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***Plasmodium falciparum* genetic diversity and malaria
diagnosis in Kenyan population**

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**by
Nderu, David Waweru**

**from
Nairobi, Kenya**

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Dean: Professor. Dr. I. B. Autenrieth
First reviewer: Professor. Dr. Thirumalaisamy P. Velavan
Second reviewer: Professor. Dr. Samuel Wagner
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1. INTRODUCTION

1.1. Epidemiology of Malaria

Malaria is a parasitic disease caused by an infective bite of female *Anopheles* mosquitoes that transmits *Plasmodium* species. In sub-Saharan Africa and tropical countries, it is a leading cause of febrile illness and mortality, particularly in children below five years (WHO, 2018). From years 2005 until 2015, the global malaria burden has reduced considerably, as a result of effective control strategies by governments. In the last few years, however, this trend has stagnated in few countries, and an increased burden in certain countries are reported. The current estimates document approximately 220 million cases, with reported 400,000 malaria deaths globally (WHO, 2018).

In Kenya, 70% of the population (~35 million people) is at risk of malaria. The national malaria burden is about eight million with ~16,000 mortalities per year, making malaria the second leading cause of death after pneumonia (KNBS, 2017, Macharia et al., 2018). Most of the cases are documented from western regions especially in the Lake Victoria basin and from Coastal Kenya. The climatic conditions in these regions serve as hotspots for malaria transmission (Figure 1). Apart from Nairobi and highlands of central Kenya, other regions experience epidemics (highlands of western Kenya) or seasonal transmission (northern and central Kenya) (NMCP et al., 2016). With these spatial differences in malaria transmission, control strategies are therefore adapted to meet the needs of a given locality (NMCP et al., 2016).

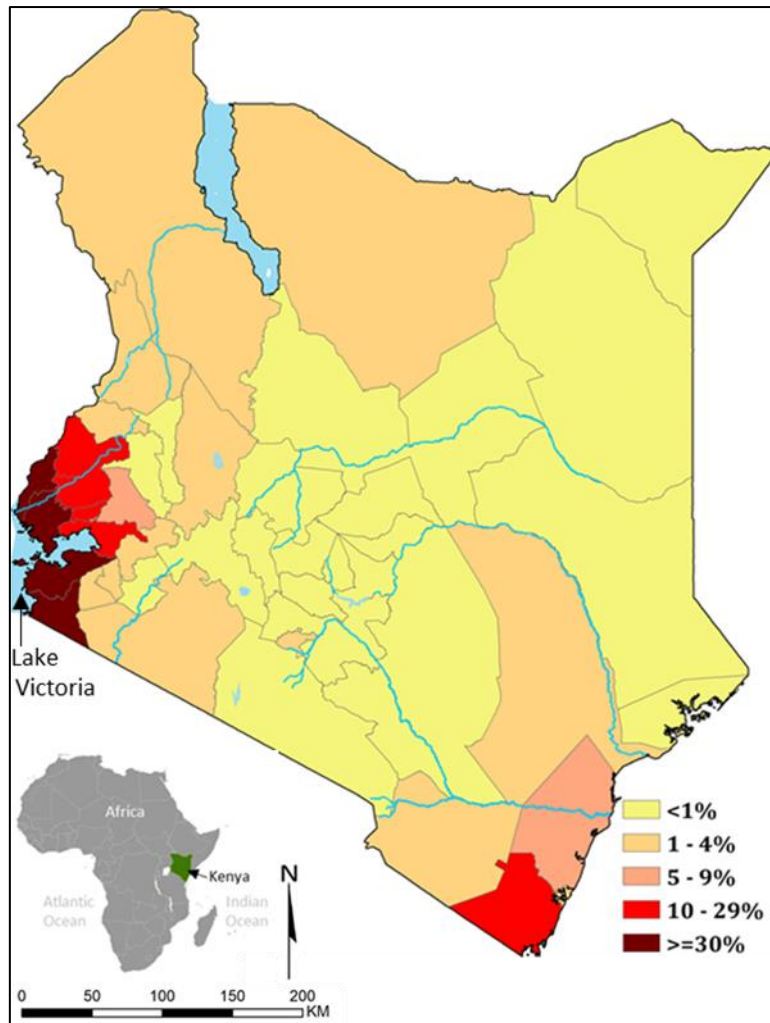


Figure 1: Map of Kenya showing *P. falciparum* prevalence ($PfPR_{2-10}$). Adopted and modified from (Macharia et al., 2018)

Three of five *Plasmodium* species known to infect human beings are present in Kenya, namely *Plasmodium falciparum*, *P. ovale* and *P. malariae*. Among them *P. falciparum* is the predominant and virulent species. It accounts for 98% of all malaria parasite infections in Kenya. The other species, *P. ovale* and *P. malariae*, occur at low prevalence. Even though *P. vivax* is not present in Kenya, it is found in few East African countries such as Ethiopia and Sudan (Howes et al., 2015). Other regions where *P. vivax* occurs include, South-East Asia, South America and the Western Pacific region (WHO, 2018, Howes et al., 2015, Howes et al., 2016). The *P. knowlesi* malaria is confined to forested areas of South-East Asia (Millar and Cox-Singh, 2015).

1.2. *Plasmodium* life cycle

The life cycle of *Plasmodium* occurs partly in humans, intermediate host, and in the female *Anopheles* mosquitoes as definitive host (Figure 2). It is initiated by an infected mosquito bite. During a blood meal, sporozoites (infective stage of *Plasmodium*) are released from the mosquito salivary glands into the skin and/or blood capillaries (Cockburn and Seder, 2018). The sporozoites sequentially migrate to the liver where the first phase of the *Plasmodium* life cycle takes place. This is commonly known as the pre-erythrocytic or liver stage.

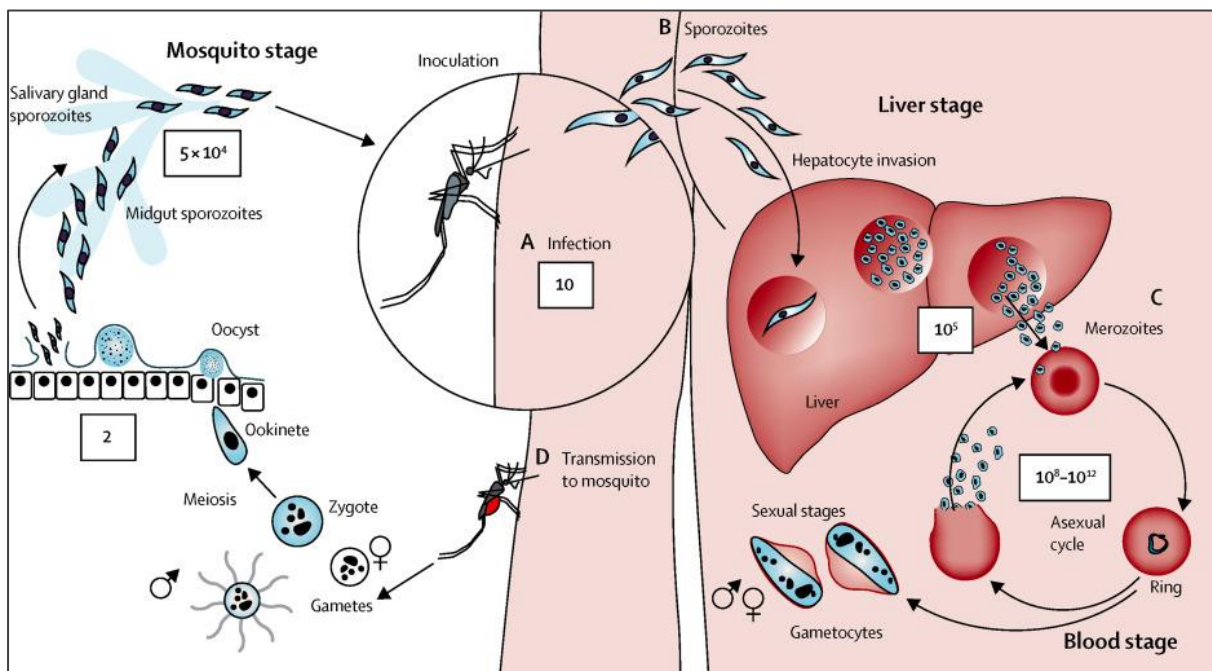


Figure 2: Schematic illustration of the *P. falciparum* life cycle. Adopted from (White et al., 2014).

In the liver, the sporozoites invade hepatocytes, multiply asexually by schizogony and mature into hepatic schizonts (White et al., 2014). The hepatic schizonts rupture and release approximately 10,000 to 30,000 merozoites into hepatic veins. This marks the end of a non-pathogenic pre-erythrocytic phase, 6–8 days post infection (White et al., 2014, Vaughan et al., 2008). In *P. vivax* and *P. ovale* however, some sporozoites can

also enter into a dormant state, called hypnozoites and may remain latent for weeks to years (Robinson et al., 2015).

The blood or erythrocytic cycle of the *Plasmodium* begins when merozoites invade erythrocytes in the hepatic veins. In the erythrocytes, merozoites reproduce asexually and develop via the ring stage (trophozoites) and mature into erythrocytic schizonts. The schizont subsequently ruptures, releasing six to 32 daughter merozoites which invade other erythrocytes in circulation and initiate another erythrocytic cycle (White et al., 2014). A complete erythrocytic cycle occurs every 24 hours for *P. knowlesi*, 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale*, and 72 hours for *P. malariae*. Although most of the merozoites invading erythrocytes enter the erythrocytic cycle, a small proportion differentiates into dormant sexual stages, male and female gametocytes. The *Anopheles* mosquito then takes up gametocytes concentrated in the skin capillaries during a blood meal (Kori et al., 2018).

In the midgut of the mosquito, male and female gametocytes transform into microgametes and macrogametes, respectively. The fusion of the male and female gametes forms a diploid zygote (Phillips et al., 2017). The zygote sequentially develops through meiosis into a motile ookinete. The ookinete transverse the mosquito midgut epithelium and transform into the oocyte. Oocytes develop further into sporoblast which then gives rise to sporozoites via budding (Kori et al., 2018). Finally, sporozoites migrate to the mosquito salivary glands for transmission to the host in the next blood meal. Mosquitoes are infective 7 to 10 days after ingesting blood containing gametocytes (Phillips et al., 2017).

1.3. Clinical malaria

Clinical or symptomatic malaria is caused by the continuous destruction of erythrocytes during the erythrocytic phase of the *Plasmodium* life cycle. Symptoms of the mildest form clinical malaria overlap with other febrile illnesses (Bartoloni and Zammarchi, 2012). These include chills, sweat, headaches, body aches, general malaise, nausea and vomiting (CDC, 2019, WHO, 2015). This form of malaria disease is referred to as uncomplicated malaria and it resolves rapidly once artemisinin-based combined therapy (ACT) is administered (WHO, 2015). If treatment is delayed however, uncomplicated malaria may progress to severe malaria or even death within a few hours (Trampuz et al., 2003).

Severe malaria is mostly caused by *P. falciparum* and in rare occasions result from *P. vivax* and *P. knowlesi* infections (WHO, 2015). It develops three to seven days after the onset of fever and manifest as serious organ failure or hematologic/metabolic abnormalities. Complications of severe malaria include cerebral malaria, severe anaemia, hemoglobinuria, acute respiratory syndrome (ARDS), abnormal blood coagulation, acute kidney injury, hyperparasitemia, hypoglycaemia and metabolic acidosis (CDC, 2019, WHO, 2015). In most cases of severe malaria involve a combination of these complications with hypoglycaemia and metabolic acidosis being the most frequent. The propensity of an uncomplicated malaria case progressing to severe malaria is dependent on several factors, namely age (1 month to 5 years in malaria hyperendemic settings or >65 years old), pregnancy, antimalarial prophylaxis and immune status (Trampuz et al., 2003). Currently, the treatment for severe malaria is intravenous or intramuscular mono artesunate until a patient can tolerate oral treatment followed by a standard ACT regimen (WHO, 2015).

1.4. Malaria diagnosis

1.4.1. Malaria rapid diagnostic tests

In the search for an accurate, cost-effective, and field applicable test for malaria, numerous parasite detection methodologies have been developed that fall into three categories. These are molecular tests, serological tests and microscopy (Tangpukdee et al., 2009). Currently, rapid diagnostic tests (RDT) are the most widely used test for malaria diagnosis, particularly in resource-limited settings where good quality microscopy (the gold standard) barely exists (Mukkala et al., 2018). This is because they are cost-effective, easy to use, fast and do not require electricity (WHO, 2015). Furthermore, RDT diagnostic performance is comparable to routine microscopy.

RDTs are ready-to-use lateral flow immunochromatographic tests that use monoclonal antibodies (mAbs) to detect malaria parasite antigens, namely *P. falciparum* histidine-rich protein 2 (PfHRP2), parasite lactate dehydrogenase (pLDH) and aldolase. Targeting these antigens allows identification of infections caused by any *Plasmodium* (pan) or related species. Detection of pLDH or aldolase identifies infections caused by all human malaria parasites. PfHRP2 and Pf-pLDH are specific for *P. falciparum* whereas Pv-pLDH is specific for *P. vivax* infection. Currently, over 200 different RDT products are commercially available. Some detect a single antigen while others target a combination of *Plasmodium* genus- and species-specific antigens. Ninety per cent of RDT that are currently in use target PfHRP2.

RDTs for malaria are made of a nitrocellulose strip enclosed in a plastic cassette. The strip is impregnated with antibodies. Figure 3 shows a schematic illustration of the principle of RDTs. Briefly, finger-prick blood (three blood drops) and a buffer are

added to the sample and buffer pads, respectively (Maltha et al., 2013). The buffer lyses the red blood cells, releasing *Plasmodium* antigens which subsequently bind to mAbs conjugated with colloidal gold.

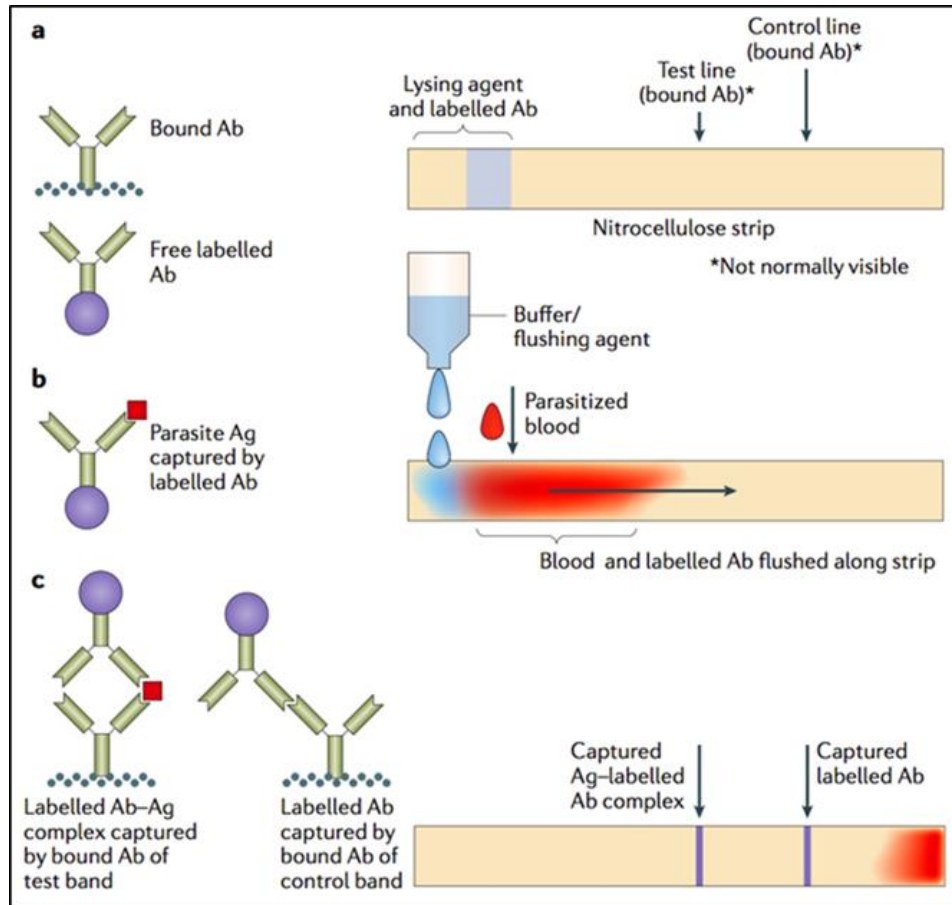


Figure 3: An illustration of the principle of RDTs. Adopted from (Bell et al., 2006)

The parasite antigen-antibody-conjugate complex migrates along the nitrocellulose strip by capillarity until it is bound by immobilised antibodies (test line). The excess complexes and conjugated mAbs move further until they reach and bind to immobilised goat-raised anti-mouse antibodies (control line). The binding of antigen-antibody-conjugate complexes and conjugated mAbs at the test line and control line concentrate the conjugate signal, thereby generating coloured lines. Development of the control line only is interpreted as a malaria negative result whereas the development of the control and test line(s) is interpreted as a malaria positive result. The test results are obtained within 15–20 minutes.

Kenya adopted RDTs for malaria in 2012 and has since become heavily dependent on these tests for the management of malaria cases. PfHRP2 detecting RDTs are preferred in sub-Saharan Africa, including Kenya, due to the predominance of *P. falciparum* and acceptable diagnostic performance relative to routine microscopy (Wu et al., 2015, Gatton et al., 2015). However, there is an increasing concern on the suitability of PfHRP2-based RDTs after it became apparent that parasite genetics influence the test's sensitivity (Baker et al., 2005, Gamboa et al., 2010).

1.4.2. *P. falciparum* histidine-rich proteins 2 and 3

PfHRP2 is a water-soluble and a heat stable protein expressed exclusively by *P. falciparum*. Although the function of PfHRP2 in *P. falciparum* is still unclear, it is hypothesised to be involved in hemozoin and actin formation, glycosaminoglycan binding, T-cell suppression, the structural modification of *P. falciparum*-infected erythrocytes, pathology and procoagulation (Sullivan et al., 1996, Benedetti et al., 2003, Ndonwi et al., 2011, Pal et al., 2016). PfHRP2 is encoded by a gene (*pfhrp2*; PF3D7_0831800) located in the sub-telomeric region of chromosome 8. *pfhrp2* has two exons, the first exon encodes a signal peptide and the second exon encodes a repetitive peptide, which mainly comprises of contiguous repeats with three amino acids, namely histidine, alanine and aspartic acid (Rock et al., 1987, Howard et al., 1986). A total of 20 different PfHRP2 amino acid repeat types have been identified to date and numerically coded as repeat types 1 to 14 and 19 to 24 (Baker et al., 2005, Baker et al., 2010). These motifs vary in length and amino acid sequence as illustrated in Figure 4 (Bharti et al., 2017).

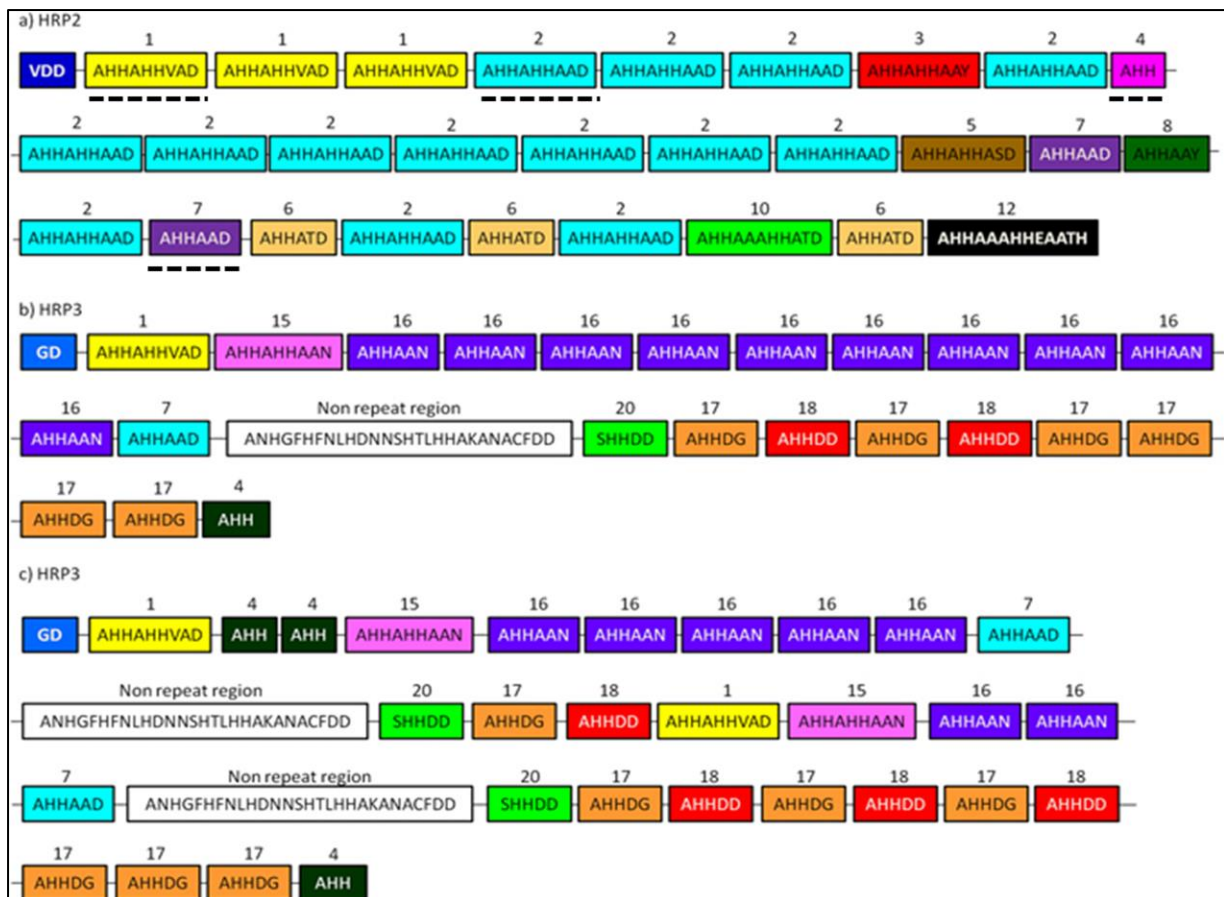


Figure 4: Schematic diagram showing the organisation of amino acid repeat types in *P. falciparum* histidine-rich protein 2 (a) and *P. falciparum* histidine-rich protein 3 (b and c). Broken line (black line) shows amino acid repeat types shared by PfHRP2 and PfHRP3. Adopted and modified from (Bharti et al., 2017).

P. falciparum isolates exhibit extensive PfHRP2 diversity within and between parasite populations (Baker et al., 2005). The high antigenic diversity emanates from variation in the number, occurrence and structural organisation of amino acid repeat types. Most of the isolates from malaria-endemic regions have distinct amino acid repeat profile with Peruvian isolates being the least diverse (Baker et al., 2005, Baker et al., 2010). Variability in amino acid repeat profile may alter PfHRP2 detectability by RDTs as exemplified by differential PfHRP2-mAb reactivity among *P. falciparum* isolates from different geographical areas (Lee et al., 2012, Lee et al., 2006). PfHRP2 diversity is therefore suggested to be a plausible explanation for any observed difference in RDT sensitivity among different settings (Baker et al., 2005).

Analyses of the relationship between individual PfHRP2 repeat types per isolate and RDT performance revealed that the number of repeat types 2 and 7 could influence RDT sensitivity (Baker et al., 2005). In addition, a binary logistic regression model, also known as Bakers' regression model, demonstrated that low values (<43) of the number of repeat type 2 multiplied by the number of repeat type 7 (type 2 × type 7) could predict RDT false negativity at <250 parasites/μl (Baker et al., 2005). Application of this model in Uganda and Senegal showed that type 2 × type 7 below 43 could reduce RDT sensitivity and cause RDT false negativity in field settings (Wurtz et al., 2013a, Kumar et al., 2012). However, a larger study showed no association thereof and thus indicated that Bakers' model cannot exclusively explain RDT false negativity (Baker et al., 2010). Other factors such as suboptimal levels of PfHRP2 should therefore be considered for decline in RDT sensitivity (Baker et al., 2011, Gatton et al., 2015, Plucinski et al., 2019).

The most apparent cause of RDT negativity is *pfhrp2* deletion. *P. falciparum* isolates lacking *pfhrp2* were first observed in the Peruvian Amazon (Gamboa et al., 2010) and later in other countries among Africa, Asia, South and Central America (Gendrot et al., 2018). *pfhrp2* deletion is usually suspected when a *P. falciparum*-infected patient test negative for PfHRP2, despite being positive for pan- or Pf-pLDH. In addition, *pfhrp2* deletion can also be suspected when the positive rate of microscopy is ≥10 to 15% higher than PfHRP2-based RDT (WHO, 2016). The WHO recommends the replacement of PfHRP2 RDTs with non-PfHRP2 RDTs only when the national prevalence of *pfhrp2* deletion exceeds five percent (WHO, 2017). The prevalence of parasites lacking *pfhrp2* vary greatly within and between regions. Reports published so far show high prevalence in some South American countries, thereby rendering

PfHRP2-based RDTs unsuitable for falciparum malaria diagnosis, especially in the Peruvian Amazon. *pfhrp2* deletion in Africa and Asia occurs at low prevalence (<10%) except in Ghana (36%) and Rwanda (23%) (Amoah et al., 2016, Kozycki et al., 2017, Gendrot et al., 2018, Kobayashi et al., 2019). *P. falciparum* isolates lacking *pfhrp2* have also been identified in a few samples (9%) collected in 2014 in Mbita, western Kenya (Beshir et al., 2017).

Since PfHRP2 and *P. falciparum* histidine-rich protein 3 (PfHRP3; also expressed exclusively by *P. falciparum*; PF3D7_1372200; Figure 4) are structurally homologous, mAbs targeting PfHRP2 can cross-react with PfHRP3 and aid detection of *pfhrp2* deficient parasites (Lee et al., 2006, Rock et al., 1987, Wellems and Howard, 1986). However, this occurs at parasite densities >1000 parasites/ μ l and may not avert RDT false negativity due to *pfhrp2* deletion. The existence of *pfhrp3* deletion in many geographical areas at frequencies higher than *pfhrp2* deletion and the presence of *P. falciparum* isolates lacking both *pfhrp2* and *pfhrp3* threaten management of malaria cases (Gendrot et al., 2018). This also raises important questions on the origin and spread of *P. falciparum* isolates lacking *pfhrp2/3*.

Studies conducted-so far show that *pfhrp2* deletion originated from multiple genetic events (Akinyi et al., 2013). The introduction and successful establishment of *pfhrp2* negative parasites in a given geographical area is hypothesised to be influenced by parasite genetic diversity and malaria treatment rate (Gatton et al., 2017). In areas where *pfhrp2* deficient parasites occur, the commonly used PfHRP2-based RDTs will fail to identify these cases and may contribute towards increasing the prevalence of *pfhrp2* lacking *P. falciparum* (Gatton et al., 2017, Watson et al., 2017). In addition, low

or reducing malaria endemicity along with the coexistence of *pfhrp2/3* deletion in *P. falciparum* drug resistant strains and insecticide-resistant *Anopheles* mosquitoes could accelerate selection and/or spatial distribution of *P. falciparum* isolates lacking *pfhrp2/3*. This suggests that changes in *P. falciparum* diversity and population structure in response to control measures may have an indirect impact on malaria RDTs. In view of the high dependence on PfHRP2-based RDTs, particularly in areas where *P. falciparum* prevalent such as Kenya, analyses of RDT performance, *pfhrp2/3* diversity and *P. falciparum* genetic diversity and population structure are of great public health importance. The present thesis presents data required to refine, adapt and improve malaria control strategies in Kenya.

1.5. Scope and specific objectives

This thesis aimed to re-evaluate the performance of RDT by microscopy and polymerase chain reaction methodologies and describe the distribution and diversity of *P. falciparum* histidine-rich proteins 2 (PfHRP2) and 3 (PfHRP3) in Kenya. Furthermore, the genetic diversity and population structure of *P. falciparum* populations from Kenyan-Ugandan border areas were analysed by genotyping *P. falciparum*-specific neutral microsatellites. The present thesis is structured as three independent chapters based on three research articles published in peer-reviewed journals. I investigated three specific objectives:

1. Comparative evaluation of a PfHRP2/pLDH malaria RDT with microscopy and nested PCR methodologies.
2. Genetic diversity of *P. falciparum* histidine rich proteins (PfHRP2 and 3) from Western and Coastal Kenya.

3. Understanding the genetic variability and population structure of *P. falciparum* isolates in the Kenyan-Ugandan border using neutral microsatellites.

2. RESULTS AND DISCUSSION

2.1.Chapter 1: Comparative evaluation of PfHRP2/pLDH malaria RDT with microscopy and nested PCR methodologies

Publication No.1

PfHRP2-PfHRP3 diversity among Kenyan isolates and comparative evaluation of PfHRP2/pLDH malaria RDT with microscopy and nested PCR methodologies.

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PfHRP2-PfHRP3 diversity among Kenyan isolates and comparative evaluation of PfHRP2/pLDH malaria RDT with microscopy and nested PCR methodologies



David Nderu^{a,b}, Francis Kimani^c, Kelvin Thiong'o^c, Maureen Akinyi^c, Evaline Karanja^d, Christian G. Meyer^{a,e,f}, Thirumalaisamy P. Velavan^{a,e,f,g,*}

^a Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany

^b School of Health Sciences, Kirinyaga University, 10300 Kerugoya, Kenya

^c Center for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya

^d Department of Biochemistry and Biotechnology, School of Biological and Life Sciences, Technical University of Kenya, Nairobi, Kenya

^e Vietnamese-German Centre for Medical Research (VG-CARE), Hanoi, Viet Nam

^f Faculty of Medicine, Duy Tan University, Da Nang, Viet Nam

^g Fondation Congolaise pour la Recherche Médicale, Brazzaville, Congo

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ABSTRACT

Rapid diagnostic tests (RDT) are valuable tools that support prudent and timely use of antimalarial drugs, particularly if reliable microscopy is not available. However, the performance and reliability of these tests vary between and within geographical regions. The present study evaluated the performance of routine malaria RDT in Kenyan febrile patients in Busia County, Kenya. A cross sectional study design was employed to recruit febrile patients attending health facilities between August and November 2016. A total of 192 febrile patients who were slide positive and negative were evaluated for their infection status by nested PCR and RDTs (PfHRP2/pLDH). In addition, *P. falciparum* diversity of the histidine-rich proteins 2 and 3, that influences the RDT test results were determined. All individuals were *P. falciparum* positive. Among the investigated 192 febrile patients, 76 (40%) were positive by microscopy, 101 (53%) by RDTs and 80 (42%) were PCR positive. The performance of the CareStart™ HRP2/pLDH (pf) RDTs was better than microscopy (Sensitivity 94%; Specificity 75%) and Nucleic acid testing (sensitivity 95%, specificity 77%) with high negative predictive values, indicating the suitability of the RDT in routine practice. Specific *pfrp2/pfrp3* deletions shown to associate with RDT false negativity was not observed. However, high genetic diversity among *pfrp2* gene was observed. Eleven new PfHRP2 and nine PfHRP3 repeats were observed. False positivity by microscopy and under reporting of infections may thus be a barrier in malaria control and elimination programs. The HRP2/pLDH(Pf) based RDT yet demonstrate to be an effective tool for malaria surveillance program.

1. Introduction

Reduction of the malaria burden over the last decade has renewed efforts towards its eventual elimination. In 2017, the World Health Organisation (WHO) estimated that 216 million malaria cases and 445,000 deaths occurred in 2016 [1], representing a significant decline in new cases (21%) and deaths (29%) compared to 2010 estimations. Despite being treatable, malaria continues to devastate developing countries, especially in sub-Saharan Africa.

In Kenya, malaria accounts for 18% of outpatient consultations and 6% of hospital admissions with children below 5 years and pregnant

women being most affected [2]. The objective of the Kenyan national malaria control programme (NMCP) is to have all malaria suspected cases tested and promptly treated with effective drugs [3]. In a move to boost malaria testing, the Kenyan NMCP introduced malaria rapid diagnostic tests (RDT) in 2012 [4]. Diagnosing of malaria in Kenya by microscopic examination of blood smears has been reduced in favour of RDT usage [2]. This trend is largely driven by microscopy's dependence on electricity, skilled manpower and scarcity of good-quality reagents and microscopes.

Malaria RDTs are lateral flow immunochromatographic tests using monoclonal antibodies (MAbs) to detect *Plasmodium* antigens, namely

* Corresponding author at: Institute of Tropical Medicine, Wilhelmstrasse 27, 72074 Tübingen, Germany.

E-mail address: velavan@medizin.uni-tuebingen.de (T.P. Velavan).

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histidine-rich protein 2 (PfHRP2), lactate dehydrogenase (LDH) and aldolase [5]. By targeting these antigens, infections caused by any human *Plasmodium* species (LDH or aldolase) and by distinct species (PfHRP2 or PfLDH for *P. falciparum* and PvLDH for *P. vivax*) may be identified [6]. RDTs vary depending on manufacturers, with some detecting *Plasmodium* (pan) antigen in combination with one or two species-specific antigens [7]. Others detect only a specific *Plasmodium* species infection with those based on the detection of PfHRP2 constituting 90% of RDTs currently used [8]. Testing is fast with visualized results obtained within 15–20 min. The low cost, ease of use and performance make these tests a key pillar in malaria control program.

Malaria diagnosis in Kenya is heavily reliant on PfHRP2-based RDTs. However, the performance of these RDTs increasingly faces reduced sensitivity, a situation that may worsen as prevalences of low parasitemia malaria increase with the improvement of malaria control strategies. Routine RDT evaluation by WHO's Foundation for Innovative New Diagnostics (FIND) and adoption of good manufacturing practices have helped to reduce the impact of RDT product quality on performance [5,9].

However, the effect of parasite factors on PfHRP2-based RDTs is yet to be defined in many malaria-endemic countries. PfHRP2 is mainly composed of three amino acids (alanine, histidine and aspartate) and it is homologous to PfHRP3 [10,11]. The amino acid repeat profile of PfHRP2 varies extensively within and between countries unlike LDH [12–14]. As a result of this, PfHRP2 diversity has been identified as a key contributor for RDT sensitivity [15]. This is supported by the differential binding of MAbs to PfHRP2 from different geographical areas [16,17]. PfHRP2 diversity has also been identified as the potential cause of RDT false negativity, particularly when parasitemia is ≤ 250 parasites/ μl . In a case study from Senegal, a low number of two PfHRP2 amino acid repeat motifs (types 2: AHHAHHAAD and type 7: AHHAAD) was identified as the cause of delayed falciparum malaria diagnosis [18]. The correlation of the number of these two motifs with RDT's limit of detection and the lack thereof have also been reported [12,15,19].

The significance of *pfrp2* deletion in falciparum malaria diagnosis is unequivocal. In South America's Peruvian Amazon high rates of *pfrp2/3* deletion rendered PfHRP2-based RDTs to be inappropriate for falciparum malaria diagnosis [20]. A similar situation has been reported from other parts of South America, Asia and Africa, however, at lower prevalences [21–24]. The recent observation of *P. falciparum* lacking *pfrp2* in Rwanda, the Democratic Republic of Congo, Eritrea as well as Kenya raises important questions on the suitability of PfHRP2-based RDTs [25–29]. Where *pfrp3* is expressed and *pfrp2* is lacking, isolates are detectable provided parasitemia is ≥ 1000 parasites/ μl . This phenomenon was reported in a genomic study from Kenya; eight isolates lacking *pfrp2* were detectable by PfHRP2-based RDTs [29]. However, this stands to be reversed as the prevalence of *pfrp3* devoid isolates increases and parasitemia decreases. The lack of information on the geographical distribution of *pfrp2/3* devoid parasites and PfHRP2-PfHRP3 diversity in Kenya clearly shows that RDT performance and the relevant parasite genetic background needs close monitoring.

Against this background, we re-evaluated the performance and reliability of RDT against microscopy and nested PCR for their sustained use in Malaria control program. In addition, we analysed the diversity of PfHRP2 and its homologue PfHRP3, for any *pfrp2/pfrp3* deletions shown to associate with RDT false negativity.

2. Materials and methods

2.1. Ethical approval

The study was approved by the Scientific and Ethics Review Unit (SERU) of Kenya Medical Research Institute (KEMRI), Nairobi (KEMRI/SERU/0152/3250). Written informed consent was obtained from each participant (for children provided by parents/guardians). All experiments were performed in accordance with good laboratory practice

(GLP) guidelines.

2.2. Study site and sample collection

The study was conducted between August and November 2016 in the Matayos Health Centre in Busia County, a malaria endemic site approximately 80 km northeast of Lake Victoria, Kenya. Busia is inhabited by 743,946 people with children < 5 years accounting for 18% [30]. It has a tropical climate (annual rainfall 750–2000 mm) with average temperatures of 21–27 °C [31]. These conditions are ideal for vector breeding which sustain perennial malaria transmission. Peak incidences occurs shortly after the long (March–June) and short (October–November) rains with increasing vector populations. Although transmission in this region is predominantly by *Anopheles gambiae* sensu stricto, *An. arabiensis* and *An. funestus* are also present at lower occurrences [32]. In 2014, malaria parasitemia prevalence was estimated to be between 20% and 40% in the Lake Victoria region, mainly caused by *P. falciparum* [33]. Coinfections of *P. falciparum* and other parasite species (*P. ovale* and/or *P. malariae*) also occur in this region. *P. vivax* does not occur [34]. Patients with fever, irrespective of age, were asked to participate in the study. A finger – prick blood sample was collected for thick and thin blood smears, dried blood spots on Whatman™ filter papers, and for CareStart™ HRP2/pLDH(Pf) RDT testing. From each participant, three to four drops of blood (40–50 μl) were applied onto a Whatman™ filter paper and allowed to dry at ambient temperature for at least one hour before being individually packed in a ziplock bag with desiccant.

2.3. Malaria diagnosis

Malaria screening was performed by microscopy, PfHRP2/pLDH (CareStart™, ACCESS BIO, INC, Korea) RDT and *P. falciparum*-specific nested PCR. Diagnoses were made by two independent laboratory technicians blinded to the study objective, clinical presentation of participants and test results of either microscopy or RDT. Thick and thin smears were Giemsa stained and examined (1000 \times magnification) [35]. Parasitemia was recorded as number of parasites per 200 white blood cells. Parasite densities were determined by multiplying parasitemia by 40, assuming that 1 μl blood contains 8000 WBCs.

Diagnosis by RDT was conducted using the PfHRP2/pLDH (CareStart™, ACCESS BIO, INC, Korea) according to the manufacturer's instructions. This RDT is a two band WHO prequalified test system, with a 91% panel detection score (PDS) and $< 1\%$ false positive rate, designed for detecting *P. falciparum* infections [9]. PDS is the proportion of *P. falciparum* positive samples identified by all PfHRP2/pLDH (CareStart™, ACCESS BIO, INC, Korea) RDT lots evaluated at 200 parasites/ μl during the sixth round of WHO product testing of malaria RDTs [9]. One of the test lines serves as control, while the second line indicates the presence of *P. falciparum*. The RDT reaction is based on two MAbs specific for *P. falciparum* antigens, namely the histidine-rich protein 2 (PfHRP2) and lactate dehydrogenase (PfLDH). Results were only considered valid if the control test line was clearly visible. If the control line was not visible, testing was repeated. All cases positive by RDT or microscopy were referred to the resident clinician for immediate treatment with artemisinin combination therapy according to local guidelines [36].

Genomic DNA was extracted from dried blood spots on Whatman™ filter paper (QIAamp DNA mini kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were tested for *P. falciparum* infection using a previously described nested PCR protocol with a limit of detection of one parasite [37,38]. Briefly, 1 μl of DNA template was added to a PCR reaction mix containing 1 \times PCR buffer (Qiagen, Hilden, Germany), 200 μM of each dNTPs, 1 unit of Qiagen Taq DNA polymerase (Qiagen, Hilden, Germany), and 100 nM of each primer in a volume of 20 μl . *Plasmodium* genus PCR reaction conditions were: initial denaturation at 95 °C for 5 mins followed by a secondary

denaturation step at 94 °C for 1 min, annealing at 58 °C for 2 mins, extension at 72 °C for 2 mins for 25 cycles and a final extension at 72 °C for 5 mins. *P. falciparum* specific PCR reactions were run for 30 cycles using the same PCR conditions. Amplicons were visualized by 1.5% agarose gel electrophoresis.

2.4. PCR amplification of *pfhrp2* and *pfhrp3* exon 2

The amplification of *pfhrp2* (exon 2) and *pfhrp3* (exon 2) was performed using a previously described semi-nested PCR [15]. The PCR master mix was prepared as required for *P. falciparum* nested PCR. Conditions were initial denaturation at 94 °C for 5 mins followed by a secondary denaturation step at 94 °C for 30 s, annealing at 50 °C for 50 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 mins. Primary and secondary PCRs were run with 27 and 32 cycles, respectively. *P. falciparum* strains 3D7 (*pfhrp2* and *pfhrp3* positive), Dd2 (*pfhrp2* negative and *pfhrp3* positive) and HB3 (*pfhrp2* positive and *pfhrp3* negative) were used as controls.

Amplicons were purified using Sephadex™ G-50 fine DNA grade (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. Sequencing was carried out using secondary PCR primers targeting the *pfhrp2* and *pfhrp3* exon 2 and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The BioEdit software was applied to assemble forward and reverse nucleic acid sequences and to deduce amino acid sequences.

2.5. PfHRP2 diversity

PfHRP2 has a rich genetic diversity (amino acid repeats) and are associated with RDT sensitivity. A total of 20 different amino acid repeats are described [12,15]. Among these 20 amino acid repeats, any changes in amino acid repeats (esp. repeat type 2: AHHAHHAAD and repeat type 7: AHHAAD) was shown to associate with declined RDT sensitivity as documented in other studies conducted across different geographical regions including South America, Africa, and Southeast Asia [15,18,19].

Based on the PfHRP2 diversity, we classified our investigated sample in four specific groups (Group A; B; I and C respectively). This classification (Bakers model) is based on multiplying the number of two amino acid repeat types (numbers of type 2 × numbers of type 7) as described previously [15]. Based on this classification, the groups were classified as very sensitive (Group A; type 2 × type 7; > 100), sensitive (Group B; type 2 × type 7; between 50 and 100), borderline or Intermediate (Group I; type 2 × type 7; between 44 and 49) and non-sensitive (Group C; type 2 × type 7; < 44) [15]. The PfHRP2 – based RDT false negativity rate was calculated, following Baker's regression model (binary logistic regression), by determining the proportion of samples with PfHRP2 types 2 × type 7 < 44 [15]. The samples classified under Group C served as an estimator for RDT false negativity (parasitemia ≤ 250 parasites/μl).

2.6. Multiplicity of infection (MOI)

The MOI was determined using a *P. falciparum* merozoite surface protein 1 – specific nested PCR (*msh1*; K1, MAD20 and R033 allelic families) as described previously [39]. Malaria cases with only one allele at each of the *msh1* allelic family loci were regarded to be monoclonal infections whereas infections with more than one allele in at least one *msh1* allelic family locus were regarded to be polyclonal infections. The total number of *msh1* alleles were divided by the number of *msh1* positive samples to determine the mean MOI. The percentage of monoclonal infections was used to estimate the probability of identifying isolates lacking *pfhrp2* and/or *pfhrp3*.

Table 1
Baseline characteristics of the study cohort.

Baseline study cohort details	Value(s)
Mean age in years (Range)	14.4 (0.3–76)
Female-male ratio	2:1
Parasite geometric mean [parasites/μl; (Range)]	7773 (80–202,880)
Sample size (n)	192
Microscopy positive	76
PCR positive	80
RDT positive	101
RDT vs. microscopy [n (%)]	
RDT positive – microscopy positive	72 (38)
RDT positive – microscopy negative	29 (15)
RDT negative – microscopy positive	04 (2)
RDT negative – microscopy negative	87 (45)
RDT vs. PCR [n (%)]	
RDT positive – PCR positive	75 (39)
RDT positive – PCR negative	26 (14)
RDT negative – PCR positive	05 (3)
RDT negative – PCR negative	86 (45)
Microscopy vs. PCR [n (%)]	
Microscopy positive – PCR positive	70 (36)
Microscopy positive – PCR negative	06 (3)
Microscopy negative – PCR positive	10 (6)
Microscopy negative – PCR negative	106 (55)

Note: RDT- Rapid diagnostic test; PCR- Polymerase chain reaction.

2.7. Data analysis

Two-tailed Chi-square test was executed to compare the *P. falciparum* positivity rates. Microscopy results were used as gold standard. The PfHRP2/pLDH RDT performance was assessed for sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV).

3. Results

A total of 192 febrile cases were recruited for evaluation of the PfHRP2/pLDH RDT. The baseline characteristics have been described in Table 1. Among the febrile individuals, *P. falciparum* was detected in 40% ($n = 76$), 53% ($n = 101$) and 42% ($n = 80$) by microscopy, RDT and nested PCR, respectively (Table 1). The detection rate of PfHRP2/pLDH RDT was significantly higher than by microscopy (p -value 0.01) as well as by nested PCR (p -value 0.03). The *P. falciparum* detection rates were similar between microscopy and PCR (p -value 0.67). All cases were caused by *P. falciparum* mono-infection and there were no invalid RDT results.

3.1. Performance of CareStart™ Malaria HRP2/pLDH(Pf) RDT

The CareStart™ Malaria HRP2/pLDH(Pf) RDT showed a similar performance compared to microscopy and nested PCR (Table 2). Thirty-three (17%) CareStart™ Malaria HRP2/pLDH(Pf) results were discordant with those of microscopy. Of the 33 discordant with

Table 2
Performance of the CareStart™ HRP2/pLDH (pf) RDT using Giemsa microscopy and *P. falciparum* nested PCR as reference methods.

PfHRP2/pLDH RDT Performance	Microscopy (95% CI)	nested PCR (95% CI)
Sensitivity (%)	94.7 (87.1–98.6)	93.8 (86.01–97.9)
Specificity (%)	75 (66.1–82.6)	76.8 (67.86–84.2)
Positive predictive value (%)	71.3 (64.3–77.3)	74.3 (67.21–84.2)
Negative predictive value (%)	95.6 (89.3–98.3)	94.5 (87.98–97.6)
Agreement (%)	82.81	83.40
Kappa Value (κ)	0.66*	0.68*
Positive likelihood ratio	3.79 (2.8–5.3)	4.04 (2.9–5.7)
Negative likelihood ratio	0.07 (0.07–0.2)	0.08 (0.03–0.2)

* p -Value < .0001.

Table 3
Distribution of the number of PfHRP2 and PfHRP3 amino acid repeat types.

Antigen	Repeat types	Amino acid repeat	Observed repeats	Occurrence (%)
PfHRP2	Type 1	AHHAHHVAD	0–7	92
		AHHAHYVAD (New)	1	4
	Type 2	AHHAHHAAD	8–15	100
	Type 3	AHHAHHAAY	0–4	84
	Type 4	AHH	0–3	40
		ASH (New)	1	3
		THH (New)	1	3
		AAH (New)	1	5
	Type 5	AHHAHHASD	0–2	68
		AHHAHHAPD (New)	1	3
	Type 6	AHHATD	1–6	100
		APHATD (New)	1	3
	Type 7	AHHAAD	3–12	100
		AHHAPD (New)	1	3
	Type 8	AHHAAAY	1–2	100
		AHHASY (New)	1	3
	Type 10	AHHAAHHATD	0–3	82
		AHHAAHHATD (New)	1	3
	Type 12	AHHAAHHHEAATH	0–1	90
		AHHAAHHHEAATP (New)	1	3
Type 13	AHHASD	0–1	8	
Type 14	AHHAHHATD	0–1	3	
Type 19	AHHAA	0	0	
	THHAA (New)	1	3	
PfHRP3	Type 1	AHHAHHVAD	0–3	96
	Type 2	AHHAHHAAD	0–1	2
	Type 4	AHH	1	98
	Type 7	AHHAAD	1	100
	Type 15	AHHAHHAAN	1	100
	Type 16	AHHAAN	7–16	100
		AHHAAK (New)	1	2
		SHHAAN (New)	1	2
	Type 17	AHHDG	2–9	100
		QHHDG (New)	1	2
		SHHDG (New)	1	8
		THHDG (New)	1	2
		AHHDE (New)	1	2
	Type 18	AHHDD	1–3	100
		EHHDD (New)	1	2
	Type 20	SHHDD	1	100
	Type	AHQDH (New)	1	2
(unknown)	LNAAN (New)	1	2	

microscopy, four were false negative (positive by microscopy and negative by RDT; 320 to 680 parasites/μl) and the remaining 29 were false positive (negative by microscopy and positive by RDT). Using nested PCR to resolve the discordance, 2 out of 4 RDT false negatives tested positive and 7 out of 29 false positives were confirmed to be true positives. Against nested PCR, the agreement was 83% which represented a discordance rate of 17% (Table 2). Overall, CareStart™ false positive rate decreased from 25% to 23%, and the false negative rate increased from 5% to 6% when nested PCR replaced microscopy as a reference method.

3.2. PfHRP2 and PfHRP3 diversity

The exon 2 of both *pfhrp2* ($n = 38$) and *pfhrp3* ($n = 49$) were Sanger sequenced. Amino acid sequences were deduced subsequently. The length of the PfHRP2 amino acid sequence was between 244 and 330 and for PfHRP3 between 160 and 229 amino acids. Overall, we identified 20 previously described PfHRP2 amino acid repeat types and nine PfHRP3 amino acid repeats (Table 3).

In PfHRP2, the repeat types 1,2,3,6,7,8,10 and 12 were observed to be more frequent, whereas repeat types 13 (8%) and 14 (3%) were rare. The repeat types 9 and 11 were absent in all isolates. Additionally, eleven new PfHRP2 repeat types that have not been described

previously were identified at low frequencies (Table 3). The classification of PfHRP2 based on the product of the number of types 2 and 7 per isolate revealed that group A, B, I and C constituted 16% (6), 71% (27), 5% (2) and 8% (3) respectively. Based on Baker's regression model, we could predict that 8% of isolates (group C) in this study would not be detected by PfHRP2-based RDTs, even at a parasite density of ≤ 250 parasites/μl [15].

Eight out of nine repeat types previously reported to occur in PfHRP3 were identified all isolates analysed as shown in Table 3 [12]. Type 2 was identified in one isolate only. In addition, nine PfHRP3 new repeat types that have not been described previously were identified at low frequencies (Table 3). Among the nine new amino acid repeats in PfHRP3, the motifs AHQDH and LNAAN could not be categorized in any of the pre-described repeat types. In addition, the newly identified motif SHHDG was observed frequently in PfHRP3.

3.3. Multiplicity of infection (MOI)

Of the 80 *P. falciparum* confirmed samples by nested PCR, *msp1* allelic families were amplified in 66 (83%) samples. K1 ($n = 55$; 83%) was the most common allelic family followed by MAD20 ($n = 30$; 45%) and RO33 ($n = 23$; 35%). Table 4 shows the occurrence of *msp1* genotypes in this study. The occurrence of mono-infection was 45% (30) whereas the occurrence of polyclonal infections was 55% (36). Based on the proportion of monoclonal infections, the estimated probability of identifying isolates lacking *pfhrp2* and/or *pfhrp3* was 45%. The mean MOI was 2.4 (95% CI: 2.0–2.7).

4. Discussion

Malaria RDTs are instrumental in increasing the proportion of successful malaria case management [2]. *P. falciparum* histidine-rich protein 2 (PfHRP2)-based RDTs are the most commonly used systems for malaria diagnosis. However, several reports on suspected sensitivity issues due to the genetic diversity of the parasite underscores the need to closely monitor the performance of PfHRP2-based RDTs.

CareStart™ Malaria HRP2/pLDH(Pf) showed a high agreement with microscopy and nested PCR and an acceptable diagnostic performance, consistent with previous studies [40,41]. Surprisingly, this test was unable to identify four malaria cases with parasitaemia between 320 and 680 parasites/μl, despite being able to simultaneously detect pLDH. Since parasitemia was above the limit of detection of RDTs (100 parasites/μl) and that two of four false negative cases were detected by nested PCR, it is likely that the observed misdiagnosis was due to methodological errors during testing. For instance, dispensing insufficient sample and/or buffer volume, reading of test results too early and/or reporting a faint test-line as negative may all be plausible reasons [5].

The specificity and positive predictive value (PPV) of the CareStart™ Malaria HRP2/pLDH(Pf) RDT were low and caused by false positivity. This is characteristic of PfHRP2-based RDTs in endemic settings [5]. Persistence of PfHRP2 after treatment of malaria and parasite clearance

Table 4
Occurrence of *P. falciparum msp1* genotypes.

<i>msp1</i> genotype	Genotype occurrence n (%)
K1	20 (30)
RO33	3 (5)
MAD20	7 (11)
K1/RO33	10 (15)
K1/MAD20	16 (24)
MAD20/RO33	3 (5)
K1/MAD20/RO33	7 (11)
Total	66 (100)

msp1: merozoite surface protein 1.

is a major cause of low specificity [42]. Other sources of PfHRP2 include expression by gametocytes and/or asexual stages at submicroscopic levels [5,6,43]. As a result, it is impossible for the CareStart™ Malaria HRP2/pLDH(Pf) test or any other PfHRP2-based RDT to reliably distinguish between active infection and positivity due to persistence of PfHRP2. This may be circumvented by detecting *Plasmodium* LDH or aldolase. Nevertheless, detection of PfHRP2 and pLDH by the CareStart™ Malaria HRP2/pLDH(Pf) RDT did not improve its specificity in the present study.

The sensitivity of a reference method has a direct impact on the performance of diagnostic tests under investigation [40]. Our reference method, microscopy, had a detection limit of 80 parasites/μl. This could have lowered the CareStart™ RDT specificity because of submicroscopic malaria infections with detectable PfHRP2-based RDT [44]. The detection of seven *P. falciparum* cases by nested PCR and RDT missed by microscopy favour this possibility and reaffirmed that microscopy is an imperfect gold standard [45]. Evaluation of the CareStart™ Malaria HRP2/pLDH(Pf) RDT against nested PCR revealed minimal changes only of sensitivity and specificity. This is inconsistent with previous studies; in fact, the nested PCR used as a reference here had a similar performance compared to microscopy [40,46]. Important to note is that CareStart™ Malaria HRP2/pLDH(Pf) exhibited high negative predictive values (NPV) and a low negative likelihood ratio as reported previously [40,41,47], indicating that negative CareStart™ Malaria HRP2/pLDH (pf) results can be relied on with sufficient confidence. In spite of a small sample size (192), these findings are significant, as the *P. falciparum* prevalence in this study (40%) was within the prevalence range (20 to 40%) reported in Western Kenya [33]. However, low specificity of the CareStart™ Malaria HRP2/pLDH(Pf) test may lead to over-prescription of antimalarial drugs and/or complicate diagnoses of other febrile conditions.

The degree of parasitemia is currently insufficient to explain PfHRP2-based RDT performance variation in malaria diagnoses [19,24,26,48]. Successful exon 2 amplification of *pfhrp2* and *pfhrp3* in all *P. falciparum* positive samples eliminated gene deletion as cause of false negativity. However, the recent identification of *P. falciparum* lacking *pfhrp2/3* in Kenya, the Democratic Republic of Congo, Eritrea and Rwanda raise fundamental questions about the sustainability of PfHRP2 – detecting RDTs in East Africa [25–27,29]. In other parts of the world, this phenomenon is indisputable, with countries such as Peru having a prevalence of 41% [20]. It is important to note that the probability of identifying isolates lacking *pfhrp2* and/or *pfhrp3* in the present study may have been reduced by *P. falciparum* polyclonal infections as reported recently [49] and the analysis of a rather small number of isolates. In view of the higher proportion of polyclonal infections than monoclonal infections, the probability of identifying isolates lacking *pfhrp2* and/or *pfhrp3* in this study was 45%.

The diversity of PfHRP2 may also determine RDT sensitivity [15,18,19]. We observed high PfHRP2 diversity among our Kenyan isolates. The repeat types identified here have also been detected in other geographical regions except for types 9 and 11 which have been detected rarely only so far [12,14,15,22,50,51]. Ten PfHRP2 repeat types (type 1 to 8, 10 and 12) identified were highly prevalent. The similarity in the occurrence of these repeat types between our study and previous studies suggests temporal persistence of similar PfHRP2 epitopes despite the observed antigen diversity. This implies that different PfHRP2-based RDT products are detecting homologous epitopes, particularly because there is a limited number of PfHRP2 – specific MAbs available at present [16,17]. This may explain the similarity of the sensitivity of CareStart™ RDT in this study with that of other RDT products evaluated previously [40,41].

Repeat types 2 and 7 constitute > 50% of repeat types found in PfHRP2 and are likely to be the main contributor of the epitopes involved in PfHRP2 detectability [15]. In 2005, Baker et al. published the first report linking RDT sensitivity to the product of the number of these two repeat types (type 2 × type 7). They showed, using a binary logistic

regression model (Baker's regression model), that PfHRP2 – based RDT's failure to detect *P. falciparum* laboratory strains at a parasitemia ≤ 250 parasites/μl was due to type 2 × type 7 that was < 43 and suggested that this model could be used to predict the false negative rate of RDTs targeting PfHRP2 in the field [15]. Evaluation of this model in field settings has provided conflicting results [12,18,19]. The present study did not investigate the impact of type 2 × type 7 below 43 on CareStart™ Malaria HRP2/pLDH(Pf) RDT false negativity rate (5%) when microscopy was used as a reference, because this RDT detects both PfHRP2 and pLDH in a single test band. Other reasons are that the PfHRP2 diversity of two RDT undetected isolates were not investigated because sequencing of the *pfhrp2* could not be achieved despite repeated attempts. The remaining two isolates had > 43 type 2 × type 7 at a parasitemia ≥ 320 parasites/μl, which is above the RDT limit of detection. Nevertheless, this study predicts that 8% of isolates from western Kenya would remain undetected at a parasitemia of ≤ 250 parasites/μl. These findings are comparable to previous studies using isolates collected from Senegal (7%) and Madagascar (9%) and globally (16%) [13,14,22].

Unlike PfHRP2, PfHRP3 was less diverse as demonstrated by the presence of all nine repeat types described previously [12]. The number of repeat types 15, 17 and 18 per sample varied the most, in line with previous reports [15,22,52]. Repeat type 2 is rarely detected in PfHRP3 and its presence in one isolate in our study was surprising. In 2010, Baker et al. reported complete absence of repeat type 2 among isolates from Africa, Asia (except a few isolates from the Philippines), South-west Pacific as well as Central and South America [12]. The Baker et al. study was based on 27 African isolates. Of this, only six isolates originated from Kenya. This is the only study to date describing PfHRP3 diversity in Kenya to the best of our knowledge. Our study shows that there is a small population of *P. falciparum* expressing PfHRP3 with repeat type 2. This suggests that presence of type 2 in western Kenya may have occurred recently. The presence of the three repeats types 1, 4 and 7 in PfHRP3 and PfHRP2 shows that CareStart™ Malaria HRP2/pLDH(Pf) can detect PfHRP3. This is useful in cases where *P. falciparum* isolates without *pfhrp2* occur provided parasitemias is ≥ 1000 parasites/μ [16,29].

Seventeen of 20 new repeat type observed here originate from a single amino acid replacement of previously published repeat sequences. Although this is the first observation among African isolates, similar observations were made in Asian isolates [50,52]. Only four repeat type variants identified in these two studies were identified in our study, including the motifs THH, AHHPAD, AHHSY and SHHDG. The first three motifs were identified in PfHRP2, and SHHDG was present in PfHRP3. The observation of repeat type variants raises questions on their future impact on the performance of PfHRP2-based RDTs. However, the low occurrence of repeat type variants suggests that they may not have a significant impact on RDT performance.

5. Conclusion

The high false positive rate indicates that the CareStart™ HRP2/pLDH(Pf) RDT may lead to over-prescription of antimalarial drugs and overestimation of the malaria burden. The management of other febrile diseases in western Kenya may, thus, be neglected or, at least, affected. Examining for other fever etiologies is required to promote prudent use of antimalarial drugs. The high negative predictive value shows that this RDT can be used to rule out *falciparum* malaria and be an effective tool for malaria surveillance program.

Conflict of interest statement

The authors have declared that they have no competing interests.

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Author's contributions

DN performed molecular experiments and drafted the manuscript. TPV designed and supervised the experiments and data analysis. FK coordinated sample collection and logistics in the field. KT, MA, EK contributed in the field diagnosis and sample collection. TPV and CGM contributed in writing the manuscript. CGM reviewed and edited the manuscript. All authors approved the final manuscript.

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2.2. Chapter 2: Genetic diversity of *P. falciparum* histidine rich proteins (PfHRP2 and 3) from Western and Coastal Kenya

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***Plasmodium falciparum* histidine-rich protein (PfHRP2 and 3) diversity in
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Plasmodium falciparum histidine-rich protein (PfHRP2 and 3) diversity in Western and Coastal Kenya

David Nderu^{1,2}, Francis Kimani³, Kelvin Thiong'o³, Evaline Karanja⁴, Maureen Akinyi³, Edwin Too³, William Chege³, Eva Nambati³, Christian G. Meyer^{1,5,6} & Thirumalaisamy P. Velavan^{1,5,6,7}

Plasmodium falciparum histidine-rich proteins 2 (PfHRP2) based RDTs are advocated in falciparum malaria-endemic regions, particularly when quality microscopy is not available. However, diversity and any deletion in the *pfhrp2* and *pfhrp3* genes can affect the performance of PfHRP2-based RDTs. A total of 400 samples collected from uncomplicated malaria cases from Kenya were investigated for the amino acid repeat profiles in exon 2 of *pfhrp2* and *pfhrp3* genes. In addition, PfHRP2 levels were measured in 96 individuals with uncomplicated malaria. We observed a unique distribution pattern of amino acid repeats both in the PfHRP2 and PfHRP3. 228 PfHRP2 and 124 PfHRP3 different amino acid sequences were identified. Of this, 214 (94%) PfHRP2 and 81 (65%) PfHRP3 amino acid sequences occurred only once. Thirty-nine new PfHRP2 and 20 new PfHRP3 amino acid repeat types were identified. PfHRP2 levels were not correlated with parasitemia or the number of PfHRP2 repeat types. This study shows the variability of PfHRP2, PfHRP3 and PfHRP2 concentration among uncomplicated malaria cases. These findings will be useful to understand the performance of PfHRP2-based RDTs in Kenya.

Malaria associated morbidity and mortality has steadily declined in recent years due to increased use of bed nets and other pertinent control measures^{1,2}. Although malaria is an easily treatable parasitic disease, the rapid development of antimalarial drug resistance considerably threatens control efforts.

Microscopic examination of stained blood smears continues to serve as the gold standard for malaria diagnosis³. However, it is not readily available in resource-limited areas due to the scarcity of skilled personnel, reliable electricity supply, good quality reagents and infrastructure³. The WHO and national malaria control programmes (NMCPs) have put in place strategies to circumvent this pitfall. One of these strategies is compulsory malaria testing by appropriate test systems, including rapid diagnostic tests (RDTs) prior to the prescription of antimalarial drugs.

Discrepancies in the performance of antigen detecting tests are attributed to a combination of factors such as parasite levels, interpretation of RDT results and/or the handling and storage of RDT kits. However, some of the inconsistencies observed with results from *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2)-based RDTs may also be explained by the deletion of the *pfhrp2* gene and its structural homologue, *pfhrp3*, in some parasite isolates.

In the 1990s, the first hand-held immunochromatographic malaria diagnostic test known as rapid diagnostic test (RDT), was developed to address the shortcomings of microscopy⁴. RDTs detect *Plasmodium* antigens using monoclonal antibodies (MAbs) impregnated on a nitrocellulose membrane⁵. About 10 µl of blood is required to perform the test. The antigens targeted by commercially available RDTs include *P. falciparum*

¹Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany. ²School of Health Sciences, Kirinyaga University, Kerugoya, Kenya. ³Center for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya. ⁴Department of Biochemistry and Biotechnology, School of Biological and Life Sciences, Technical University of Kenya, Nairobi, Kenya. ⁵Vietnamese-German Centre for Medical Research (VG-CARE), Hanoi, Vietnam. ⁶Faculty of Medicine, Duy Tan University, Da Nang, Vietnam. ⁷Fondation Congolaise pour la Recherche Médicale, Brazzaville, Congo. Correspondence and requests for materials should be addressed to T.P.V. (email: velavan@medizin.uni-tuebingen.de)

histidine-rich protein 2 (PfHRP2), lactate dehydrogenase (LDH) and aldolase. PfHRP2 is *P. falciparum*-specific, aldolase is genus-specific, and LDH is available in three formats, namely *P. falciparum*-specific, *P. vivax*-specific and genus-specific⁶.

Currently, there are more than 200 commercially available malaria RDT brands. The RDTs differ between manufacturers, depending on the antigen or combination of antigens that can be detected⁷. The WHO recommendation on the RDT format to be used in a given geographical area depends on the predominant *Plasmodium* species. For regions where *P. falciparum* is predominant such as sub-Saharan Africa, the WHO recommends the use of PfHRP2-based RDTs. Eighty-three percent of RDTs procured in 2016, globally, were supplied to African countries². Ninety percent of these RDTs target PfHRP2⁸.

The national malaria control programme of Kenya adopted the use of PfHRP2 detecting RDTs in 2012^{9,10}. Future use of this test is threatened in many malaria-endemic areas including Kenya by the deletion of the gene coding for *P. falciparum* PfHRP2 and extensive antigen diversity that contributes to variation of the sensitivity of these tests^{11–17}. There has been a considerable increase in the number of countries with *P. falciparum* isolates devoid of *pfhrp2* and/or *pfhrp3* over the last eight years. Recent entrants include Mozambique, Eritrea, Rwanda and Kenya^{8,12,15,18–23}. It is therefore important to monitor parasite factors that can undermine malaria RDT-based diagnosis and, in the long run, safeguard the efficacy of antimalarial drugs and promote prompt and appropriate management of febrile illnesses.

The present study aimed to investigate the diversity of PfHRP2 and its homologue PfHRP3 as well as variation of PfHRP2 levels in uncomplicated malaria cases from two malaria-endemic regions located in Western and Coastal Kenya.

Results

Exon 2 of both *pfhrp2* and *pfhrp3* was detected in all the 400 samples analysed in this study. Of this, 244 *pfhrp2* and 267 *pfhrp3* PCR products were successfully sequenced and their amino acid sequences deduced for an assessment of PfHRP2 and PfHRP3 diversity, respectively. The remaining 156 *pfhrp2* and 133 *pfhrp3* PCR products were excluded from further analysis because the nucleotide sequences for these amplicons could not be obtained despite repeated attempts. PfHRP2 and PfHRP3 amino acid sequence diversity among Kenyan *P. falciparum* isolates was characterised by differences in the frequency, occurrence and structural organisation of different amino acid repeat types.

PfHRP2 diversity. A total of 228 different PfHRP2 amino acid sequences were identified among 244 PfHRP2 sequences deduced in this study. The size of PfHRP2 was between 206 and 317 amino acids. Overall, PfHRP2 had a total of 20 to 37 amino acid repeat types per isolate. The organization of the amino acid repeat types in PfHRP2 was highly diverse. Thus, 94% (214/228) of PfHRP2 sequences occurred once only. The remainder (14) were shared among 30 isolates of which 12 sequences were identified in 2 isolates and 2/14 sequences occurred in 3 isolates.

Thirteen previously reported PfHRP2 amino acid repeat types were identified in *P. falciparum* isolates from Kenya as shown in Table 1¹⁶. The frequency of these repeat types was similar among Western and Coastal Kenyan isolates. Table 2 shows the occurrence of PfHRP2 repeat types in this study. Repeat types 2 and 7 were identified in all isolates, whereas repeat types 1, 3, 5, 6, 8, 10 and 12 were observed in 80% to 99% of the isolates. Repeat type 4 (27%) occurred in a few isolates only. Repeat types 13 (8.2%), 14 (6.6%) and 19 (1.2%) were rare. All isolates lacked repeat types 9 and 11. Repeat type 14 did not occur in Tiwi, Coastal Kenya, and repeat type 19 was identified in only three isolates (3%) from Busia, Western Kenya. Most of the PfHRP2 repeat types had a similar occurrence within and between Western and Coastal Kenya, except for four repeat types (Table 2). Type 6 was significantly more prevalent and type 10 was significantly less prevalent in Western than in Coastal Kenya. Type 14 was significantly more prevalent in Msambweni than in Tiwi. Thirty-nine new PfHRP2 repeat types, which have not been reported previously, were identified at low frequencies with repeat type AHHA AH (5.7%) being the most common one (Table 3).

We classified *P. falciparum* isolates into groups A, B, I and C based on the product of the number of repeat type 2 and type 7 (type 2 × type 7), as described in the methods section (PfHRP2 and PfHRP3 diversity), to determine their distribution on basis PfHRP2 diversity. Our study revealed that most of the isolates were in group B (type 2 × type 7; ranges from 50 to 100) (Table 4). The occurrence of group A (type 2 × type 7; >100) was significantly higher in Mbita than in Busia and Nyando, Western Kenya. In the Coastal region, however, the occurrence of group C (type 2 × type 7; <43) was significantly higher in Tiwi than in Msambweni.

The structural organisation of the PfHRP2 repeat types was highly variable. Nevertheless, three characteristic features were observed. The repetitive region of most of the PfHRP2 sequences began with repeat type 1 in 96% to 99% of the *P. falciparum* isolates, ended with type 12 in 85.5% to 96.8% of *P. falciparum* isolates and had a semi-conserved PfHRP2 repeat type motif composed of repeat types 2, 3, 5, 7 and 8 in 50% (121/244) of the isolates as illustrated in Fig. 1a. Twenty-eight percent of the isolates shared parts of this motif composed of types 7, 8 and 2. These two motifs were not identified in 55 isolates.

PfHRP3 diversity. A total of 124 different PfHRP3 amino acid sequences were deduced from 267 *pfhrp3* (exon 2) nucleotide sequences obtained in this study. The size of PfHRP3 ranged from 160 to 247 amino acids, whereas the total number of amino acid repeat types per isolate ranged between 18 and 33 types. Repeat types 1, 4 and 7 identified in PfHRP2 were also present in PfHRP3. The number of repeat types 16, 17 and 18 per isolates varied most (Table 1). Apart from type 2, which occurred only in one isolate from Nyando, the other repeat types had an occurrence of ≥97%. We identified 20 new PfHRP3 repeat types that have not been reported previously at low frequencies (Table 3). The amino acid sequence SHHDG was the most common (9.7%) novel PfHRP3 repeat type.

Repeat types	Repeat sequence	PfHRP2	PfHRP3	Malaria-endemic region		Overall
				Western	Coastal	
Type 1	AHHAHHVAD	+	+	0–6	0–9	0–9
				0–4	1–3	0–4
Type 2	AHHAHHAAD	+	+	7–18	6–16	6–18
				0–1	0	0
Type 3	AHHAHHAAY	+	–	0–3	0–3	0–3
Type 4	AHH	+	+	0–6	0–6	0–6
				1	1	1
Type 5	AHHAHHASD	+	–	0–3	0–3	0–3
Type 6	AHHATD	+	–	0–8	0–6	0–8
Type 7	AHHAAD	+	+	2–12	1–12	1–12
				0–1	1	0–1
Type 8	AHHAAY	+	–	0–3	0–3	0–3
Type 10	AHHAHHATD	+	–	0–3	0–3	0–3
Type 12	AHHAHHHEAATH	+	–	0–1	0–1	0–1
Type 13	AHHASD	+	–	0–2	0–2	0–2
Type 14	AHHAHHATD	+	–	0–2	0–2	0–2
Type 15	AHHAHHAAN	–	+	0–1	0–1	0–1
Type 16	AHHAAN	–	+	6–18	9–15	6–18
Type 17	AHHDG	–	+	3–10	3–8	3–10
Type 18	AHHDD	–	+	1–4	1–3	1–4
Type 19	AHHAA	+	–	0–1	0	0–1
Type 20	SHHDD	–	+	1	1	1

Table 1. Comparison of the range of individual PfHRP2 and PfHRP3 repeat types in malaria-endemic sites of Kenya. The plus (+) and minus (–) signs show the presence or absence, respectively, of individual amino acid repeats in PfHRP2 and PfHRP3. The range of individual amino acid repeats in PfHRP3 is shown in bold.

Repeat types	Western				Coastal			Regional Total			Overall
	Nyando n (%)	Busia n (%)	Mbita n (%)	<i>p</i> -value ^a	Msambweni n (%)	Tiwi n (%)	<i>p</i> -value ^a	Western n (%)	Coastal n (%)	<i>p</i> -value ^b	
Type 1	30 (96.8)	116 (99.1)	24 (96.0)	ns	37 (94.9)	32 (100)	ns	170 (98.3)	69 (97.2)	ns	239 (98.0)
Type 2	31 (100)	117 (100)	25 (100)	ns	39 (100)	32 (100)	ns	173 (100)	71 (100)	ns	244 (100)
Type 3	29 (93.5)	109 (93.2)	21 (84)	ns	33 (84.6)	29 (90.6)	ns	159 (91.9)	62 (87.3)	ns	221 (90.6)
Type 4	8 (25.8)	28 (23.9)	7 (28.0)	ns	10 (25.6)	13 (40.6)	ns	43 (24.9)	23 (32.4)	ns	66 (27.0)
Type 5	25 (80.6)	91 (77.8)	19 (76.0)	ns	35 (89.7)	26 (81.3)	ns	135 (78)	61 (85.9)	ns	196 (80.3)
Type 6	31 (100)	116 (99.1)	25 (100)	ns	32 (82.1)	32 (100)	0.014	172 (99.4)	64 (90.1)	0.001	236 (96.7)
Type 7	31 (100)	117 (100)	25 (100)	ns	39 (100)	32 (100)	ns	173 (100)	71 (100)	ns	244 (100)
Type 8	30 (96.8)	111 (94.9)	24 (96.0)	ns	38 (97.4)	31 (96.9)	ns	165 (95.4)	69 (97.2)	ns	234 (95.9)
Type 10	27 (87.1)	97 (82.9)	23 (92.0)	ns	38 (97.4)	31 (96.9)	ns	147 (85)	69 (97.2)	0.007	216 (88.5)
Type 12	30 (96.8)	100 (85.5)	24 (96.0)	ns	36 (92.3)	29 (90.6)	ns	154 (89)	65 (91.5)	ns	219 (89.8)
Type 13	2 (6.5)	9 (7.7)	3 (12.0)	ns	3 (7.7)	3 (9.4)	ns	14 (8.1)	6 (8.5)	ns	20 (8.2)
Type 14	1 (3.2)	13 (11.1)	2 (8.0)	ns	8 (20.5)	0	0.007	16 (9.2)	8 (11.3)	ns	24 (9.8)
Type 19	0	3 (2.6)	0	ns	0	0	ns	3 (1.7)	0	ns	3 (1.2)

Table 2. Comparison of the occurrence of individual PfHRP2 repeat types within and between two malaria-endemic regions in Kenya. The 244 isolates analysed were distributed as follows; Nyando 31, Busia 117, Mbita 25, Msambweni 39 and Tiwi 32 isolates. The superscript letters show the *p*-values of comparison of the occurrence of repeat types within (^a) and between (^b) malaria-endemic regions in Kenya. Statistically significant difference ($p < 0.05$) shown in bold. ns: not significant.

Structurally, PfHRP3 was more conserved than PfHRP2. Eighty-one out of 124 (65.3%) different PfHRP3 sequences occurred only once, whereas 43/124 (34.7%) different PfHRP3 sequences were shared by 2–17 isolates each. In addition, the organisation of repeats was conserved between isolates as shown in Fig. 1b. A non-repetitive sequence was located between two PfHRP3 repetitive motifs.

PfHRP2 concentration in whole blood samples. PfHRP2 levels were measured in samples from 96 uncomplicated malaria cases with a median parasite density of 21,400 parasites/μl (interquartile range, IQR: 7,781–34,180 parasites/μl) and a mean haemoglobin level of 10.48 g/dl (95% CI 10.13–10.82 g/dl). The

PfHRP2				PfHRP3			
Repeat Types	Known repeats	Novel Repeats	(%)	Repeat Type	Known repeats	Novel Repeats	(%)
Type 1	AHHAHHVAD	AHHAHHVAY	0.8	Type 1	AHHAHHVAD	AHHAHHGAE	0.3
		AHHAHHVPD	0.4			AHSHHHVAD	0.7
		AHHTHHVAD	0.4			AHQAHHVAD	0.3
Type 2	AHHAHHAAD	AHHADHAAD	0.4			AQHAAHVAD	0.3
		AHHAHHAAH*	4.1	Type 7	AHHAAD	AHHADD	0.3
		AHHAHHADD	1.2	Type 15	AHHAHHAAN	AHHAHHAPH	0.3
		AHHAHHADH	0.4	Type 16	AHHAAN	AHHAAH*	0.7
		AHHAHHAPD	0.8			AHHADN	0.3
		AHHAHHAPH	0.4			AHHAPH	0.3
Type 4	AHH	AHQ	0.4			AHHASN*	1.5
		ADH	0.4			AHHTAN	0.3
Type 5	AHHAHHASD	AHHAPHASD	0.4			AHQADN*	0.3
		AHHDHHASD	0.4			AYHASH	0.3
Type 6	AHHATD	AHHATH	0.4	Type 17	AHHDG	AHHDE	0.3
Type 7	AHHAAD	AHHAPD	0.4			AHHDH	0.3
		AHHAAH*	5.7			AHYDG	0.3
		AHHADD*	2.0			PHHDG	0.7
		AHHAHD	0.4			PHQDG	0.3
		AHHAND	1.2			SHHDG	9.7
		AHHANH	0.4	Type 18	AHHDD	APHDD	0.3
Type 8	AHHAAY	AHHADY	0.4				
Type 10	AHHAHHATD	AHHAHHADND	0.4				
		AHHAHHAND	0.8				
		AHHAHHATG	0.4				
		AHHAHHHATD	1.2				
		AHHAHHHATD	0.4				
Type 12	AHHAHHHEAATH	ADHAHHDDAATH	0.4				
		ADHAHHHEAATH	0.4				
		AHHAHHDDHATH	0.4				
		AHHAHHDEAATH	0.8				
		AHHAHHHEAAA	0.8				
		AHHAHHHEAASH	0.4				
		AHHAHHHESATH	0.4				
		AHHAHHHHAATH	0.4				
		AHHAHHPEAATH	0.4				
		AHHAAPHHEAATH	0.4				
		AHHADAHHDAATH	0.4				
Type 13	AHHASD	AHHASH	0.4				

Table 3. List of new PfHRP2 and PfHRP3 amino acid repeat types identified in Kenya. The asterisks (*) shows new repeat types with >1 copy per isolate. The single-letter amino acid code in bold shows the position where the novel repeat types differ from the known repeat types. % shows the occurrence of the novel repeat types.

PfHRP2 groups	Western				Coastal			Regional total			Overall Total n (%)
	Nyando n (%)	Busia n (%)	Mbita n (%)	<i>p-value</i> ^a	Msambweni n (%)	Tiwi n (%)	<i>p-value</i> ^a	Western n (%)	Coastal n (%)	<i>p-value</i> ^b	
Group A	0	10 (8.5)	5 (20.0)	0.025	2 (5.1)	1 (3.1)	ns	15 (8.7)	3 (4.2)	ns	18 (7.4)
Group B	27 (87.1)	90 (76.9)	17 (68.0)	ns	30 (76.9)	21 (65.8)	ns	134 (77.5)	51 (71.8)	ns	185 (75.8)
Group I	0	8 (6.8)	2 (8.0)	ns	5 (12.8)	2 (6.3)	ns	10 (5.7)	7 (9.9)	ns	17 (7.0)
Group C	4 (12.9)	9 (7.7)	1 (4.0)	ns	2 (5.1)	8 (25.8)	0.035	14 (8.1)	10 (14.1)	ns	24 (9.8)

Table 4. Comparison of the occurrence of PfHRP2 groups (Baker model) within and between malaria-endemic regions of Kenya. Groups A, B, I and C constitute PfHRP2 sequence whose Baker repeat type 2 × type 7 number is >100, ranges from 50 to 100, ranges from 44 to 49 and <43, respectively. The superscript letters show the *p-values* of the comparison group occurrence within (^a) and between (^b) malaria-endemic regions of Kenya. Statistically significant difference ($p < 0.05$) shown in bold. ns: not significant.

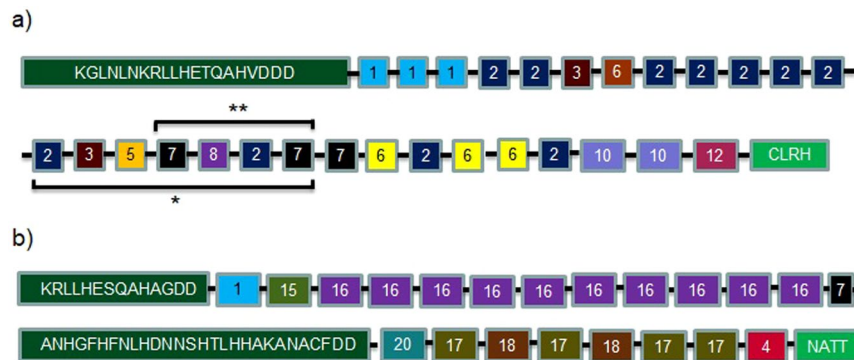


Figure 1. Schematic diagram of the structural organisation of PfHRPs 2 and 3 amino acid repeat types in Kenya. (a) *P. falciparum* histidine-rich protein 2. (b) *P. falciparum* histidine-rich protein 3. *Semi-conserved amino acid repeat motif; **partial amino acid repeat motif.

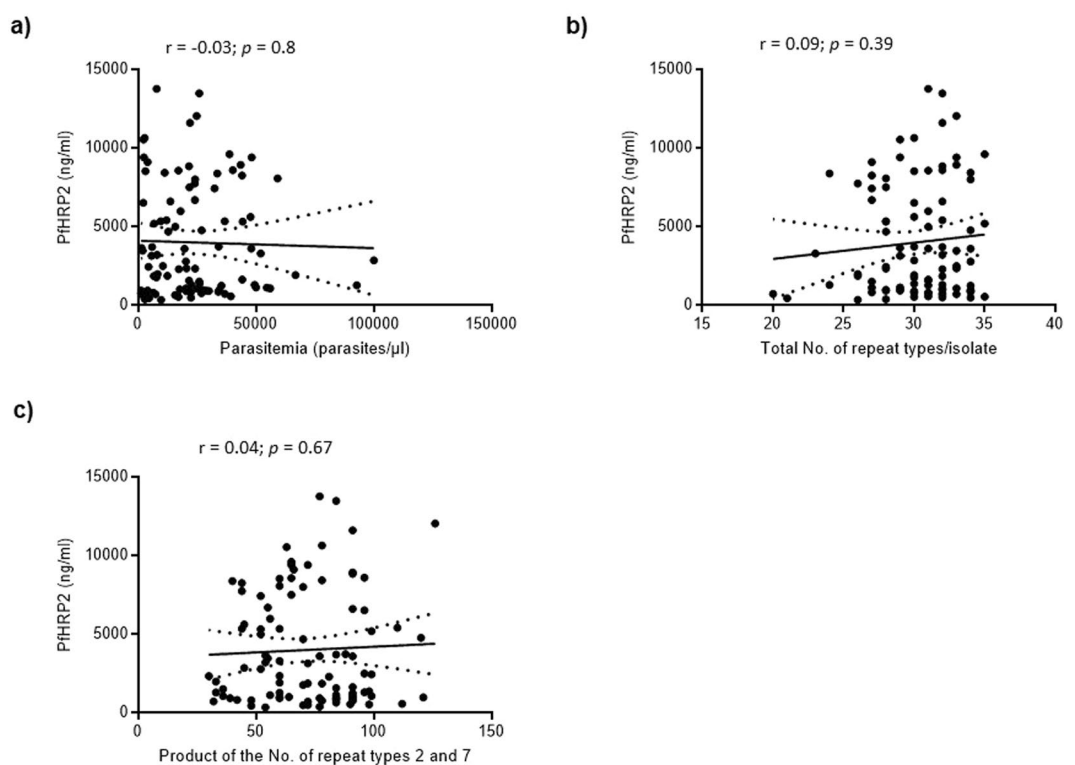


Figure 2. Results of Spearman's correlation analysis between PfHRP2 levels and three *P. falciparum* parameters. (a) PfHRP2 vs. Parasitemia. (b) PfHRP2 vs. Total number of PfHRP2 repeat types per isolate. (c) PfHRP2 vs. Product of the number of repeat types 2 and 7. Statistical significance set at p -value < 0.05.

concentration of PfHRP2 in whole blood among the participants was highly variable. It ranged from 339.3 ng/ml to 13,766 ng/ml with a median of 2,470 ng/ml (IQR: 980.8 ng/ml–6,670 ng/ml). PfHRP2 levels did not correlate with parasitemia, the number of individual PfHRP2 repeat types per isolate and the product of the number of repeat types 2 and 7 per isolate (Fig. 2).

Discussion

PfHRP2 targeting RDTs are an important pillar of malaria control programmes and promote access to malaria diagnosis where microscopy is not available. Future use of these tests in malaria-endemic countries is threatened by the spread of isolates that do not express PfHRP2^{18,24,25}. Since most of the commercially available RDTs target PfHRP2 expressed solely by *P. falciparum*, analysis of *pfhrp2/3* genetic diversity is of public health importance.

We analysed the diversity of *pfhrp2* and *pfhrp3* among 400 isolates collected at five different time points (2007–2016) in Coastal and Western Kenya. Our study shows that *pfhrp2* and *pfhrp3* deletion did not occur among these isolates. This is consistent with two previous reports^{16,17}. In 2017, however, Beshir *et al.* published the first report of *pfhrp2* deletion in Mbita, Kenya¹⁵. We analysed 58 samples collected from this area in 2007, seven years before

the Beshir *et al.* sample collection in 2014. Absence of *pfhrp2/3* deletion in the present study strongly suggests that the reported *pfhrp2* and *pfhrp3* deletion may have occurred only recently in Mbita. It is important to note that the analysis of a small sample size in our study and the inclusion of symptomatic malaria cases may have influenced the results of this study. The latter is highly plausible considering that the Beshir *et al.* study analysed isolates obtained from asymptomatic malaria cases. Moreover, differences in the complexity of infection (COI) could have limited the detection of *pfhrp2/3* deletion as reported recently²⁶.

At the amino acid sequence level, the structural organisation of repeat types was highly diverse. Ninety-four percent of the different PfHRP2 sequences identified in this study occurred only once. Similar findings have been reported in other malaria-endemic countries with Peruvian isolates being the least diverse^{16,17}. Nevertheless, several characteristics were shared between isolates. Majority of the PfHRP2 sequences started with repeat type 1 and terminated with type 12, unlike in isolates from Senegal where type 12 was uncommon²⁷. Similarly, 50% of the isolates had a previously described motif of repeat types 2, 3, 5, 7, 8, 2 and 7, which has been identified in 44% of *P. falciparum* isolates globally¹⁶. An additional 27% of our isolates had part of this motif (7, 8, 2 and 7), which is predominant in isolates from Madagascar²⁸. The motif of types 2, 4, 5, 6, 7 and 8 found in Indian isolates was absent¹³.

In contrast to the diverse structural organisation described here, subtle differences were observed in the occurrence and number of PfHRP2 repeat types per isolate between and within Kenyan malaria-endemic sites. We found types 2 and 7 in all isolates, however, types 9 and 11 were completely absent in all isolates as reported elsewhere^{13,16,27–32}. Type 4 was found in a few isolates (27%) and the rare types 13 and 14 were found in <8% of isolates^{27,29–31,33}. Our data show for the first time the occurrence of type 19 (3 isolates) in Kenya. Other countries where type 19 was found are Uganda, Senegal, Mali and the Philippines^{16,30,33}. The prevalence of the other repeat types identified were >80% consistent with earlier reports.

PfHRP3, on the other hand, showed lower variation than PfHRP2 in its structural organisation, occurrence and number of repeat types. This is reflected by the omnipresence of eight PfHRP3 repeat types identified previously¹⁷, the presence of a conserved repeat type organisation and a lower proportion (65%) of different PfHRP3 repeat type profiles. The identification of type 2 in the PfHRP3 of one isolate from Nyando confirmed our previous observation of the occurrence of this repeat in one isolate from Busia, Western Kenya, during the evaluation of a malaria RDT³⁴. This strengthens our earlier hypothesis that type 2 presence in PfHRP3 may have occurred recently among Kenyan *P. falciparum* isolates. Beyond the Kenyan borders, the presence of type 2 in PfHRP3 has been reported from India at a prevalence of 2.9%³³.

Another characteristic feature of histidine-rich proteins 2 and 3 from Kenyan isolates was the presence of repeat types that have not been described previously. Here, we identified 59 new repeat types arising from replacement of ≥ 1 amino acid of the previously described PfHRP2 and PfHRP3 repeat types. Majority of these new repeat types (39/59) were identified in PfHRP2, consistent with its higher diversity. A similar phenomenon was observed among isolates from the Chinese-Myanmar border where novel PfHRP2 repeat types originated from replacement of a single amino acid of eight amino acid repeats types, compared to the replacement ≥ 1 amino acid in 14 repeat types reported in this study³¹. Five additional repeats types (novel) have also been identified in Indian isolates³³. Our study corroborates the existence of repeat types that are yet to be described and shows that they have a low prevalence. It also shows that there is a limited distribution of the new repeat types between different geographical regions. This is exemplified by the observation of the novel repeats types AHHAHHVAY (PfHRP2) and SHHDG (PfHRP3) among Kenyan isolates that were previously found in isolates from the China-Myanmar border and India, respectively^{31,33}. Nevertheless, the new PfHRP2 repeat type AHHAH (6%) and the new PfHRP3 repeat type SHHDG (10%) were most frequent in this study.

Currently it is of major importance in how far PfHRP2 diversity may affect malaria diagnoses based on the detection of PfHRP2. Baker *et al.* demonstrated, using a binary logistic regression model (Baker model), that the observed inter-study sensitivity variation of PfHRP2-based RDTs is linked to the product of the number of type 2 and type 7 (type 2 \times type 7), especially with parasite densities of ≤ 250 parasites/ μ l¹⁷. In 2010, Baker *et al.* showed that RDT sensitivity does not correlate with type 2 \times type 7 of isolates from different geographical areas¹⁶. Studies by Kumar *et al.* (2012) and Wurtz *et al.* (2013) have, however, associated type 2 \times type 7 below 43 (group C) with RDT false negativity and reduced limited of detection, respectively^{13,14}. This study was not able to investigate this relationship in Kenyan isolates owing to the lack of PfHRP2-based RDT testing of the samples analysed here. Nevertheless, we utilized PfHRP2 classification of isolates based on type 2 \times type 7 to determine the distribution of Kenyan isolates on basis of PfHRP2 diversity¹⁷. Our data reveals that most of Kenyan isolates (76%) are in group B (type 2 \times type 7; ranges from 50 to 100). This finding is congruent with studies from Madagascar (69%) and Senegal (71%)^{27,28} and is comparable to our recent observation of 71% group B isolates in a small size of 38 samples³⁴.

PfHRP2-specific monoclonal antibodies have been reported to also detect PfHRP3, a PfHRP2 homologue^{17,35}. This cross-reactivity originates from the presence of repeat types 1, 2, 4 and 7, which are also found in PfHRP2. Consistent with this observation, we identified repeat types 1, 4 and 7 in PfHRP2 and PfHRP3 of Kenyan isolates apart from one isolate that exhibited these repeat types in addition to type 2. This implies that PfHRP3 may be useful in modulating the impact of PfHRP2 antigenic polymorphism in the context of malaria diagnosis using PfHRP2-based RDTs^{15,36,37}. Whether cross-reactivity offers a diagnostic advantage remains to be validated due to the current lack of PfHRP3-specific monoclonal antibodies.

Along with genetic deletion and diversity of histidine-rich proteins 2 and 3, suboptimal antigen levels can contribute to false negative results of RDTs³⁸. We observed a significant variation of PfHRP2 concentrations among uncomplicated malaria cases from Busia County, Kenya. Furthermore, the level of PfHRP2 did not correlate with parasitemia or the number of PfHRP2 repeat types per isolate. While we could not assess RDT sensitivity directly, our findings suggest that repeat type is not likely to be associated with RDT sensitivity, as previously observed by Baker *et al.*¹⁶. A previous study demonstrated, *in vitro*, that PfHRP2 expression varies between strains, the erythrocytic stages of *P. falciparum* and the mature schizonts account for most of the PfHRP2 released³⁹. This

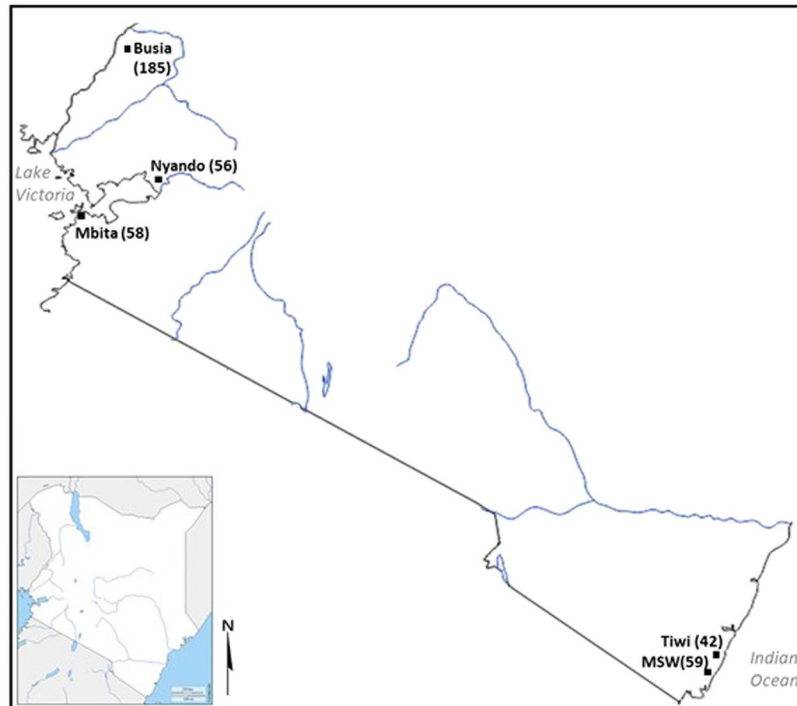


Figure 3. Map of Kenya showing the geographical location of the study sites. MSW is an abbreviation for Msambweni. The number of *P. falciparum* isolates recruited per study site shown in parentheses.

presents a methodological challenge when investigating factors influencing PfHRP2 levels in the host. Additional confounding factors include PfHRP2 antibody cross-reaction with PfHRP3, PfHRP2 expression by gametocytes, slow antigen clearance and residual PfHRP2 from previous infections^{6,40–42}. Nevertheless, the potential impact of PfHRP2 levels on sensitivity needs also to be considered during the evaluation of RDTs.

Taken together, this study shows extensive diversity of *Plasmodium falciparum* histidine-rich proteins 2 and 3 in Kenyan isolates. It also highlights the existence of additional amino acid repeat types which extends PfHRP2 and PfHRP3 antigenic variability. Information from this study will be useful to understand the performance of PfHRP2-detecting RDTs in this setting.

Methods

Study sites and sample selection. Four hundred *P. falciparum* isolates collected from symptomatic malaria cases in the frame of antimalarial drug efficacy trials conducted between 2007 and 2016, in malaria-endemic sites situated in Western (Mbita 2007, Nyando 2015 and Busia 2016) and Coastal (Tiwi 2008 and Msambweni 2013) Kenya were analysed in this study (Fig. 3). An inclusion criterion of >2,000 to 200,000 parasites/ μ l by microscopy was used in these studies. Samples were obtained before antimalarial drug administration and stored as dried blood spots (DBS) on filter papers, except isolates collected in Busia (whole blood in EDTA). Samples were included in the study after *P. falciparum* infection was confirmed using a *P. falciparum*-specific 18S ribosomal RNA (rRNA) nested PCR.

Ethical Statement. The study was approved by the Scientific & Ethics Review Unit (SERU) of Kenya Medical Research Institute (KEMRI) Nairobi (KEMRI/SERU/0152/3250) and written informed consent was obtained from the parents/guardians of all children and from adult participants. All experiments were performed in accordance with relevant guidelines and regulations.

Molecular analysis. Genomic DNA was extracted using QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Individual PCRs targeting *P. falciparum* 18S rRNA, *pfhrp2* (exon 2) and *pfhrp3* (exon 2) were performed as described previously (Table S1)^{17,43}. Briefly, 1 μ l of DNA template was added into a master mix containing 1 \times PCR buffer (Qiagen, Hilden, Germany), 200 μ M of each dNTP, 1 unit of Qiagen Taq DNA polymerase (Qiagen, Hilden, Germany), and 100 nM of each primer in a total volume of 20 μ l. Genomic DNA of three *P. falciparum* strains was used as controls, namely 3D7 (*pfhrp2*⁺ and *pfhrp3*⁺), Dd2 (*pfhrp2*⁻ and *pfhrp3*⁺) and Hb3 (*pfhrp2*⁺ and *pfhrp3*⁻).

pfhrp2 and *pfhrp3* PCR products were purified using SephadexTM G-50 fine DNA grade (GE Healthcare, Buckinghamshire, UK) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. PCR products were sequenced in the forward and reverse direction. DNA sequence chromatograms were visually inspected to resolve discordant base-calling. BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) was used to assemble the nucleic acid sequences using

P. falciparum 3D7 *pfhrp2* (PF3D7_0831800) and *pfhrp3* (PF3D7_1372200) DNA sequences as the references as well as deduce amino acid sequences of PfHRP2 and PfHRP3.

PfHRP2 and PfHRP3 diversity. The diversity of PfHRP2 and PfHRP3 was described based on the frequency, occurrence and organisation of histidine-rich protein repeat types (numerically coded as repeat types 1 to 24) as described by Baker *et al.*^{16,17}. The product of the number of PfHRP2 repeat types 2 and 7 (type 2 × type 7) was used to classify *P. falciparum* isolates into four groups, namely group A (very sensitive), group B (sensitive), group I (borderline) and group C (non-sensitive) when the number of type 2 × type 7 was above 100, ranged from 50 to 100, ranged from 44 to 49, and less than 43, respectively. This was conducted to determine the distribution of *P. falciparum* isolates based on PfHRP2 diversity.

PfHRP2 quantification by enzyme-linked immunosorbent assay (ELISA). The measurement of PfHRP2 levels in whole blood samples of uncomplicated malaria cases from Busia County was conducted using a commercial sandwich ELISA kit (Malaria Ag Celisa™, Cellabs, Sydney, Australia) in accordance with the manufacturer's instructions. The optical density (OD) was measured at 450 nm in a PHOmo reader (Autobio Diagnostics Co. Ltd, Zhengzhou, China). The OD cut-off level was set by calculating the mean OD + 3 SD of three negative controls included in each ELISA run. OD above and below the cut-off was regarded to be positive and negative, respectively. PfHRP2 concentration was calculated using a standard curve prepared using serial dilutions (1:1) of recombinant PfHRP2 in phosphate buffered saline (PBS).

Data Analysis. Chi-square and Fisher's exact tests were used to compare the occurrence of amino acid repeat types between and within malaria-endemic sites. Kruskal Wallis and Mann-Whitney U tests were applied for analysis of non-parametric data. Significance was observed at a *p-value* < 0.05.

Data Availability

Data analysed in this publication are available from the corresponding author on reasonable request. *Plasmodium falciparum* histidine-rich proteins 2 and 3 nucleic acid sequences are available in GenBank (*pfhrp2*: accession numbers MH230283 - MH230526; *pfhrp3*: accession numbers: MH230527 - MH230790).

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Author Contributions

D.N. performed molecular experiments and drafted the manuscript. T.P.V., designed and supervised the experiments and data analysis. F.K. coordinated sample collection and logistics in the field. K.T., E.N., M.A., E.T., W.C., E.K., contributed in the field diagnosis, sample collection, processing and shipment. T.P.V. contributed in writing the manuscript. C.G.M. reviewed and edited the manuscript. All authors approved the final manuscript.

Additional Information

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2.3. Chapter 3: Genetic variability and population structure of *P. falciparum* in Kenyan-Ugandan border areas

Publication No. 3

Genetic diversity and population structure of *P. falciparum* in Kenyan-Ugandan border areas

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Genetic diversity and population structure of *Plasmodium falciparum* in Kenyan–Ugandan border areas

David Nderu^{1,2}, Francis Kimani³, Evaline Karanja⁴, Kelvin Thiong'o³, Maureen Akinyi³, Edwin Too³, William Chege³, Eva Nambati³, Laura N. Wangai², Christian G. Meyer^{1,5,6} and Thirumalaisamy P. Velavan^{1,5,6,7}

1 Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany

2 School of Health Sciences, Kirinyaga University, Kerugoya, Kenya

3 Center for Biotechnology Research and Development, Kenya Medical Research Institute, Nairobi, Kenya

4 Department of Biochemistry and Biotechnology, School of Biological and Life Sciences, Technical University of Kenya, Nairobi, Kenya

5 Vietnamese-German Centre for Medical Research, Hanoi, Vietnam

6 Faculty of Medicine, Duy Tan University, Da Nang, Vietnam

7 Fondation Congolaise pour la Recherche Médicale, Brazzaville, Republic of Congo

Summary

Kenya has, in the last decade, made tremendous progress in the fight against malaria. Nevertheless, continued surveillance of the genetic diversity and population structure of *Plasmodium falciparum* is required to refine malaria control and to adapt and improve elimination strategies. Twelve neutral microsatellite loci were genotyped in 201 *P. falciparum* isolates obtained from the Kenyan–Ugandan border (Busia) and from two inland malaria-endemic sites situated in western (Nyando) and coastal (Msambweni) Kenya. Analyses were done to assess the genetic diversity (allelic richness and expected heterozygosity, [H_e]), multilocus linkage disequilibrium (I_A^L) and population structure. A similarly high degree of genetic diversity was observed among the three parasite populations surveyed (mean $H_e = 0.76$; $P > 0.05$). Except in Msambweni, random association of microsatellite loci was observed, indicating high parasite out-breeding. Low to moderate genetic structure ($F_{ST} = 0.022$ – 0.076 ; $P < 0.0001$) was observed with only 5% variance in allele frequencies observed among the populations. This study shows that the genetic diversity of *P. falciparum* populations at the Kenyan–Ugandan border is comparable to the parasite populations from inland Kenya. In addition, high genetic diversity, panmixia and weak population structure in this study highlight the fitness of Kenyan *P. falciparum* populations to successfully withstand malaria control interventions.

keywords *Plasmodium falciparum*, microsatellites, genetic diversity, population structure, malaria, Kenya

Introduction

Malaria is a major public health problem accounting for about 219 million morbidities and 435 000 mortalities reported in 2017 globally [1]. Ninety per cent of the disease burden occurs in sub-Saharan Africa, in particular in children below 5 years. In Kenya, malaria is the second leading cause of morbidity and mortality [2]. The continuous fight against malaria has led to a remarkable reduction in disease incidence and fatalities in recent years [3–5]. These achievements are largely attributed to the improved malaria control policy, including widespread use of artemisinin-based combination therapies (ACTs) and the scale-up of mosquito vector control [3–5].

Malaria treatment guidelines have undergone numerous changes in the last three decades. In the late 1990s,

sulfadoxine–pyrimethamine (SP) replaced chloroquine (CQ) as first-line treatment for uncomplicated *P. falciparum* malaria in Kenya, due to treatment failure and the advent of CQ resistance [6]. SP efficacy was short-lived, also due to the rapid development of drug resistance. Since 2006, artemether–lumefantrine (AL) has been the antimalarial drug of choice in Kenya [7, 8] and in other countries of sub-Saharan Africa. The changes in drug policy have elicited notable changes in *P. falciparum* population genetics. For instance, the frequency of non-synonymous mutations in *P. falciparum*'s kelch 13 gene has increased, even though these mutations are not associated with delayed parasite clearance [9], whereas the frequency of distinct single-nucleotide polymorphisms conferring resistance to CQ and SP has declined after the withdrawal of these drugs in 1999

and 2006 respectively [10, 11]. Continued SP use in intermittent preventive treatment in pregnancy (IPTp) has recently been linked to the resurgence of SP resistance markers in Kenya [10, 11]. The shift from CQ and SP to AL in uncomplicated malaria treatment has increased the genetic diversity of the Kenyan *P. falciparum* population because of the reduced drug selection pressure [12].

Plasmodium falciparum genetic diversity was expected to decline with malaria transmission and disease prevalence reduction. However, this has not been the case despite the intensified deployment of vector control measures such as use of insecticide-treated nets (ITNs) and insecticide residual spraying (IRS). Analyses conducted 5 and 10 years after ITN introduction showed no change in the genetic diversity and population structure of *P. falciparum* populations from inland Kenya [13, 14]. Increasing asymptomatic malaria prevalence with higher gametocytaemias [15], mosquito vector resistance to pyrethroids [16], changing mosquito biting behaviour [17, 18] and high gene flow [19–21] are some of the prominent factors informing the resilience of *P. falciparum* genetic variability and population structure.

Plasmodium falciparum genetic factors are also dependent on transmission intensity. *P. falciparum* populations from low malaria transmission settings such as South America and Southeast Asia are characterised by low genetic diversity with strong linkage disequilibrium (LD) and defined structures of parasite populations [22]. In sub-Saharan high transmission regions, *P. falciparum* strains exhibit high genetic diversity, non-defined population structures and weak LD. Nevertheless, parasite inbreeding and subtle population fragmentation have been observed in a few high transmission areas despite the prevailing high genetic diversity, in particular in Senegal, Niger, Republic of Djibouti, Zimbabwe, Republic of Congo and Kenya [20, 22–24].

The level of genetic diversity and panmixia contributes to the fitness of *P. falciparum* to counter malaria control interventions such as candidate malaria vaccines [25] as well as the emergence and dispersal of antimalarial drug-resistant parasites. Insight into the genetic diversity and population structure of *P. falciparum* populations is vital to refine and effectively implement malaria control and elimination strategies. This study describes the genetic diversity and population structure of *P. falciparum* populations from a distinct region of the Kenyan–Ugandan border (Busia) in comparison to *P. falciparum* populations from two inland areas in Kenya, namely Msambweni in coastal Kenya and Nyando in western Kenya.

Methods

Ethical considerations

The study was approved by the Scientific & Ethics Review Unit (SERU) of Kenya Medical Research Institute (KEMRI) Nairobi (KEMRI/SERU/0152/3250 and SSC2276). Written informed consent was obtained from the parents/guardians of all children. All experiments were performed in accordance with good laboratory practice guidelines.

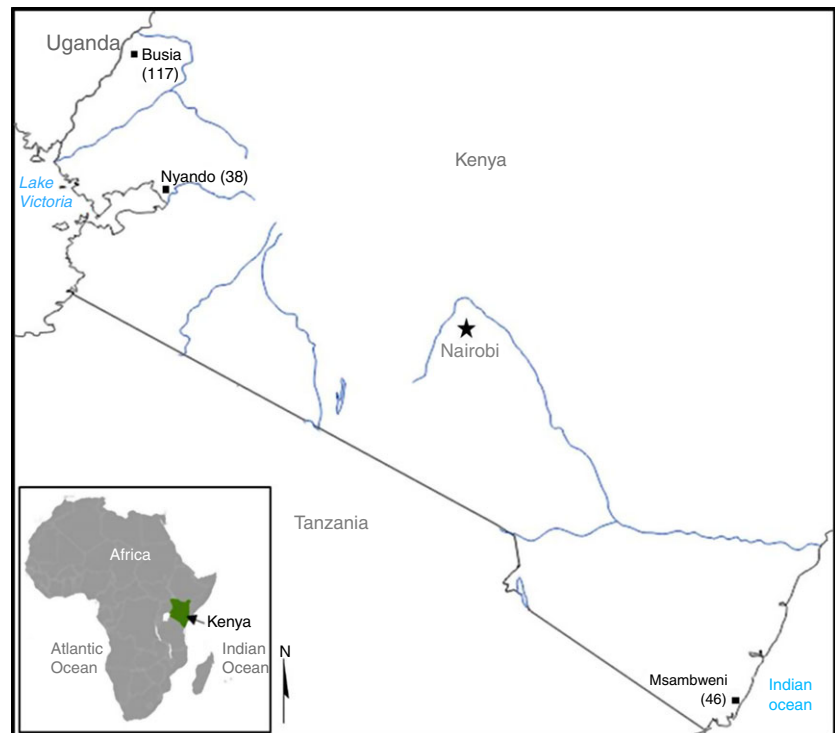
Study sites and study samples

The study was conducted in malaria-endemic areas situated in western (Busia and Nyando) and coastal (Msambweni) Kenya regions (Figure 1). Busia is located in Busia County and borders Uganda; Nyando is located in Kisumu County and Msambweni in Kwale county. According to the national population census of 2009, the population of Busia is 743 946 of Kisumu 968 909 and of Kwale 649 931 people [26]. Agriculture and fishing are the main economic activities of these counties owing to their proximity to Lake Victoria (Busia and Kisumu Counties) and the Indian Ocean (Kwale County).

Malaria transmission is perennial in the three study areas with peak incidences shortly after the rainy seasons (March to June and October to November). The prevalence of malaria in western Kenya ranges from 20% to 40% [27], whereas in the coastal region, it ranges between 5% and 20% [28]. Most of the malaria cases are due to *P. falciparum* infection. Co-infections of *P. falciparum* and other *Plasmodium* species (*P. malariae* and/or *P. ovale*) occur as well [29]. *P. vivax* is not present. Three *Anopheles* mosquito vectors transmit malaria in Kenya sympatrically; these include *A. gambiae sensu stricto*, *A. arabiensis* and *A. funestus* [30]. In the coastal region, however, *A. merus* is an important secondary vector for malaria transmission [30].

Two hundred and one *P. falciparum* isolates obtained from febrile children visiting health facilities in 2013 (Msambweni), 2015 (Nyando) and 2016 (Busia) were analysed in this study (Figure 1). Patients from all study sites were recruited during the rainy season. Parasitaemias ranged from >2000 to 200 000 parasites/ μ l as assessed by microscopy. Genomic DNA was extracted from dried blood spots on filter papers, except for Busia (EDTA whole blood), using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. A PCR method targeting the *P. falciparum* 18S ribosomal RNA (rRNA) gene was

Figure 1 Map of Kenya showing study sites. The sample size of each study site is shown in parentheses. Black lines: country borders, blue lines: rivers, star: the capital city of Kenya.



performed to confirm *P. falciparum* positivity as described previously [31].

Plasmodium falciparum microsatellite loci genotyping

Twelve neutral microsatellite (MS) loci were used to genotype *P. falciparum* isolates using a hemi-nested PCR method described previously [32, 33]. The MS loci included Poly- α (Chromosome, Chr, 4), TA81 (Chr 5), TA42 (Chr 5), TA87 (Chr 6), TA1 (Chr 6), TA109 (Chr 6), TA40 (Chr 10), 2490 (Chr 10), ARA2 (Chr 11), PfG377 (Chr 12), PfPK2 (Chr 12) and TA60 (Chr 13). From each sample, FAM, HEX and PET-labelled PCR products were pooled together with GeneScan™ 500 Liz size standard (Applied Biosystems, Foster City, CA, USA) for capillary electrophoresis by the 3130xl Genetic Analyzer (Applied Biosystems) platform. The Peak Scanner™ software version 1.0 (Applied Biosystems) was used to visualise the electropherograms and automatically determine MS allele lengths relative to the GeneScan™ 500 Liz size standard. MS alleles per locus were scored if the electropherogram peak height was ≥ 200 relative fluorescent units (RFU). Where more than one peak was observed per MS locus, only the minor peak(s) with $\geq 30\%$ height of the predominant allele peak was/were scored as additional allele(s). *P. falciparum* strain

3D7 genomic DNA and PCR grade water were used as a positive and negative controls, respectively, for each run.

Data analysis

As the asexual stages of *P. falciparum* are haploid, the number of multiple *P. falciparum* parasite strains in a given individual was determined by the number of alleles he/she carries among the investigated MS loci [32]. In brief, a single or monoclonal infection is defined by the observation of a single allele across all MS loci, whereas multiple or polyclonal infections are defined by the observation of > 1 allele in at least one MS locus. The MS locus with the highest number of alleles was used to determine the number of multiple infections of a sample. Kruskal–Wallis tests were applied to compare the mean number of infections between study sites. Chi-square tests were used to compare the proportion of multiple infections between study sites.

ARLEQUIN software version 3.5.2.2 (<http://cmpg.unibe.ch/software/arlequin35/>) was used to analyse the genetic diversity and population structure of Kenyan *P. falciparum* isolates using the predominant alleles. Genetic diversity was estimated by the allele frequencies, number of alleles per MS locus (A), allelic richness (A_R) and the expected heterozygosity (H_e). Since A is

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dependent on the sample size, A_R was calculated using the hierfstat package of the R software, based on the smallest sample size, to normalise data. H_e is the probability that any two MS locus alleles observed in a population are from different *P. falciparum* isolates. H_e was determined by using the formula $H_e = \frac{n}{n-1} (1 - \sum p_i^2)$ where p_i was the frequency of the different alleles of a given MS locus and n was the number of samples. The non-random association of MS loci was estimated by the multilocus linkage disequilibrium (LD). Multilocus LD was determined by the index of association (I_S^A) using LIAN software, <http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query> [34]. The paired Wright's fixation index (F_{ST}) and analyses of molecular variance (AMOVA) were used to assess the population structure of Kenyan *P. falciparum* isolates. The sum of genetic variability between parasite populations was classified and interpreted as follows: low differentiation, (F_{ST} value: 0–0.05), moderate differentiation (F_{ST} value: >0.05–0.15), great differentiation (F_{ST} value: >0.15–0.25) and very great differentiation (F_{ST} value: >0.25) [35]. Nei's genetic distance was used to assess the genetic relatedness among populations. Significant differences were observed at P -values < 0.05.

Results

Plasmodium falciparum genetic diversity

A total of 201 *P. falciparum* isolates were genotyped using 12 neutral microsatellite (MS) loci. High allelic

polymorphism was observed with an overall mean of 10.14 ± 1.07 alleles (Table 1). The total number of different alleles circulating at the various study sites did not differ significantly. Furthermore, the difference between the total number of different alleles and the mean allelic richness did not reach statistical significance. This showed that the sample size difference among study sites did not influence the determination of allelic polymorphism. On average, the MS locus TA40 had the highest number of alleles (17.33), while loci PfG377 and 2490 were the least polymorphic ones.

The genetic diversity of Kenyan *P. falciparum* isolates was estimated by calculating the expected heterozygosity (H_e) based on the allele frequencies of the predominant alleles as described in the Methods section. The overall H_e ranged from 0.45 (TA42) to 0.90 (TA40) and the mean H_e was 0.76 (Table 1). The mean H_e of individual study sites was not statistically different. Poly- α had the highest H_e (0.92 in Busia) whereas 2490 and PfG377 in Busia, as well as TA42 in Nyando, had the lowest H_e (0.4). Figure 2a,b show the distribution and frequency of alleles of the 12 MS loci. A few alleles were observed to be predominant in at least one study site compared to the others. Since the blood stages of *P. falciparum* are haploid, we used the MS locus with the highest number of alleles per sample to determine the number of infections per sample and calculated the mean number of infections as well as the proportion of samples with polyclonal infections as described in the Methods section. The overall mean of the number of infections was 1.8 (95% CI: 1.6–1.8) and the overall proportion of polyclonal

Table 1 Genetic diversity of *Plasmodium falciparum* isolates from western (Busia and Nyando) and coastal (Msambweni) Kenya

Loci	$A(A_R)$			H_e			Mean		
	Busia	Nyando	Msambweni	Busia	Nyando	Msambweni	A	A_R	H_e
Poly- α	18 (18)	18 (13.35)	11 (10.39)	0.92	0.88	0.85	15.67	13.91	0.88
PfPK2	14 (10)	10 (10.22)	13 (12.22)	0.87	0.73	0.90	12.33	10.81	0.83
TA81	10 (10)	10 (8.04)	8 (7.62)	0.85	0.87	0.80	9.33	8.55	0.84
ARAII	11 (9)	9 (8.52)	8 (7.82)	0.81	0.82	0.86	9.33	8.45	0.83
TA87	13 (10)	10 (10.62)	10 (9.77)	0.88	0.87	0.88	11	10.13	0.87
TA40	23 (18)	18 (14.53)	11 (10.47)	0.89	0.91	0.89	17.33	14.33	0.90
TA42	7 (7)	7 (5.24)	7 (6.42)	0.47	0.41	0.47	7	6.22	0.45
2490	5 (6)	6 (3.54)	6 (5.77)	0.42	0.58	0.67	5.67	5.14	0.56
TA1	13 (13)	13 (10.28)	9 (8.79)	0.86	0.87	0.87	11.67	10.69	0.87
TA60	10 (6)	6 (8)	7 (6.65)	0.80	0.83	0.78	7.67	6.88	0.80
TA109	13 (7)	7 (8.32)	8 (7.6)	0.81	0.78	0.81	9.33	7.64	0.80
PfG377	6 (5)	5 (4.5)	5 (4.5)	0.43	0.57	0.54	5.33	4.77	0.51
Mean	12 (10)	10 (8.77)	9 (8.19)	0.75	0.76	0.78	10.14	8.96	0.76
SEM	1.5 (1.3)	1.27 (0.95)	0.67 (0.63)	0.056	0.045	0.041	1.07	0.90	0.046

A, number of alleles; A_R , allelic richness; H_e , unbiased expected heterozygosity; SEM, standard error of mean, P -value > 0.05.

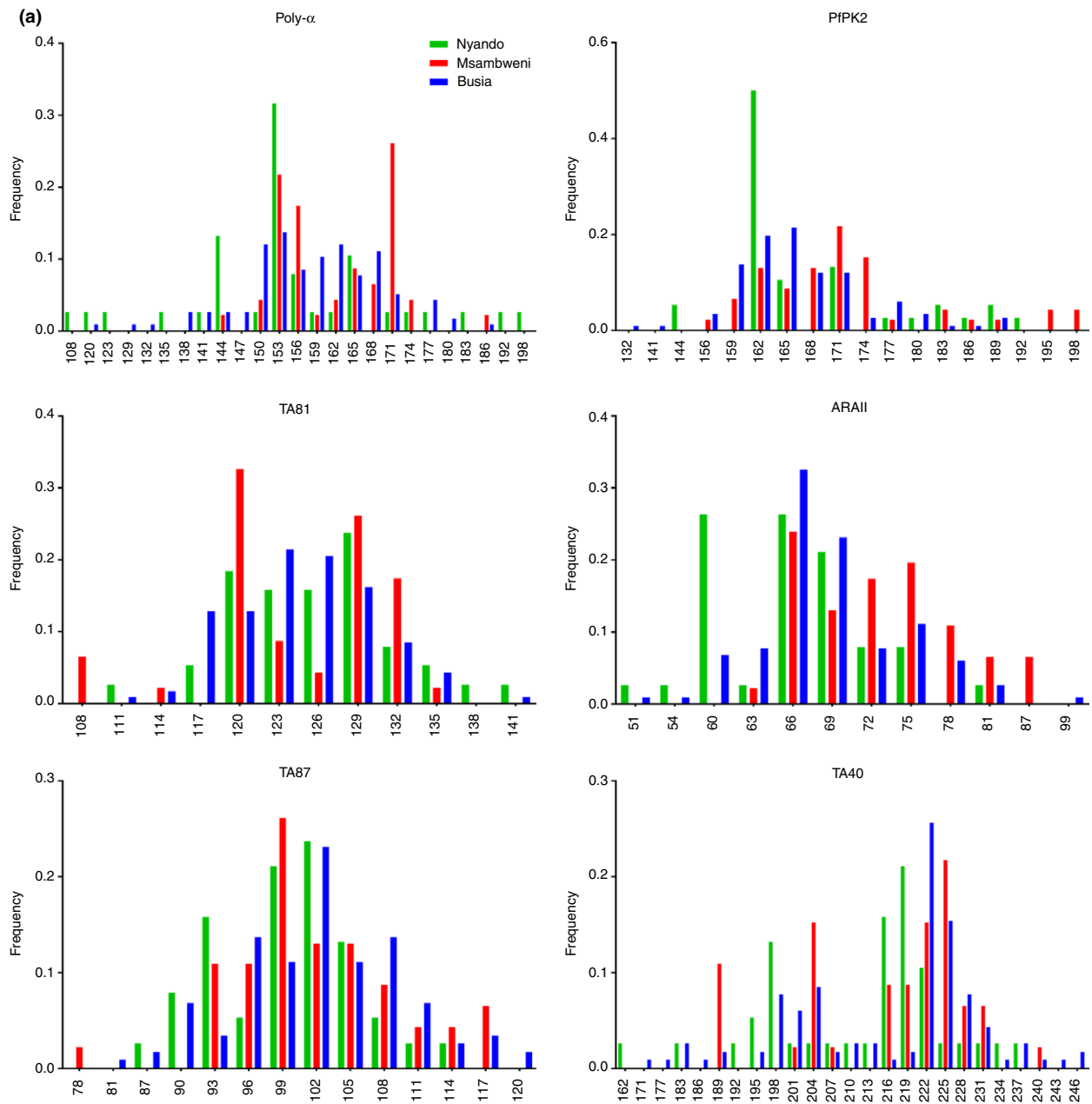
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Figure 2 Allele frequency and size variability of *Plasmodium falciparum* neutral microsatellite loci. X-axis is the normalised allele size (base pairs) and y-axis is the standardised proportion per locus.

infections was 51%. The mean of the number of infections and proportion of polyclonal infections was similar among the study sites ($P > 0.05$). The mean of the number of infections among the samples from Nyando, Busia and Msambweni was 1.6 (95% CI 1.3–1.9), 1.9 (95% CI

1.7–2.1) and 1.6 (95% CI 1.3–1.8) respectively. 45% of samples from Nyando, 56% from Busia and 43% from Msambweni had *P. falciparum* polyclonal infections.

Multilocus index of association analysis was performed to assess the non-random association of all MS loci in

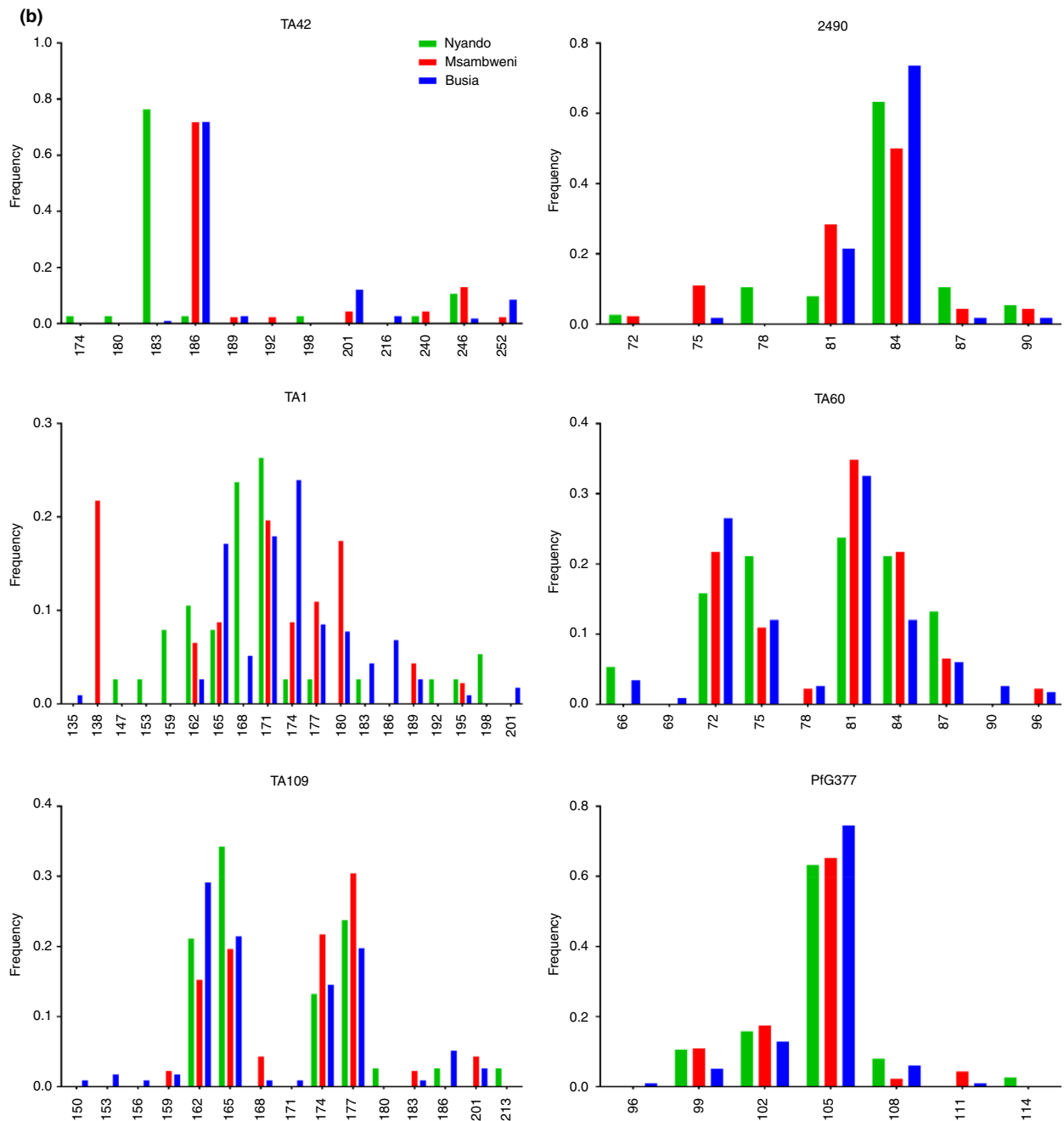


Figure 2 Continued

the individual study sites. Statistical significance of linkage disequilibria (LD) was tested using 10 000 Monte Carlo simulations. The LD ranged from 0 in Busia to 0.04 in Msambweni. Significant multilocus LD was only

observed in Msambweni (Table 2). This resulted from the repeated occurrence of four MS haplotypes. Three MS haplotypes were shared by two isolates (4%) each, whereas the remaining haplotype was shared by three

D. Nderu *et al.* Genetic diversity and population structure of *P. falciparum***Table 2** Multilocus linkage disequilibrium in *Plasmodium falciparum* isolates from Kenya

Test factor	Busia	Nyando	Msambweni
V_D	1.90	2.05	2.64
V_E	1.84	1.91	1.86
I_S^A	0.00	0.01	0.04
P -value	0.19	0.196	0.0001

Statistical significance at P -value < 0.05 . I_S^A , index of association calculated by Monte Carlo simulations at 10 000 per mutations under the null hypothesis $V_D = V_E$; V_D , observed variance; V_E , expected variance.

isolates (6%). There were no matching MS haplotypes in Busia and Nyando, indicating that these parasite populations are panmictic.

Genetic structure analyses performed by paired Wright's fixation index (F_{ST}) showed a significantly low genetic differentiation in Busia *vs.* Msambweni ($F_{ST} = 0.022$; $P < 0.0001$) and significant moderate genetic differentiation in Busia *vs.* Nyando ($F_{ST} = 0.076$; $P < 0.0001$) as well as in Nyando *vs.* Msambweni

($F_{ST} = 0.079$; $P < 0.0001$). This shows that there were differences in the frequency of alleles among the study sites. Analysis of molecular variance (AMOVA) also demonstrated significant genetic structuring of Kenya *P. falciparum* isolates ($P = 0.01$). Overall, 95% of the variance in allele frequencies observed was within populations, whereas 5% of the variance was observed across populations (Table 3). The highest allele frequency variance (8%) among paired study sites was observed in Busia *vs.* Nyando and Nyando *vs.* Msambweni, and the lowest allele frequency variance (2%) occurred in Busia *vs.* Msambweni (Table 4), indicating that the Kenyan *P. falciparum* populations studied are closely related. A short Nei's genetic distance and a high gene flow (number of migrants [Nm]) determined here support these findings. The Nei's genetic distance between Busia and Nyando was 0.294, 0.072 between Busia and Msambweni and 0.34 between Nyando and Msambweni. The overall Nm was 9.08 (Table 3). Interestingly, the Nm between Busia and Msambweni was 3.7-fold higher than between Busia and Nyando as well as between Nyando and Msambweni (Table 4).

Table 3 Analysis of molecular variance in Kenyan *Plasmodium falciparum* population (Busia, Nyando and Msambweni)

Source of variation	df	Sum of squares	Mean squares	Estimated variance	Percentage of variance (%)	Nm
Among Population	2	37.94	18.97	0.25	5	
Among individual	198	900.83	4.55	4.55	95	9.08
Within individual	201	0.00	0.00	0.00	0	
Total	401	938.77		4.80	100	

df, degrees of freedom; Nm, number of migrants; P -value = 0.01.

Table 4 Analysis of molecular variance in paired *Plasmodium falciparum* populations from Kenya

Populations	Source of variation	df	Sum of squares	Mean squares	Estimated variance	Percentage of variance (%)	Nm
Busia and Nyando	Among population	1	25.97	25.97	0.37	8	
	Among individual	153	691.15	4.52	4.52	92	6.04
	Within individual	155	0	0.00	0.00	0	
	Total	309	717.12		4.89	100	
Busia and Msambweni	Among population	1	11.26	11.26	0.10	2	
	Among individual	161	731.8	4.55	4.55	98	22.35
	Within individual	163	0	0	0	0	
	Total	325	743.06		4.65	100	
Nyando and Msambweni	Among population	1	21.03	21.03	0.39	8	
	Among individual	82	378.7	4.62	4.62	92	5.86
	Within individual	84	0	0.00	0	0	
	Total	167	399.73		5.01	100	

df, degrees of freedom; Nm, number of migrants; P -value = 0.01.

Discussion

Malaria control strategies have changed tremendously over the last three decades with the sole aim of reducing the disease burden and safeguard gains, particularly in sub-Saharan Africa where 90% of the global disease burden occurs [30, 36]. The introduction and/or withdrawal of malaria interventions have shaped *P. falciparum* genetic factors. Insight into the genetic diversity and population structure of *P. falciparum* populations is vital for refinement of malaria control and elimination strategies. This study describes the genetic diversity and population structure of *P. falciparum* in the Kenyan–Ugandan border region (Busia, Busia County) in comparison to two inland malaria-endemic sites of Kenya, Nyando (western Kenya) and Msambweni (coastal Kenya).

Unexpectedly, we observed a lower proportion of polyclonal infections (51%) than previously reported in western Kenya. In 2018, Zhong *et al.* reported the prevalence of polyclonal infections to be 75% in western Kenya [37]. This was a slight reduction from the $\geq 80\%$ prevalence reported by previous studies [13, 14, 19, 38]. Since the prevalence of polyclonal infections is proxies of malaria transmission, our findings suggest that malaria transmission intensity has decreased in line with the lower malaria prevalence in Kenya over the last three decades [4]. However, this trend has not been observed in the genetic diversity of the Kenyan *P. falciparum* population as exemplified in the present and in previous studies [13, 14, 19, 20, 38].

High *P. falciparum* genetic diversity has remained unchanged in Kenya even after the introduction of artemisinin-based combination therapy (ACT) and insecticide-treated nets (ITN) [12–14]. In fact, the parasite genetic diversity increased after ACTs were introduced [12]. Explanations for the persisting high genetic diversity are the increasing number of asymptomatic malaria cases with higher gametocytaemias, vector resistance against pyrethroids which sustain transmission and the removal of anti-malarial drug selection pressure following the replacement of the less effective sulphadoxine–pyrimethamine in 2006 by the highly efficacious ACT [8, 12, 15, 16, 39, 40]. It is also worth noting that the *P. falciparum* genetic diversity reported here is similar to the genetic diversity of parasite populations from other countries in sub-Saharan Africa [22, 41], and it is higher than the genetic diversity of populations from low malaria-endemic settings in the Pacific Region, Southeast Asia and South America [22, 42–46].

Multilocus linkage disequilibrium (LD) analysis revealed that *P. falciparum* populations in the Kenyan–Ugandan border region and in Nyando are panmictic. This was

demonstrated by the lack of a significant index of association (I_S^A). In contrast, a significant I_S^A was observed in the *P. falciparum* population from Msambweni with the repeated occurrence of four MS haplotypes. This indicates inbreeding in Msambweni and respective absence at the Kenyan–Ugandan border and in Nyando. The level of multilocus non-random association in this study is lower than previously reported in areas with intense transmission, including Kenya [13, 14, 20, 22, 38]. The recent increase (2011–2014) and subsequent decline (from 2015 on) of malaria prevalence are likely to have altered the frequency of minor alleles [4, 5], thus contributing to the lower LD observed here. This is demonstrated by significant multilocus LD in Asembo, western Kenya before deployment of ITNs in 1996, followed by non-significant and significant multilocus LDs in 2001 and 2007, respectively [13, 14]. The lower parasite prevalence in coastal (10–29%) and western ($\geq 30\%$) Kenya offers an alternative explanation for the observed significant multilocus LD in Msambweni, which was not seen in Busia and Nyando [4]. Apparently, parasite inbreeding increases with a reduction transmission [22].

The distance from Busia to Nyando is 163 km, and from Busia to Msambweni, it is 987 km. However, our data show that the parasite populations from these areas are not isolated as previously described in Africa [22]. This becomes apparent by low to moderate genetic differentiation and Nei's genetic distance of parasite populations (Busia and Msambweni) separated by the longest geographical distance. These results are also supported by the low allele frequency variance (2%) in Busia *vs.* Msambweni compared to 8% in both Busia *vs.* Nyando and Nyando *vs.* Msambweni. In 2016, Ingasia *et al.* reported similar findings in Kenyan *P. falciparum* populations separated by a similar geographical distance (≈ 1000 km) as populations from Busia and Msambweni which have a low genetic differentiation ($F_{ST} = 0.027$; $P < 0.0001$) [20]. Busia is a socio-economically important gateway to Uganda, a landlocked country. It accounts for most of human travel between Kenya and Uganda. Therefore, parasite migration via humans is the likely reason for the limited genetic structure observed here [20, 47]. The high parasite migration ($Nm > 3$) in this study shows that there are limited barriers to hinder genetic flow, particularly between the Kenyan–Ugandan border region and Msambweni. Studies conducted in the islands of Lake Victoria and Vanuatu demonstrate human-mediated *P. falciparum* dispersal and gene flow [21, 48]. The existence of high *P. falciparum* effective population sizes in sub-Saharan Africa offers an additional explanation for the weak parasite population structure observed in this study [22].

In conclusion, this study shows that the genetic diversity of *P. falciparum* populations in the Kenyan–Ugandan border area and inland Kenya is similar. The high panmixia and limited genetic population structure highlight the genetic fitness of Kenyan *P. falciparum* populations to counter the impact of current malaria control interventions. Therefore, continued surveillance of *P. falciparum* genetics and prompt refinement of malaria control and elimination strategies is needed to accelerate the realisation of a malaria-free Kenya.

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Corresponding Author Thirumalaisamy P. Velavan, Institute of Tropical Medicine, Wilhelmstrasse 27, 72074 Tübingen, Germany. Tel.: +49 7071 2985981; Fax: +49 7071 294684; E-mail: velavan@medizin.uni-tuebingen.de

3. DISCUSSION

Recent stagnation of malaria reduction has heightened discussions on the approach and efficacy of current malaria control strategies as well as *P. falciparum* genetic diversity in an effort to refine and adapt malaria control/ elimination programmes. This dissertation describes the diagnostic performance of PfHRP2/pLDH-based rapid diagnostic test (RDT) and genetic diversity of *P. falciparum* populations in Kenya.

3.1. Comparative evaluation of Carestart™ PfHRP2/pLDH (Pf) RDT

The Carestart™ PfHRP2/pLDH (Pf) RDT is one of the RDTs currently deployed by the national malaria control programme of Kenya to promote testing and prompt treatment of confirmed cases of malaria with antimalarial drugs, especially with artemisinin-based combined therapies. The first Chapter of my dissertation documents that Carestart™ has acceptable diagnostic performance in Kenya and in congruence with previous studies (Wanja et al., 2016, Osanjo et al., 2017, Adu-Gyasi et al., 2018). However, high false positive rate and low positive predictive values were observed. This could have been caused by the detection of persisting PfHRP2 after parasite clearance. On average, it takes 35 to 45 days for a patient to become negative after parasite clearance (Grandesso et al., 2016, Dalrymple et al., 2018). RDT false positives may also be attributed to PfHRP2 from *P. falciparum* sub-microscopic infections and/or gametocytes, the sexual stages of *Plasmodium* parasites. The consequences of RDT false positivity include over prescription of antimalarial drug, overestimation of malaria burden and impaired diagnosis of the cause(s) of febrile illnesses. Even though simultaneous RDT detection of PfHRP2 and pLDH could help avert this bottleneck as well as discern current and convalescent infections, PfHRP2- and PfHRP2/pLDH-based RDTs have comparable false positive rate (Adu-Gyasi et

al., 2018). RDTs targeting PfHRP2 are preferred to pLDH-based RDTs because of superior sensitivity and thermal stability (Li et al., 2017, Chiodini et al., 2007).

In our investigations, microscopy, the gold standard for malaria diagnosis, had a limited of detection (LOD) of 80 parasites/ μ l. The LOD by microscopy may have influenced the sensitivity of Carestart™ RDT in the present study (Wanja et al., 2016). This is likely owing to the plausible occurrence of sub-microscopic *P. falciparum* infections with detectable PfHRP2 (Bell et al., 2005). In fact, we identified seven RDT and nested PCR (nPCR) positive cases missed by microscopy supporting this possibility and reiterating that microscopy is an imperfect gold standard (Kattenberg et al., 2011). The evaluation of Carestart™ with nested PCR as the reference method showed minimal changes in the RDT sensitivity and specificity. This is inconsistent with previous studies, thereby implying nested PCR performance in our study was comparable to microscopy (Swarthout et al., 2007, Wanja et al., 2016). Nevertheless, high negative predictive value and low negative likelihood ratio clearly demonstrate that negative Carestart™ RDT results can be used to rule out *P. falciparum* infections with sufficient confidence (Osanjo et al., 2017, Wanja et al., 2016, Singh et al., 2010).

Of note, seven falciparum malaria cases with parasitemias ranging from 320 to 680 parasites/ μ l were not detected by Carestart PfHRP2/pLDH RDT. Detection of *P. falciparum* infections with similar parasitemias in this study suggests that RDT false negativity could have been caused by other factors other than low parasitemia. Parasite genetics has been identified as a key factor that may alter RDT sensitivity, for instance, PfHRP2 gene deletion or diversity (Baker et al., 2005, Gamboa et al., 2010). The relationship between PfHRP2 diversity and RDT false negativity could not

be investigated in this study since Carestart™ RDT detects both PfHRP2 and pLDH in a single test line. Despite this, our findings show that Carestart™ PfHRP2/pLDH (Pf) RDT is still an effective diagnostic tool for malaria surveillance in Kenya.

3.2. *P. falciparum* histidine-rich proteins 2 and 3 diversity in Kenya

Diversity of PfHRP2 is currently recognised as a threat to the sensitivity of PfHRP2-based RDTs, particularly *pfhrp2/3* deletion (Gendrot et al., 2018, Gamboa et al., 2010). With increasing cases of false negativity being associated with *pfhrp2/3* deletion, national malaria control programmes and health care providers require information on the status of PfHRP2 diversity (Gendrot et al., 2018). This will promote correct interpretation of RDT negative results.

In Chapter two, we characterised PfHRP2 diversity of *P. falciparum* isolates collected in western and coastal Kenya from 2007 to 2016. This study demonstrated high PfHRP2 diversity in the Kenyan *P. falciparum* population with 94% of the PfHRP2 sequences occurring only once as reported elsewhere (Baker et al., 2010, Baker et al., 2005). This heterogeneity emanated from differences in the occurrence, number and organisation of PfHRP2 amino acids repeat types between isolates. Despite the apparent high PfHRP2 diversity, there were noticeable similarities among the *P. falciparum* isolates. These included the complete absence of PfHRP2 repeat types 9 and 11, the omnipresence of repeat types 2 (AHHAHHAAD) and 7 (AHHAAD) and most of the PfHRP2 sequences started and terminated with repeat types 1 and 12, respectively. We also identified a semi-conserved PfHRP2 amino acid repeat motif comprising repeat types 2, 3, 5, 7, 8, 2 and 7 in 50% of the isolates (Baker et al., 2010). A further 27% of the isolates had a motif, comprising repeat types 7, 8, 2 and 7,

frequently observed in Malagasy isolates (Mariette et al., 2008). However, a motif comprising repeat types 2, 4, 5, 6, 7, and 8 observed in Indian isolates was absent among Kenyan isolates (Kumar et al., 2012).

The frequency of thirteen previously identified PfHRP2 repeat types in *P. falciparum* populations from the different study sites in western and coastal Kenya was comparable (Baker et al., 2010). Repeats types, 1 to 8, 10 and 12, were highly prevalent (>80%) whereas repeat types 13 and 14 were rare ($\leq 8\%$) akin to isolates from other geographical settings (Atroosh et al., 2015, Deme et al., 2014, Bharti et al., 2017, Li et al., 2015, Wurtz et al., 2013b). In Senegal, however, repeat type 12 is uncommon, unlike the present study (Wurtz et al., 2013b). Repeat type 19 was observed for the first time in three Kenyan isolates. Besides Kenya, repeat type 19 has been observed in a few other malaria-endemic countries, namely Senegal, Uganda, Mali and the Philippines (Baker et al., 2010, Deme et al., 2014, Bharti et al., 2017). The similarities between PfHRP2 in our study and studies from other countries imply that monoclonal antibodies (mAbs) incorporated in RDTs recognise the same or similar PfHRP2 epitopes. This is probably because there is a limited number of commercially available PfHRP2-specific mAbs (Lee et al., 2006, Lee et al., 2012). Further, it provides a plausible explanation for comparable sensitivity of Carestart™ RDT in this study and recent studies (Osanjo et al., 2017, Wanja et al., 2016).

PfHRP2 repeat types 2 and 7 are most likely to influence the sensitivity of RDTs targeting PfHRP2. This is because they constitute >50% of total number repeat types per isolate and are therefore the most likely contribute to the epitopes involved in PfHRP2 detection (Baker et al., 2005). Baker et al. initial findings suggested that the

sensitivity of PfHRP2-based RDT is dependent on the number repeat type 2 multiplied by the number of repeat type 7 (type 2 × type 7) at parasitemias ≤ 250 parasites/ μ l. Using a binary logistic regression model (Bakers' regression model), Baker et al. showed that *P. falciparum* isolates with type 2 × type 7 value below 43 were not detectable at this threshold and thus could predict RDT false negativity (Baker et al., 2005). A study from India and another case study from Senegal supported this finding (Wurtz et al., 2013a, Kumar et al., 2012). However, analyses of larger and more diverse field samples have revealed that the Bakers' model cannot exclusively explain RDT false negativity (Baker et al., 2010). Low PfHRP2 levels could be an alternative explanation (Plucinski et al., 2019).

In this study, the relationship between PfHRP2 levels and PfHRP2 diversity and parasite density was also examined. We observed that PfHRP2 levels in blood samples from *P. falciparum*-infected individuals does not correlate with *P. falciparum* parasitemia, type 2 × type 7 values and the total number of PfHRP2 repeats per isolate. These findings are not surprising because of existing technical difficulties that could impede robust analyses, namely *P. falciparum* infections are not synchronised and PfHRP2 concentration in blood samples reflect that total parasite biomass (Dondorp et al., 2005, Tjitra et al., 2001). Furthermore, PfHRP2-specific mAbs cross-reactivity with PfHRP3 could also have undermined our analyses, particularly at parasite densities >1000 parasites/ μ l. This phenomenon is hypothesised to be driven by similarity between PfHRP2 and PfHRP3. Both protein have amino acid repeat types 1, 2, 4 and 7 (Lee et al., 2006, Beshir et al., 2017).

Unlike PfHRP2, PfHRP3 was less diverse in the present study in line with previous studies (Baker et al., 2005, Wurtz et al., 2013b). This is exemplified by a lower proportion of isolates with distinct PfHRP3 repeat type profile and omnipresence of previously described repeat types. In addition, PfHRP3 amino acid repeats types were organised into two semi-conserved repetitive motifs separated by a single non-repetitive region. Isolates from other parts of the world exhibit a similar characteristic (Baker et al., 2010, Bharti et al., 2017). There are, however, a few isolates from Africa, the Southwest Pacific region and Asia with two non-repetitive regions intervening PfHRP3 repetitive regions (Baker et al., 2010, Bharti et al., 2017).

A total of 78 novel repeat types were identified in this study. Of these, 50 and 28 were identified in PfHRP2 and PfHRP3, respectively. These repeat types resulted from the replacement of at least one amino acid and had low frequencies. This is the first report of this phenomenon in sub-Saharan Africa. Similar findings have been reported in the Chinese-Myanmar border and India (Bharti et al., 2017, Li et al., 2015). In both instances, the novel repeat types arose from the replacement of a single amino acid unlike in the present study. Only four out of 78 novel repeat types identified in this study have been reported previously (Bharti et al., 2017, Li et al., 2015). These amino acid repeat types are THH, AHHAPD, AHHASY and SHHDG. The limited geographic distribution and low frequencies of the novel repeat types suggest that they may not have a profound impact on the diagnostic performance of RDTs targeting PfHRP2. Emergence of the novel repeat types also signals expanding PfHRP2 and PfHRP3 diversity.

Existence of *P. falciparum* isolates lacking the genes encoding PfHRP2 and PfHRP3 in natural parasite population represents an emerging challenge for falciparum malaria diagnosis. These parasites have been observed in several malaria-endemic countries in Africa, Asia, South- and Central-America (Gendrot et al., 2018). None of the *P. falciparum* isolates screened in this study lacked either *pfhrp2* or *pfhrp3* (PfHRP3 gene) contrary to a recent report showing *pfhrp2* deletion in a few Kenyan isolates (Beshir et al., 2017). The study by Beshir et al involved samples collected in 2014, seven years after our sample collection, from one of the study sites (Mbita) included in the current study. Findings from the current study as well as Beshir et al. suggest that *pfhrp2* deletion in Kenyan isolates occurred recently. These findings also indicate that *pfhrp2/3* deletion does not pose an immediate threat to falciparum malaria diagnosis by PfHRP2-based RDTs in Kenya. It is important to bear in mind that estimation of the prevalence *pfhrp2/3* deletion in malaria-endemic settings is determined by the proportion of multiple infections (Sepúlveda et al., 2018, Cheng et al., 2014).

3.3. Genetic diversity and population structure of *P. falciparum* in Kenyan-Ugandan border areas.

Chapter three estimated the probability of identifying *pfhrp2/3* deletion and determined of the number of infections per individual, the proportion of multiple polyclonal infections, the genetic diversity and population structure of *P. falciparum* in the Kenyan-Ugandan borders areas by genotyping 12 neutral microsatellite (MS) loci distributed across the genome of malaria parasites. The number of alleles per MS locus indicated the number of infections per individual. This is because *P. falciparum* asexual stages are haploid. Monoclonal infections were defined by the observation of

only one allele for all MS loci whereas polyclonal infections were defined by the observation of >1 allele in at least one MS locus. Furthermore, the proportion of monoclonal infections was used to estimate the probability of identifying isolates lacking *pfhrp2/3*.

The prevalence of multiple infections in the current study was 51%. This indicates that the probability of identifying *pfhrp2/3* deletion in parasite populations from Kenya was 49%. It also shows that the prevalence of multiple infections in the study sites investigated here was surprisingly lower than reported previously. In 2018, Zhong et al. reported that the proportion of polyclonal infections was 75% (Zhong et al., 2018); a slight decline from the ≥80% prevalence reported in earlier studies (Bonizzoni et al., 2009, Zhong et al., 2007, Gatei et al., 2015, Gatei et al., 2010). Since the prevalence of multiple infections is a proxy for malaria transmission intensity, our findings suggest that transmission has declined in agreement with reduction in malaria parasitemia prevalence over the last three decades (Macharia et al., 2018).

We have shown that *P. falciparum* genetic diversity in Kenya has, however, remained unchanged, regardless of changing malaria control strategies, at levels comparable to that of parasite populations from other areas in sub-Saharan Africa (Zhong et al., 2007, Bonizzoni et al., 2009, Ingasia et al., 2016, Gatei et al., 2015, Gatei et al., 2010, Anderson et al., 2000, Oyebola et al., 2014). Temporal analyses have demonstrated high and stable genetic diversity even after the introduction of artemisinin-based combination therapy (ACT) and insecticide-treated nets (ITN) (Gatei et al., 2015, Gatei et al., 2010, Chebon et al., 2016). This shows that current malaria interventions have not reduced *P. falciparum* genetic diversity. In fact, *P. falciparum* diversity

increased after ACTs replaced the less efficacious antimalarial drug, sulphadoxine–pyrimethamine (SP), in Kenya due to reduced drug selection pressure (Chebon et al., 2016). Increasing *Anopheles* mosquito resistance to pyrethroids and large asymptomatic parasite reservoir with higher gametocytaemia are plausible explanations for the persistent high *P. falciparum* genetic diversity (Ototo et al., 2017, Zhou et al., 2016, MOH, 2016, Wanjala and Kweka, 2018, Ondeto et al., 2017).

Parasite populations from Nyando and the Kenya–Ugandan border area are panmictic as demonstrated by random association of multiple loci in the present study except Msambweni where significant non–random association (index of association [I_S^A]) was observed. Repeated occurrence of four MS haplotypes suggest that *P. falciparum* inbreeding occurs in Msambweni. The level of non–random association observed in Msambweni is lower than previously reported in studies from malaria–endemic regions including Kenya (Gatei et al., 2015, Gatei et al., 2010, Ingasia et al., 2016, Anderson et al., 2000, Zhong et al., 2007). Recent changes in malaria prevalence could have altered the frequency of minor alleles and caused non–random association of MS alleles in the present study (Snow et al., 2015, Macharia et al., 2018). This hypothesis is supported by the observation of significant multilocus linkage disequilibrium (LD) in 1996 and 2007 after the deployment of insecticide–treated nets (ITNs) in Kenya (Gatei et al., 2015, Gatei et al., 2010). Lower malaria prevalence in coastal Kenya than in the Lake Victoria region may have also contributed to the significant LD in Msambweni and lack thereof in Busia and Nyando (Macharia et al., 2018). It is apparent that parasite inbreeding and malaria transmission intensity are inversely related (Anderson et al., 2000).

Kenyan *P. falciparum* populations investigated here are connected despite being geographically distant (163–1000 Km) as previously described in Africa (Anderson et al., 2000). This was shown by low to moderate genetic differentiation and small Nei's genetic distance. The parasite populations, Busia vs Msambweni, separated by the longest distance (1000 Km) were more genetically close than Busia vs Nyando (163 Km) and Nyando vs Msambweni (877 Km). This is congruent with the low allele frequency variance observed in Busia vs Msambweni (2%) compared to 8% of both Busia vs Nyando and Nyando vs Msambweni. Comparable findings have been reported in Kenyan *P. falciparum* populations separated by a similar geographical distance as populations from Busia and Msambweni (Ingasia et al., 2016). High human-mediated *P. falciparum* migration accounts for the weak parasite population fragmentation in the present and previous studies (Ingasia et al., 2016, Schultz et al., 2010). This is likely because Busia is an economically important gateway to Uganda, a landlocked country, and accounts for most of the human travel between Kenya and Uganda. Thus, implying that *P. falciparum* populations in Kenya have limited gene flow barriers. Human-mediated parasite dispersal and gene flow have been reported previously in the islands of Lake Victoria and Vanuatu (Lum et al., 2004, Mulenge et al., 2016). The existence of large *P. falciparum* effective population sizes in sub-Saharan Africa further explains the weak parasite population fragmentation in our study (Anderson et al., 2000).

In conclusion, malaria remains a major public health burden in the tropics where it claims a child below five years every two minutes. Current malaria control programmes are failing in some regions due to emergence of resistance against antimalaria drugs and insecticides as well as *P. falciparum* lacking gene encoding antigen targeted by

malaria RDTs. My dissertation aimed to comparatively evaluate the performance of a malaria RDT and describe *P. falciparum* diversity in Kenya. The findings of the first and second publications demonstrate that CareStart™ HRP2/pLDH(Pf) RDT is still an effective diagnostic tool for the malaria surveillance program and *pfhrp2/3* exon 2 deletion does not pose an immediate threat to malaria RDT diagnosis in Kenya. They also highlight the extensive diversity of both PfHR2 and PfHRP3. The findings of the third publication show *P. falciparum* populations in Kenyan-Ugandan border areas are genetic diverse, panmictic and have a weak population structure. These findings highlight the fitness of Kenyan *P. falciparum* populations to withstand malaria control interventions. Collectively, this dissertation provides information to promote prompt refinement of malaria control and elimination strategies and recommends continued surveillance of *P. falciparum* genetics *en route* to a malaria-free Kenya.

4. SUMMARY

Malaria is a public health burden in Kenya and *P. falciparum* contributes largely towards malaria related mortality and morbidity. In recent years, rapid diagnostic tests (RDT) that target *P. falciparum* histidine-rich protein 2 (PfHRP2) have been widely employed for malaria diagnosis. The circulating *P. falciparum* isolates lacking both *pfhrp2* and *pfhrp3* threaten the effectiveness of RDT malaria diagnosis based on *pfhrp2*. Against this background, utilizing a Kenyan study cohort, this dissertation (i) evaluated malaria diagnosis by RDT (Carestart™ PfHRP2/pLDH (Pf) RDT) against microscopy and nucleic acid testing methods; (ii) characterized and interpreted *pfhrp2* and *pfhrp3* among circulating parasites; (iii) determined *P. falciparum* multiple infection rate, genetic diversity and population structure in western and coastal Kenya. The investigations revealed CareStart™ RDT was comparable with other reliable methodologies. This RDT was demonstrated to be an effective tool for malaria diagnosis. Among endemic regions, especially in western and coastal Kenya, *pfhrp2* and *pfhrp3* omnipresence as well as a high parasite genetic diversity and limited population structure were documented. Collectively, these findings will be useful in understanding the performance of PfHRP2-based RDTs in Kenya and their dependability as tools in malaria control initiatives.

5. GERMAN SUMMARY (ZUSAMMENFASSUNG)

Malaria ist eine Belastung für die öffentliche Gesundheit in Kenia, und *P. falciparum* trägt in hohem Maße zur Sterblichkeit und Morbidität im Zusammenhang mit Malaria bei. In den letzten Jahren wurden weit verbreitet Schnelltests (rapid diagnostic tests „RDT“), die das histidinreiche Protein 2 (*P. falciparum* histidine-rich protein 2; PfHRP2) des *P. falciparum* erkennen, für die Malariadiagnose eingesetzt. Im Umlauf befindliche *P. falciparum*-Stämme, die weder Pfhrp2 noch Pfhrp3 besitzen, bedrohen die Malariadiagnose durch RDT. Vor diesem Hintergrund und unter Verwendung einer kenianischen Studienkohorte (i) bewertet diese Dissertation die Malariadiagnose mittels RDT (Carestart™ PfHRP2/pLDH (Pf) RDT) anhand von Mikroskopie- und Nukleinsäuretestmethoden; (ii) charakterisiert und interpretiert sie *pfhrp2* und *pfhrp3* der verbreiteten Parasiten; (iii) ermittelt sie Mehrfachinfektionsraten, genetische Vielfalt und Populationsstruktur der *P. falciparum* im Westen und an der Küste Kenias. Die Untersuchungen ergaben, dass die RDT mit anderen zuverlässigen Methoden vergleichbar sind. RDTs haben sich als wirksames Instrument für die Malariadiagnose erwiesen. Parasiten beobachtet wurden. In den endemischen Regionen, insbesondere im Westen und an der Küste Kenias, wurde die Allgegenwart der Pfhrp2 noch Pfhrp3 sowie eine hohe genetische Vielfalt und eine begrenzte Populationsstruktur der Parasiten dokumentiert. Zusammengefasst, werden diese Ergebnisse hilfreich sein, um die Effektivität von PfHRP2-basierten RDTs in Kenia und infolgedessen ihre Zuverlässigkeit als Diagnosemittel bei Initiativen zur Malariakontrolle zu verstehen

6. BIBLIOGRAPHY

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7. DECLARATION OF CONTRIBUTIONS

The doctoral dissertation entitled “*Plasmodium falciparum* genetic diversity and malaria diagnosis in Kenyan population” is now submitted to the members of the PhD Board at the Faculty of Medicine, University of Tübingen is a record of an original work by Mr. David Waweru Nderu and by co-authors from the Institute of Tropical Medicine, University of Tübingen, Kenya Medical Research Institute, Technical University of Kenya and Kirinyaga University. Three publications (Publication Nr.1: Parasitology International 2018 Aug 20;67(6):793-799. PMID: 30138695; Publication Nr.2: Scientific Reports. 2019 Feb 8;9(1):1709. PMID: 30737461; Publication Nr.3: Tropical Medicine and International Health. 2019 Feb 28. PMID: 30816614) are accomplished by Mr. Nderu as a first author and remains the backbone of this dissertation. We declare that Mr. David Waweru Nderu contributed substantially to all three manuscripts with respect to study design, experimental design, data analyses and writing of the manuscripts. We also state the individual contribution of all the authors in each publication as follows;

Contributions of PhD candidate and other co-authors:

Publication 1: David Waweru Nderu performed molecular experiments and drafted the manuscript. Thirumalaisamy P. Velavan designed and supervised the experiments and data analysis. Francis Kimani coordinated sample collection and logistics in the field. Kelvin Thiong’o, Maureen Akinyi and Evaline Karanja contributed in the field diagnosis and sample collection. Thirumalaisamy P. Velavan and Christian G. Meyer contributed in writing the manuscript. Christian G. Meyer reviewed and edited the manuscript. All authors approved the final manuscript.

Publication 2: David Waweru Nderu performed molecular experiments and drafted the manuscript. Thirumalaisamy P. Velavan designed and supervised the experiments and data analysis. Francis Kimani coordinated sample collection and logistics in the field. Kelvin Thiong’o, Eva Nambati, Maureen Akinyi, Edwin Too, William Chege and Evaline Karanja contributed in the field diagnosis, sample collection, processing and shipment. Thirumalaisamy P. Velavan contributed in writing the manuscript. Christian G. Meyer reviewed and edited the manuscript. All authors approved the final manuscript.

Publication 3: David Waweru Nderu performed molecular experiments, data analysis and drafted the manuscript. Thirumalaisamy P. Velavan designed and supervised the experiments and interpreted the data. Francis Kimani and Laura N. Wangai coordinated sample collection and logistics in the field. Eva Nambati, Kelvin Thiong’o, Maureen Akinyi, Edwin Too, William Chege and Evaline Karanja contributed in the field diagnosis, sample collection, processing and shipment. Thirumalaisamy P. Velavan contributed in writing the manuscript. Christian G. Meyer reviewed and edited the manuscript. All authors approved the final manuscript.

Yours sincerely,

David Waweru Nderu
(PhD candidate)



Prof. Dr. Thirumalaisamy P. Velavan
(Primary supervisor)



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David Waweru Nderu