sec 56 ºC 30sec 72 ºC 1min 72 ºC 10min		
	A261	5’-TAATCTTCGAT TAAATGGATT-3’			