High-throughput molecular tests for routine surveillance of malaria potentially missed by rapid diagnostic tests

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Dekan

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Summary

Malaria remains one of the most common infectious diseases in the world and still a public health problem particularly in sub-Saharan Africa despite the remarkable progress made during the past decades in cutting the number of cases by around 50 %. Improvement in diagnosis and the introduction of artemisinin-based therapies have contributed substantially to this development.

Currently available diagnosis tools for the identification of *P. falciparum* include the gold standard TBS microscopy and RDT. Despite many advantages, currently deployed RDTs have severe limitations in detecting low-density parasitemia. *P. falciparum* isolates lacking the *hrp*2 and *hrp*3 genes escape RDT detection posing a serious threat to the currently deployed test-treat-track approach for malaria recommended by WHO. False positive RDTs based on circulating HRP2 protein after successful asexual blood stage clearance following treatment have been described. RDTs with high sensitivity and specificity for monitoring non-falciparum species are missing. Therefore, improved diagnostic tools for rapid surveillance of malaria are essential for approaching the final aim of malaria elimination.

The main goal of this PhD thesis is to develop, validate and implement novel tools and techniques for high-throughput molecular detection that will support a comprehensive surveillance of malaria by (i) measuring prevalence of *P. falciparum* parasites potentially escaping detection by RDTs, (ii) monitoring of *kelch13* gene mutations conferring artemisinin resistance, (iii) monitoring impact of preventive treatment programs in pregnant women, (iv) understand the interaction of co-infections like HIV on *P. falciparum*, (v) assess the epidemiology of *Plasmodium* spp. co-infections with *P. falciparum*.

Manuscript 1: Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests

This manuscript describes the use of malaria rapid diagnostic tests (RDTs) strips as a source for total nucleic acids at large-scale in order to screen for SNPs in an artimisinin-associated drug resistance marker among thousands of healthy, malaria asymptomatic individuals.

We compared four extraction protocols side by side and focused on developing the one with superior performance and taking into account the cost and ability to co-extract RNA.

Next, we developed the ENAR (Extraction of Nucleic Acids from RDTs) approach for large-scale molecular malaria surveillance using thousands of RDTs collected during the malaria indicator survey (MIS) 2018 on Bioko Island, Equatorial Guinea. We found several, known and new, nonsynonymous SNPs in the propeller region of the *kelch 13* gene among isolates circulating on the Island.

Manuscript 2: ELIMU-MDx: A Web-Based, Open-Source Platform for Storage, Management and Analysis of Diagnostic qPCR Data

In order to manage properly the vast amount of qPCR data generated during Controlled Human Malaria Infections and other epidemiological studies, we developed a web-based and open-source software for storage, management and analysis of diagnostic qPCR data.

The ELIMU platform is being used in house for large-scale analysis and interpretation of diagnostic qPCR data.

Manuscript 3: A multiplex qPCR approach for detection of *pfhrp2* and *pfhrp3* gene deletions in multiple strain infections of *Plasmodium falciparum*

Here, we developed a qPCR-based assay suitable for high-throughput identification of *pfhrp*2 and *pfhrp*3 gene deletions in *P. falciparum* isolates, in response to the emerging threat of *P. falciparum* strains lacking *pfhrp2* and/or *pfhrp3* genes and therefore not detected by PfHRP2-based RDTs. The assay is particularly interesting because it allows to detect "masked" *pfhrp*2/3 deletions in multiple strains *P. falciparum* co-infections. This feature of the assay allows to study the epidemiology of *pfhrp*2/3 deletions in malaria endemic regions where the prevalence of multiple strains *P. falciparum* infections is high.

Manuscript 4: Malaria prevalence in pregnant women established by molecular tests after introduction of intermittent preventive treatment on Bioko Island, Equatorial Guinea

Women living in malaria endemic regions particularly in first and second pregnancies have higher risk of malaria, with serious adverse effects for the woman and the child.

We describe here the prevalence and molecular characteristics of *Plasmodium* infections in pregnant women covering the first, second and third trimester of pregnancy, by utilising the RDTs collected during the MIS 2018 on Bioko Island. We found that the prevalence of malaria infection in pregnant women was lower than in non-pregnant women on the Island, underscoring that implementation of preventive measures targeting this vulnerable population is effective. Although less pregnant women were infected with *P. falciparum*, they were more likely to suffer from anaemia. Using the MIS 2018, we were able to identify malaria-infected women in their first trimester who should be encouraged to come early to antenatal care facilities.

Manuscript 5: HIV infection negatively impacts the vaccine efficacy of whole irradiation attenuated sporozoite based malaria vaccines when evaluated by homologous controlled human malaria infections

Malaria and HIV are co-endemic in many regions of Sub-Saharan Africa. Deployment of a malaria vaccine in the general population will require that the vaccine is safe and efficacious in all volunteers, irrespective of their HIV infection status. Here, we have vaccinated a group of HIV positive and HIV negative volunteers in Tanzania with irradiation attenuated purified *Plasmodium falciparum* sporozoites to compare the safety, immunogenicity and protective efficacy against homologous controlled human malaria infection. This is the first time that HIV positive volunteers have undergone controlled human malaria infection providing the opportunity to understand under highly controlled conditions the interaction between HIV and malaria. This trial has been registered at ClinicalTrials.gov Identifier: NCT03420053.

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List of Abbreviations

ACT	Artemisinin-based combination therapy
CHMI	Controlled human malaria infection
CSA	Chondroitin sulfate A
DNA	Deoxyribonucleic acid
DVI	Direct venous inoculation
ELIMU	Electronic lab information & management utensil
EMA	European Medicines Agency
ENAR	Extraction of nucleic acids from RDTs
HIV	Human immunodeficiency virus
IPTp	Intermittent preventive treatment during pregnancy
IRS	Indoor residual spraying
ITN	Insecticide-treated net
LAMP	Loop mediated isothermal amplification
LBW	Low birth weight
LOD	Limit of detection
MIS	Malaria indicator survey
NA	Nucleic acid
NAT	Nucleic acid amplification technique
PfHRP2	*P. falciparum* histidine rich protein 2
PfSPZ	*P. falciparum* sporozoites
pLDH	*Plasmodium* lactate dehydrogenase
PMR	Parasite multiplication rate
qPCR	Quantitative polymerase chain reaction
RDT	Rapid diagnostic test

RNA	Ribonucleic acid
RT-qPCR	Reverse transcription qPCR
SNP	Single nucleotide polymorphism
TBS	Thick blood smear
uRDT	ultrasensitive RDT
VE	Vaccine efficacy
WHO	World Health Organization

Introduction

1. Current situation of Malaria

Malaria is one of the most common infectious diseases in the world and remains a public health problem particularly in sub-Saharan Africa despite the remarkable progress made during the past decades (1). Six species of Plasmodium infect humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium knowlesi, Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* (2). Worldwide, approximately 219 million cases of malaria and 435 000 related deaths occurred in 2017 and most of these cases and deaths were in children under the age of five years and pregnant women in Africa (3). *P. falciparum* is the most dangerous species causing the highest level of morbidity and mortality (1). *P. falciparum*-infected erythrocytes adhere to the endothelium of capillaries, leading to obstruction of the microcirculation and local tissue anoxia in vital organs such as the brain (cerebral malaria), the kidneys (renal failure) or the intestines (bacteraemia) (4, 5). Another manifestation of severe malaria is severe anaemia in high transmission settings, which is usually the consequence of destruction of erythrocytes of all age during schizogony, insufficient erythropoiesis and accelerated splenic removal of unparasitised red blood cells (6, 7). Sub-Saharan Africa is the most malaria-affected continent because of the presence of highly efficient transmitting vectors *Anopheles gambiae* and *Anopheles funestus* and the predominance of *P. falciparum* (8, 9) **(Figure 1)**.

In addition to morbidity and mortality, the disease has a significant economic and social burden for endemic countries (10-12). Indeed, a comparison of income in malaria affected and non-affected countries indicated more than a fivefold difference in Gross Domestic Product (10). Furthermore, the cognitive abilities and school performance are impaired in children following malaria infection (13, 14).

Approximately 125 million pregnant women are at risk of malaria each year with around 32 million of them living in Africa (15). *P. falciparum* and *P. vivax* are most commonly implicated in the occurrence of adverse pregnancy outcomes (15), however, the effect in pregnancy of the other four species are largely unknown (16, 17).

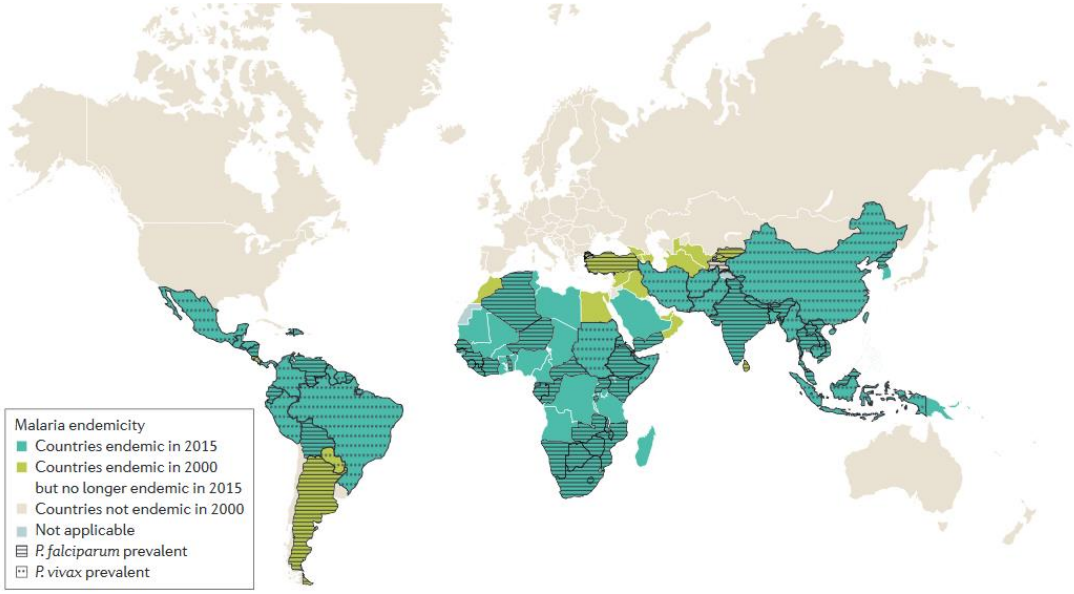

Figure 1. A map of malaria-endemic region (reproduced from Phillips MA *et al*., Malaria. Nature Reviews Disease Primers. 2017; 3:17050, with the publisher's permission)

2. *Plasmodium* spp. life cycle and pathogenesis

2.1 Life cycle

The *Plasmodium* parasite has a complex life cycle that alternates between the intermediate human host and the definitive host Anopheles mosquito **(Figure 2).**

2.1.1 Parasite

In 1880, Charles Louis Alphonse Laveran discovered the malaria parasite *Plasmodium* (18). *Plasmodium* belongs to the phylum Apicomplexa, the order Haemosporida, the family of *Plasmodiidae,* and to the genus *Plasmodia*. Apicomplexans are obligate intracellular parasites

with a complex life cycle involving both asexual sporozoite and merozoite stages and sexual gametocyte stage (19).

2.1.2 Vector

In 1897, Ronald Ross discovered that mosquitoes transmitted malaria. The female mosquitoes that transmit malaria belong to the phylum Arthropoda, the order Diptera, the family of *Culicidae*, and to the genus *Anopheles*. Among 25 anopheline species in the world, *An. gambiae* complex and *An. funestus* represent the primary vectors in Africa (8, 9). The most effective vectors are characterized by their long lifespan, adaptation to the environment, rapid reproduction and anthropophily (9). Two applicable measures for malaria vector control are insecticide-treated nets (ITNs) and indoor residual spraying (IRS) (20).

2.1.3 Life cycle

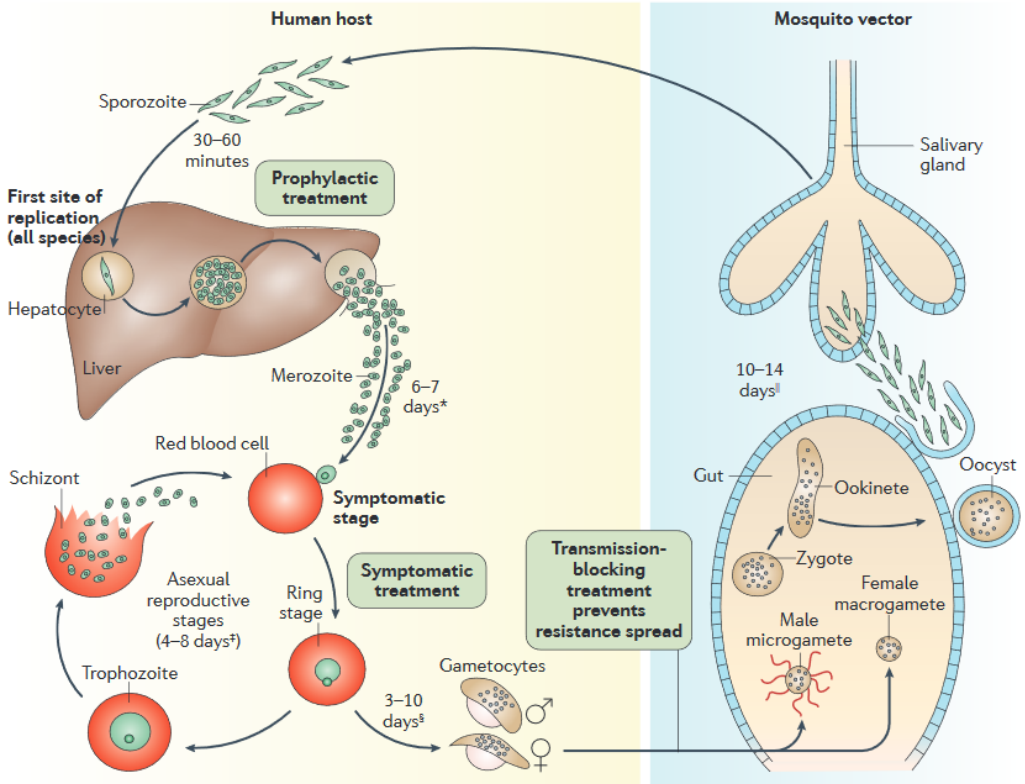

Figure 2. Overview of the Life cycle of Plasmodium spp. (reproduced from Phillips MA *et al*., Malaria. Nature Reviews Disease Primers. 2017; 3:17050, with the publisher's permission)

Malaria parasite development requires the *Anopheles* mosquitoe and human host to complete the life cycle. The cycle starts when a feeding female *Anopheles* mosquito inoculates around 1-100 motile sporozoites into the dermis of a human host (21). The fate of these sporozoites is not clearly known, but they can take 1–3 hr to exit the dermis. The sporozoites rapidly reach the liver within 30-60 minutes transported in the bloodstream, and traverse through Küpffer cells and several hepatocytes before finally infecting a suitable hepatocyte (22). In the hepatocyte, the sporozoites undergo many asexual replications and differentiate into trophozoites and subsequently schizonts. This hepatic phase of infection is asymptomatic and last 6-10 days (9, 23). A successful sporozoite releases 10000-40000 infectious merozoites into the bloodstream, where they invade circulating erythrocytes and initiate the clinically important intra-erythrocytic cycle lasting 24 to 72 hours depending on the *Plasmodium* species. In the case of *P. vivax* and *P. ovale*, some merozoites enter into a dormant stage or hypnozoite that can persist in the liver for weeks or years before re-entering the bloodstream, and cause disease relapse (24). Others merozoites differentiate into female and male gametocytes through a process not yet completely understood of sexual differentiation in 3-10 days (25). Gametocytes concentrate in skin capillaries and are ingested by the mosquito vector during a blood meal (25). In the gut of the mosquito, each male gametocyte produces eight microgametes after three rounds of mitosis; the female gametocyte matures into a macrogamete. At least one male and one female stage V gametocytes have to be ingested by a female *Anopheles* mosquito during a blood meal to continue the cycle. The gametocytes male and female fuse into a diploid zygote in the mosquito's midgut. The zygote develops into a motile ookinete that penetrate the mosquito's midgut epithelial cells to form an oocyst. Oocysts undergo cycles of replication and form sporozoites. It takes around 10-14 days from fertilization to sporozoites maturation (25).

Upon release, sporozoites migrate from the abdomen to the salivary glands of the mosquito, where they can infect a human host and thereby perpetuate the life cycle.

2.2 Pathogenesis of *P. falciparum* infections

The pre-erythrocytic stage in hepatocytes is clinically silent. The erythrocytic asexual stage induces all the clinical manifestations. Symptoms of malaria are unspecific such as fever, headache, fatigue, muscle and joint aches, chills, perspiration and vomiting (9).

In young children and non-immune individuals, the clinical manifestation is more patent and can rapidly become life threatening. They are more susceptible to respiratory distress, cerebral malaria and severe anaemia. Factors that affect the severity of malaria infection include host immune status and the causative *Plasmodium* species (26, 27).

The rupture of infected erythrocytes releases hemozoin and stimulate pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1 and leads to clinical symptoms (28-30). The pathogenesis of anaemia in *P. falciparum* malaria is multifactorial. In malaria naïve individuals and without comorbidities, red blood cell loss is an important mechanism of anaemia (6). On the contrary, the reduction of erythropoiesis is likely to play an important role in patients living in endemic areas and with chronic low-density *P. falciparum* infections (31).

Cytoadherence plays an important role in the pathogenesis of severe malaria. During the intra-erythrocytic maturation, the parasite exports several proteins (32) that are important for cytoadherence to endothelial cells and rosetting (4, 33). These proteins are encoded by members of large polymorphic multigene families such as the var, the subtelomeric variable open reading frame (stevor) or repetitive interspersed family (rif) families (34, 35). The most characterized of these proteins is the multigene family *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) (36). Other proteins that are exported to the surface of infected

red blood cells include the Ring-Infected Erythrocyte Surface Antigen (RESA) (37) and the knob-associated histidine-rich protein (KAHRP) (38).

Sequestration constitutes an immune evasion strategy for the parasite by preventing splenic clearance of infected erythrocytes (23). It follows the adhesion of PfEMP1 to host cell surface receptors such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1) (39), chondroitin sulphate A (CSA), hyaluronic acid (HA), and CD36 (40). *P. falciparum*-infected red blood cells are also able to adhere to uninfected ones forming conglomerates or "rosettes". Cytoadherence and rosetting result in the clogging of vascular structures of various organs such as the brain, intestine, kidney and lung (5, 41).Pregnant women, especially during their first and second pregnancies have an increased susceptibility to malaria compared to non-pregnant women and are also more attractive to mosquitoes (42). The vulnerability is more important among women who have not yet developed adequate pregnancy-specific immune responses against the subpopulation of parasites that sequester in the placenta (43). Different approaches have been used to investigate the immunity observed in pregnancy across many studies indicated a parity-dependent acquisition of antibody against placental parasites or CSA-binding laboratory isolates (44). Higher levels of antibodies in multigravidae compared with primigravidae have been reported from many countries (45-47). Although the vast majority of pregnant women infected are asymptomatic (48), malaria can result in low birth weight (LBW) and maternal anaemia (49), increasing maternal, neonatal and infant mortality (50). The contribution of malaria as a cause of preterm delivery and intrauterine growth retardation is remarkably high in malaria endemic areas of Africa (49, 51).

Malaria infection during pregnancy may impair placental development due to maternal hormonal and inflammatory disturbances. Furthermore, transport of nutrient by the placenta could be impaired and result in foetal growth restriction (52). Acute infection and high

parasitaemia are associated with stillbirth and preterm delivery whereas chronic placental inflammation is associated with foetal growth retardation (51).

Similarly, anaemia is more common in pregnant women compared to non-pregnant women. Although anemia in pregnancy is multifactorial in Sub-Saharan countries (hemodilution, iron and folate deficiency, intestinal helminth infection and heamoglobinopathies), the contribution of malaria is still significant (53).

Malaria during pregnancy is caused by the massive accumulation of *P. falciparum*-infected erythrocytes in the placenta. Selective sequestration of infected erythrocytes into the placenta occurs through adhesion of members of the PfEMP1 family, which are encoded by the highly diverse *var* gene family (54) to CSA expressed on syncytiotrophoblast cells lining the placental intervillous spaces (45). This makes primigravidae particularly vulnerable to placental infection with the consequences of LBW and maternal anaemia (43, 44). Specific humoral immunity targeting these parasite variants is acquired in later pregnancies and confers protection against the consequences of malaria in pregnancy (43, 55).

3. Diagnosis

The diagnosis tools currently available for the identification of *Plasmodium* species include thick and thin blood smear microscopy, rapid diagnostic tests (RDTs) and nucleic acid amplification techniques such as polymerase chain reaction (PCR) (56, 57) or loop mediated isothermal amplification (LAMP) (58).

3.1 Microscopy

Light microscopy involves the visualization of the malaria parasites in a thick or thin blood smear. Thick blood smear allows the identification of the different species and stages and quantification of parasite density. The lower limit of detection is around 50 parasites/µL in expert hands (59). Microscopy slides can be preserved for a long time and offer the possibility

of quality control and training of lab technicians. However, microscopy requires at least one hour to deliver result and requires good equipment and a well-trained staff (60).

3.2 Rapid diagnostic test

RDTs are lateral flow immuno-chromatographic antigen-detection tests, which rely on the capture of dye-labeled antibodies to produce a visible band on a strip of nitro-cellulose (61). Currently, the majority of RDTs in the market detect the *P. falciparum* specific antigen histidine rich protein 2 (PfHRP2) and are relatively more sensitive and heat stable compared to lactate dehydrogenase (pLDH) or aldolase-based RDTs (62). The limit of detection for RDTs is around 100 parasites/µL (63). RDTs have a number of advantages over microscopy including rapidity, simplicity and ease of manipulation. Disadvantages of RDTs include the lack of sensitivity in detecting low parasitaemia, the impossibility of quantifying parasite density, the difficulty in differentiating *Plasmodium* species and false-positive results after parasite clearance due to the persistence of parasite antigens (64, 65). Furthermore, a number of reports have raised the issue of *pfhrp*2/*pfhrp*3 gene deletion in circulating field populations resulting in false negative PfHRP2-based RDTs that may have serious consequences for diagnosis and treatment (66).

3.3 Polymerase chain reaction

Nucleic acid amplification tests enable sensitive detection of low density malaria infections (below 1 parasite/µL) (67). Nucleic acid tests can be used for qualitative or quantitative parasite detection, determination of the multiplicity of infection, genotyping to distinguish recrudescence from reinfection, and detection of drug resistance mutations (68).

The 18S ribosomal RNA gene has unique sequences that enable the identification of all 5 malaria species infecting humans and is therefore commonly targeted for amplification (69, 70).

Species diagnosis provides therapeutic orientation and allows to anticipate relapses in *P. vivax* and *P. ovale* infections.

3.4 Diagnosis of malaria in pregnancy

The diagnosis of malaria in pregnancy is important because of the devastating consequences of the disease in pregnant women. Current diagnostic tools include microscopy, RDTs and histology. *P. falciparum* infections can be difficult to diagnose during pregnancy, particularly in asymptomatic women with low peripheral blood parasite density while harbouring parasites in the placenta (44, 71). The gold standard for placental malaria diagnosis is histology of placental tissue which allows to distinguish active, chronic and past infections (72).

4. Malaria prevention and surveillance

4.1 Prevention

Different approaches are used to prevent and control malaria. These strategies include vector control measures, chemoprevention, early diagnosis and effective treatment of malaria cases.

Vector control represent an essential component of malaria prevention and includes the use of mosquito larvicides, indoor residual spraying (IRS) of houses and insecticide-treated mosquito nets (ITNs). Sleeping under a treated mosquito reduces physical contact between mosquitoes and humans and provides at the same time an insecticidal effect. In areas of moderate to high transmission in Africa, deployment of ITNS reduced all-cause mortality by around 20% in under five years old children (73). Together with ITNs, IRS represent the second core vector control measure and involves spraying the inside of houses with an insecticide with a long residual activity (20).

ITN use is recommended as part of the antenatal package provided during pregnancy and leads to improved birth outcomes (74). Major challenges remain the resistance of vectors to insecticides and the brevity of insecticides' effect (75).

Chemoprevention is currently reserved for children in seasonal malaria transmission areas, pregnant women in Africa and for non-immune individuals traveling to endemic areas. Seasonal malaria chemoprevention (SMC) is the intermittent administration of full treatment courses of an antimalarial to children in areas of highly seasonal transmission during the malaria season. Currently, intermittent preventive treatment during pregnancy using sulfadoxine-pyrimethamine (IPTp-SP) defined as the administration of curative dose of SP at predefined intervals during routine antenatal care as recommended by the World Health Organization (WHO). Challenges to IPTp-SP include modest coverage and increasing resistance of *P. falciparum* to SP (76-78).

4.2 Surveillance

Improved surveillance for malaria cases and deaths helps ministries of health to determine which areas or population groups are most affected while enabling countries to monitor changing disease trends. A strong malaria surveillance system also helps a country in designing effective health interventions and evaluate the impact of its malaria control programme. The annual World malaria report, brings together all the data reported by countries to WHO, including their surveillance data (79).

Recently, renewed focus on global malaria elimination has seen a rapid increase in the measurement of key malaria indicators through nationally representative household surveys. These malaria indicator surveys (MIS) measure indicators related to the Roll Back Malaria (RBM) Global Malaria Action Plan, the Millennium Development Goals (MDG), and the President's Malaria Initiative (PMI) targets. Information is collected on the ownership and use of insecticide-treated mosquito nets (ITNs), indoor residual spraying (IRS) of insecticides, prompt and effective treatment of fever in young children, and the prevention of malaria in pregnant women. Most MIS also include biomarker tests for malaria using microscopy or RDT and haemoglobin to diagnose anaemia (80).

4.3 Vaccines

More than 35 years of research efforts after the first malaria vaccination trials in the 1970s (81, 82), Mosquirix also known as RTS,S/AS01 remains the only vaccine that has received a positive opinion from the European Medicines Agency's (EMA) Committee for Medicinal Products for Human Use (CHMP) (83).

Difficulty in developing a malaria vaccine could be related to several factors including the complex life cycle of the parasite, the genetic diversity of malaria parasites and the absence of sterilizing immunity (84, 85). In addition, most of the antimalarial antigens selected as vaccine candidates are targets of natural immunity and therefore have significant genetic polymorphism (85).

Depending on the stages of development of the parasite, malaria vaccine candidates can be classified into 3 groups: pre-erythrocytic vaccines, blood stage vaccines and "other" vaccines including transmission-blocking vaccines and vaccines against malaria during pregnancy.

4.3.1 Pre-erythrocytic vaccines

Pre-erythrocytic vaccines target the clinically silent sporozoite and liver stages of *P. falciparum* to prevent hepatocyte invasion and the development of the blood stage. The brief passage of sporozoites into the bloodstream before the invasion means that humoral and cellular responses are limited at this stage (23). RTS,S is a recombinant vaccine that contains an adjuvant. It is composed of a *P. falciparum* circumsporozoite protein fused with hepatitis B surface antigen (rts), and combined with hepatitis B surface antigen (s) (86, 87).

In phase 3 clinical trials in 11 African countries RTS,S showed a 26% reduction in clinical malaria cases in the youngest children and a 36% reduction in children under 17 months (88). RTS,S has received a positive opinion from the EMA for children aged 5-17 months and is currently being implemented in pilot studies in Ghana, Kenya and Malawi as part of routine childhood vaccination (89).

Another promising pre-erythrocytic candidate vaccines include irradiated whole *P. falciparum* sporozoites (PfSPZ) or infective sporozoites administered under chemoprophylaxis (chemoprophylaxis vaccination or Cvac). Several clinical trials have shown that the whole irradiated sporozoite vaccine PfSPZ manufactured by Sanaria® is safe and well tolerated and offers promising protection against malaria when administered intravenously (90-94). A Phase 3 clinical trial to evaluate its efficacy is planned for 2020 in Equatorial Guinea (95).

4.3.2 Blood stage vaccines

Erythrocyte vaccines are intended to prevent disease by preventing the invasion of red blood cells by merozoites by inducing the production of antibodies against surface antigens of *Plasmodium*-infected erythrocytes. An example of this type of vaccine is the MSP3-based vaccine located on the surface of the merozoite, which was associated with a reduction in malaria incidence in children in a Phase 2 trial (96).

Vaccine candidates in this group have generally had little success in clinical trials (97, 98). A first generation *P. falciparum* reticulocyte binding protein homolog 5 (PfRH5) vaccine is being tested in clinical trials in Oxford and Tanzania (98).

4.3.3 Other vaccines

Transmission-blocking vaccines also known as "altruistic vaccines" induce antibodies against gametocytes, thus blocking fertilization (99). *P. falciparum* Pfs25 antigen is the most targeted at this stage (100, 101).

Vaccines against pregnancy-associated malaria could be useful to prevent placental malaria and reduce the burden of the disease during pregnancy. PAMVAC is a vaccine candidate based on a recombinant fragment of VAR2CSA, the protein responsible for binding to the placenta via CSA. PAMVAC was safe, well-tolerated and induced functionally active antibodies in healthy malaria-naive volunteers and will next be assessed in women before first pregnancies in endemic areas (102, 103).

The next step in malaria vaccine development will be to combine several vaccine approaches with different combinations of antigens from different stages and strains of the parasite.
The absence of known biological correlate of protection correlates for immunity requires costly clinical trials to demonstrate the efficacy of a vaccine (104). Although remarkable progress has been made, the development of continuous malaria vaccine is still costly and complex.

Controlled human malaria infection (CHMI), the deliberate inoculation of volunteers with *Plasmodium* spp. sporozoites, is increasingly being used to assess the efficacy of malaria vaccines and contributes to accelerate vaccine development (105). Post CHMI, volunteers are closely monitored for signs and symptoms of malaria. Parasitaemia in whole blood is closely monitored and volunteers are treated before they develop high parasite density. Recently, more sensitive quantitative polymerase chain reaction (qPCR) is being used as a primary diagnostic test. CHMI has been shown to be safe and very well tolerated and a reliable tool to test vaccines and drug's efficacy in many clinical trials (106, 107).

5. Aims of the thesis

Rapid, sensitive and accurate malaria diagnosis is the cornerstone of surveillance and response approaches which are needed to reach the final goal of malaria elimination. The overall goal of this thesis is to develop novel, molecular-based, sensitive, robust and high-throughput methods to monitor *Plasmodium* spp. infections that might escape RDT-based malaria detection.

Aim 1: develop and evaluate a high-throughput procedure to extract *Plasmodium* nucleic acids including RNA and DNA from malaria rapid diagnostic tests regularly used in malaria indicator surveys.

Aim 2: evaluate a novel quantitative polymerase chain reaction-based method for detecting *hrp2* and *hrp3* gene deletions in *Plasmodium falciparum* field isolates, including mixed infections prevalent in sub-Saharan countries.

Aim 3: describe the prevalence of malaria in pregnant women after introduction of intermittent preventive treatment on Bioko Island using RDTs collected during the malaria indicator survey conducted on Bioko Island, Equatorial Guinea in 2018.

Aim 4: Understand the impact of HIV-co-infections on asexual blood stage parasitemia during controlled human malaria infection in Tanzanian volunteers.

Chapter 2

Tools and techniques for high-throughput molecular malaria surveillance

This chapter contains the following manuscript submitted to *Scientific Reports*:

Etienne Guirou, Tobias Schindler, Salome Hosch, Olivier Tresor Donfack, Charlene Aya Yoboue, Silvan Krähenbühl, Anna Deal, Glenda Cosi, Linda Gondwe, Grace Mwangoka, Heavenlight Masuki, Nahya Salim, Maxmillian Mpina, Jongo Said, Salim Abdulla, Stephen L. Hoffman, Carlos Cortes Falla, Wonder P. Phiri, Carl Maas, Guillermo A. Garcia, Marcel Tanner and Claudia Daubenberger.
Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests

This chapter contains also the following publication:

Silvan Krähenbühl, Fabian Studer, **Etienne Guirou**, Anna Deal, Philipp Mächler, Salome Hosch, Maximilian Mpina, Sarah Mswata, Claudia Daubenberger & Tobias Schindler.
ELIMU-MDx: a web-based, open-source platform for storage, management and analysis of diagnostic qPCR data. *Biotechniques* 2019 doi: 10.2144/btn-2019-0064.

Molecular malaria surveillance using a novel protocol for extraction and analysis of nucleic acids retained on used rapid diagnostic tests

Running title: ENAR: Extraction of Nucleic Acids from RDTs

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Abstract

The use of malaria rapid diagnostic tests (RDTs) as a source for nucleic acids that can be detected by nucleic acid amplification techniques has several advantages, including the small amount of blood and cost-effectiveness regarding sample collection as well as simplified storage and shipping conditions at room temperature. We have developed systematically and evaluated extensively a procedure to extract total nucleic acids from used malaria RDTs. The co-extracted DNA and RNA molecules from small volumes of dried blood retained on the RDTs allows detection and quantification of *P. falciparum* parasites from asymptomatic patients with parasite densities as low as 1 Pf/µL blood using reverse transcription quantitative PCR. Around the extraction protocol, we have developed the Extraction of Nucleic Acids from RDTs (ENAR) approach, a complete workflow for large-scale molecular malaria surveillance. Using RDTs collected during a malaria indicator survey, we demonstrated that ENAR provides a powerful tool to analyse nucleic acids from thousands of RDTs in a standardized and high-throughput manner. We found several, known and new, non-synonymous single nucleotide polymorphism in the propeller region of the *kelch 13* gene among isolates circulating on Bioko Island, Equatorial Guinea.

Keywords

Nucleic Acid Extraction, Molecular Malaria Surveillance, Rapid Diagnostic Test (RDT), Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR), Artemisinin resistance, *kelch* 13

Introduction

Malaria remains a global public health issue with an estimated 228 million cases resulting in 405'000 deaths in 2018 [1]. *P. falciparum* (*Pf*) is the most pathogenic malaria species accounting for the vast majority of malaria cases and deaths. Malaria surveillance, the continuous and systematic collection, analysis and interpretation of epidemiological data, is the basis of intervention programs to control malaria and provides the framework for effective allocation of resources [2]. A critical surveillance measure, which reflects the malaria transmission intensity closely, is the parasite rate; the proportion of the population found to carry parasites in their peripheral blood [3,4]. Malaria rapid diagnostic tests (RDTs) have become the most widely used technique to measure parasite rates in endemic countries. In sub-Saharan Africa, RDTs have almost completely replaced light microscopy for malaria diagnosis, with an estimated 75% of malaria tests conducted using RDTs in 2017 [1]. Among the advantages of RDTs are the relatively low costs, fast result turnaround time, wide availability and simplicity in use. However, there are also disadvantages, which include the rather low sensitivity, which results in poor performance among asymptomatic individuals [5] and the widespread emergence of *pfhrp2* deletions in certain regions [6].

Nucleic amplification techniques (NATs), such as polymerase chain reaction (PCR), do not only show higher sensitivities than RDTs [5,7] they also allow further characterization of *Pf* isolates using molecular markers. Surveillance of drug-resistant *Pf* strains, based on analysis of resistance-associated molecular markers, is a widely used and valuable epidemiological tool [8]. In sub-Saharan Africa, treatment of malaria relies heavily on artemisinin-based combination therapy (ACT) and implementation of surveillance programs for early detection of emerging artemisinin resistant *Pf* strains will be key to prevent the spread across the continent [9]. Artemisinin resistant *Pf* strains were first reported in Cambodia [10,11] and remain a public health concern in South East Asia, but have not yet been found to be widespread in Africa, South America or Oceania [12]. Non-synonymous mutations in the

propeller region of the *Pf kelch* 13 gene (pfk13) were discovered as molecular markers for artemisinin resistance [13].

The use of the blood retained on RDTs as as a source for nucleic acids (NAs) that can be used as an input for NAT-based resistance markers screening would have several advantages, including the simplicity and cost-effectiveness regarding sample collection as well as simplified storage and shipping conditions under room temperature (RT). Over the past decade, several reports have been published describing the use of DNA extracted from used RDTs for molecular analysis of malaria parasites (studies summarized in Table S1) [14–24]. However, most studies that tried to address the question of using RDT as source of DNA were conducted with small sample sizes and focused rather on demonstrating the feasibility of extracting DNA than fitting this approach for molecular surveillance of malaria parasites at larger scale. We identified three key areas, which are critical in order to develop a surveillance tool based on molecular analysis of used RDTs: i) accessing a representative collection of RDTs and effective selection and sorting strategies for RDTs of interest. ii) high-throughput extraction and analysis of NA from RDTs with minimal hands-on time and focus on reproducibility and quality control throughout the entire extraction process. iii) increasing recovery of *Pf* NAs during extraction process in order to include asymptomatic individuals with low parasite density infections. This report outlines a strategy and protocols for collecting, sorting and processing RDTs to extract the retained NA at large-scale in order to screen for single nucleotide polymorphism (SNP) in an artimisinin-associated drug resistance marker among thousands of healthy, malaria asymptomatic individuals. We developed systematically and evaluated extensively a procedure to extract NA from RDT. The “Extraction of Nucleic Acids from RDTs” (referred to as ENAR) approach is supported by custom-made software solutions allowing to analyse thousands of RDTs in a standardized, reproducible and high-throughput manner.

We implemented the ENAR approach within the 2018 malaria indicator survey (MIS) conducted on Bioko Island, Equatorial Guinea. Island-wide interventions have resulted in a substantial reduction in

malaria, achieving a reduction in parasitaemia of over 75% over 13 years [25]. Despite these achievements, malaria transmission remains stable and recently a *Pf* isolate of African origin with artimisinin-resistance, including a novel non-synonymous mutation in pfk13, was identified in a 43-year-old man returning to China from Equatorial Guinea [15].

Material and methods

Pf reference samples

Pf reference samples were used to test the performance of the ENAR procedure. Experiments with *Pf* reference samples were conducted using Carestart™ HRP2/pLDH Combo RDTs (Access Bio, Inc., Somerset, NJ, USA). Serial dilutions of the WHO International Standard for *Pf* DNA Nucleic Acid Amplification Techniques (NIBSC code: 04/176, herein referred to as PfIS) [27] were used to quantify *Pf* parasitaemia by (RT)-qPCR. Whole blood was spiked with different parasite densities, ranging from 10'000 to 0.1 Pf/µL and 5 µL of this suspension applied onto RDT.

Additionally, ten-fold serial dilutions, ranging from 10'000 to 0.1 Pf/µL, of freshly cultured *Pf* strains PfNF54, PfDD2 and PfHB3 were prepared and 5 µL were applied onto RDTs. 5 µL of stage V gametocytes were obtained from *in vitro* parasite culture as described previously [28]. RDTs probed with these stage V gametocytes were extracted using the ENAR protocol after a three-week storage period at RT.

School-based survey in Mkuranga district

Carestart™ HRP2/pLDH Combo RDTs were used to determine the parasite rate among asymptomatic children from three primary schools in the Mkuranga district of Coastal Tanzania. Extraction protocol A, which is based on the Quick-DNA™ Miniprep Kit (Zymo Research Corporation, Irvine CA, USA), was used to extract DNA from a total of 190 RDTs collected during this school-based survey. *Pf* was detected by amplifying the acidic terminal sequence of the var genes (PfvarATS) [29].

Sample collection, analysis and storage during CHMI studies

RDTs were collected during two CHMI studies conducted to evaluate Sanaria's PfSPZ Vaccine in Bagamoyo, Tanzania (Clinical Trials.gov registration numbers NCT02613520 and NCT03420053,

respectively). The first CHMI was conducted in 2016 (referred to CHMI-1) the second CHMI was conducted in 2018 (referred to CHMI-2). Fresh venous whole blood collected in EDTA tubes was analysed by RDTs within 45 min after blood collection. During CHMI-1, 20 µL was applied to BinaxNOW® Malaria RDT (Alere, Cologne, Germany) and during CHMI-2, 5 µL was applied to Carestart™ HRP2/pLDH Combo RDT. The RDTs were read according to the manufacturers guide and then stored in a box at RT until extraction of NA.

The same samples were used to monitor parasitaemia during CHMI by thick blood smear microscopy and qPCR as described elsewhere [30,31]. All samples were processed and analysed at the laboratories of the Bagamoyo branch of the Ifakara Health Institute in Tanzania.

Malaria indicator survey on Bioko Island, Equatorial Guinea

The 2018 Malaria indicator survey was carried out between August and October 2018 on a representative sample of 13'505 individuals from 4774 households selected from all communities across Bioko Island. All consenting permanent residents and short-term visitors were tested for malaria using the CareStartTM Malaria HRP2/pLDH Combo RDT. Used RDTs were stored at RT in plastic bags containing desiccants and transported to the Swiss Tropical and Public Health Institute for further molecular analysis.

Nucleic acid extraction methods from RDTs

The cassettes of the RDTs were opened, the entire RDT strip removed, and cut into four small pieces in order to fit into a 1.5 mL micro-centrifuge tube. A set of cleaned forceps and scissors were used with special attention given to prevent cross-contamination between samples. After processing a sample, scissors and forceps were cleaned in 10% sodium hypochlorite and wiped with ethanol sprayed tissues and dried before processing the next sample. The four nucleic extraction protocols tested, named A-D, were all based on silica columns.

Protocol A – ZR Quick-DNA™ Miniprep Kit: The protocol is based on the Quick-DNA™ Miniprep Kit (Zymo Research Corporation, Irvine CA, USA). Briefly, 1 mL of Genomic Lysis Buffer was added to the pre-cut RDT strip in a 1.5 mL micro-centrifuge tube and incubated at 95 °C for 20 minutes. The mixture was then transferred onto the extraction column and the manufacturers guide was followed for extraction. DNA was eluted in 50 µL of DNA Elution Buffer.

Protocol B – ZR Quick-DNA™ Miniprep Plus Kit: The protocol is based on the Quick-DNA™ Miniprep Plus Kit (Zymo Research Corporation, Irvine CA, USA). Briefly, 600 µL of Solid Tissue Buffer (Blue) and 40 µL of Proteinase K was added to the pre-cut RDT strip in a 1.5mL micro-centrifuge tube and incubated at 55 °C for 60 minutes. The supernatant was transferred to a clean 1.5 mL micro-centrifuge tube and 640 µL of Genomic Lysis Buffer was added and mixed thoroughly. The mixture was then transferred onto the extraction column and the manufacturers guide was followed for extraction. DNA was eluted in 50 µL of DNA Elution Buffer.

Protocol C – NukEx Pure RNA/DNA Kit: The protocol is based on NukEx Pure RNA/DNA Kit (Gerbion GmbH, Kornwestheim, Germany), which co-extracts DNA and RNA. Briefly, a working solution of 500 µL of Binding Buffer, 4 µL of Poly A and 50 µL of Proteinase K was added to the pre-cut RDT strip in a 1.5 mL micro-centrifuge tube and incubated at 60 °C for 10 minutes. The supernatant was transferred onto the NukEx Spin Column and the manufacturers guide was followed for extraction. Total NA were eluted in 50 µL of Elution Buffer.

Protocol D – Zainabadi et al. extraction method for DBS: The protocol is based on a recently published extraction protocol for total NA from dried blood spots [32]. Identical buffer compositions were used, and the protocol was adapted to extraction of NA from RDT strips. Briefly, the pre-cut RDT strip was incubated in 900 µL lysis buffer at 60 °C for 2 hours. The supernatant was transferred onto Omega HiBind RNA Mini Columns (Omega Bio-Tek, Norcross, USA) and NA extracted as described. NA were eluted in 50 µL of Elution Buffer (Quick-DNA™ Miniprep Kit, Zymo Research Corporation, Irvine CA, USA).

High-throughput extraction protocol of NAs from RDTs (ENAR protocol)

We adapted protocol D to extract NA from used RDTs in a high-throughput manner. The main modification included a horizontal incubation of the entire uncut RDT strip by using sterile, RNase-/DNase-free 12-channel reservoirs (Axygen, Corning Inc, USA) and switching to a 96-well format for extraction. By removing the cutting step, the hands-on time during the extraction process is significantly reduced, and the risk of cross-contamination by carryover during the cutting is minimized. Up to eight 12-channel reservoirs, with a total of 96 samples, were processed in one batch. Lysis was conducted by adding 900 µL lysis buffer to each RDT strip placed in the 12-channel reservoir followed by incubation at 60 °C for 2 hours with gentle shaking. All supernatants were then transferred to Omega E-Z 96 wells DNA plates (Omega Bio-Tek, Norcross, USA), washed with Wash Buffer 1 and 2 and finally eluted into a 96 well plate (DNA LoBind Plates, Eppendorf) with 50 µL pre-warmed (60 °C) Elution Buffer (Zymo Research Corporation, Irvine CA, USA). With these adaptations to the protocol, NA from 96 RDTs are extracted in about three hours, with minimal hands-on time needed. One positive control (RDT spiked with 5 µL blood containing 200 Pf/µL) and one negative control (Lysis Buffer only) were included with each extraction plate to control for plate-to-plate consistency and cross-contamination. A standard operating procedure (SOP) for ENAR can be found as supplementary file 1.

Detection and quantification of *Plasmodium* spp. parasites

We used the PlasQ assay, a multiplex qPCR assay for *Plasmodium* spp. and *Pf* detection and quantification to analyse the NA extracted from RDTs [31]. The PlasQ assay targets the Pan-*Plasmodium* 18S DNA and RNA (Pspp18S) [33,34] and the *Pf*-specific acidic terminal sequence of the var genes (PfvarATS) [29]. The human *rnasep* gene (HsRNaseP) [33] served as an internal control to assess the quality of NA extraction and qPCR amplification. To run the PlasQ as a RT-qPCR assay, targeting

both DNA and RNA templates, we added 1x Luna WarmStart RT Enzyme Mix (New England Biolabs, Ipswich, USA) and did start the qPCR program with a reverse transcription step at 55 °C for 15 min. All qPCR and RT-qPCR assays were run on a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). Samples were analysed in duplicates with positive (PfNF54 DNA), negative (malaria negative individual) and non-template (molecular biology grade H_2O) controls added to each qPCR run.

Multiplex pre-amplification of *Plasmodium* spp. DNA

The *Plasmodium* spp. 18S rDNA and pfk13 genes of all PlasQ positive samples were amplified in a multiplex reaction by conventional PCR. Briefly, 3 µL of extracted NAs were amplified in a total volume of 20 µL using 1x HOT FIREPol® MultiPlex Mix (Solis Biodyne, Tartu, Estonia). Using 0.25 µM of the published primers, AGT GGA AGA CAT CAT GTA ACC AG and CCA AGC TGC CAT TCA TTT GT, 986 bp of the pfk13 propeller region were amplified [26]. Simultaneously, 1407-1469 bp of the pan-*Plasmodium* 18S rDNA were amplified using 0.5 µM of GRA ACT SSS AAC GGC TCA TT [35] and AGC AGG TTA AGA TCT CGT TCG [33]. The conditions of the multiplex PCR were the following: 95 °C for 12 minutes; 25 cycles of 95 °C for 20 seconds, 57 °C for 40 seconds and 72 °C for 1 minute 45 seconds; and 72 °C for 10 minutes.

Detection of gametocytes and *Plasmodium* spp. species identification

Gametocyte-specific RT-qPCR assay: A previously published RT-qPCR assay for identification of *Pf* gametocytes based the PF3D7_0630000 transcript was used [36]. Briefly, 2 µL of extracted NA were added to 8 µL reaction mix consisting of 0.6 µM of primers, 0.3 µM probe and Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, USA). The qPCR program included reverse transcription for 15 min at 53 °C, followed by polymerase activation for 1 min at 95 °C and 45 cycles with 15 s at 95 °C and 45 s at 53 °C.

Plasmodium spp. species identification: Non-*falciparum Plasmodium* species identification based on the 18S rDNA gene was performed. 2 µL of the product from the *Plasmodium* spp. multiplex pre-amplification were added to the master mix containing 1x Luna® Universal Probe qPCR Master Mix, 0.8 µM forward (GTT AAG GGA GTG AAG ACG ATC AGA) and 0.8 µM reverse primers (AAC CCA AAG ACT TTG ATT TCT CAT AA) to amplify a 157- to 165-bp segment of the *Plasmodium* spp. 18S rDNA gene [37]. Species-specific probes were selected to differentiate between the species. *P. malariae* was detected using a Yakima Yellow-labelled MGB probe (CTA TCT AAA AGA AAC ACT CAT) [38], *P. ovale* spp. using a novel designed Texas Red-labelled and LNA-modified probe (GGA [LNA-A]AT [LNA-T]TC TTA GAT TGC TTC CT[LNA-T] CAG), *P. vivax a* Cy5-labelled probe (GAA TTT TCT CTT CGG AGT TTA) [39] and *P. knowlesi* a Cy5-labelled probe (CTC TCC GGA GAT TAG AAC TCT TAG ATT GCT) [40]. The conditions for the qPCR were: 95 °C for 3 minutes and 45 cycles of 95 °C for 15 seconds and 57 °C for 45 seconds.

Genotyping of pfk13 propeller region

In a second PCR reaction with a 15 µL total volume, 1.5 µL of the product from the *Plasmodium* spp. multiplex pre-amplification was amplified using 1x HOT FIREPol® MultiPlex Mix (Solis Biodyne, Tartu, Estonia) and 0.33 µM forward (TGA AGC CTT GTT GAA AGA AGC A) and reverse (TCG CCA TTT TCT CCT CCT GT) primers. Except for an annealing temperature of 58 °C, the PCR conditions were similar to the first reaction. The 798 bp product of the second PCR was evaluated using agarose gel electrophoresis and samples which failed amplification were repeated. Amplicons were sequenced by Microsynth (Microsynth AG, Balgach, Switzerland).

Data analysis and statistics

All (RT)-qPCR assays were run in duplicates and initial data analysis of the (RT)-qPCR data was conducted using CFX Maestro Software (Bio-Rad Laboratories, California, USA). In case of one

replicate interpreted as positive and the other replicate negative, then the assay had to be repeated and the result was considered positive if two positive replicates were obtained out of the total four replicates. All (RT)-qPCR data generated were managed and analysed by a custom-designed laboratory management and information system named ELIMU-MDx [41]. The ELIMU-MDx platform supports automated quality control, management and analysis of qPCR data. Oligo design and sequence analysis was performed using Geneious Prime 2019.1.1 (https://www.geneious.com). Statistical analysis and visualization of data was conducted using R version 3.5.1 based on packages *dplyr*, *ggpubr*, *ggplot2*, *gridextra, reshape2* and *scales*.

Results

Blood stored on RDTs is a source of *Pf* DNA

First, we conducted a literature search of reports describing the use of NA extracted from RDTs as input templates for NAT based detection of malaria parasites (Table S1). A total of 11 studies were published between 2006 and 2019. All studies were limited to the extraction of DNA and used a variety of different extraction methods. Most extraction protocols were based either on the Chelex method (n=7) or silica column based DNA extraction kits (n=6). One study extracted DNA from the entire RDT strip, all other studies used only predefined fragments of the RDT strip. These previous studies demonstrated that *Pf* DNA can be recovered from RDTs and amplified by NATs. Several studies genotyped drug resistance associated markers using Sanger or next generation sequencing.

As the majority of these studies extracted DNA from RDTs of febrile clinical malaria cases, indicating high parasite densities, we first conducted a study to test feasibility of detecting Pf DNA from RDTs of asymptomatic individuals. We employed RDTs collected in a malaria survey conducted among asymptomatic children from three primary schools in the Mkuranga district of Coastal Tanzania. DNA was extracted from 190 RDTs and *Pf* DNA was recovered from 90.8% (59/65) of PfHRP2-positive, from 100% (5/5) of PfHRP2/pLDH-positive and from 11.7% (14/120) negative RDTs (Fig 1A).

Encouraged by the outcome of the school-based survey, we aimed to improve the extraction method from RDTs further. As a proxy for the amount of extracted NAs, the Cq value of the human *rnasep* gene (HsRNaseP target), which is the internal control of the previously published PlasQ assay, was used to assess the overall performance of four different extraction procedures (Fig 2B). Side by side comparison of the four extraction procedures, named Protocol A-D, confirmed the superior performance of protocol B and D. Taking the costs and the fact that protocol D co-extracts RNA into consideration, we decided to focus on development of protocol D, which was subsequently renamed to ENAR (Extraction of Nucleic Acids from RDTs). In order to identify the part of the RDT strip with

most *Pf* NA accumulated, we analysed the sample pad (proximal part), the detection area (middle part) and the absorption pad (distal part) using ENAR. In RDTs probed with fresh blood, *Pf* NAs are found in all three parts, with more than 87% of total DNA enriched in the middle part. RDTs probed with frozen blood that is associated with red blood cell lysis resulted in an equal distribution of NA along the entire RDT strip (Fig S1).

Detection and quantification of *Pf* parasites based on ENAR protocol

We then evaluated the ENAR protocol with cultured *Pf* strains including the strains PfDD2, PfHB3 and PfNF54 by preparing ten-fold serial dilutions in whole blood with parasite densities corresponding to 0.1 – 1000 Pf/µL. RDTs were probed with 5 µL of diluted cultures, the NA extracted by ENAR and analysed by qPCR and RT-qPCR (Fig 1C). Only the RT-qPCR assay resulted in detection of all three strains with the 1 Pf/µL parasite density. Furthermore, the Pspp18S based RT-qPCR assay even detected two (PfDD2 and PfNF54) out of the three *Pf* strains at density of 0.1 Pf/µL. This result demonstrates that the ENAR clearly co-extracts DNA and RNA. The *Pf* 18S ribosomal RNA, detected by the Pspp18S RT-qPCR assay, is highly and constant expressed during the life cycle of the parasite [42,43], while the acidic terminal sequence of the var genes (PfEMP1), detected by the PfvarATS assay, has lower RNA levels [44]. The ability of the ENAR protocol to co-extract DNA and RNA was also demonstrated with the following experiment. Five µL of an *in vitro* generated stage V gametocyte culture was applied onto RDTs and stored at RT for three weeks before NA were extracted by ENAR. The gametocyte-specific transcript PF3D7_0630000 was reverse transcribed and amplified using a published assay which does not require DNase treatment for specific detection of gametocytes [36]. Extracted NA from 5 µL undiluted and 1:100 diluted stage V gametocytes amplified specifically the gametocyte marker, while the control without reverse transcription step did not result in amplification (Fig S2).

The PfIS, an international standard with known parasite density, was used to explore the feasibility of quantifying *Pf* parasites extracted by ENAR. In total, 51 individual RDTs containing 5 µL PfIS with different parasite densities, ranging from 0.1 to 10'000 Pf/µL of the PfIS, were prepared. A high reproducibility and a reversed correlation between parasite densities and Cq values were observed for both targets, the *Pf* specific PfvarATS and the pan-*Plasmodium* target Pspp18S (Fig 1D). Based on the slope, RT-qPCR efficiencies of 75.4% and 124.3% were calculated for PfvarATS and Pspp18S, respectively. RDTs negative for PlasQ assay amplification (Cq > 45, colored in red) carried mostly dilutions representing parasite densities ≤ 1 parasite/µL. Two exceptions were observed where the Pspp18S assay failed to amplify two RDTs probed with higher parasitaemia levels (5 and 10 Pf/µL, respectively). RDTs probed with 1 parasite/µL were detected in 4 (PfvarATS) and 7 (Pspp18S) out of 10 RDTs tested.

In summary, based on experiments conducted with standardized Pf reference samples we conclude that ENAR is able to recover DNA and RNA, which results in an increased sensitivity of the RT-qPCR compared to the qPCR-based detection methods. The lower limit of detection (LOD) for RT-qPCR-based amplification of NA from RDTs is around 1 Pf/µL, although 10x lower parasitaemia levels can be detected as demonstrated with freshly cultured *Pf* parasites. RDTs are a reliable source of NA and extraction by ENAR followed by analysis using RT-qPCR assays allows quantification of *Pf* parasites.

Evaluation of ENAR protocol using Controlled Human Malaria Infection studies as a platform

Blood collected from volunteers undergoing Controlled Human Malaria Infection (CHMI) studies represent well-characterized samples since the parasite strain, the timing and dosing of infection is known. Therefore, blood samples collected from volunteers undergoing CHMI are highly suitable to develop and validate novel malaria diagnostic tools [45].

The ENAR protocol was evaluated with venous blood samples collected during CHMIs assessing the efficacy of Sanaria's PfSPZ Vaccine in clinical trials in Bagamoyo, Tanzania in malaria pre-exposed volunteers. RDTs were probed with blood and stored as part of two CHMIs, the first CHMI was conducted in 2016 (CHMI-1) and the second CHMI in 2018 (CHMI-2). As part of the standard diagnostic procedures during the CHMIs, whole blood was collected in EDTA tubes and DNA was extracted from a total of 180 µL whole blood. A DNA-based qPCR assay was run and parasitaemia quantified (defined as WB-qPCR). Parasite densities as low as 0.05 Pf/µL are detected with the WB-qPCR protocol. During both CHMIs fresh blood from asymptomatic subjects collected 9 to 18 days post CHMI was tested with RDTs (Table 1). CHMI-1 and CHMI-2 used two different types of RDTs which required 20 µL and 5 µL of whole blood, respectively. RDTs collected during CHMI-1 were stored for 605 days on average (storage defined as > 18 months), while RDTs collected during CHMI-2 were stored for 18 days on average (< 1 month) before processing following the ENAR protocol. During the entire storage period, RDTs were kept at RT in a closed box and protected from light. NA were extracted from the RDTs using the ENAR protocol and parasites were detected and quantified by RT-qPCR using the PlasQ assay.

Table 1. Overview of blood samples collected during two CHMIs and stored on RDTs.

Impact of long-term storage on detection rate of parasite NA extracted by ENAR

First, we analysed the impact of RDT storage time on the parasite detection rates. Long-term storage (> 18 months) negatively affects the *Pf* detection rate in samples with parasite density between 1 and 10 Pf/µL in blood used to probe the RDT but has no negative impact on samples with initial parasite density greater than 10 Pf/µL (Fig 2A). Long-term storage negatively affects rather the detection rate based on the Pspp18S target (33% vs 100%, Fisher's exact test p = 0.06) than the PfvarATS target (66% vs 100%, Fisher's exact test p = 0.46). Interestingly, the parasite densities estimated from RDTs

with shorter storage time (< 1 month) are closer to the reference parasite densities assessed by WB-qPCR using 180 µL freshly prepared blood than the estimates from RDTs with longer storage time (> 18 months) (Fig 2B). This is an additional indicator that NAs conserved on RDTs might undergo degradation over time.

Clinical sensitivity and parasite quantification based on ENAR approach

If the data of both CHMIs is combined, the overall detection rate was 54% for the ENAR-based RT-qPCR when compared to WB-qPCR, which was significantly higher than detection by microscopy (9%) or PfHRP2 antigen capture by RDT (12%) using the identical samples.

In order to understand the contribution of RNA to the detection rates in this clinical sample set, we decided to compare RT-qPCR with qPCR. Detection rates of RT-qPCR in relation to parasite densitiy reveals an improved diagnostic performance over the whole range of *Pf* densities compared to qPCR (Fig 2C). RT-qPCR is significantly more sensitive than qPCR for the Pspp18S assay (27% vs 47%, McNemar test p=0.0026), but not for the PfvarATS assay (47% vs 47%, McNemar test p=1.0). Interestingly, among the long-term stored RDTs collected in 2016, the detection rate of the Pspp18S assay was also significantly higher for RT-qPCR compared to qPCR (52% vs 22%, McNemar test p =0.01). Even after long-term storage a significant proportion of (fragmented) RNA can be still extracted and used for RT-qPCR amplification.

Parasite densities determined by WB-qPCR versus densities obtained with ENAR-based RT-qPCR method showed significant positive correlation underlying the quantitative character of our approach (Fig 2D). The correlation was stronger with the PfvarATS assay (r^2 = 0.72) than with the Pspp18S assay (r^2 = 0.39).

Implementation of ENAR protocol within malaria indicator survey

Next, we implemented the ENAR approach within a malaria indicator survey in which we aimed to screen for SNP in the pfk13 propeller region to study the prevalence and type of mutations potentially associated with artemisinin resistance. We started our project with the 2018 malaria indicator survey on Bioko Island which included more than 13'000 individuals (Fig 3A). Instead of disposing the RDTs after probing and reading, used tests were labeled with a barcode with the intention to connect each RDT with other survey data collected in questionnaires (Fig 3B). For each of these barcode-labeled RDTs an extra informed consent for molecular analysis was obtained from the participants or their legal guardians. For the sorting and selection of distinct RDTs that we wanted to analyse, we developed the *RDTselect* app (https://github.com/Sparclex/barcode-value-finder), a browser-based mobile phone application which identifies barcode-labeled RDTs based on an input list containing all barcodes of a certain selection (Fig 3C). To enable tracking of an individual RDT throughout the ENAR extraction process the *RDTallocator* app (https://github.com/Sparclex/position-allocator) was programmed. The barcodes are scanned with a mobile phone camera and the *RDTallocator* app allocates the associated RDT strip to the next available position in a 96-well plate (Fig 3C). Next, after opening the RDT shell the entire uncut RDT strip is removed with sterile, single use forceps (Fig 3D), incubated with lysis buffer in a 12-well long-format plate (Fig 3E), and finally NA are extracted with a high-throughput 96-well format of the ENAR protocol (Fig 3F). All extracted samples undergo initial screening for presence of *Plasmodium* spp. parasites and quality control using the PlasQ RT-qPCR assay (Fig 3G). All RT-qPCR data generated were managed and analysed by a custom-designed laboratory management and information system. ELIMU-MDx is designed for automated quality control, management and analysis of qPCR data [41] (Fig 3H). Samples positive for *Pf* were subjected to amplification and sequencing of pfk13 for identification of SNPs associated with drug resistance (Fig 3I).

A total of 2690 out of 13'270 (20.3%) RDTs were extracted by ENAR and analysed for *Plasmodium* spp. parasites by RT-qPCR. The demographic information of the entire MIS population and the selected volunteers for the molecular analysis are given in Table 2. Only volunteers with body temperature < 37.5 °C were included. Intentionally, in our sample set volunteers with a positive RDT and pregnant women are over-represented.

Table 2. Demographic information of MIS participants.

Malaria infections among asymptomatic MIS participants are characterized by *Pf* infections with mainly low parasite densities

Applying the approach described in Figure 3, 30.8% (828/2690) of analysed RDTs were tested positive for *Plasmodium* spp. NA (Table 3). A qPCR-based species identification revealed that 92.9% were *Pf*, 4.0% *P. malariae* and 1.0% *P. ovale* spp. No *P. vivax* or *P. knowlesi* NA were found. In this asymptomatic population the *Pf* infections had on average parasite densities of 29.2 Pf/µL, with densities being the highest among children below the age of five years (Fig 4a). The rather low parasitaemia levels of asymptomatic individuals in combination with the little amount of blood available have implications for pfk13 genotyping. Samples with parasitaemia levels below 50 Pf/µL are being rarely amplified successfully for pfk13 sequencing (Fig 4b). In order to increase the efficiency of pfk13 genotyping process from RDTs, pre-selection based on their RDT result is advised. For example, 84.5% of RDTs positive for both, PfHRP2 and pLDH, carried parasite densities high enough to result in successful amplification of the pfk13 propeller region.

Table 3. ENAR-based identification of malaria parasites using PlasQ RT-qPCR assay.

Low prevalence of SNPs in the pfk13 propeller region among *Pf* parasite isolates on Bioko

Sequence analysis of the pfk13 propeller region revealed a low prevalence of SNPs (Table 4). 97.6% (283/290) of Bioko's *Pf* isolates carried the wildtype allele. Two isolates had the A578S and one the V589I non-synonymous SNPs, which have been described in sub-Saharan Africa before [12,46] and which are not associated with artemisinin resistance [47]. The P553L SNP was first described in Cambodia [13]. This SNP has previously been found at low prevalences in East Africa, in Kenya and Malawi [48] and was recently also found in an isolate from a Chinese national returned from Angola [49]. To our knowledge the V517I SNP has never been described before. Compared to the other three known SNPs, the V517I SNP had the lowest PROVEAN [50] score, indicating no or neutral effects on the biological function of the kelch 13 protein. Two synonymous SNPs, namely, V510V and C469C, were also found.

Table 4. *Pf*k13 propeller polymorphisms observed in MIS population on Bioko Island.

Discussion

This report presents the development of an approach for large-scale, high-throughput and cost efficient molecular surveillance of malaria parasites based on extraction of NA from RDTs. During the development of ENAR, special attention was given to the evaluation of its reproducibility and the impact of long-term storage on the detectability of the NAs. Using samples from CHMI studies as a standardized platform allowed us to conclude that NAs can be reliably recovered and amplified from RDTs, even after long-term storage at RT. The small amount of blood in combination with low parasite density is a challenge when it comes to detecting *Pf* in asymptomatic patients. Therefore, we aimed to maximize the amount of NA recovered from RDTs by expanding the pool of possible target molecules for detection to RNA by using RT-qPCR. Even after a storage period of over 18 months at RT the detection rate of the RT-qPCR assay was still significantly higher compared to qPCR only, indicating long-term preservation of DNA and RNA.

We aimed to transform the ENAR approach into an amendable tool for larger scale surveillance studies by increasing extraction and analysis throughput. The ENAR approach was successfully integrated into the 2018 MIS on Bioko Island. More than 13'000 individuals gave their extra consent for storage and molecular analysis of their RDT. This high acceptance rate was also described by others [21] and can be attributed to the convenient way of blood collection by finger prick and the small blood volume, usually 5 to 10 µL, applied onto RDTs. With a total of 2750 RDTs, we analysed blood from more than 20% of MIS participants. This was made possible by the development of custom-made software solutions for sorting and identification of RDTs and by reducing the processing time significantly by using the entire RDT strip instead of cutting it into pieces.

Robust (quantitative) data, as generated by ENAR, in combination with a large-scale MIS adds substantial value to our understanding of malaria endemicity on Bioko Island without the need of conducting additional expensive and time consuming epidemiological studies. We did not find evidence

for presence of *P. vivax* on Bioko Island. This is contradictory to previous studies when a surveys carried out in 1996 and 1998 found two [51] and one [52] case of *P. vivax,* respectively.

We decided to screen for SNPs in the propeller region of the pfk13 gene among asymptomatic individuals to obtain data of possible artemisinin resistant *Pf* strains circulating on the island. We found that 1.7% (5/290) of the analysed *Pf* isolates had non-synonymous SNPs in the pfk13 propeller region, which is comparable to prevalences found in other African countries [46]. Among the five isolates with non-synonymous SNPs, two isolates had the A578S, one the V589I, one the P553L and one the V517I SNP. The A578S and V589I allele had been reported in the region already [53,54], while with V517I we found one new allele, which has not been reported yet at all. Interestingly, the P553L SNP is the only mutation we found which was previously associated with delayed parasite clearance [12]. Although the prevalence of pfk13 SNPs seems to be low at the moment, the spread of *Pf* parasites with pfk13 SNP needs to be closely monitored. An molecular surveillance approach as presented may offer an unique opportunity to support policy makers regarding the choice and change of drugs for malaria treatment [55].

Based on the presented results we conclude that ENAR provides a powerful tool for molecular malaria surveillance and could be reliably used for retrospective quantitative and in-depth molecular studies of malaria.

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Competing interests

SL Hoffman is salaried and full-time employee of Sanaria Inc, the developer and sponsor of Sanaria® PfSPZ Vaccine. He was not responsible for the collection, recording or entry of the parasitological data used in this study. The other authors have no conflicts of interest.

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Ethics approval and consent to participate

For the school-based survey in Mkuranga district, sample collection was approved by the Senate Research and Publication Committee (SRPC) of the Muhimbili University of Health and Allied Sciences and the respective authorities at Mkuranga district.

Both clinical trials were performed in accordance with Good Clinical Practices (GCP). CHMI-1 (Clinical Trials.gov: NCT02613520) protocol was approved by IRBs of the Ifakara Health Institute (IHI) (Ref. No. IHI/IRB/ No: 32-2015), the National Institute for Medical Research Tanzania (NIMR) (NIMR/HQ/R.8a/Vol.IX/2049), and the Ethikkommission Nordwest- und Zentralschweiz (EKNZ)

Switzerland (reference number 15/104). The protocol was also approved by the Tanzania Food and Drug Authority (TFDA) (Auth. No. TZ15CT013). CHMI-2 (Clinical Trials.gov: NCT03420053) protocol was approved by IHI's IRB (Ref. No. IHI/IRB/ No: 32-2015), NIMR (NIMR/HQ/R.8a/Vol.IX/2049), EKNZ (reference number 15/104) and TFDA (Auth. No. TZ15CT013). The 2018 malaria indicator survey was approved by the Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of the London School of Hygiene & Tropical Medicine. Written informed consent was obtained from all adults and from parents or guardians of children who agreed to participate. Only samples for which an additional consent for molecular analysis was obtained were included in this study.

Abbreviations

Pf (*P. falciparum*), pfk13 (*Pf kelch* 13), RDT (rapid diagnostic test), DBS (dried blood spot), ENAR (extraction of nucleic acids from RDT), CHMI (controlled human malaria infection), NA (nucleic acids), NAT (nucleic acid amplification technique), PfIS (WHO International standard for *P. falciparum* NAT), LOD (limit of detection), RT (room temperature), qPCR (quantitative polymerase chain reaction), PlasQ (multiplex qPCR assay for quantification of *P. falciparum* and *Plasmodium spp.* Parasites), SNP (single nucleotide polymorphism), Pf/µL (*Pf* parasites per µL blood)

Figure legends

Figure 1. Extraction and detection of *Pf* NAs from used RDTs. A) Recovery rates of *Pf* DNA from RDTs collected in asymptomatic Tanzanian school children. B) Comparison of extraction perfromance of four protocols based on Cq values of the human *rnasep* gene. C) Association of parasite densities and Cq values of freshly prepared *Pf* strains (PfDD2, PfHB3 and PfNF54). Grey colour indicates failed detection. D) Correlation between parasite densities of selrial diluted PfIS and Cq values for PlasQ targets. Red coloured dots represent samples with failed amplification.

Figure 2. Evaluation of ENAR protocol using samples collected during CHMI studies. A) *Pf* detection rates grouped by parasite density and storage time. B) Quantification ratio between densities derived from ENAR and densities derived from whole blood qPCR (WB-qPCR). C) Diagnostic sensitivity of rapid diagnostic test (RDT), ENAR followed by qPCR detection (qPCR) and ENAR followed by RT-qPCR detection (RT-qPCR) in relation to parasite density. Rolling means of 10 observations, using WB-qPCR as a gold standard, are shown with 95% CIs (shaded areas). D) Correlation of parasite densities obtained from DNA extracted from fresh whole blood and NA extracted by ENAR.

Figure 3. Adaptation of ENAR protocol for analyzing large numbers of barcoded RDTs. A) Malaria indicator survey is conducted which includes a detailed questionnaire. B) Malaria prevalence is determined by RDT followed by storage of barcode labelled RDTs. C) Sorting and tracking of RDTs using smartphone apps. D-F) High throughput protocol for extraction of NA from RDTs using the ENAR approach. G) Detection and quantification of *Pf* and non-*Pf* malaria parasite. H) Automated analysis of qPCR data using ELIMU-Mdx. I) Genotyping of pfk13 propeller region for drug resistance monitoring.

Figure 4. Parasite densities among asymptomatic individuals and implication for sequence analysis. A) Age group dependent parasite densities. B) Association between parasite density and successful amplification of pfk13 for sequence analysis.

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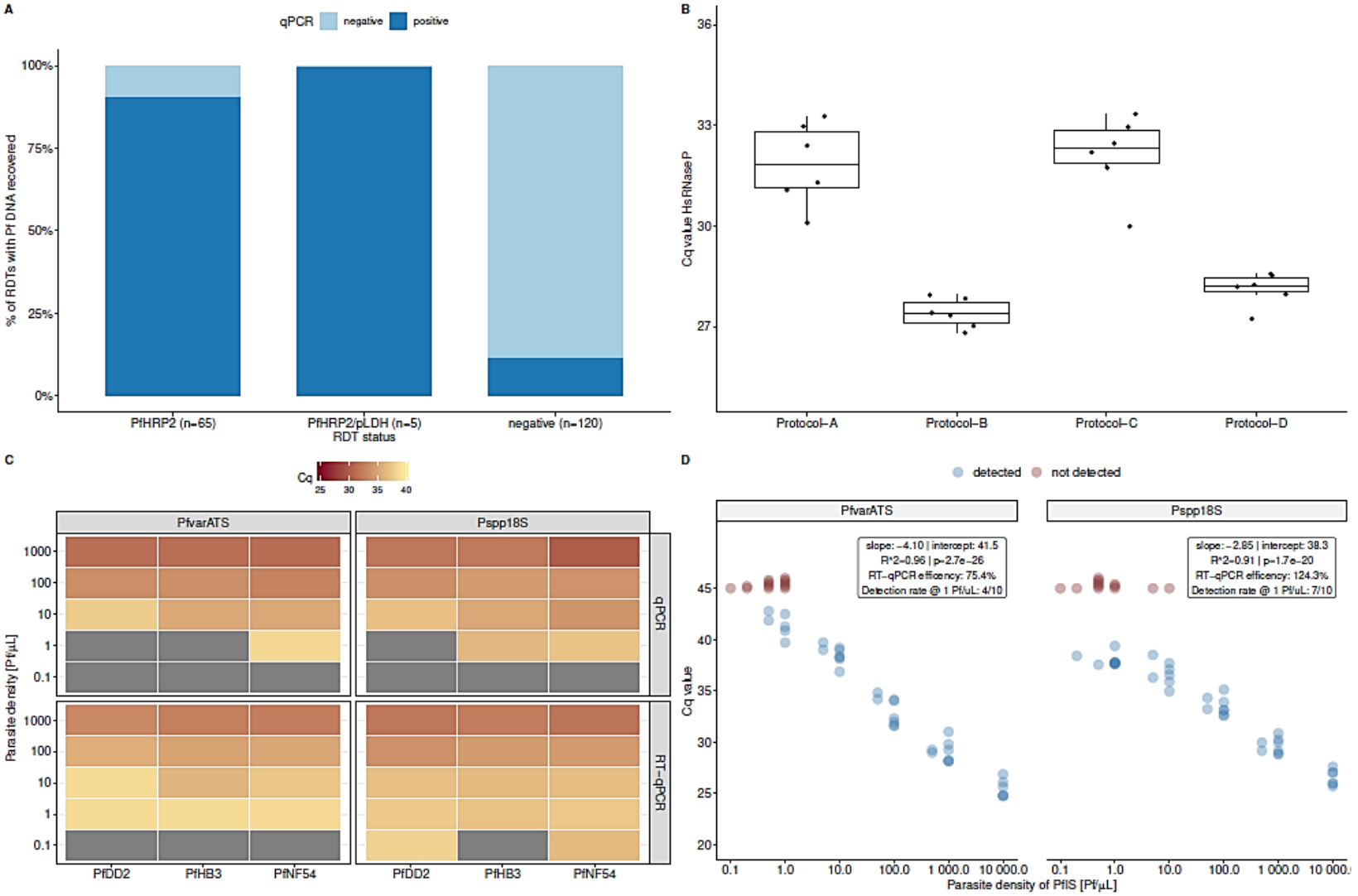
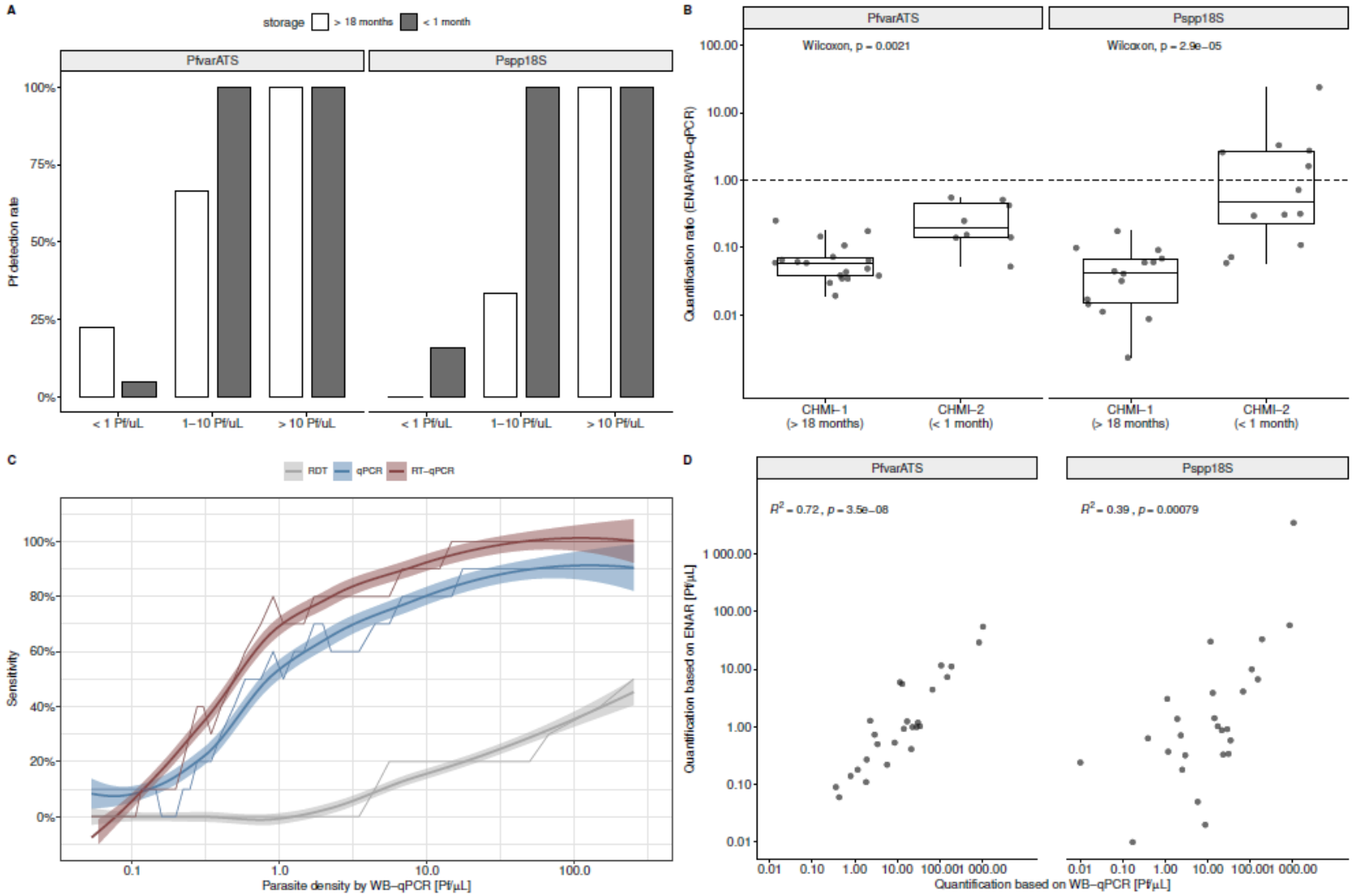
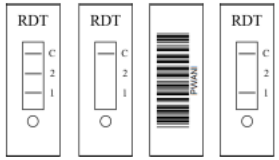

A
qPCR
negative
positive
% of RDTs with Pf DNA recovered
100%
75%
50%
25%
0%
PfHRP2 (n=65)
PfHRP2/pLDH (n=5)
negative (n=120)
RDT status
B
Cq value HsRNaseP
36
33
30
27
Protocol-A
Protocol-B
Protocol-C
Protocol-D
C
Cq
25
30
35
40
PfvarATS
Pspp18S
qPCR
RT-qPCR
Parasite density [Pf/µL]
1000
100
10
1
0.1
PfDD2
PfHB3
PfNF54
D
detected
not detected
PfvarATS
Pspp18S
slope: -4.10 | intercept: 41.5
R^2=0.96 | p=2.7e-26
RT-qPCR efficency: 75.4%
Detection rate @ 1 Pf/uL: 4/10
slope: -2.85 | intercept: 38.3
R^2=0.91 | p=1.7e-20
RT-qPCR efficency: 124.3%
Detection rate @ 1 Pf/uL: 7/10
Cq value
45
40
35
30
25
20
0.1
1.0
10.0
100.0
1 000.0
10 000.0
Parasite density of PfIS [Pf/µL]


A
storage > 18 months < 1 month
PfvarATS
Pspp18S
Pf detection rate
100%
75%
50%
25%
0%
< 1 Pf/uL
1–10 Pf/uL
> 10 Pf/uL
B
PfvarATS
Pspp18S
Wilcoxon, p = 0.0021
Wilcoxon, p = 2.9e–05
Quantification ratio (ENAR/WB–qPCR)
100.00
10.00
1.00
0.10
0.01
CHMI–1 (> 18 months)
CHMI–2 (< 1 month)
C
RDT
qPCR
RT–qPCR
Sensitivity
100%
80%
60%
40%
20%
0%
0.1
1.0
10.0
100.0
Parasite density by WB–qPCR [Pf/µL]
D
PfvarATS
Pspp18S
R^2 = 0.72 , p = 3.5e–08
R^2 = 0.39 , p = 0.00079
Quantification based on ENAR [Pf/µL]
1 000.00
100.00
10.00
1.00
0.10
0.01
0.01 0.10 1.00 10.00 100.00 1 000.00
Quantification based on WB–qPCR [Pf/µL]

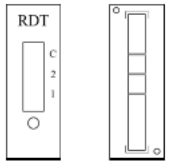
A. Malaria indicator survey with high population coverage

B. Malaria diagnosis using RDT followed by storage of barcode-labelled RDTs

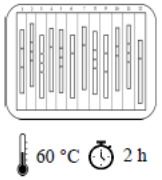


C. Identification and tracking of distinct RDTs with mobile phone apps *RDTselect* and *RDTallocator*

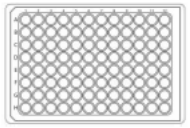
D. RDT strip is removed from shell with single use forceps to avoid cross-contamination.



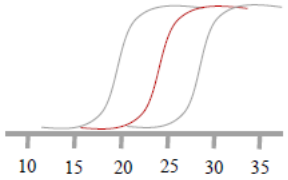
E. Horizontal incubation of entire uncut RDT strip for total recovery of NA



F. High-throughput purification of NA using 96-well format *ENAR* protocol

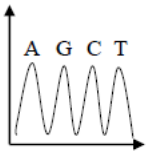


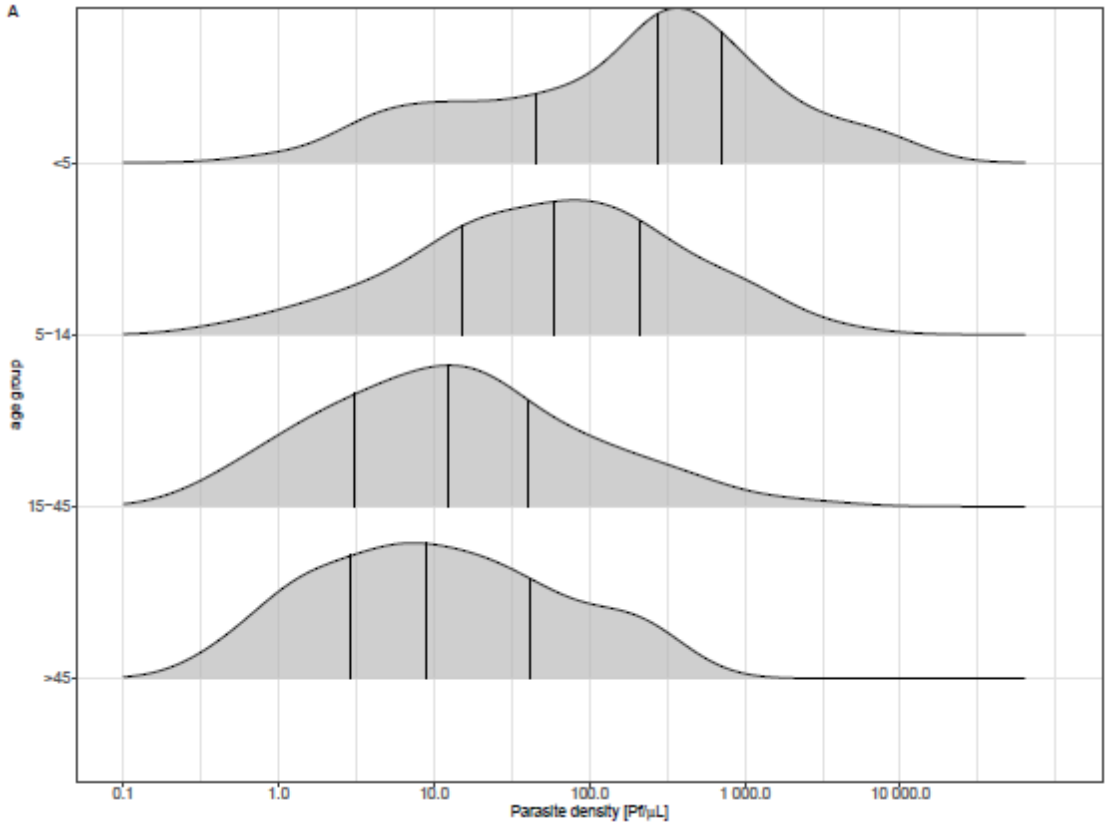
G. Systematic identification and quantification of *Plasmodium spp.* parasites using PlasQ assay



H. Automated qPCR data management, quality control and analysis using *ELIMU-MDx* platform

I. Sequencing of pfk13 propeller region for drug resistance monitoring of local *P. falciparum* strains




A
<5
5−14
15−45
>45
age group
0.1
1.0
10.0
100.0
1 000.0
10 000.0
Parasite density [Pf/µL]

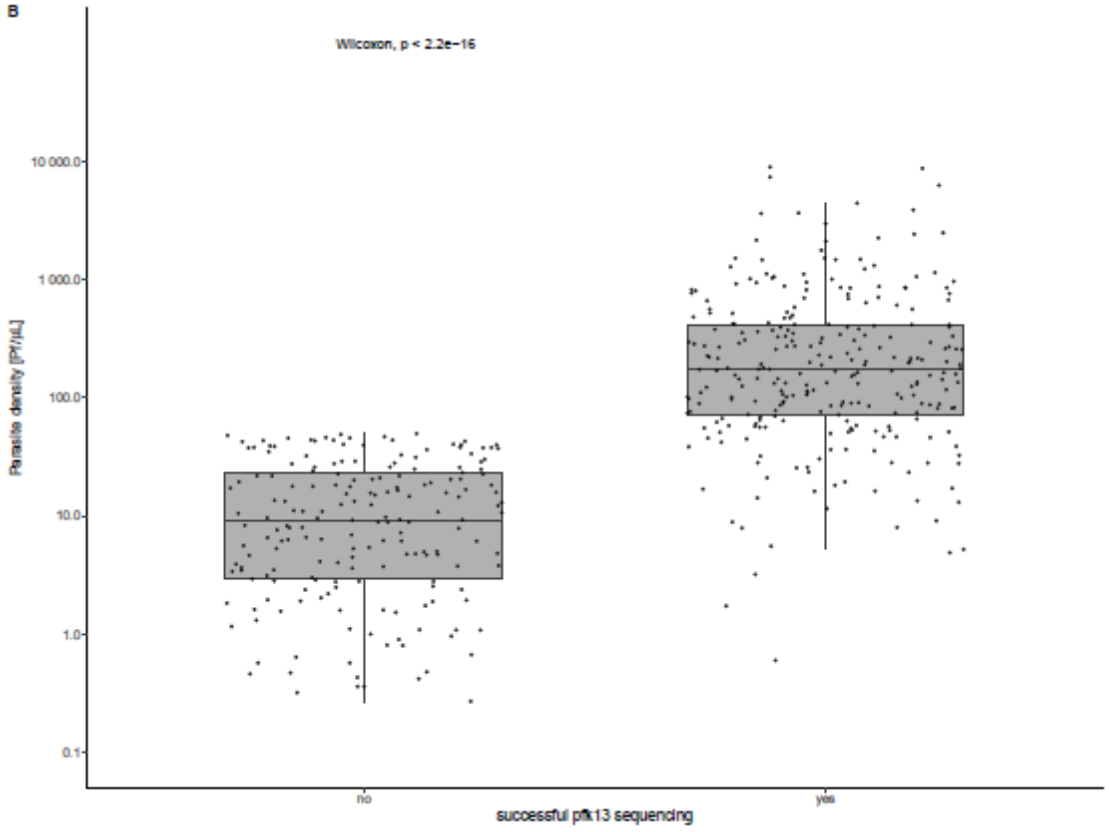

B
Wilcoxon, p < 2.2e−16
10 000.0
1 000.0
100.0
10.0
1.0
0.1
Parasite density [Pf/µL]
no
yes
successful pfk13 sequencing

Table 1. Overview of blood samples collected during two CHMIs and stored on RDTs.

Study (Year)	**CHMI-1 (2016)** **> 18 months storage**	**CHMI-2 (2018)** **< 1 month storage**
RDT brand	BinaxNOW® Malaria RDT	CareStart™ Malaria (Pf/PAN) Combo
Number of RDTs collected	71	50
Blood volume on RDT	20 µL	5 µL
Storage time in days (mean and range)	605 (596-616)	18 (10-48)
Storage conditions	RT	RT
Sampling days post CHMI (mean and range)	14.0 (10.5-18.0)	12.7 (9.0-18.0)
% positive by WB-qPCR	38.0% (27/71)	62.0% (31/50)
WB-qPCR parasite density (parasites/µL, geom. mean and range)	4.7 (0.05-840.0)	0.3 (0.01-1041.0)

Table 2. Demographic information of MIS participants.

	All individuals (n=13270)	Selected individuals (n=2690)
Gender		
female	7155 (53.9 %)	1569 (58.3 %)
male	6115 (46.1 %)	1121 (41.7 %)
Age (years)		
Mean (SD)	21.2 (± 17.7)	27.1 (± 21.0)
Age group		
<5	2308 (17.4 %)	276 (10.3 %)
5-14	3719 (28.0 %)	660 (24.5 %)
15-45	5758 (43.4 %)	1208 (44.9 %)
>45	1485 (11.2 %)	546 (20.3 %)
District		
Baney	1519 (11.4 %)	400 (14.9 %)
Luba	1093 (8.2 %)	268 (10.0 %)
Malabo	10121 (76.3 %)	1814 (67.4 %)
Riaba	537 (4.0 %)	208 (7.7 %)
RDT result		
negative	11842 (89.2 %)	1623 (60.3 %)
pLDH	43 (0.3 %)	39 (1.4 %)
PfHRP2	871 (6.6 %)	653 (24.3 %)
pLDH+PfHRP2	462 (3.5 %)	367 (13.6 %)
Pregnancy status		
currently pregnant	237 (1.8 %)	225 (8.4 %)
gave birth to live baby	918 (6.9 %)	128 (4.8 %)
Hemoglobin (g/dL)		
Mean (SD)	12.4 (± 1.79)	12.1 (± 1.88)
Anemia status		
no	8874 (65.7 %)	1593 (57.6 %)
mild	2711 (20.1 %)	634 (22.9 %)
moderate	1777 (13.2 %)	502 (18.1 %)
severe	110 (0.8 %)	34 (1.2 %)

Table 3. ENAR-based identification of malaria parasites using PlasQ RT-qPCR assay.

	number of samples (%)
RDTs analysed by PlasQ	2690
Positive for PlasQ RT-qPCR	828 (30.8%)
Plasmodium spp. identification	
Positive for *P. falciparum*	769 (92.9%)
P. falciparum with >100 Pf/µL	227 (29.5%)
Positive for *P. malariae*	33 (4.0%)
Positive for *P. ovale* spp.	8 (1.0%)
Positive for *P. knowlesi*	0 (0.0%)
Positive for *P. vivax*	0 (0.0%)
Pf/Pm co-infections	16 (1.9%)

Table 4. *Pf*k13 propeller polymorphisms observed in MIS population on Bioko Island.

Kelch13 propeller genotyping		**PROVEAN score**
P. falciparum strains sequenced	290	
PfNF54 allele	283 (97.6%)	
Non-synonymous SNPs		
A578S (G1732T)	2 (0.69%)	-1.962
V589I (G1765A)	1 (0.35%)	-0.663
V517I (G1549A)	1 (0.35%)	-0.562
P553L (C1659T)	1 (0.35%)	-1.721
Synonymous SNPs		
V510V (G1530A)	1 (0.35%)	
C469C (C1407T)	1 (0.35%)	

Table S1. Summary of published studies using DNA extracted from RDTs for molecular analysis of malaria parasites.

Publication/Year	**Veron et al. 2006**	**Ishengoma et al. 2011**	**Cnops et al. 2011**	**Morris et al. 2013**	**Papa-Mze et al. 2015**	**Nabet et al. 2016**	**Ndiaye et al. 2017**	**Boyce et al. 2018**	**Robinson et al. 2019**	**Nag et al. 2019**	**Nguyen et al. 2019**
Origin of RDTs	French Guiana	Tanzania	Belgium	Tanzania	Senegal, Comoros Islands	Mali	Senegal	Uganda	Kenya	Guinea-Bissau	Gabon
Storage time and temperature	6-36 months at room temperature	28-65 days at room temperature	1 week – 16 month at room temperature	1-9 months at room temperature	not specified	14 months at room temperature	<1 month at RT	2 years at room temperature	14 months at −20 °C	3-18 months at room temperature	0-6 months at room temperature
Number of samples for clinical evaluation	40	165	121	855	204	134	572	299	141	2488	669
Brand of RDT used for clinical evaluation	Optimal®, ICT Now®	ParaHIT®f, Paracheck Pf®	12 different RDT brands were assessed, OptiMAL and SD BIOLINE Malaria Ag P.f® and P.f/Pan® were used to determine sensitivity	Paracheck-Pf, SD BIOLINE Malaria Ag P.f® and P.f/Pan®	SD BIOLINE Malaria Ag P.f® and P.f/Pan®	SD BIOLINE Malaria Ag P.f® and P.f/Pan®	SD BIOLINE Malaria Ag P.f® and P.f/Pan®	SD BIOLINE Malaria Ag P.f® and P.f/Pan®	SD BIOLINE Malaria Ag P.f® and P.f/Pan®	not specified	VIKIA® Malaria Ag Pf/Pan, Paracheck Pf, SD BIOLINE Malaria Ag P.f® and P.f/Pan®
Part of RDT used for clinical evaluation	proximal part	proximal part	proximal part	distal part	proximal part	entire strip	proximal	distal part	central part	not specified	proximal part
Extraction method	Phenol/chloroform extraction performed better than Chelex method or QIA amp DNA mini blood kit	Chelex method	Simple boil and spin method outperformed QIAamp DNA mini kit	Chelex method outperformed simple boil and spin method and ABI-based extraction	QIAamp DNA Mini kit	Automated extraction using NucliSENS EasyMAG instrument	Chelex method	Chelex method	Chelex method and robotic extractions using QIAsymphony gave comparable results	Chelex method	QIA amp DNA mini blood kit
Parasite detection	18S rDNA nested PCR	18S rDNA nested PCR	18S rDNA qPCR	18S rDNA nested PCR, cytochrome b nested PCR, and 18S rDNA qPCR	Nested PCR and HRM-qPCR for genotyping	18S rDNA qPCR	pfcrt PCR-SSOP-ELISA	pfldh qPCR	pgmet-tRNA qPCR	18S rDNA PCR	18S rDNA qPCR
Quantification of parasites	no	no	no	no	no	yes	no	no	yes	no	yes
Analytical sensitivity	50 Pf/µL with 15 µL diluted blood from malaria patient	1 Pf/µL with 10 µL diluted blood from malaria patient	0.02 Pf/µL with diluted blood from malaria patient	2 Pf/µL with 5 µL diluted P. falciparum culture	not specified	not specified	not specified	not specified	0.05 Pf/µL based on detection on a single RDT	not specified	70 Pf/µL with 5 µL diluted blood from malaria patient
Clinical evaluation (detection rates)	94.3% detection rate for samples with parasite densities ≥ 400 parasites/µL	91.0% detection rate for samples positive by microscopy	100% detection rate of clinical samples with parasite densities ≥ 1 parasites/µL	No difference in detection rates compared to DBS samples from febrile patients	98.5% amplification rate for genotyping among positive RDTs	79.8% detection rate among positive RDTs collected from symptomatic febrile patients	94.7% detection rate among positive RDTs	96.0% amplification rate among RDTs from patients with severe malaria	81.25% agreement with DBS from children with positive RDT s	74.0% detection rate among positive RDTs from suspected malaria cases	96.6% detection rate during screening for a clinical trial
Non-*P. falciparum* species detected by NAT	P. vivax	no	P. vivax, P. ovale, P. malariae	no	no	no	no	no	no	no	no

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ELIMU-MDx: a web-based, open-source platform for storage, management and analysis of diagnostic qPCR data

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ABSTRACT

The Electronic Laboratory Information and Management Utensil for Molecular Diagnostics (ELIMU-MDx) is a user-friendly platform designed and built to accelerate the turnaround time of diagnostic qPCR assays. ELIMU-MDx is compliant with the MIQE guidelines and has extensive data-import capabilities for all major qPCR instruments by using the RDML data standard. This platform was designed as an open-source software tool and can be accessed through the web browser on all major operating systems.

METHOD SUMMARY

ELIMU-MDx is an open-source web-application developed using PHP to analyze, manage, validate and store user-provided qPCR data in a MySQL database.

KEYWORDS

diagnostic • ELIMU-MDx • infectious diseases • MIQE • qPCR • RDML

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The global market of *in vitro* diagnostics, comprising tools to detect, diagnose or monitor diseases, is estimated to be worth US$40–45 billion [1]. One of the fastest growing areas is the identification of infectious diseases using molecular diagnostics, which is becoming an integral part of medical practice and public health worldwide [2]. Molecular diagnostic tools, especially nucleic acid amplification techniques, provide faster, more sensitive and often more cost-effective diagnoses than traditional culture methods for microbiological analysis [3]. Quantitative PCR (qPCR) is a well-established method for the detection, quantification and typing of bacteria, viruses, fungi and protozoa in the areas of clinical and veterinary diagnostics, as well as food safety [4]. Among the biggest advantages of qPCR-based diagnosis are the universality in designing and developing new assays as well as the widespread availability of the technology. Moreover, qPCR also allows quantification and genotyping of pathogens. With the rapid spread of qPCR into routine diagnostics, standardization and validation processes of qPCR assays and data had to be addressed [4]. A first step was the publication of the MIQE guidelines [5], which has led to a certain degree of standardization in the validation process of qPCR assays across different laboratories. In order to standardize the qPCR data format the real-time PCR data markup language (RDML) [13], which stores the data in a compressed extensible markup language file, was designed [6]. RDML stores the raw data acquired by the qPCR instrument as well as the information required for its interpretation, such as sample annotation and cycling protocol. A comprehensive review of 27 open-access software packages and tools for qPCR data analysis reported that most tools use their own file format and only a fraction of the currently existing tools support the standardized data exchange format RDML [7]. The lack of a common standard for qPCR data makes it difficult to share and analyze data across different qPCR platforms.

The high-throughput character of qPCR analysis in infectious disease diagnosis easily leads to a rapid accumulation of raw data that needs to be quality controlled and interpreted in order to correctly identify and quantify infectious agents. Therefore, access to well-designed, user-friendly and semi-automated software facilitating the storage, quality control (QC) and analysis of diagnostic qPCR data is needed.

Controlled human malaria infection (CHMI), the deliberate exposure of human volunteers to fully infectious malaria parasites, has proven to be safe and well tolerated, and accelerates the evaluation of malaria vaccines candidates in clinical trials [8,9]. Volunteers participating in CHMIs must be monitored closely to ensure volunteer safety. Blood is drawn twice daily for up to 2 weeks and screened for *Plasmodium falciparum* parasites by thick blood smear microscopy, and after the first appearance of blood-stage parasites the antimalarial treatment is initiated. Nowadays, more sensitive qPCR is increasingly used in CHMI to closely monitor parasitemia levels, which improves the safety of participants [10]. In order to manage the vast amount of qPCR data generated during a series of CHMI studies with more than 250 individuals included and conducted in Tanzania and Equatorial Guinea, we decided to design and build a platform that facilities the analysis and interpretation of diagnostic qPCR data. We identified several key features which in our opinion would be required for such software:

• Highly adaptable and integrative: a so-

lution that can be easily adapted to a variety of qPCR assays, supports semi-automated data analysis and integrates into existing sample workflow;
- Data traceability throughout analysis process: the use of an audit trail and community data standards to ensure high data integrity;
- Accessibility and reliability of software: Platform-independent, open-source and low-maintenance software that could be deployed rapidly to new laboratories and other projects using qPCR analysis as a monitoring tool.

To address these needs, we developed the web-based, open-source platform ELIMU-MDx (Electronic Laboratory Information and Management Utensil for Molecular Diagnostics).

MATERIAL & METHODS

ELIMU-MDx development

ELIMU-MDx combines a relational SQL database with a user-friendly web interface programmed in PHP 7.2 using the framework Laravel 5.8 and the tool Laravel Nova 2.0. The platform is accessed through a web browser (Google Chrome, Firefox and Safari were successfully tested) and needs to be hosted on a nginx or apache server. It validates user-provided data and stores it in a MySQL 5.7 database, and where appropriate constraints ensure data consistency.

Analysis of qPCR data by ELIMU-MDx

qPCR data generated for diagnostic purposes have different requirements to software than data generated for other qPCR applications. Most importantly, the consistency and reliability of the data needs to be ensured. Each qPCR run needs to have controls in place to test for contamination (preventing false-positive results) and to test the performance of extraction and qPCR reaction (preventing false-negative results). All data uploaded to ELIMU-MDx is quality controlled based on several controls and predefined criteria. Only samples that pass all QC thresholds are considered for further analysis. Additionally, a sample measured multiple times with the same assay is automatically considered as technical replicates, even if the data are derived from different experiments. This allows users to repeat sample analysis if needed.

ELIMU-MDx processes qPCR data derived from both major qPCR detection technologies. Assays using dye-based (e.g., SYBR or Eva-Green dyes) or TaqMan-based detection can be analyzed. The quantification cycle (Cq) values, the number of cycles needed to reach a fluorescence threshold, is extracted from the RDML file and used for qualitative and quantitative interpretation of the qPCR assay. In case of a dye-based detection, only Cq values from samples whose amplicon has a melting temperature within a predefined range are considered for further analysis. Since the melting temperatures are not included in the RDML version 1.1 from Bio-Rad (CA, USA), CFX Maestro (Version 4.1.2433.1219) and RDML version 1.2 from MyGo Pro PCR Software (Version 3.4; IT-IS Life Science, Republic of Ireland), they need to be uploaded separately in addition to the RDML file.

The current version of ELIMU-MDx supports absolute quantification of the qPCR target molecules using the slope and intercept derived from the linear relationship between Cq values and serially diluted standards of known nucleic acid concentrations.

The assay definition file (ADF) includes all assay-specific parameters that together define the assay. ADF is used by ELIMU-MDx for data analysis using different thresholds and parameters depending on the actual experimental settings. The advantage of defining all assay parameters in one single file is its universality and easy adaption to new qPCR assays. An example for an ADF is provided as an excel file (Supplementary file 1).

RESULTS & DISCUSSION

ELIMU-MDx overview

The structure of ELIMU-MDx is shown in Figure 1. ELIMU-MDx consists of three modules – the sample module, the assay module and the data module – which are connected by an experiment. Each of the modules is supplied with data and parameters from predefined components. These components contain sample-, assay- or data-specific information that are necessary to interpret and document the experiments. The ADF, which contains all parameters necessary to analyze samples measured with the appropriate qPCR assay, is linked through the assay to the experiment. The experiment is considered the central node of ELIMU-MDx and combines the sample and assay metadata with qPCR raw data. Upload of standardized data is followed by initial QC and automated analysis and eventually results in a downloadable result file, which can be used for generating reports or statistical computing and data visualization by any statistical software.

Examples of the ELIMU-MDx user interface are illustrated in Figure 2. All features are accessible through a sidebar (Figure 2A). The oligo details are shown as an example for the level of detail that is provided by ELIMU-MDx's virtual freezer (Figure 2B). As part of the built-in sample inventory, the storage position of each sample can be displayed (Figure 2C). Results are automatically displayed after uploading the qPCR RDML files and samples with inconclusive results can be easily identified using the custom-build filters (Figure 2D). The integrated audit trail records all major events and therefore ensure high data integrity (Figure 2E).

ELIMU-MDx is built as an open-source web-app

ELIMU-MDx is a user-friendly platform designed and built to accelerate the turnaround time of diagnostic qPCR assays. The advantages of web-based applications include rapid deployment, cross-platform compatibility and easy application development [11]. A detailed guide to setup the platform, with step-by-step instructions, is provided at https://sparclex.github.io/elimu. The low-maintenance structure is an additional advantage, particularly in resource-limited settings. The source code is open source and distributed under a MIT license and is publicly available for download. ELIMU-MDx is unlike most commercially available qPCR software in that it is not a black box. All features of ELIMU-MDx are completely disclosed and can be improved or changed by other members of the scientific community or even used separately in other software.

The ELIMU-MDx can be accessed through https://elimu.ch, where we have created a specific user account for demonstration purposes (login: demo.user@elimu.ch, password: elimu2019).

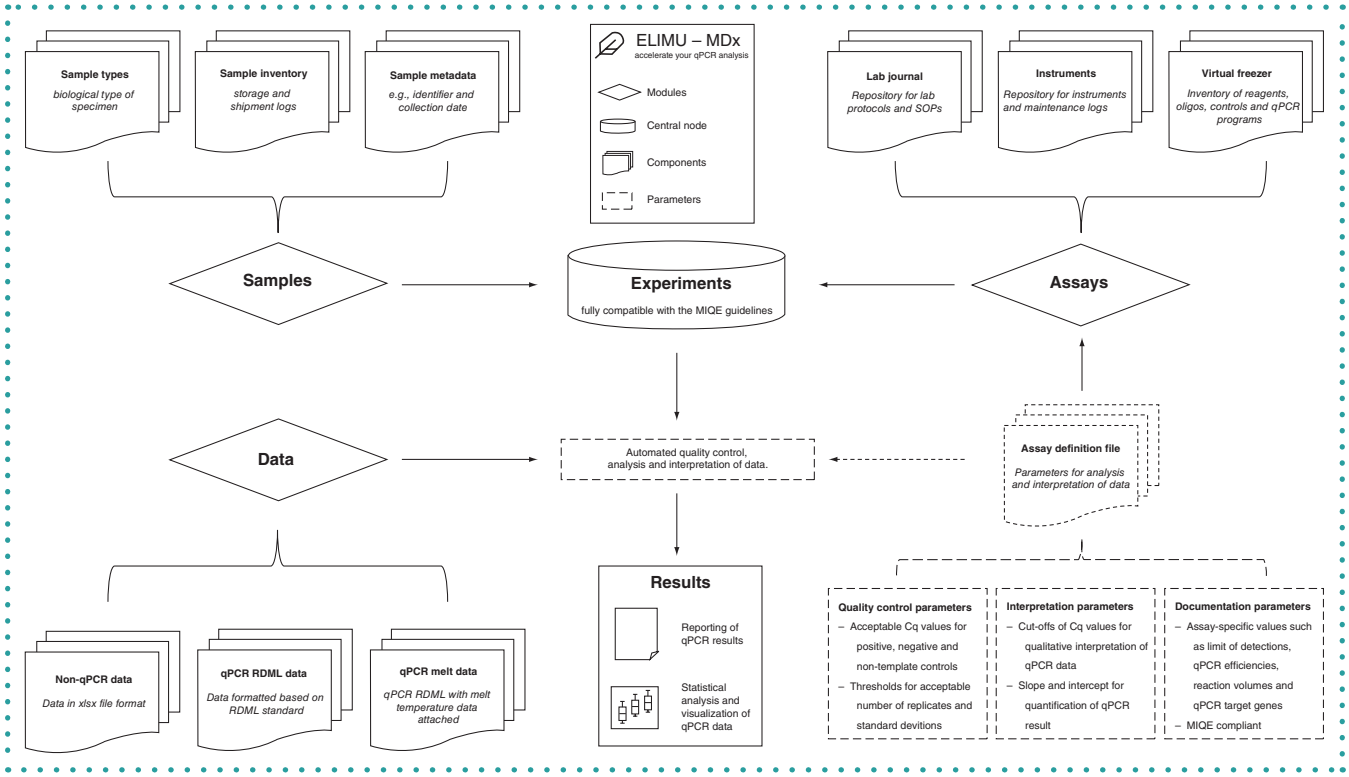

Figure 1. Structure of ELIMU-MDx. The sample, assay and data modules are connected to create an experiment, which is the central node of the platform. Assay-specific parameters are provided through the assay definition file.
Cq: Quantification cycle; RDML: Real-time PCR data markup language; SOP: Standard operating procedure.

ELIMU-MDx uses the RDML data standard, making it qPCR instrument independent

ELIMU-MDx supports RDML, a structured and universal data standard for exchanging qPCR data. RDML files contain all information to understand the experimental setup, reanalyze the data and interpret the results. The use of the RDML data standard allows using data from different qPCR instruments. The platform was successfully tested with RDML files version 1.1 and 1.2 from the Bio-Rad CFX96 Real-Time System and the MyGo Pro qPCR instrument.

Semiautomated data QC & analysis improves sample-to-result turnaround times

The use of the standardized data format of RDML files allowed us to automate QC, analysis, and validation of qPCR data. Initial QC includes the automated analysis of the positive, negative and nontemplate control of each qPCR run uploaded based on provided cut-off values. The internal control of each sample is used to validate the extraction and amplification performance. Based on the Cq values provided by the RDML file and a set of assay-specific parameters, as defined by the ADF, QC as well as qualitative and/or quantitative analysis is performed.

The ELIMU-MDx platform was extensively tested with diagnostic qPCR data generated during CHMI studies in Tanzania (total of 478 samples) and Equatorial Guinea (total of 2907 samples). To demonstrate the advantages and speed of ELIMU-MDx, we used three separate qPCR runs generated during CHMI studies for a head-to-head comparison with data-management solutions based on Microsoft (MS; WA, USA) Excel and a previously used custom-built MS Access database (Figure 3). At each of the four main steps of qPCR data management, the use of ELIMU-MDx allows for faster processing of qPCR data. With ELIMU-MDx the initial QC of the qPCR run is automatically performed during the upload of the qPCR data and by using a single, standardized RDML file containing the qPCR raw data, the upload procedure to ELIMU-MDx is simple and faster. Once the data are uploaded, the Cq values are interpreted and the result is linked to the sample. At this step, after a manual review of the data, the results are summarized and ready to be reported. Overall, the entire process from initial QC to a final report took approximately 15 min using ELIMU-MDx. This is a significant reduction in time spent on QC and analysis of the qPCR data when compared with the two other data analysis tools.

ELIMU-MDX simplifies documentation, statistical analysis & visualization of qPCR experiments

The combination of assay-specific information, as provided by the ADF, sample metadata and data provided by the RDML file, contains all information required by the MIQE guidelines. Table 1 lists all information documented for each qPCR experiment as defined by the ELIMU-MDx platform.

The ELIMU-MDx platform also serves as a laboratory information and management system. We added general features of a laboratory information and management system to the ELIMU-MDx platform. A unique storage position can be assigned to each sample to keep track of sample storage. If samples are shipped, all details of the shipment will be logged. Apart from qPCR data, all other types of results can ▶

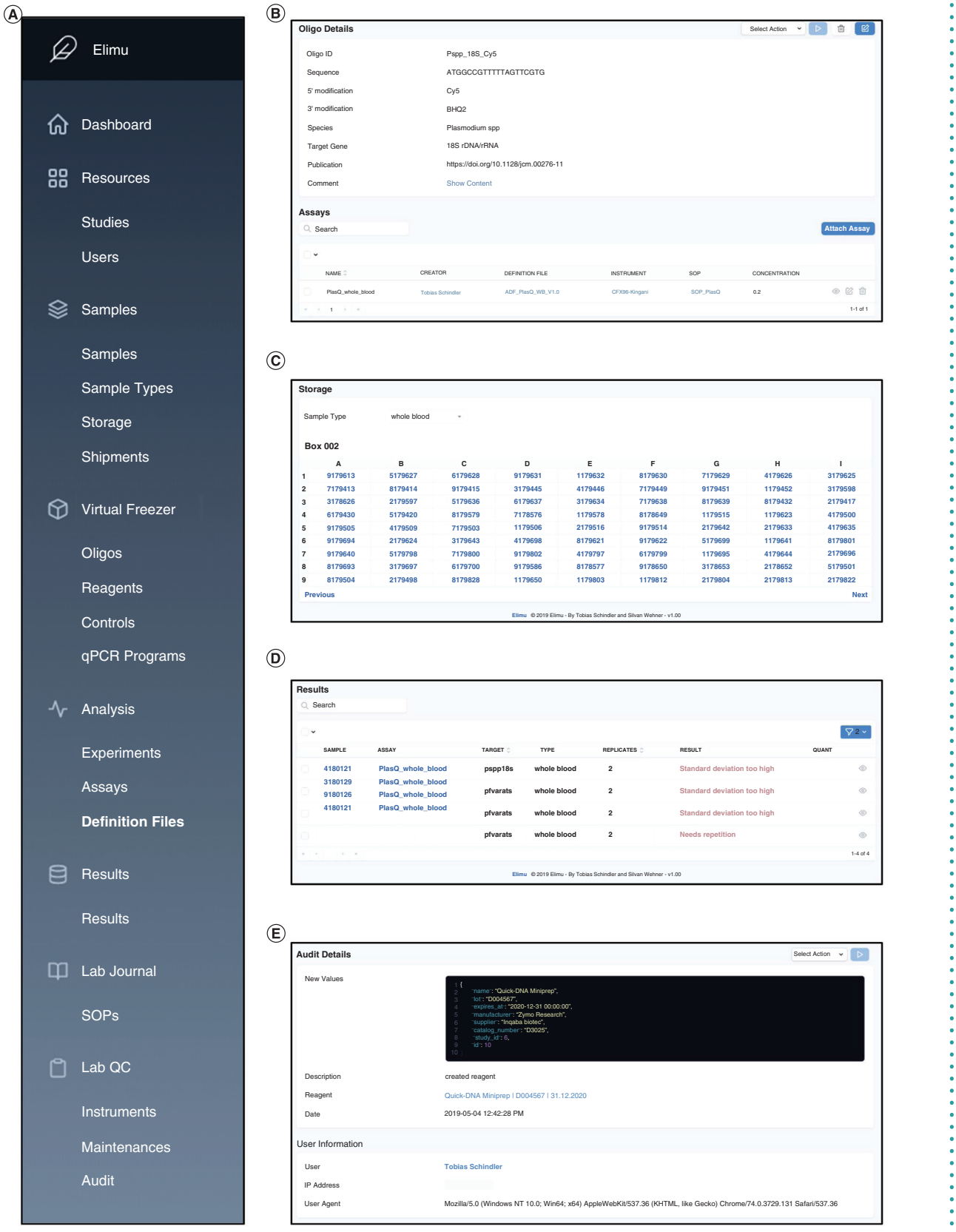

Figure 2. User interface of ELIMU-MDx. (A) Side bar for navigation within ELIMU-MDx. (B) Oligo details as an example for an ELIMU-MDx component. (C) Automatically assigned storage position for samples. (D) Samples that need special attention can be filtered in the results section. (E) ELIMU-MDx audit trail. Cq: Quantification cycle; SOP: Standard operating procedure.

Table 1. Information documented for each experiment and sample.

Modules	Component	Description
Sample module	Sample metadata	Required is a unique identifier, collection date and biological type of sample. Optional information such as demographics can be added
	Sample inventory	Contains information related to storage and shipment of samples
Assay module	Lab journal	Contains detailed information for the associated protocols and standard operating procedures
	Instruments	A detailed repository for qPCR instruments, which also includes maintenance logs
	Virtual freezer	Contains information for: Oligonucleotides: sequences, link to original publication Reagents: qPCR reagents with lot number and expiry date and extraction kits for nucleic acids Controls: name and concentration of positive controls qPCR programs: Cycling conditions and detection channels used. qPCR instrument software name and version
Data module	qPCR raw data	Quantification cycle values and melting temperatures provided by RDML file
	qPCR metadata	Metadata for each run are saved within the RDML file
Assay definition file	Analysis parameters	Assay-specific parameters that are used to analyze and interpret the qPCR data. For details, refer to the ELIMU-MDx user guide
	Documentation parameters	Additional assay-specific parameters, e.g., qPCR efficiencies, which are used for documentation purposes only

be uploaded and attached to the samples, and therefore linked with the qPCR result. Importantly, the structured data generated by ELIMU-MDx allows use of the R language and environment for statistical computing and visualization of qPCR data without extensive data cleansing.

Integrity & traceability of qPCR data in ELIMU-MDx

All qPCR data uploaded to ELIMU-MDx are protected from accidental or intentional modifications. The original qPCR raw data, as a RDML file, are attached to the results. The Cq values cannot be changed or deleted; the only option is to exclude replicates. Exclusion of Cq values is visually indicated and can be reversed. Each modification of the data is attributed to a user. The use of personal accounts that are password protected and the automatic creation of an audit trail that records and logs all major changes are in line with US FDA regulations on electronic records and electronic signatures (FDA regulation 21 CFR Part 11).

qPCR is a powerful tool that is used in different areas of infectious disease diagnostics, and it has become a key technology for the detection of viruses, bacteria, fungi and parasites by increasingly replacing traditional diagnostic techniques [4]. For laboratories, especially if they generate diagnostic data for clinical trials, the adequate storage, curation and analysis of qPCR data can be a challenge. Currently available software solutions, commercial or open source, are not well suited for the management and analysis of diagnostic qPCR data. Often a combination of several tools is used with an inadequate documentation of this essential step of the qPCR workflow.

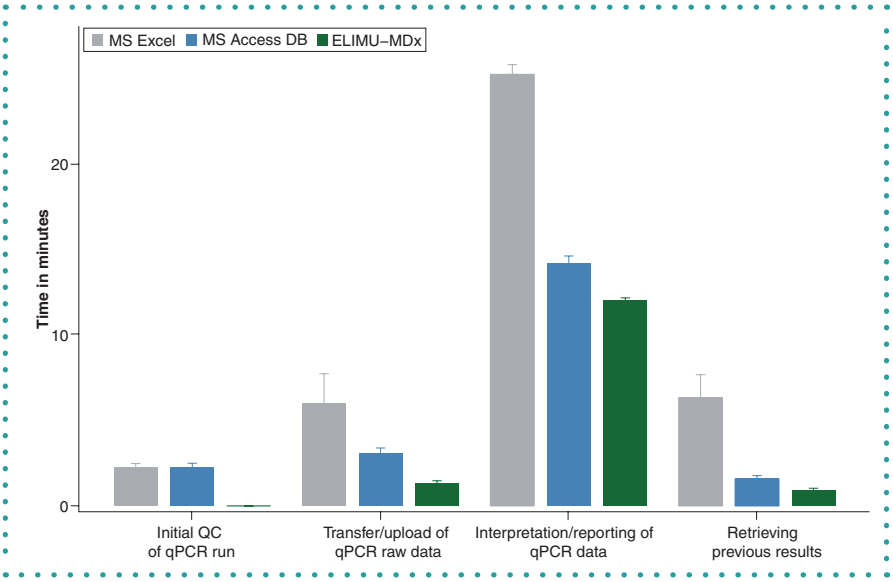

Figure 3. Analysis of qPCR data using ELIMU-MDx. Time spent on different steps of qPCR data management compared between Microsoft Excel, a custom-built Microsoft Access database and ELIMU-MDx.
QC: Quality control.

We have built the ELIMU-MDx platform as a response to the large amount of qPCR data generated during clinical trials, including CHMI studies. Due to its integration into the sample workflow and automation of the qPCR QC and preliminary analysis, the use of ELIMU-MDx accelerates the sample-to-result turnaround time significantly. The use of the RDML standard allows analysis of the qPCR data in a streamlined and trans- ▶

parent way without losing the association between the final result (interpretation of the qPCR data) and the raw data (fluorescent data of the qPCR run). We also placed importance on the documentation of the qPCR experiments by attaching metadata from the samples and assays to the qPCR data. Maintaining an audit trail is a regulatory compliance requirement; therefore, we integrated an automated, time-stamped audit trail that allows the reconstruction of all events related to the creation, modification or deletion of electronic records.

ELIMU-MDx is open source, licensed under the permissive MIT license. Disclosing the software code used for data analysis should be the standard and will help to increase the reproducibility of data analysis [12]. Because ELIMU-MDx is an open-source project, contributions and feedback from the community are highly encouraged and will be an important factor in improving and adaptation of ELIMU-MDx over time.

FUTURE PERSPECTIVE

With the rapid spread of qPCR-based technologies for infectious disease diagnostics, the development and implementation of software for qPCR data analysis will continue to gain importance, particularly in the framework of clinical intervention studies that need to follow good laboratory practices. Open access software tools that enable reproducible data analysis, audit trails and long-term storage of raw and analyzed qPCR data are likely to become essential in experimental clinical research and diagnostics in the foreseeable future.

AVAILABILITY & REQUIREMENTS

Software name: ELIMU-MDx
Software home page: https://www.elimu.ch
Software source: https://github.com/Sparclex/elimu
Open-source license: MIT license
Operating systems(s): OS independent, designed as a web-app
Supported browsers: Firefox, Google Chrome and Safari.
Programming languages: PHP
Other requirements: https://sparclex.github.io/elimu/dev-docs/requirements.html
Any restrictions to use by non-academics: no limitations

AUTHOR CONTRIBUTIONS

Concept and design: SK, FS, CD, TS. Programming: SK. Beta testing, troubleshooting and providing feedback: PM, EG, AD, SH, MM, SM, TS. Drafting the manuscript: SK, TS, CD. All authors read and approved the final manuscript.

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

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No writing assistance been used in the creation of this manuscript.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0064

DATA SHARING STATEMENT

The source code of ELIMU-MDx is fully disclosed and available.

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Chapter 3

A multiplex qPCR approach for detection of *pfhrp*2 and *pfhrp*3 gene deletions in multiple strain infections of *Plasmodium falciparum*

This chapter contains the following publication:

Tobias Schindler, Anna C. Deal, Martina Fink, **Etienne Guirou**, Kara A. Moser, Solomon M. Mwakasungula, Michael G. Mihayo, Said A. Jongo, Prosper P. Chaki, Salim Abdulla, Paulo C. Manrique Valverde, Katherine Torres, Jose R. Bijeri, Joana C. Silva, Stephen L. Hoffman, Dionicia Gamboa, Marcel Tanner & Claudia Daubenberger.

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A multiplex qPCR approach for detection of *pfhrp2* and *pfhrp3* gene deletions in multiple strain infections of *Plasmodium falciparum*

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The rapid and accurate diagnosis of *Plasmodium falciparum* malaria infection is an essential factor in malaria control. Currently, malaria diagnosis in the field depends heavily on using rapid diagnostic tests (RDTs) many of which detect circulating parasite-derived histidine-rich protein 2 antigen (PfHRP2) in capillary blood. *P. falciparum* strains lacking PfHRP2, due to *pfhrp2* gene deletions, are an emerging threat to malaria control programs. The novel assay described here, named qHRP2/3-del, is well suited for high-throughput screening of *P. falciparum* isolates to identify these gene deletions. The qHRP2/3-del assay identified *pfhrp2* and *pfhrp3* deletion status correctly in 93.4% of samples with parasitemia levels higher than 5 parasites/µL when compared to nested PCR. The qHRP2/3-del assay can correctly identify *pfhrp2* and *pfhrp3* gene deletions in multiple strain co-infections, particularly prevalent in Sub-Saharan countries. Deployment of this qHRP2/3-del assay will provide rapid insight into the prevalence and potential spread of *P. falciparum* isolates that escape surveillance by RDTs.

Malaria is an infectious disease with an estimated 219 million cases globally and was responsible for 435'000 deaths in 2017. More than 90% of these malaria cases and deaths occurred in sub-Saharan Africa with *Plasmodium falciparum* as the most pathogenic malaria parasite species, accounting for the vast majority of clinical malaria cases[1].

Advances have been made in malaria control which have contributed to the decline in malaria prevalence observed worldwide with improved diagnostic tests and better access to malaria treatment contributing significantly to this development[1]. The rapid and accurate diagnosis and treatment of malaria cases is an essential factor in the control of malaria. Rapid diagnostic tests (RDTs) are becoming the most widely used method to diagnose malaria infections in the field with 245 million RDTs distributed worldwide in 2017[1]. In sub-Saharan Africa an estimated 75% of malaria tests conducted in 2017 were based on RDTs[1]. Malaria RDTs are based on an immuno-chromatographic assay using a lateral-flow device which allows the detection of malaria antigens in usually 5 to 15 µL of capillary blood[2]. RDTs provide results within 20 minutes and can be employed by inexperienced health workers operating in resource-limited settings[3]. RDTs recognizing circulating histidine-rich protein 2 (PfHRP2) for sensitive and specific detection of *P. falciparum* make up more than 90% of RDTs currently in

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Target gene	Size	Oligo name	Oligo sequence [5′ to 3′]	Fluorophores	Conc. in 5 × PrimerMix[a]
pfrnr2e2 (PF3D7_1015800)	107 bp	IC-PfRNR2E2 fwd	AGTATCCAAAACACTATAATTCCAAGTAC	—	1.5 µM
		IC- PfRNR2E2 rev	ATTTTCTCCTTTCTTAACAGTTTCTTCC	—	1.5 µM
		IC-PfRNR2E2 Cy5	CCTTTTAGTTGGCCCGAATTTACAA	Cy5-BHQ2	1.125 µM
pfhrp2 (PF3D7_0831800)	286 bp	PfHRP2 fwd[b]	GTATTATCCGCTGCCGTTTTTGCC	—	1.5 µM
		PfHRP2 rev[b]	TCTACATGTGCTTGAGTTTCG	—	1.5 µM
		PfHRP2 TxRd	TTCCGCATTTAATAATAACTTGTGTAGC	TexasRed-BHQ2	0.375 µM
pfhrp3 (PF3D7_1372200)	289 bp	PfHRP3 fwd	ATATTATCCGCTGCCGTTTTTGCT	—	1.5 µM
		PfHRP3 rev	CCTGCATGTGCTTGACTTTCGT	—	1.5 µM
		PfHRP3 YY	CTCCGAATTTAACAATAACTTGTTTAGC	YakimaYellow-BHQ2	0.75 µM

Table 1. Oligonucleotide sequences used for qHRP2/3-del assay. [a]All oligonucleotides are premixed as a 5× primer mix. [b]Oligonucleotide sequences obtained from Abdallah *et al.*[32].

use[4]. The relatively high abundance and stability of PfHRP2 in the blood of infected patients and expression by *P. falciparum* during the erythrocytic stage makes this antigen a valuable biomarker for malaria infection[5]. PfHRP3, a protein also expressed by *P. falciparum* with high level of structural similarity to PfHRP2, might be also recognized by some of the monoclonal antibodies used in the RDTs[6]. RDTs are critical diagnostic tools for identifying symptomatic malaria infections; however, due to the reduced performance in infections with low parasite density, its use for the diagnosis of malaria infection in asymptomatic individuals is rather limited[7].

Recent studies report on reduced diagnostic performance of PfHRP2-based RDTs which were attributed to genetic diversity of the *pfhrp2/3* genes[6], differences in expression level of PfHRP2/3 antigen in parasite field strains[8] or isolates lacking *pfhrp2* and/or *pfhrp3* genes[9]. *P. falciparum* isolates lacking *pfhrp2* and/or *pfhrp3* genes are found around the world, with different proportions of the circulating *P. falciparum* population affected. The regions with the highest proportions of *P. falciparum* strains carrying *pfhrp2* deletions are South America and sub-Saharan Africa[10]. Since malaria control programmes depend on reliable diagnosis of malaria cases using RDTs, parasites lacking *pfhrp2/3* genes pose a threat to malaria control and local elimination efforts[11].

The presence or absence of *pfhrp2/3* genes is usually determined by amplifying these genes by polymerase chain reaction (PCR). Several different (nested) PCR protocols have been published and a deletion is reported if there is no amplification of the *pfhrp2/3* genes in the presence of an amplification in at least two *P. falciparum* single copy genes[12]. The conventional nested PCR methods are time consuming, requiring separate reactions for each target gene amplification as well as gel electrophoresis for visualization of the PCR products. Additionally, there are methodological issues related to this approach which assumes identical PCR performance of the *pfhrp2/3* and the reference genes. Particularly at lower parasitemia levels with a small number of DNA target molecules present, unavoidable stochastic effects can play a major role and might lead to false reporting of *pfhrp2/3* deletions. Furthermore, none of the published methods detecting *pfhrp2/3* deletions can identify "masked" deletions in multiple strain infections with only one out of several *P. falciparum* strains carrying a *pfhrp2* and/or *pfhrp3* gene deletion[13]. These limitations of recommended molecular monitoring methods could result in an underestimation of the prevalence of *P. falciparum* strains with *pfhrp2/3* deletions, especially in regions with high proportions of multiple strain co-infections.

This paper presents a novel, quantitative PCR-based method for detecting *pfhrp2* and *pfhrp3* gene deletions suitable for high throughput screening of *P. falciparum* isolates. The qHRP2/3-del (quantitative detection of *pfhrp2* and *pfhrp3* deletion) assay was developed as a multiplex assay, with the ability to amplify individually and specifically the *pfhrp2* and *pfhrp3* genes together with a single copy gene, the *P. falciparum* ribonucleotide reductase R2_e2 (*pfrnr2e2*)[14], as an internal reference. The quantitative nature of the qHRP2/3-del assay provides the basis for estimating the proportions of *P. falciparum* strains carrying *pfhrp2* and *pfhrp3* deletions in regions with multi-clonal malaria infections.

Results

Design and evaluation of the novel qHRP2/3-del assay. We aimed at improving the detection of *pfhrp2* and *pfhrp3* gene deletions by developing a quantitative PCR-based assay able to detect and quantify *pfhrp2* and *pfhrp3* genes in a single reaction. Given the high nucleotide sequence similarity and the repetitive structure of the *pfhrp2* and *pfhrp3* genes, nucleotide regions serving as targets for primers and probes were limited (Supplementary File 1). The primer and probe combinations selected for our assay (Table 1) bind to a region spanning exon 1 and exon 2 of both genes. Absence of amplification will therefore indicate a deletion of the entire genes or partial gene deletions including exon 1, the intron and first 96 base pairs of exon 2. Although there are chromosome breaking points outside the amplified regions, in particular the section that contains the repeats and epitopes detected by RDTs, analysis of field isolates suggest that the selected regions are highly predictive for *pfhrp2/3* deletions in field strains[9,12,15,16].

We designed a multiplex qPCR assay using three differently labelled TaqMan assays detecting the *pfhrp2* (PF3D7_0831800) and *pfhrp3* (PF3D7_1372200) genes with the single copy gene *pfrnr2e2* (PF3D7_1015800) as the internal control. The sequence alignment of the *pfhrp2* and *pfhrp3* genes highlighting the oligo binding regions is shown in Supplementary File 1.

The multiplexed assays correctly identify *P. falciparum* strains carrying known deletions of *pfhrp2* (PfDD2 strain) and *pfhrp3* (PfHB3 strain) as well as a strain without deletion (PfNF54 strain) (Fig. 1A). The multiplexed assays show comparable characteristics in terms of sensitivity and qPCR performance. Using DNA extracted from

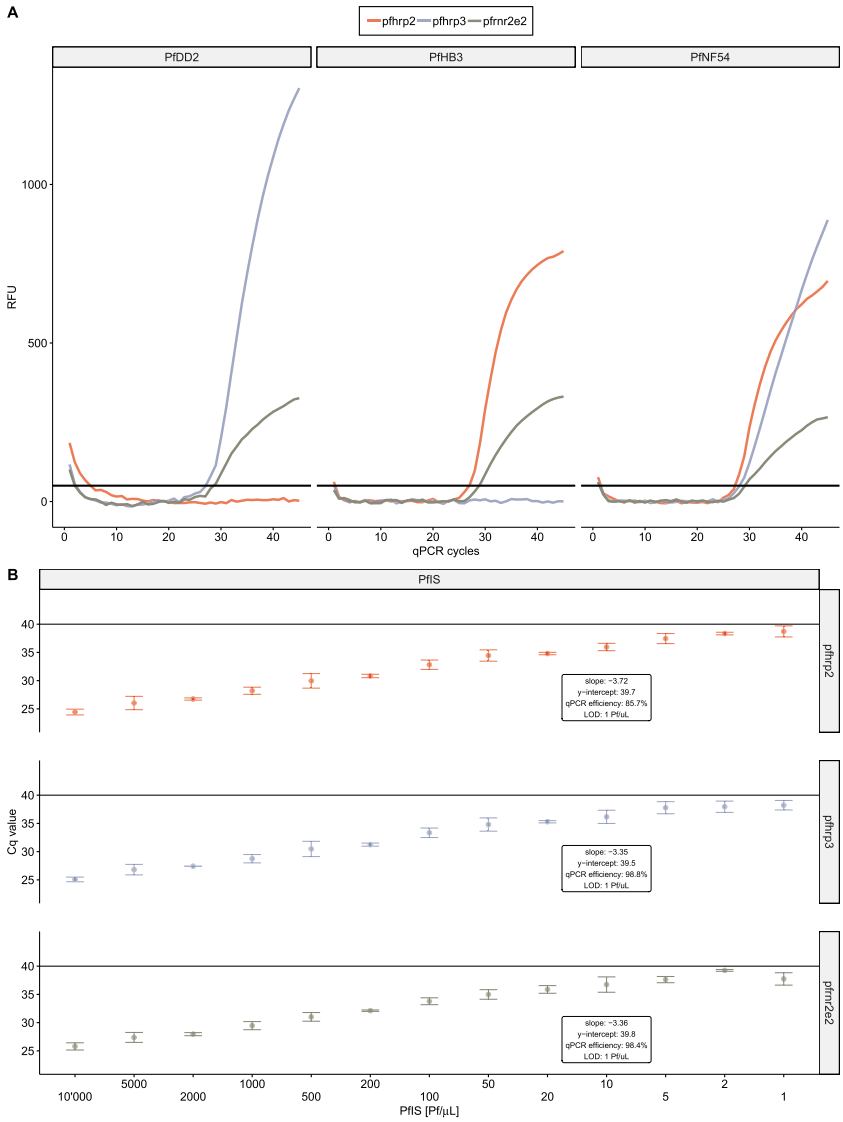

Figure 1. Multiplex detection of *pfhrp2* and *pfhrp3* genes using the qHRP2/3-del assay. (**A**) The qHRP2/3-del assay amplifies *pfhrp2*, *pfhrp3* and *pfrnr2e2* target sequences in a multiplex qPCR reaction and correctly identifies strains carrying either a *pfhrp2* deletion (PfDD2), a *pfhrp3* deletion (PfHB3) or no deletion (PfNF54). (**B**) Performance characteristic of each individual amplification assay, run within the multiplex qHRP2/3-del assay, is shown. Correlation with high linearity between serially diluted WHO international standard for *P. falciparum* NATs (PfIS) and Cq values was obtained and used to calculate the qPCR efficiency. Cq values above 40 (black line) are considered negative.

cultured parasites, all three targets are detected in samples with parasitemia as low as 1 parasite/µL and an inverse linear correlation between Cq values and parasite densities ranging from 1 to 10'000 parasites/µL was observed. The qPCR efficiencies were calculated as 85.7%, 98.8% and 98.4% for the amplification of *pfhrp2*, *pfhrp3* and *pfrnr2e2*, respectively (Fig. 1B). The qHRP2/3-del assay was next tested using purified DNA from eight culture adapted *P. falciparum* strains from Africa (Pf3D7, PfNF54, PfNF166.C8), South and Central America (Pf7G8, PfHB3), South East Asia (PfNF135.C10, PfDD2) and Papua New Guinea (PfFC27) with known deletion status of the *pfhrp2* and *pfhrp3* genes. The Cq values for amplification of *pfrnr2e2* were comparable between the eight strains amplified and no significant differences of Cq values for the *pfhrp2* gene and *pfhrp3* gene across the strains carrying the genes was observed. Sequence alignments of PfNF135.C10, Pf3D7, Pf7G8, PfNF54 and PfNF166.C8 did not reveal sequence variation in the oligo binding regions of *pfhrp2* (Supplementary File 2) or *pfhrp3* (Supplementary File 3) supporting these findings. DNA derived from five non-*falciparum Plasmodium* species (*P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae*, *P. knowlesi*, *P. vivax*) was tested with the qHRP2/3-del assay and did not result in amplification of any target demonstrating the specificity for *P. falciparum*. In summary, we developed a *P. falciparum*-specific multiplex qPCR assay that allowed the simultaneous amplification of the *pfhrp2*, *pfhrp3* and *pfrnr2e2* genes in a single reaction with high efficiency and ability to correctly identify *pfhrp2* and *pfhrp3* gene deletions.

Sample set	Description of sample set	Number of *P. falciparum* positive samples[b]	Parasitemia in parasites/µL (Median/IQR)	Amplification rate by qHRP2/3-del assay[c]
CHMI[a]	CHMI in TZ with PfNF54 strain (no deletion)	49	51.1 (1.5–152.5)	78%
PE	Peruvian samples around Iquitos city. High proportion of *pfhrp2/3* deletions	68	592.4 (186.7–1982.0)	99%
EG	Blood donors with asymptomatic malaria infection living on Bioko Island, Equatorial Guinea	47	4.8 (1.0–45.3)	51%
TZ	Sampling of symptomatic volunteers at two health facilities in Southern Tanzania	90	38.8 (0.7–808.6)	62%
Combined		**254**	**75.7 (2.2–571.6)**	**73%**

Table 2. Field samples used for evaluation of qHRP2/3-del assay. [a]Controlled Human Malaria Infection. [b]All confirmed by diagnostic qPCR assays. [c]Positive for internal control of assay (*pfrnr2e2*).

Analysis of *P. falciparum* field strains with qHRP2/3-del assay. The qHRP2/3-del assay was next tested using a collection of 254 *P. falciparum* isolates originating from East Africa, Central-West Africa and Latin America (Table 2). The infection status and parasitemia levels were well established in these samples by using published diagnostic qPCR assays routinely used in the laboratories in Tanzania[17], Equatorial Guinea[18] and Peru[19]. The overall median parasitemia in these samples was 75.7 parasites/µL (IQR: 2.2–571.6), which is below the LOD of 100 parasites/µL for PfHRP2-based RDTs[20,21]. First, the ability of the *pfrnr2e2* singly copy gene to serve as internal assay control and to quantify parasitemia levels was assessed. Out of the 254 samples, 186 (73.2%) amplified the *pfrnr2e2* singly copy gene. Failure in amplification of *pfrnr2e2* was associated with low parasitemia levels (Fig. 2A). In samples with parasitemia levels of 3 parasites/µL and above, more than 95% of all samples were amplified successfully. In samples with parasitemia >100 parasites/µL, the lower limit of detection for PfHRP2-based RDTs, all qPCR reactions were positive for *pfrnr2e2*. Parasitemia levels determined by using the amplification of *pfrnr2e2* correlated closely with parasite densities obtained from *P. falciparum* diagnostic qPCR assays (Fig. 2B), this is supported by the findings of the Bland-Altman plot which demonstrates a high order of agreement (Fig. 2C). The average ratio of parasite quantification based on diagnostic qPCR assays and qHRP2/3-del assay is 0.8 (95% CI: −1.7–3.3). In summary, the qHRP2/3-del assay amplifies 95% of samples with parasitemia levels of 3 parasites/µL and above and can be used to reliably quantify parasite levels in field samples.

Identification of *pfhrp2* and *pfhrp3* gene deletions using qHRP2/3-del assay. Next, we wanted to establish the performance of the qHRP2/3-del assay in comparison with nested PCR. Samples with known *pfhrp2/3* deletion status obtained from four different sources were included. Serial dilutions of DNA purified from PfDD2 (*pfhrp2* deletion), PfHB3 (*pfhrp3* deletion) and PfIS (no deletion) served as controls. Samples from CHMI using PfNF54 (no deletion) were added to test the specificity of the qHRP2/3-del assay. Two sample sets genotyped by nested PCR, one from Tanzania (TZ) dominated by *P. falciparum* strains without deletions and one from Peru (PE), with a high proportion of *pfhrp2/3* deletions were analysed. The Peruvian sample set consisted of 54 samples with both genes deleted and 7 samples with only one gene deleted. The qHRP2/3-del assay defines a deletion as failure of amplification of the *pfhrp2/3* genes (Fig. 3A, y axis, Cq set to 45) in samples which are positive for the internal control, *pfrnr2e2* (Fig. 3A, x axis). Sensitivity is defined as the proportion of correctly identified *pfhrp2/3* deletions, while specificity is the proportion of correctly identified strains without *pfhrp2/3* deletions. All control samples with known deletion status were identified as expected (Fig. 3A, first panel). Importantly, the qHRP2/3-del assay correctly identified samples with parasitemia levels ranging from 1–10'0000 parasites/µL, demonstrating the dynamic range of at least 5 logs of this assay. In samples collected from volunteers that have undergone CHMI with PfNF54 (CHMI, n = 38), one sample that is positive for *pfhrp2/3* genes was wrongly detected as a double deleted parasite, resulting in a reduced specificity (Fig. 3A, second panel). A high sensitivity was achieved with the Peruvian samples (PE, n = 67), *pfhrp2* and *pfhrp3* deletions were detected with sensitivity of 94.4% and 94.9%, respectively (Fig. 3A, third panel). The low specificity of 76.9% and 87.5% for *pfhrp2* and *pfhrp3*, respectively, is based on the incorrect detection of deletions in three samples. Among the samples from Tanzania (TZ, n = 56), no *pfhrp2/3* deletions were detected by the nested PCR. In contrast, the qHRP2/3-del assay identified three deletions, resulting in a specificity of 93.8% (Fig. 3A, fourth panel).

Grouping the samples with missed deletions (reducing the sensitivity) and the false deletions (reducing the specificity) by parasitemia levels revealed a high proportion of false deletions among the samples with the lowest parasitemia levels (Fig. 3B). Based on these findings, the inclusion criteria for samples to be analysed by qHRP2/3-del assay was changed. The threshold for the *pfrnr2e2* gene amplification was reduced from Cq < 40 to Cq < 37.5, corresponding to parasitemia levels of 5 parasites/µL. Based on these new inclusion criteria, the qHRP2/3-del assay obtained results from 106 samples out of 127 samples (inclusion rate of 83.5%) (Fig. 3C). 12 samples were not amplified by the qHRP2/3-del assay and an additional 9 samples excluded based on the new inclusion criteria. Samples which were not amplified by the qHRP2/3-del assay were mainly ultra-low parasite density samples from Tanzania (11 out of 12).

In 99 out of 106 samples (93.4%), the *pfhrp2/3* deletion status was identical when compared between qHRP2/3-del assay and nested PCR. This is reflected in the near perfect agreement between these two PCR based diagnostic methods for each of the amplified targets. Cohen's kappa was calculated as 0.89 and 0.91 for *pfhrp2* and *pfhrp3*, respectively. Out of the seven samples which were misidentified in four samples both *pfhrp* genes were affected, while in two samples the *pfhrp2* and in one sample the *pfhrp3* status was misclassified. For four misidentified samples with higher parasitemia levels the possibility of sample mix-up or cross-contamination

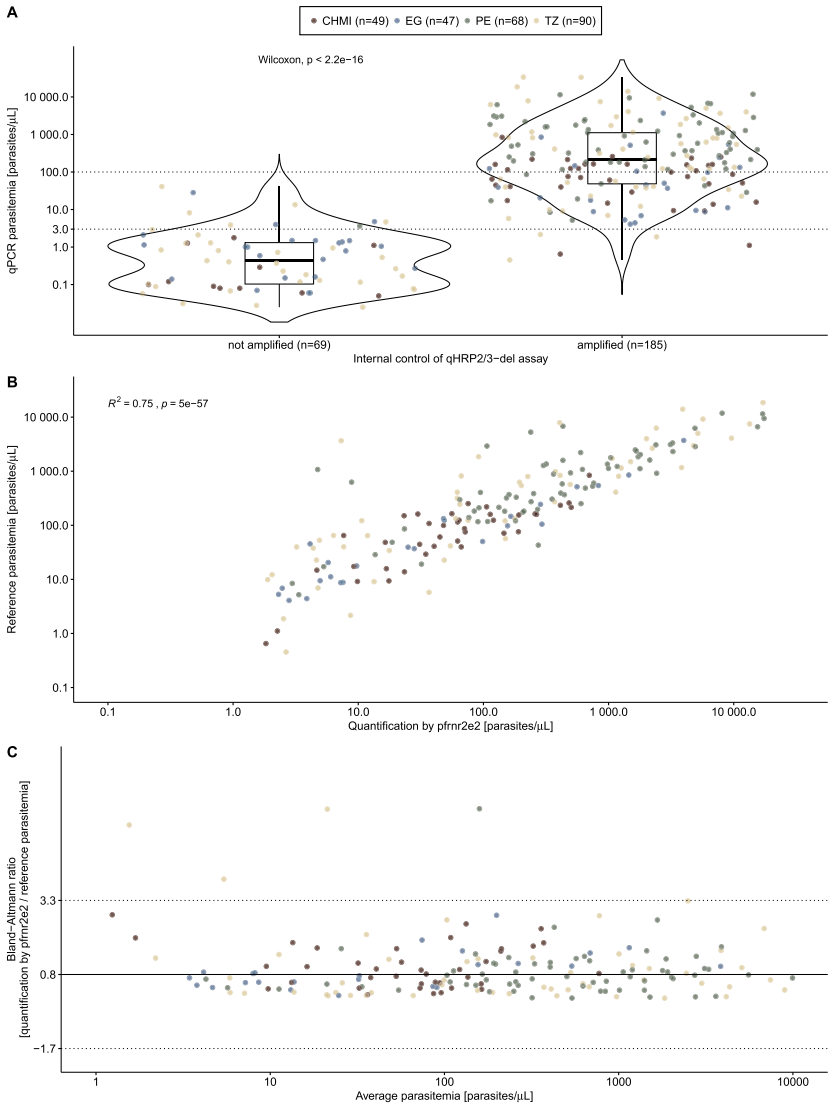

Figure 2. Detection and quantification of field samples using qHRP2/3-del assay. (**A**) Amplification rate of *pfrnr2e2* target, the internal control of qHRP2/3-del assay and association with parasitemia levels. Wilcoxon-Mann-Whitney test was used for comparison of parasitemia between groups. The dashed line at 3 parasites/µL represents the parasitemia at which more than 95% of the samples were amplified, while the dashed line at 100 parasites/µL represents the LOD of RDTs at which all samples are successfully amplified. (**B**) Correlation of parasitemia levels, obtained from diagnostic qPCR assays, and parasitemia, derived from the internal control of qHRP2/3-del assay, is shown. The color represents the different sample sets and R^2 the Spearman's rank correlation coefficient. (**C**) Bland-Altman plot of average parasitemia (x-axis) and ratio of parasitemia levels calculated between internal control of qHRP2/3-del assay and diagnostic qPCR assays (y-axis). Average ratio (black line) and 95% limits of agreement (dashed line) are depicted.

cannot be excluded, since these samples were located next to each other on the DNA plate which was shipped. In summary, the qHRP2/3-del assay specificity (94.4% and 96.0% for *pfhrp2* and *pfhrp3*, respectively) and sensitivity (94.2% and 94.6% for *pfhrp2* and *pfhrp3*, respectively) were above 90%. The negative predictive value (NPV) was calculated as 94.5% and 94.1% and the positive predictive value (PPV) as 94.2% and 96.4%, for *pfhrp2* and *pfhrp3*, respectively (Fig. 3D).

Multiple strain *P. falciparum* infections are masking *pfhrp2* and *pfhrp3* deletions. In many malaria endemic regions, particularly in sub-Saharan Africa, infections with multiple strains of *P. falciparum* are common[22]. A blood sample carrying multiple *P. falciparum* strains with and without *pfhrp2/3* deletions will result in failure to detect the deletion by nested PCR if the parasitemia level of the strain without deletion is sufficiently high for amplification. This limitation leads most likely to an underestimation of the prevalence of *pfhrp2/3* gene deletions in regions with high prevalence of multiple strain infections. We reasoned that the qHRP2/3-del assay could offer a solution by calculating the difference between the Cq values obtained for amplification of *pfhrp2* or *pfhrp3* and *pfrnr2e2*. To demonstrate the ability of the qHRP2/3-del assay to correctly identify and quantify

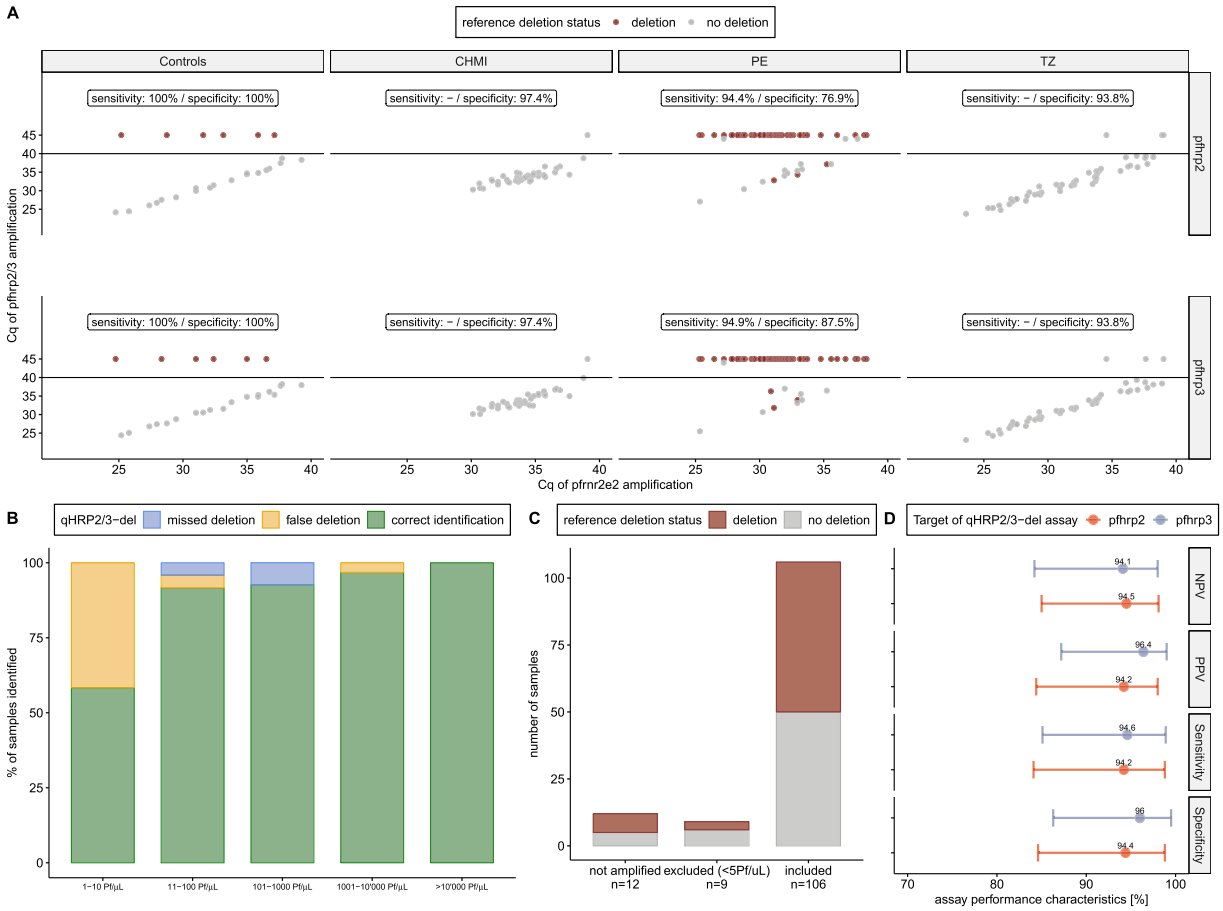

Figure 3. Diagnostic performance of qHRP2/3-del assay. (**A**) Samples with Cq values for *pfhrp2* and *pfhrp3* amplification >40 (shown on y-axis, black line indicates cut-off) are considered to carry a *pfhrp2/3* deletion. Reference deletion status, based on nested PCR, is color coded (red = deletion, grey = no deletion). (**B**) Proportion of correctly and incorrectly identified *pfhrp2/3* deletion status grouped by parasitemia. (**C**) Number of samples included for analysis by qHRP2/3-del assay (n = 106), excluded due to ultra-low parasitemia (n = 9) and not amplified (n = 12). (**D**) Analytical validation of qHRP2/3-del assay performance was assessed by comparing it to nested PCR. Standard parameters such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) including their 95% confidence intervals are shown.

"hidden" or "masked" *pfhrp2/3* gene deletions in mixed infections, we first tested defined mixtures of DNA from PfNF54 (no *pfhrp2/3* deletions) and PfDD2 (*pfhrp2* deletion) or PfHB3 (*pfhrp3* deletion) in a range of different ratios. For each combination of strain mixtures, PfDD2/PfNF54 or PfHB3/PfNF54, 10 mixtures were prepared containing varying ratios of strains with and without a *pfhrp2/3* deletion (Fig. 4A). The contribution from PfDD2 and PfHB3 strains to these mixtures ranged from 0.1% to 88% and 0.1% to 86%, respectively. In seven mixtures, the strain with a deletion constituted the minority (with less than 50% abundance) and in three mixtures the majority (with more than 50% abundance). None of these mixtures failed to amplify the *pfhrp2/3* genes, even if the strain carrying the deletion constituted the majority in the mixture. A positive correlation between abundance of isolate carrying a deletion and an increase of ΔCq (Cq of *pfhrp2* or *pfhrp3* minus Cq of *pfrnr2e2*) is observed (Fig. 4B). The qHRP2/3-del assay does not only successfully identify "masked" *pfhrp2/3* deletions but can also discriminates between mixtures where the strain with the deletion constitutes the majority or minority (Fig. 4C). A ΔCq cut-off value of 2.0 was chosen to identify "masked" *pfhrp* gene deletions. Applying this cut-off to our sample collections revealed that two isolates each from Tanzania and Peru have high ΔCq values for both *pfhrp* genes indicative of the presence of "masked" *pfhrp2/3* deletions (Fig. 4D). Three additional samples from the Peruvian collection had a ΔCq value > 2 for the *pfhrp2* gene only. No ΔCq values above 2 were found in Equatorial Guinean isolates and among samples collected from volunteers undergoing CHMI (Fig. 4D). These experiments demonstrate that by calculating the ΔCq values between Cq for *pfrnr2e2* and *pfhrp2* or *pfhrp3*, "masked" deletions can be identified.

Discussion

P. falciparum strains carrying *pfhrp2/3* deletions are an emerging threat to malaria control and elimination programs around the world. Novel analysis tools enabling high-throughput screening of *P. falciparum* populations from the field are needed. The currently published methods, mostly based on nested PCR, have clear limitations in that these methods are extremely time consuming, prone to detection of incorrect deletions at low parasitemia levels and unable to identify "masked" deletions in multiple strain co-infections.

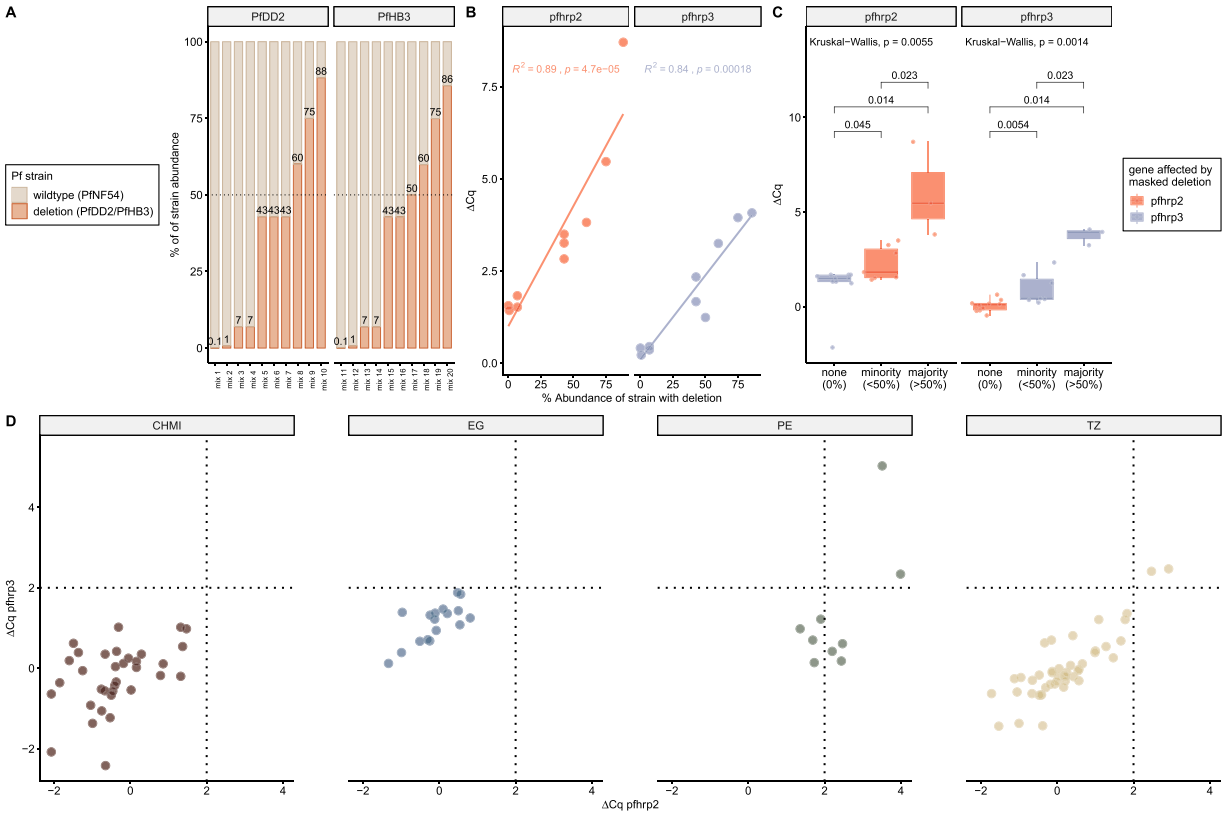

Figure 4. Identification of masked *pfhrp2/3* deletions in multiple strain infections. (**A**) Mixtures containing two strains, one with a *pfhrp* deletion (PfDD2 or PfHB3) and no deletion (PfNF54), were generated. (**B**) Correlation between abundance of strain carrying deletion and ΔCq is shown for both targets, *pfhrp2* (red) and *pfhrp3* (blue). (**C**) The ΔCq approach distinguishes between strain mixtures not carrying deletions, mixtures with minority abundance as well as majority abundance of strains with deletions. Statistical comparison was performed using the Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney for pairwise comparisons. (**D**) The ΔCq approach of the qHRP2/3-del assay was applied to four sample collections to identify "masked" *pfhrp2/3* deletions. The control group, based on samples from CHMI, did not reveal isolates with increased ΔCq values. The dashed lines represent the ΔCq cut-off values for *pfhrp2* (x-axis) and *pfhrp3* (y-axis).

The sensitivity and specificity of the PfHRP2/3-del assay is comparable to the widely used nested PCR. However, the novel qHRP2/3-del assay is well suited for high throughput screening of *P. falciparum* isolates with approximately 30 samples analyzed in less than two hours - including DNA extraction and data analysis. Two additional major advantages of the qHRP2/3-del assay are obvious: firstly, the ability to quantify parasitemia levels and therefore include samples based on parasitemia and secondly, to identify "masked" deletions in multiple strain infections.

The identification of *pfhrp2/3* deletions in samples with low parasitemia levels is difficult since the absence of amplification could be due to lack of sufficient template leading to incorrect reports of deletions. The conventional method depends on the successful amplification of at least two single copy reference genes to ensure sufficient template in the PCR reaction. This procedure is time-consuming and labour-intensive. The qHRP2/3-del assay uses an alternative inclusion criterion, based on the Cq value of its internal control. The pre-defined exclusion criteria of all samples that have parasitemia below 5 parasites/μL will improve the quality, reproducibility and comparability of malaria parasite survey data obtained with the qHRP2/3-del assay.

The ability to detect "masked" *pfhrp2/3* deletions is probably the most interesting feature of the qHRP2/3-del assay, because it will allow studying the epidemiology of *pfhrp2/3* deletions in malaria endemic regions with a high proportion of infections caused by multiplicity of infections, particular sub-Saharan Africa[22]. The qHRP2/3-del assay correctly identified infections that contain two strains, one with a deletion and the other one without a deletion, based on a difference in the Cq values derived from the amplification of the *pfhrp2/3* gene targets and the *pfrnr2e2* control. However, currently we cannot exclude that nucleotide sequence variations located in the binding sites of the oligonucleotides used in the PfHRP2/3-del assay could potentially also lead to variation in ΔCq values. The ΔCq application of our novel PfHRP2/3-del assay in additional studies including a larger sample size will improve our understanding of the relevance of "masked" *pfhrp2* and *pfhrp3* gene deletions and their impact on reliability of malaria RDT test results.

Two Tanzanian isolates had an increased ΔCq value for both *pfhrp* genes, indicating the presence of *pfhrp2/3* deletions in the East African nation. This was recently confirmed when *pfhrp2* and *pfhrp3* deletions were

identified in Tanzania and Uganda[23].Together with findings from Kenya[24], the Democratic Republic of Congo[25], Rwanda[26] and Mozambique[27] there is strong evidence for the existence of *pfhrp2/3* deletions in this region. Therefore, establishing programs which systematically monitor *pfhrp2/3* deletions and their impact on the performance of RDTs is advised.

Conclusion

The qHRP2/3-del assay presented here is suitable for high-throughput screening of *P. falciparum* strains to identify *pfhrp2/3* gene deletions in different malaria endemic settings, including areas with high a proportion of multiple strain co-infections. With growing availability of qPCR instruments in reference laboratories in sub-Saharan countries, this assay could be used as surveillance method to monitor over time the potential expansion of *P. falciparum* strains carrying *pfhrp2* and *pfhrp3* deletions.

Methods

P. falciparum isolates from tanzania, equatorial guinea and peru. In this study a total of 205 *P. falciparum* isolates collected from three different malaria endemic regions, East Africa, West-Central Africa and South America were included. The samples from East Africa (n = 90) were collected in rural southern Tanzania (TZ) as part of a malaria baseline survey[28]. The West-Central African isolates (n = 47) were identified among blood donors living in Malabo, Equatorial-Guinea (EG)[18]. Both samples sets were analyzed locally, at the Bagamoyo branch of the Ifakara Health Institute and the laboratory of the Equatorial Guinea Malaria Vaccine Initiative using harmonized protocols. Briefly, genomic DNA was isolated either from 6 circles with 2 mm diameter of dried blood spots (Tanzania) or 180 µL whole blood (Equatorial Guinea) using the Quick-DNA Miniprep kits (Zymo Research, Irvine, USA). *P. falciparum* was identified and quantified using published qPCR protocols based on varATS[29]. Extracted DNA (n = 68) from Peruvian isolates (PE), collected between 2008–2009 and 2015–2016 around Iquitos city, was shipped to the Swiss Tropical and Public Health Institute for *pfhrp2/3* characterization by qHRP2/3-del assay.

Additional parasite isolates and laboratory strains. Forty-nine PfNF54 isolates from Controlled Human Malaria Infections (CHMI) conducted in Bagamoyo, Tanzania (ClinicalTrials.gov: NCT02613520[30]) as well as genomic DNA isolated from 8 laboratory strains with known *pfhrp2/3* deletion status (Pf3D7, Pf7G8, PfDD2, PfHB3, PfNF135.C10, PfNF166.C8, PfNF54 and PfFC27) were used as controls. The 1st WHO International Standard *for Plasmodium falciparum* DNA Nucleic Acid Amplification Techniques (NIBSC code: 04/176, herein referred to as PfIS) was used to assess the performance of the qHRP2/3-del assay. Non-falciparum *Plasmodium* species, including *P. malariae (Pm)*, *P. ovale curtisi (Poc)*, *P. ovale wallikeri (Pow)*, *P. vivax (Pv)* and *P. knowelsi (Pk)* and an additional 28 samples from malaria negative individuals living in Tanzania were used to assess specificity of the assay.

Detection of the _pfhrp2_ and _pfhrp3_ genes by conventional nested PCR. *P. falciparum* positive samples collected in Tanzania were selected for detection of *pfhrp2* and *pfhrp3* genes by nested PCR. As a reference gene, the *msp2* gene was amplified using a previously described protocol[31]. All isolates with successful *msp2* amplification were analyzed for the presence of *pfhrp2* and *pfhrp3* genes using primers spanning exon 1, the intron, and exon 2[32]. All PCR products were separated and visualized on a 2% agarose gel. Cultured parasite isolate PfDD2 (*pfhrp2* deletion) was used as a control for all nested PCR experiments on *pfhrp2* while PfHB3 (*pfhrp3* deletion) was used as a control for all nested PCR experiments on *pfhrp3*. PfNF54 (no *pfhrp2/3* deletion) was used as a positive control for both *pfhrp* genes. *Pfhrp2/3* deletion status of the Peruvian *P. falciparum* isolates was analyzed previously following the procedures described elsewhere[9]. Results were shared to be used for the evaluation of the qHRP2/3-del assay.

Design of qHRP2/3-del assay. Published *pfhrp2/3* primer sequences for conventional PCR were adapted to the qPCR platform using EvaGreen® qPCR Mix (Solis BioDyne, Tartu, Estonia). The primers were tested with different DNA concentrations extracted from PfNF54, PfDD2 and PfHB3 strains, corresponding to parasitemia levels of 1 and 100 parasites/µL. The best performing primer pairs, in terms of specificity and sensitivity, were then used in combination with newly designed TaqMan® hydrolysis probes. The *pfhrp2/3* oligo sequences were systematically optimized using the trial-and-error approach. As the internal control of the qHRP2/3-del assay we amplify a *P. falciparum* specific 107 bp long sequence of the ribonucleotide reductase R2_e2 (*pfrnr2e2*), a distantly related paralog of the canonical eukaryotic small subunit ribonucleotide reductase R2, that is unique to apicomplexan species[14]. The performance of *pfrnr2e2* as a biomarker for detection and quantification of *P. falciparum* was tested by direct comparison with parasitemia levels obtained from a 18 S rDNA based qPCR assay[33]. A sensitivity of 89.1% for samples with parasitemia >1 parasite/µL and a Bland-Altman ratio of 0.99 (95% CI: −0.012–2.5) demonstrate its robustness and accuracy as internal control (Supplementary File 4). Genomic sequences for *pfrnr2e2* (PF3D7_1015800), *pfhrp2* (PF3D7_0831800) and *pfhrp3* (PF3D7_1372200) of Pf3D7 strain were obtained from PlasmoDB. A *pfhrp2/3* sequence alignment including five reference strains from West-Africa (Pf3D7, PfNF54), Guinea (PfNF166.C8), Brazil (Pf7G8) and Cambodia (PfNF135.C10) revealed no SNPs in oligo binding regions suggesting a high degree of conservation within the target region of the *pfhrp2/3* genes (Supplementary Files 2 and 3). The *pfhrp2*, *pfhrp3* and *pfrnr2e2* sequences for Pf7G8, PfNF135.C10, PfNF166.C8 and PfNF54 were obtained from whole genome sequencing[34]. The Geneious version 8.1.9 software (Biomatters Ltd, Auckland, New Zealand) was used for sequence alignments and oligo designs. Relevant information concerning the oligos used in the qHRP2/3-del assay is summarized in Table 1.

Sample analysis with qHRP2/3-del assay. Amplification and qPCR measurements were performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The thermal profile used for qHRP2/3-del assay is as follows: Taq polymerase activation for 5 min at 95 °C, followed by 45 cycles of

15 s at 95 °C and 35 s at 57.5 °C. 2 µL DNA was added to 8 µL reaction master mix containing 1x Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, USA) and 1x qHRP2/3-del Primer Mix (Table 1). All qPCR assays were run in triplicates with appropriate controls including Non-Template Control and DNA from PfDD2, PfHB3 and PfNF54 as controls for the *pfhrp2/3* deletion status.

Data management and statistical analysis. *Preliminary analysis of qPCR data*. Cq values were obtained from the Bio-Rad CFX96 Manager 3.1 software (Bio-Rad Laboratories, California, USA) after setting the threshold manually. Cq values were transferred and linked to the samples' metadata using a custom-designed database for storage and analysis of qPCR data. Only samples with a Cq $\leq$ 40.0 for the internal control, *pfrnr2e2*, were considered eligible for analysis of *pfhrp2/3* deletion status. ΔCq were calculated by subtraction of *pfrnr2e2* Cq values from *pfhrp2* or *pfhrp3* Cq values.

Analytical performance of qHRP2/3-del assay and quantification of parasitemia. Based on the PfIS a serial dilution ranging from 0.01–10'000 parasites/µL was prepared and used to assess the performance of the qHRP2/3-del assay. The slope, y-intercept, qPCR efficiency and R^2 was established for each target. The Limit of Detection (LOD) was defined as the lowest PfIS parasitemia with a positive amplification in 4 out of 6 replicates. Parasitemia was estimated using linear regression derived from serial dilution of the PfIS and the *pfrnr2e2* target which serves as the internal control of the qHRP2/3-del assay.

Graphical representation and statistical analysis. We used R version 3.5.1 for creating ggplot2-based graphs using the packages *ggpubr*, *gridextra* and *scales*. The Diagnostic test evaluation calculator (freely available at https://www.medcalc.org/calc/diagnostic_test.php) was used for analytical validation of qHRP2/3-del assay performance. Cohen's kappa including 95% confidence intervals, providing a measure of agreement, was calculated using STATA version 12.0 software (Stata Corp LP; College Station, Texas, USA). P values < 0.05 were considered as significant for all statistical analysis.

Ethical approval and informed consent. The samples analyzed in this study were collected in different studies. All studies were approved by the appropriate institutions and informed consent was obtained from all participants. The CHMI samples were collected during a clinical study, registered at Clinical Trials.gov (NCT02613520), and conducted under a U.S. FDA IND application. The study was performed in accordance with Good Clinical Practices. All samples analyzed in this publication were obtained according to the approved study protocol. The protocol was approved by the institutional review boards of the Ifakara Health Institute (IHI/IRB/No: 32–2015), and the National Institute for Medical Research Tanzania (NIMR) (NIMR/HQ/R.8a/Vol.IX/2049), by the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (Ref. No. 15/104), and by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013). For the Tanzanian sample collection ethics approval for the study was granted by the institutional review boards of Ifakara Health Institute (IHI/IRB/No: 18–2015) and by NIMR (NIMR/HQ/R.8a/Vol.IX/2015). For the sample collection from Equatorial Guinea approval was given by the Ministry of Health and Social Welfare. The collection, transport and storage of the blood samples from Peru was approved by the Human Ethics Committee from Universidad Peruana Cayetano Heredia (UPCH 52707 & 59751).

Data Availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information Files.

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Author Contributions

Study concept and design: T.S., A.C.D., C.D. Acquisition of data: T.S., A.C.D., M.F., E.G., S.M.M. Analyses and interpretation of data: T.S., A.C.D., D.G., P.M.V., C.D. Drafting the manuscript: T.S., A.C.D., C.D. Technical or material support: K.M., J.C.S., D.G., P.M.V., K.T., S.A., S.L.H., M.T. Sample collection and enrollment of patients: M.G.M., S.A.J., P.P.C., J.R.B., P.M., K.T. Study supervision: C.D. All authors read and approved the final manuscript.

Additional Information

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Chapter 4

Malaria prevalence in pregnant women established by molecular tests after introduction of intermittent preventive treatment on Bioko Island, Equatorial Guinea

This chapter contains the following manuscript to be submitted to Malaria Journal:

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Malaria prevalence in pregnant women established by molecular tests after introduction of intermittent preventive treatment on Bioko Island, Equatorial Guinea

Malaria prevalence in pregnant women established by molecular tests after introduction of intermittent preventive treatment on Bioko Island, Equatorial Guinea

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Abstract

Background: Women living in malaria endemic regions particularly in first and second pregnancies have higher risk of malaria, with serious adverse effects for the woman and the child. To prevent these adverse effects, intermittent preventive treatment with sulfadoxine-pyrimethamine is recommended during pregnancy. Although rapid diagnostic tests (RDTs) are increasingly used during malaria indicator surveys, the test lacks sensitivity for the detection of low parasite densities. We describe here the prevalence and molecular characteristics of *Plasmodium* infections in the subpopulation of pregnant women, by utilising the RDTs collected during a malaria indicator survey (MIS) on Bioko Island, Equatorial Guinea.

Methods: We used data and finger-pricked blood samples applied on CareStart^{TM} RDTs collected during the MIS 2018 from August to October on Bioko Island from 13505 volunteers. Haemoglobin concentrations were measured by HemoCue Hb301 analyzer and results were recorded. Nucleic acids were extracted from the used, collected RDTs for downstream quantitative polymerase chain reaction (qPCR) assays and Sanger sequencing.

Results: We found a higher proportion of infections in non-pregnant (15%, 32/208) compared to pregnant women (9%, 21/227) detected by both, RDT and qPCR (p=0.05). Four out of 21 (19%) and 5 out of 32 (15%) of malaria infections were detected by qPCR only in pregnant and in non-pregnant women, respectively. Six out of 17 (35 %) and 4 out of 27 (15 %) malaria infections were detected by RDT only, in the pregnant and non-pregnant women, respectively, indicative of false positive RDTs. Six, four and four women were infected with malaria during their first, second and third trimester of pregnancy measured by qPCR. *Plasmodium*-infected pregnant women without clinical symptoms and signs had significantly lower haemoglobin concentrations compared to malaria negative, pregnant women.

Conclusions: Our study shows that prevalence of malaria infection in pregnant women in 2018 might be lower than in non-pregnant women, indicate for positive impact of preventive measures targeting this population. *Plasmodium*-infected pregnant women that were asymptomatic were more likely to suffer from anaemia underscoring the negative effects of sub-patent malaria infections in pregnancy.

Keywords: Prevalence, Malaria, Pregnancy, Intermittent preventive treatment, Quantitative polymerase chain reaction, Equatorial Guinea

Background

In 2018, it has been estimated that around 11 millions of pregnancies suffered from a *Plasmodium falciparum* malaria infection in 38 countries in Sub-Saharan Africa (1). Women during their first and second pregnancies are more susceptible to malaria compared to non-pregnant adults in malaria endemic countries (2). Increased susceptibility to *P. falciparum* infection during pregnancy is related to the sequestration of malaria infected erythrocytes (IE) into the placenta (3). The accumulation of IE in the intervillous space is enabled by a combination of slow blood flow and binding of IE to the glycosaminoglycan chondroitin sulfate A (3). The parasite encoded protein VAR2CSA - belonging to the variable antigen *P. falciparum* erythrocyte membrane protein-1 PfEMP1 protein family - interacts specifically with CSA expressed on syncytiotrophoblast cells lining the placental intervillous spaces (4). After repeated pregnancies, clinical signs and symptoms and prevalence of malaria infection during pregnancy decline significantly (2). This acquired immune protection correlates with increasing titers of antibodies targeting VAR2CSA that block the binding to CSA and enable phagocytosis of IE (3)(5).

Severe anaemia, cerebral malaria, preterm delivery or stillbirth usually affects pregnant women in low transmission settings. Those living in high transmission settings mainly suffer from maternal anaemia and low birth weight of the offspring (6). Malaria associated maternal anemia is a major driver of low birth-weight of newborns and WHO estimated that malaria infections in pregnancy (MIP) have resulted in an estimated number of 872´000 babies born with low birth-weight in 2018 (1). Children with low birth weight have a higher risk of death, stunting and poor cognitive development later in live (7). Exposure to malaria *in utero* might promote immune tolerance increasing the risk of malaria disease in the infant after birth (8)(9)(10). Although *P. falciparum* causes most of malaria related morbidity and mortality in Sub-Saharan Africa, non-*falciparum* malaria infections also contribute to morbidity in endemic areas and have been described on Bioko Island (11)(12)(13).

Protection from malaria in pregnancy currently relies on the use of long-lasting insecticide treated bed nets (LLINs), intermittent preventive treatment using sulfadoxine-pyrimethamine (IPTp-SP) and prompt diagnosis and effective treatment (14). IPTp-SP was introduced on Bioko in 2005 and has since been implemented in government health facilities through the National Malaria Control Program in collaboration with Medical Care Development International (15).

Diagnosis and surveillance of malaria have traditionally relied on thick and thin blood smear microscopy (16). Recently, rapid diagnostic tests (RDTs) that are simple to perform, give rapid results, and are deployable in rural settings have been introduced as malaria diagnostic tool (17). Most RDTs are based on the detection of circulating histidine-rich protein 2 (PfHRP2) and PfHRP3, selectively expressed by *P. falciparum* (18). RDTs are increasingly used in health care settings for prompt diagnosis, but also to measure malaria prevalence during malaria indicator surveys (15). The limit of detection of microscopy in expert hands is around 50 parasites/μL, while PfHRP2-based RDTs reliably detect parasite densities above 100 parasites/μL (19). RDTs therefore lack sensitivity for the detection of low parasitemia which is particularly the case in asymptomatic malaria infections in adults (20). Furthermore, *P. falciparum* isolates lacking the histidine-rich protein 2 (*pfhrp*2*)* and/or *pfhrp*3 genes, resulting in false-negative RDTs have been reported potentially compromising the usefulness of PfHRP2/HRP3 based RDTs for malaria diagnosis (21)(22). The specificity of PfHRP2/HRP3 based RDTs suffers from the extended clearance time of the circulating PfHRP2 antigen after successful antimalarial treatment (23). Therefore, diagnostic tools with higher sensitivity and specificity such as a nucleic acid (NA) based approaches could complement studies investigating malaria prevalence particularly in subclinical infections (20). Here we used RDTs collected from 13505 individuals covering an age range of 0 – 83 years and inhabiting 4774 selected households during the MIS on Bioko Island in 2018. We compare malaria diagnosis based on RDT and using NA extracted from these RDT, subjected to qPCR, to describe the prevalence and molecular characteristics of *Plasmodium* infections in pregnant women covering the first, second and third trimester of pregnancy.

Methods

Study area and population

The study is based on data and samples collected during the MIS conducted on Bioko Island, Equatorial Guinea between August 2018 and October 2018. Bioko Island is home to the capital city Malabo and located 240 km from the city of Bata on the mainland and 32 km off the coast of Cameroon. The surface area of the island is around 2000 km^2 with a population of approximately 335000 people. The MIS survey design and procedures were described previously (24). Briefly, all households members present at the time of visit were eligible to participate. Survey questions were programmed using DroidDB software, and run on Android-based tablets to collect a wealth of information including pregnancy status, antimalarial therapy and history of travel (25). We included into the study all women identified as pregnant during the survey and contrasted them to non-pregnant women individually matched by age to the pregnant women.

Sample collection and definitions

Finger pricked blood samples were used to screen for ongoing malaria infection using the CareStart^{TM} Malaria HRP2/pLDH (pf/PAN) combo test (ACCES BIO, Inc. 65 Clyde Road Somerset, NJ 08873 U.S.A) and haemoglobin concentration was measured with the use of a HemoCue Hb301 analyzer (HemoCue AB, Angelholm, Sweden). Using the World Health Organization's classification, severe anaemia was defined as a haemoglobin < 7 g/L and 8 g/dL in pregnant and non-pregnant women, respectively (26). Used RDTs were collectively stored at room temperature in plastic bags containing desiccants until transport to the Swiss Tropical and Public Health Institute for further analysis.

Extraction of nucleic acids

The Electronic Laboratory Information and Management Utensil for Molecular Diagnostics (ELIMU-MDx) platform was used to identify and sort the RDTs from pregnant and non-pregnant

women (32). Extraction of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was done using the newly developed Extraction of Nucleic Acids from RDTs (ENAR) protocol (33). Each 96-well extraction plate included one negative control consisting of the Lysis buffer, and one positive control RDT at the density of 200 parasites/μL (whole blood was spiked with WHO International Standard parasites and applied on RDTs). Total nucleic acids were recovered in 50 μL of elution buffer (Quick-DNA™ Miniprep Kit, Zymo Research Corporation, Irvine CA, USA) and stored in 96-wells DNA plates at -20°Celsius. Extraction of nucleic acids was performed within three months of sample collection.

Nucleic acid based malaria diagnosis

The multiplex PlasQ PCR assay targeting the pan-*Plasmodium* 18S DNA (*Pspp*18S) and the *P. falciparum*-specific *var* acidic terminal sequence genes (*Pfvar*ATS) was used to screen for presence of malaria and to quantify *P. falciparum* density (27)(13). For the identification of *Plasmodium* species, we used the PlasID qPCR assay (13). Briefly, to overcome the low amount of nucleic acids starting material for the PlasID qPCR assay, we amplified the *Pspp18S* gene by conventional PCR. We performed a Species identification qPCR assay based on the 18S rRNA gene. The assay detects all human *Plasmodium* species except *P. falciparum*. The following primer combinations was used for the pre-amplification of 18S: Pspp18S_RT-HRM_F GRA ACT SSS AAC GGC TCA TT (28), and Pspp_18S_R: AGCAGGTTAAGATCTCGTTCG (PlasQ RT-qPCR). Briefly, 2 μL of the product from the pre-amplification were added to the master mix containing 1x Luna® Universal Probe qPCR Master Mix and 1x Species ID primer mix with a total volume of 10 μL. The probes for the species were labelled with following fluorophores: *P. malariae*: Yakima Yellow, *P. ovale*: Texas Red, P. vivax: Cy5, *P. knowlesi*: Cy5. The conditions for the qPCR were: 95 °C for 3 minutes and 50 cycles of 95 °C for 15 seconds and 60 °C for 45 seconds. Fluorescence intensity was measured after each cycle. Positive controls for *P. ovale* and *P. malariae*, *P. falciparum* DNA as a negative control and a non-template control were included

in all runs. In case of a positive Cy5 result, the sample would be run with the same conditions in a multiplex assay using the *P. vivax* Cy5 probe and a probe for *P. knowlesi* labelled with Quasar 705. Following nucleotide sequences were used: Pk_18S_Cy5: CTC TCC GGA GAT TAG AAC TCT TAG ATT GCT (Cy5)(29), PlasID_Pv18S_Cy5: GAA TTT TCT CTT CGG AGT TTA T (30), Pm18SMGBVIC: CTA TCT AAA AGA AAC ACT CAT (Yakima Yellow)(30), and Po18S_TxRd_v3.0: GGA 5AT6T CTT AGA TTG CTT CCT 6CAG (Texas Red, 5 = LNA-A, 6 = LNA-T, unpublished). Samples were run in duplicate and considered positive if one replicate out of two showed a clear amplification curve with Cq < 42. All qPCR and RT-qPCR data were uploaded to ELIMU-MDx for an automated quality control step and analysis (31).

Data analysis and statistics

Categorical variables were described with absolute and relative frequencies, and discrete variables with medians and interquartile ranges (IQR). Proportions were compared by means of Pearson Chi square test and continuous variables by Student's t-test. Statistical analyses were performed using Stata version 14 (StataCorp LP, College Station, TX) and GraphPad Prism version 7 (GraphPad Software, La Jolla, USA).

Results

We utilized RDTs collected from 227 pregnant and 208 age-matched non-pregnant control women during the malaria indicator survey on Bioko Island in 2018**.** Study population and malaria diagnostic tests used are summarized in **Figure 1**. Sociodemographic characteristics of the women and malaria prevention measures are shown in **Table 1**. From these RDTs, RNA and DNA was extracted following our newly developed protocol (Guirou et al., manuscript submitted).

Figure 1 - Overview of study population and outcome of malaria diagnosis

Table 1 - Sociodemographic characteristics and malaria prevention used by cohort analysed

Prevalence of *Plasmodium spp* infection detected by RDT and qPCR

Plasmodium was detected by qPCR and RDT combined in 15/227 (7%) and 28/208 (13%) pregnant and non-pregnant women, respectively (**Figure 2)**. Positive RDTs that tested negative by qPCR were found in 6/227 (28%) in the pregnant and 4/208 (12%) non-pregnant women (**Figure 3)**. RDTs missed 4/21 (19%) and 5/32 (16%) of malaria infections in the pregnant and non-pregnant women, respectively, since they had positive qPCR results **(Figure 3)**. In addition to *P. falciparum*, four *P. malariae* infections were detected. Three of the infections were mixed with *P. falciparum* and one was a *P. malariae* mono-infection. RDT failed to detect the only *P. malariae* mono-infection. No *P. ovale* nor *P. vivax* infection was detected **(Figure 1)**.

Figure 2 - Proportions of *P. falciparum* infections detected by RDT and PlasQ PCR in pregnant and non-pregnant women

Figure 3 – Venn Diagram comparing the outcomes of RDT testing with qPCR results based on amplification of 18S and PfvarATS

Parasite density by PlasQ qPCR

P. falciparum densities measured by PlasQ qPCR in samples collected from pregnant (n = 21) and non-pregnant women (n = 32) are shown in **Figure 4**. Overall medians of parasite densities were low (< 100 parasites/µL) in all RDTs tested (Range: 0.3 - 2165 parasites/ µL) and did not significantly vary between the pregnant and the control groups (Mann Whitney test, p = 0.29). Parasite densities in samples tested positive by both RDT and qPCR were significantly higher compared to samples tested positive by qPCR only (Mann Whitney test, p = 0.038). Conversely, infections missed by RDT were of low parasite density (Range: 0.4 to 94 parasites/µL) (**Figure 4A**). Six, 4 and 4 malaria infected pregnant women were in their first, second, and third trimester of pregnancy, respectively. Median parasite density was slightly lower in the first compared to the second and third trimesters of pregnancy (**Figure 4B**).

Figure 4 - Parasite densities in infected women

Anemia in malaria infected pregnant women

Mean haemoglobin concentration was 10.9 ± 1.4 g/dL among the pregnant and 11.8 ± 1.5 g/dL in the non-pregnant group. Haemoglobin concentration was significantly lower in malaria infected (10.0 ± 0.3 g/dL) compared to non-malaria infected pregnant women (11.0 ± 0.1 g/dL), t-test, p = 0.012, in the pregnant but not in the control group (t-test, p = 0.21) (**Figure 5**).

Figure 5 – Anemia in pregnant women is associated with malaria infection

Factors associated with *Plasmodium* infection

Next, we tried to understand risk factors associated with malaria infection in pregnant women. The proportion of women who reported sleeping under a mosquito net the night preceding the survey was higher in the pregnant compared to the control group (**Table 1**). The proportion of women

who reported being sick was 26.7% in qPCR-positive and 9.4% in qPCR-negative pregnant women. *Plasmodium* infection was associated with sickness within two weeks prior to survey in the pregnant (Chi-square test, p = 0.036) but not in the control group (Chi-square test, p = 0.76).

Discussion

We used RDT in combination with NA based diagnostic assays to determine the prevalence of *P. falciparum* and non-*falciparum* species in pregnant and age matched non-pregnant women in samples collected as part of MIS2018. The sensitivity of the CareStartTM Malaria HRP2/pLDH (pf/PAN) combo test is around 100 parasites/µL and we set out to understand how many infections might be missed, particularly in pregnant women, by using a more sensitive qPCR based diagnostic test developed by our group (Guirou et al., manuscript submitted). Molecular testing added to the 44 RDT positive women an additional 9 cases that have been missed by RDT (17%) supporting the idea that the PlasQ qPCR is more sensitive than RDT.

Ten women tested positive by RDT only and in the absence of confirmation by qPCR, we consider these tests as false positive. We did not find any association between RDT false-positivity and prior treatment in pregnant women in contrast to previous reports (23)(32). One possible explanation is that the RDT detected HRP2 released from IE that sequestered in the placenta with very low numbers of parasites circulating in the periphery - therefore remaining largely undetectable in peripheral blood (33). Overall, the prevalence of *Plasmodium* infection (RDT and qPCR positive samples combined) in our cohort was 12% which is in agreement with malaria prevalence reported (15). We found a higher proportion of infections in non-pregnant (15%, 32/208) compared to pregnant women (9%, 21/227) detected by both, RDT and qPCR (p=0.05). These result indicate that the IPTp-SP program implemented on Bioko Island through the National Malaria Control Program reduces malaria infections in this highly vulnerable population. The implementation of targeted preventive interventions by the Bioko Island Malaria control program including distribution of bed nets, administration of IPTp-SP, and delivery of educational

messages to pregnant women has been conducted since 2005 (15). Among the pregnant women eligible for IPTp-SP at the time of the survey, 69, 38, and 36% took at least one, two and three doses of SP, respectively. These figures compare favourably to reports from other malaria endemic countries where lower numbers of pregnant women are reached by the IPTp-SP (34)(1).

Four *P. malariae* infections were detected in our cohort confirming that *P. malariae* is prevalent on Bioko Island (13)(35). Little is known about the effect of *P. malariae* infections in pregnancy, warranting further investigations of impact of non-*falciparum* species on clinical signs and symptoms of the population living on Bioko Island (11). No *P. ovale* and *P. vivax* parasites were detected in the 43 women tested.

Clearly, the major limitation of our study is the small volume of blood applied on RDT (~ 5 µL) which reduces the sensitivities of the qPCR assays used. Medians of parasite densities were below the reported lower limit of detection of the CareStart^{TM} Malaria HRP2/pLDH (100 parasites/µL) when assessed by PlasQ qPCR and ranged from 0.3 to 2165 parasites/µL. *P. falciparum* parasite densities were not significantly higher in pregnant women compared to non-pregnant women and parasite densities did also not significantly vary according to parity or age of pregnancy. Albeit our total number of *P. falciparum* positive pregnant women is low, it is important to note that more qPCR-positive women were detected during the first compared to the second and third trimesters of pregnancy, supporting previous reports highlighting the importance of malaria infection early during pregnancy (36)(34).

Despite the low prevalence of malaria infection, *Plasmodium*-infected, pregnant women had significantly lower haemoglobin concentrations, confirming that malaria infection in pregnancy - even in the absence of clinical symptoms and at low parasite density - constitute an important risk factor for maternal anaemia (37). In contrast, there was no association between malaria infection and haemoglobin concentration in the non-pregnant women. Hence, malaria in pregnant women on Bioko Island is a driver for anaemia emphasizing the importance of preventing malaria and prompt treatment in this vulnerable group.

Conclusions

Our study shows that the prevalence of malaria infection in pregnant women was lower than in non-pregnant women on Bioko Island. This finding underscores that implementation of preventive measures targeting this vulnerable population is effective. Using the MIS2018, we are identify malaria infected women in their first trimester. These women should be encouraged to come to health facilities early. Although less pregnant women were infected with *P. falciparum*, they were more likely to suffer from anaemia.

List of abbreviations

IPTp-SP: intermittent preventive treatment of malaria in pregnancy using sulfadoxine-pyrimethamine; MIS: malaria indicator survey; PfHRP2: *Plasmodium falciparum* histidine-rich protein 2; pLDH: *plasmodium* lactate dehydrogenase; qPCR: quantitative polymerase chain reaction; RDT: rapid diagnostic test

Declarations

Ethics approval and consent to participate

The survey was approved by the Ministry of Health and Social Welfare of Equatorial Guinea and the Ethics Committee of the London School of Hygiene & Tropical Medicine. Written informed consent was obtained from all adults and from parents or guardians of children who agreed to participate. Only samples for which an additional consent for molecular analysis was obtained were included in this study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EAG, TS and CD conceived the study and prepared the manuscript. OTD, WPP, CDM, CCF and GAG collected the MIS data. EAG, CY and SH performed the laboratory work. EAG and TS analysed the data with input from OTD and KK. CD and EAG prepared the manuscript with support from TS, KK, and MT. All authors read and approved the final manuscript.

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Table 1 - Sociodemographic characteristics and malaria prevention used by cohort analysed

	Pregnant women (N = 227)		Non-pregnant women (N = 208)		
Age, Median (IQR)	26 (21- 31)		25 (21 - 31)		
	n	%	n	%	p-value
Parity					
Primi-secundiparae	107	48.4	127	62.9	0.002
Residence					
Malabo	180	79.3	169	81.3	0.6
Baney	32	14.1	29	13.9	0.95
Luba	8	3.5	6	2.9	0.72
Riaba	7	3.1	4	1.9	0.42
Socioeconomic status					
1-Wealthiest	39	17.2	25	12.0	0.12
2-Wealthy	44	19.4	40	19.2	0.95
3-Medium	54	23.8	44	21.2	0.51
4-Poor	40	17.6	52	25.0	0.06
5-Poorest	50	22.0	47	22.6	0.88
Mosquito net use	137	60.4	89	42.8	0.001
≥ 2 doses of IPT-SP[a]	43	58.1			
≥ 3 doses of IPT-SP	20	27.0			

[a] Doses of IPTp-SP received by women in the second and third trimester of pregnancy

IQR: interquartile ranges, Socioeconomic status defined by using principal components analysis of household assets and amenities.

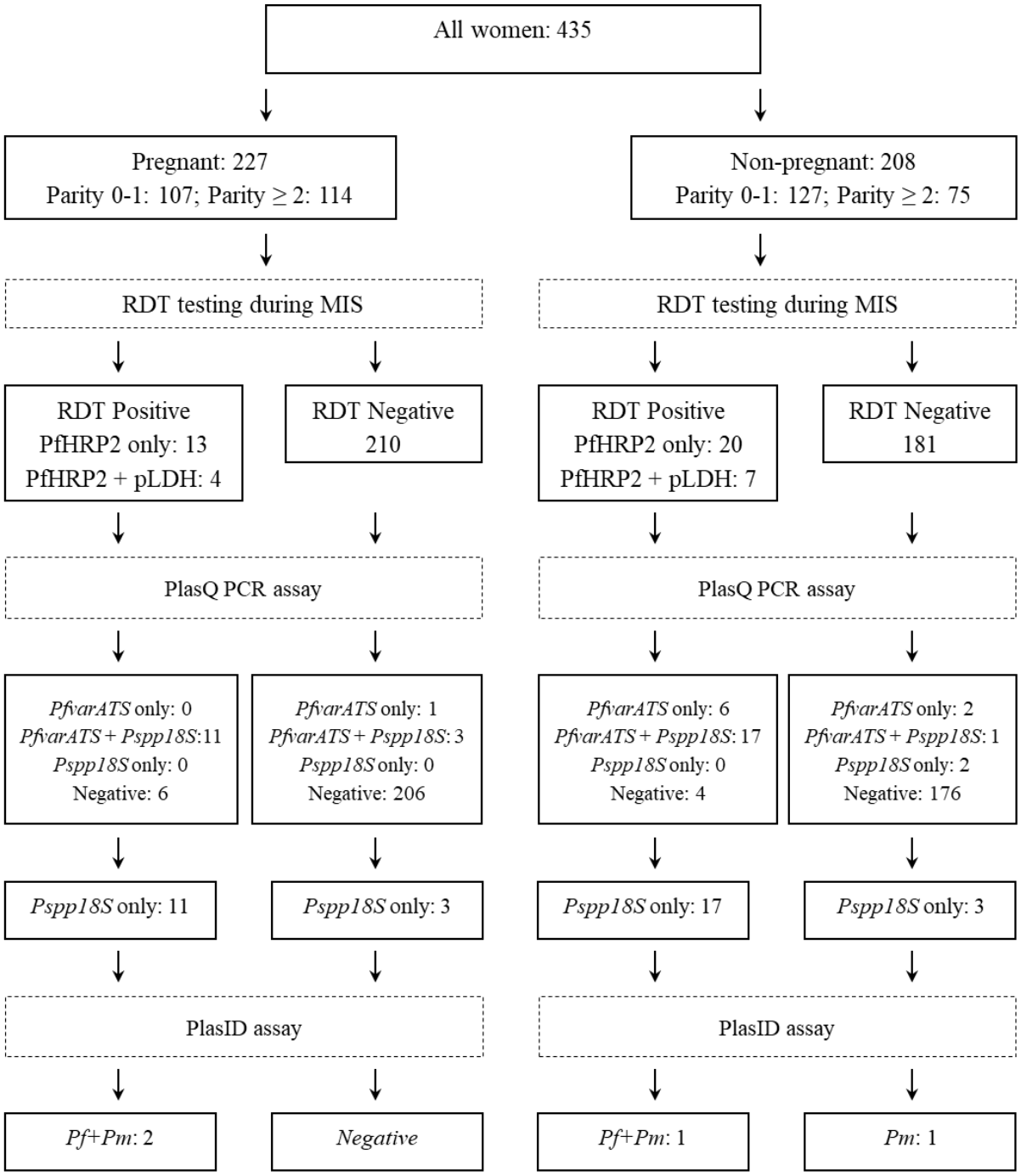

Figure 1 - Overview of study population and outcome of malaria diagnosis
RDT collected from pregnant and age matched non-pregnant women were collected and screened for *P. falciparum* and non-*falciparum* species using the PlasQ and PlasID PCR assays. MIS: malaria indicator survey, *Pf: P. falciparum*, *Pspp: Plasmodium* species, *Pm: P. malariae*.

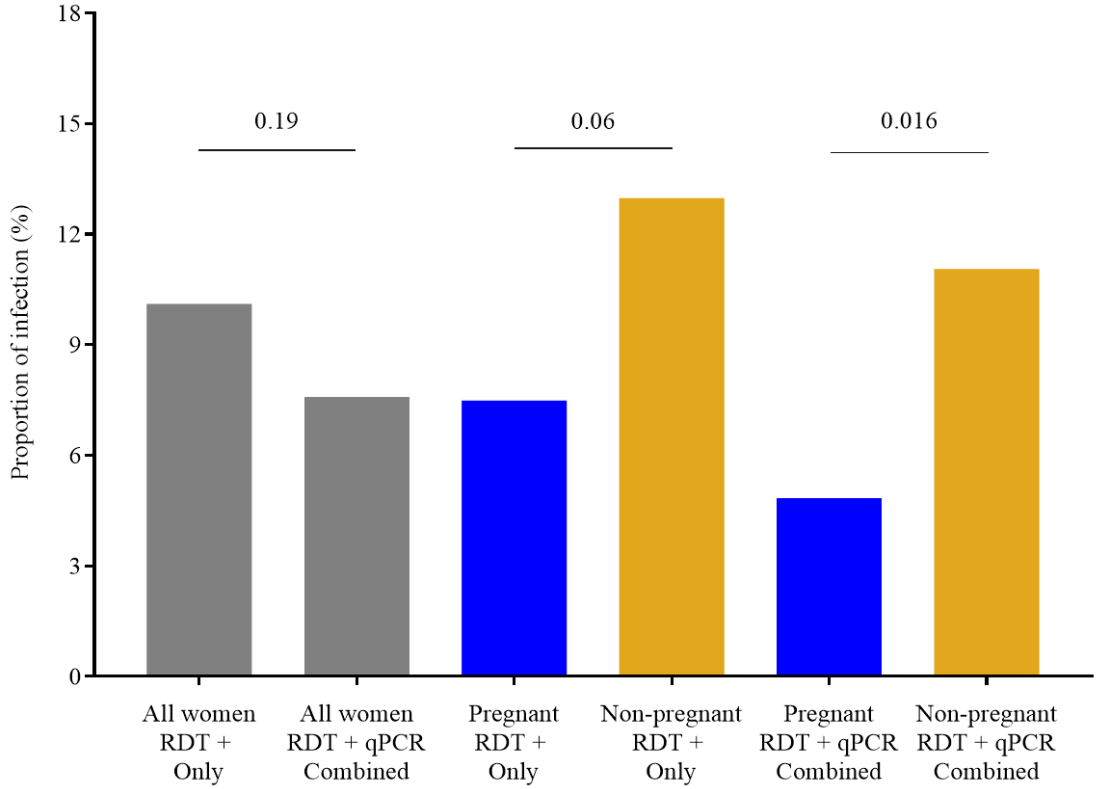

Figure 2 - Proportions of *P. falciparum* infections detected by RDT and PlasQ PCR in pregnant and non-pregnant women
Samples were categorised according to positivity by RDT (all RDT positive) and qPCR (all qPCR positive). The proportion of infection detected by qPCR was significantly higher in non-pregnant group (Chi-square test).

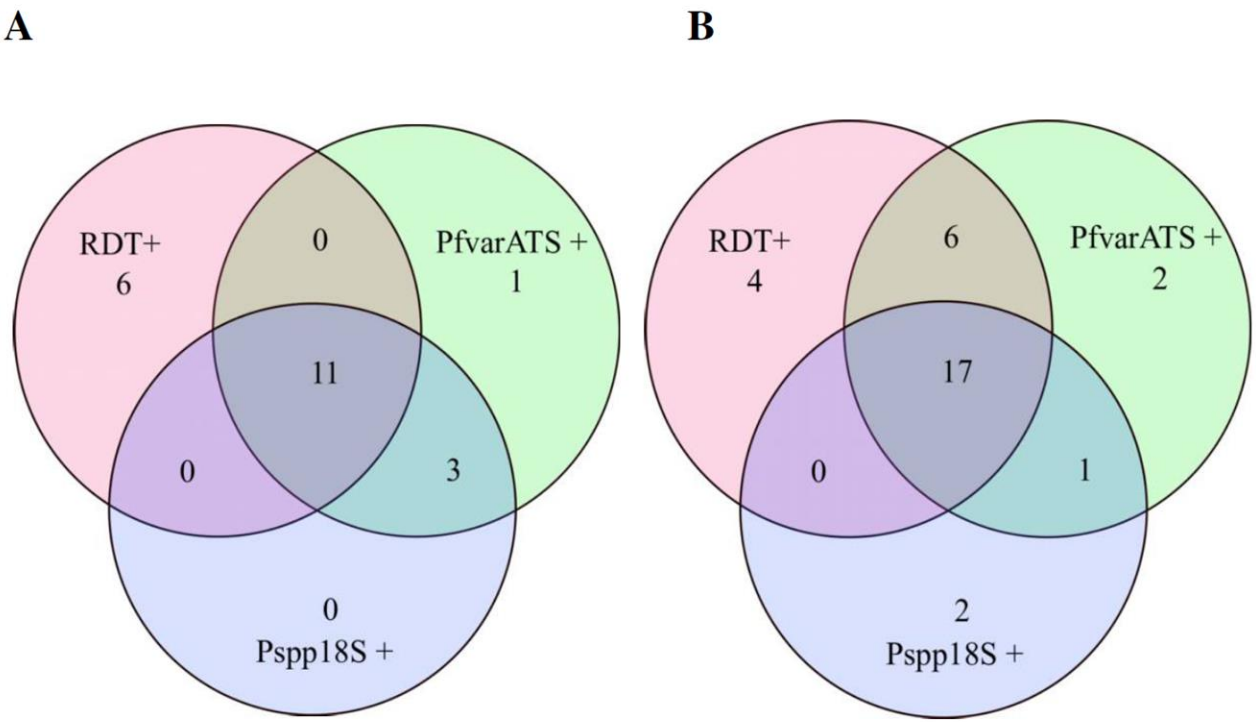

Figure 3 – Venn Diagram comparing the outcomes of RDT testing with qPCR results based on amplification of 18S and PfvarATS.

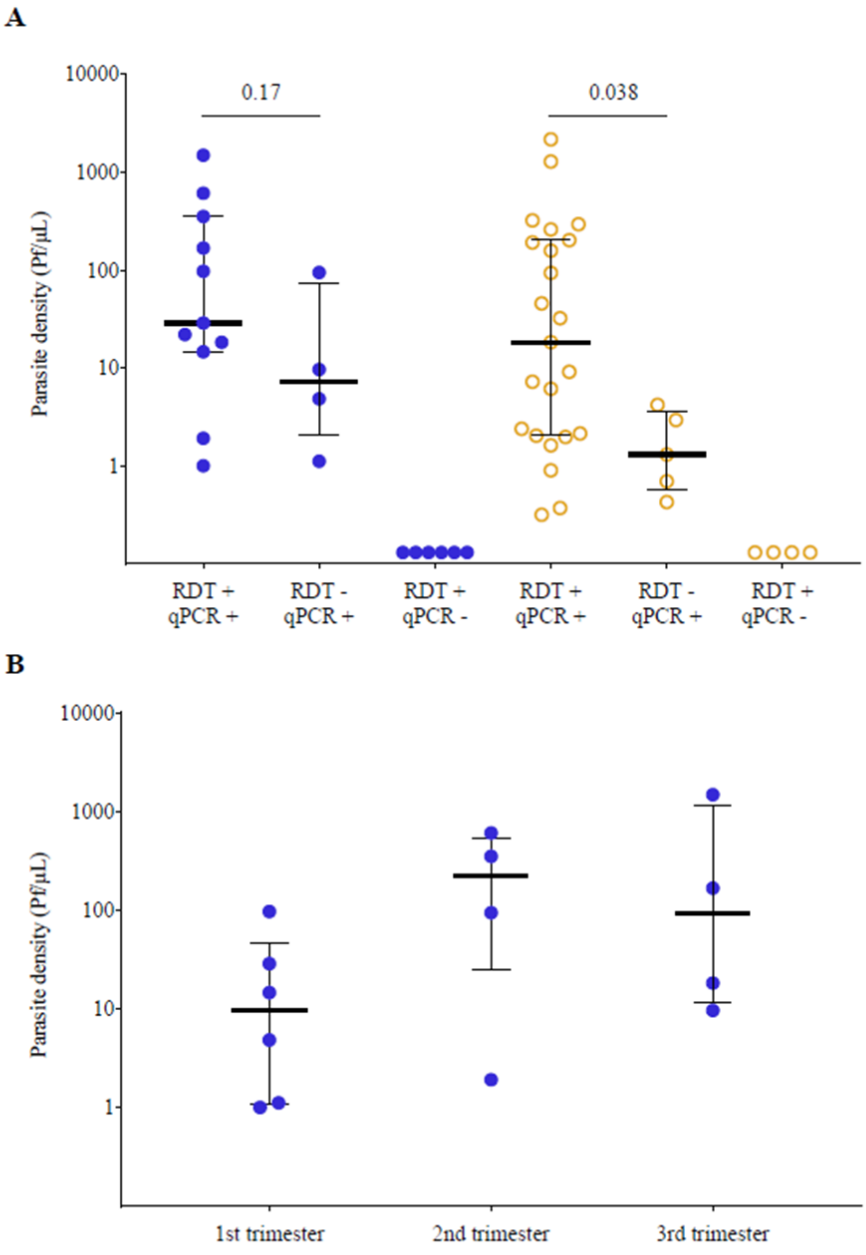

Figure 4 - Parasite densities in infected women

Plasmodium positivity by RDT and PlasQ qPCR in pregnant (filled circles) and non-pregnant women (white circles) is shown. Each circle represents one sample. Thick lines indicate medians, and thin lines interquartile ranges. Medians of parasite densities were higher in samples positive by RDT compared to samples only positive by qPCR. (**Figure 4A**). Six, four and four pregnant women were in the first, second and third trimester of pregnancy, respectively. Each filled circle represents one sample from a pregnant woman. Medians of parasite densities did not significantly vary according to the trimester of pregnancy (Kruskal-Wallis test, p = 0.20) (**Figure 4B**).

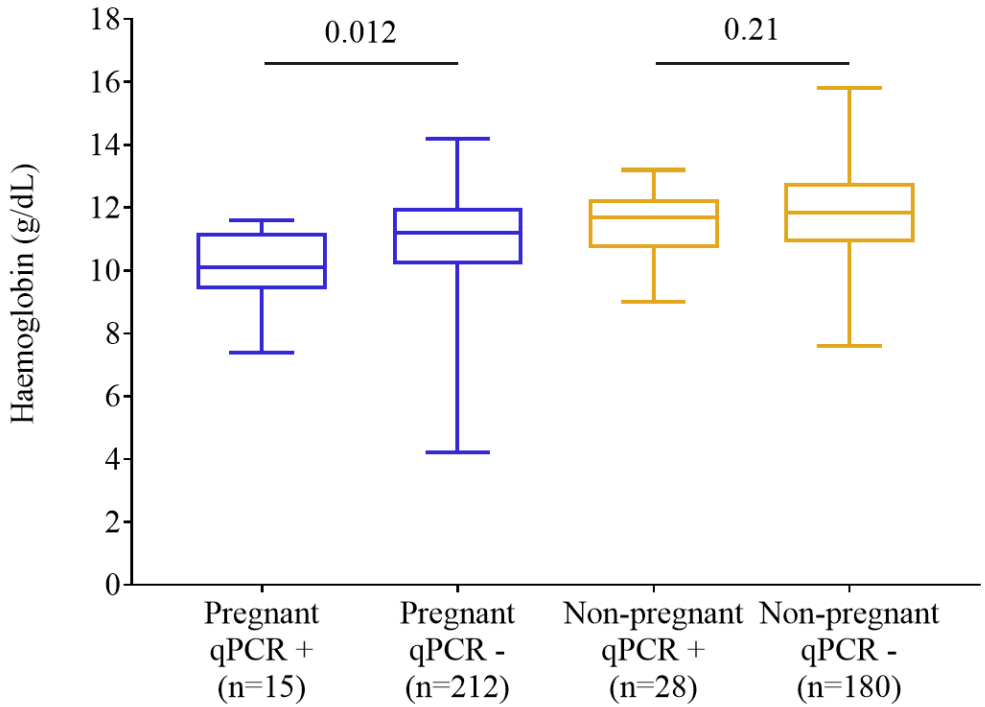

Figure 5 – Anemia in pregnant women is associated with malaria infection
Mean haemoglobin concentrations (g/dL) in *Plasmodium*-infected and uninfected women is shown. Mean haemoglobin was significantly lower in malaria infected pregnant compared to malaria negative women (Student's t-test, p=0.012). Two malaria uninfected multiparous pregnant women who were in their third trimester of pregnancy had severe anaemia (haemoglobin < 7 g/dL).

Chapter 5

HIV infection negatively impacts the vaccine efficacy of whole irradiation attenuated sporozoite based malaria vaccines when evaluated by homologous controlled human malaria infections

This chapter contains the following working manuscript:

Etienne Guirou *et al*. **HIV infection negatively impacts the vaccine efficacy of whole irradiation attenuated sporozoite based malaria vaccines when evaluated by homologous controlled human malaria infections.**

HIV infection negatively impacts the vaccine efficacy of whole irradiation attenuated sporozoite based malaria vaccines when evaluated by homologous controlled human malaria infections

This is a working manuscript, which is currently not ready to be submitted to a peer reviewed journal since the clinical study has not been published by the study sponsor, Sanaria Inc.

Abstract

Malaria and HIV are co-endemic in many regions of Sub-Saharan Africa. Deployment of a malaria vaccine in the general population will require that the vaccine is safe and efficacious in all volunteers, irrespective of their HIV infection status. Here, we have vaccinated a group of HIV positive and HIV negative volunteers in Tanzania with irradiation attenuated purified *Plasmodium falciparum* sporozoites to compare the safety, immunogenicity and protective efficacy against homologous controlled human malaria infection. This is the first time that HIV positive volunteers have undergone controlled human malaria infection providing the opportunity to understand under highly controlled conditions the interaction between HIV and malaria. This trial has been registered at ClinicalTrials.gov Identifier: NCT03420053.

Keywords: Controlled Human Malaria Infection (CHMI), Human immunodeficiency virus (HIV), Rapid diagnostic test (RDT), quantitative polymerase chain reaction (qPCR), thick blood smear (TBS) microscopy, Plasmodium falciparum, PfSPZ Challenge

Introduction:

Malaria remains a disease of global health importance, despite the gains made during the last decade in reducing morbidity and mortality. The latest estimates of the burden of malaria indicated that 228 million cases and over 405 000 deaths from malaria globally in 2018 deaths (1). Vector control by impregnating bed nets and spraying indoor surfaces of houses with insecticides is the primary intervention for decreasing malaria transmission at the community level. However, the increased resistance of mosquitoes to insecticides such as pyrethroids is a challenge to current vector control measures (2). Despite encouraging progress made in some areas of Africa, malaria elimination will likely require an effective vaccine ((3)). Based on previous evidence that attenuated whole *Plasmodium falciparum* sporozoite (PfSPZ) induced high level, durable protection (4). Sanaria Inc. developed aseptic irradiation attenuated cryopreserved PfSPZ as vaccine candidate. PfSPZ-based vaccines were safe, well tolerated and protective in many studies ((5) and could be used in mass vaccination programs for malaria elimination. Because HIV infection shows a significant geographic overlap with malaria in sub-Saharan Africa (6), it is important to assess the safety, tolerability and efficacy of the vaccine in the HIV infected individuals. Both malaria and HIV case management involve combination therapies, and interactions between antiretroviral treatment and antimalarials also deserve attention (7). Clinical development is time consuming and costly, therefore Controlled human malaria infection (CHMI) offers an opportunity to select pre-erythrocytic vaccine candidates with potential efficacy using experimental controlled conditions (8). CHMI entails the deliberate inoculation by syringe and needle of live infectious sporozoites to healthy human volunteers. Such infections have been recently carried out in many malaria endemic countries including Bagamoyo, Tanzania (9). Following inoculation, parasitemia are closely monitored by thick blood smear microscopy and by quantitative polymerase chain reaction (qPCR) to ensure that volunteers are treated before the appearance of malaria symptoms. Here we report for the first time on the safety, immunogenicity and vaccine efficacy (VE) against CHMI of irradiation attenuated whole *P. falciparum* sporozoite based vaccine (PfSPZ Vaccine) in HIV negative and HIV positive Tanzanian adults.

Material and Methods:

Study design and population

This was a single center randomized, double-blind, placebo-controlled trial designed to assess the safety, tolerability, immunogenicity and efficacy of Sanaria's radiation attenuated PfSPZ Vaccine in HIV negative and HIV positive volunteers in Bagamoyo, Tanzania (Clinical Trials.gov: NCT03420053). In total, 21 male and female adult volunteers aged from 18 to 45 years were enrolled and 18 of them underwent CHMI. Nine volunteers comprising six vaccinees and three placebo controls were enrolled into each group. HIV positive volunteers were on stable anti-retroviral therapy (ART) for at least 3 months with a CD4+ cell count above 500 cells/μL at screening. Volunteers in the vaccine group received 900'000 aseptic, purified, metabolically active, live, radiation attenuated cryopreserved *P. falciparum* sporozoites (PfSPZ) administered intravenously at 0, 2, 4, 6 and 28 days. Controls received parallel injections with normal saline to keep blinding.

Controlled Human Malaria Infection

Homologous CHMI (PfSPZ Challenge) was conducted by intravenous administration of 3200 infectious aseptic, purified, cryopreserved NF54 *P. falciparum* sporozoites approximately 3 weeks after the last dose of PfSPZ candidate vaccine to assess protective efficacy. Parasitemia was checked twice daily from day 8 to 14 and then once daily through day 20 post CHMI. Volunteers diagnosed with malaria were treated and discharged. Volunteers not positive by the end of the observation period (day 20), were discharged and scheduled for a follow-up clinic visit on day 28 post CHMI. Those who remained negative throughout day 28 of follow-up were presumptively treated with antimalarials.

Parasite detection and quantification

Thick blood smear microscopy (TBS) microscopy was performed as previously described (10) The PlasQ qPCR assay was used in parallel to TBS to determine and quantify parasite density during the CHMI study as described (11).

CD4+ counts and HIV-1 viral load assessment

Throughout the immunization period and the CHMI, among the HIV+ volunteers the CD4+ counts and the HIV viral loads were closely monitored. CD4+ counts were obtained by FACS (BD Biosciences, California, USA). The Xpert HIV-1 VL assay (Cepheid, California, USA) an

automated molecular test which uses the GeneXpert systems was used to assess HIV viral loads in 1 mL plasma according to the manufacturer's instructions.

Anti-PfCSP antibody assays

Blood for immunogenicity testing was drawn prior to the first immunization and 2 weeks after the final immunization. Serum was separated and frozen at -80°C within 4 hours of collection. IgG antibodies to Pf circumsporozoite protein (PfCSP) were assessed by ELISA as described (10).

Data management and statistical analysis

The qPCR assays were run on a Bio-Rad CFX 96 Real-Time instrument using CFX Maestro Software (Bio-Rad Laboratories, California, USA). qPCR raw data was managed and analysed by our in-house qPCR-specific laboratory management and information system ELIMU-MDx (12). Parasite densities based on the *P. falciparum*-specific PfvarATS qPCR assays were used to calculate parasite multiplication rates (PMRs). PMRs were modelled using a linear model fitted to log10-transformed qPCR data as previously published(13). PMR was calculated for all volunteers that developed blood-stage parasitaemia which lasted for at least two 48-hour cycles. Data was analysed and plots created using Graphpad Prism software version 9.

Results:

HIV infection is associated with reduced immunogenicity and vaccine efficacy of PfSPZ Vaccine

All volunteers successfully finalized the vaccination period without any safety signals observed. Three weeks past last vaccination, the volunteers underwent CHMI using 3200 fully infectious purified sporozoites. As shown in Figure 1, all placebos developed asexual blood stage parasitemia detected by qPCR, but not by TBS microscopy. While 5 out of 6 vaccinated volunteers were protected in the HIV negative cohort (n=6), all HIV positive vaccinees succumbed to malaria infection and developed asexual blood stage parasites. It is well known that HIV infection results in immunosuppression. We therefore compared the anti-CSP antibody responses in vaccinees versus placebos in both cohorts (Figure 2). Surprisingly, no difference in antibody titers binding to CSP could be observed in HIV positive and HIV negative volunteers 2 weeks past last vaccination, representing the peak time point of serum antibody responses induced after whole sporozoite based vaccination.

HIV infection status does not impact the blood-stage malaria parasite growth dynamics

Next we wanted to understand if asexual blood stage parasites grow faster in HIV positive versus HIV negative volunteers during CHMI. In Table 1, the prepatent period as measured by qPCR and TBS is shown for HIV positive and HIV negative volunteers (vaccinees and placebos combined). The peak parasitemia and parasite multiplication rates are given. No significant difference in the prepatent period, peak parasitemia and parasite multiplication rates could be established. Figure 3 shows the growth patterns of asexual blood stage parasitemia over time as measured by qPCR for each of the volunteers stratified by vaccination and HIV infection status.

Table 1:

	HIV- volunteers positive for Pf during CHMI (n=4)	HIV+ volunteers positive for Pf during CHMI (n=8)	P value
Prepatent period by qPCR (average and range)	10.0 (9.5-11.0)	12.3 (8.5-18.0)	0.18
Prepatent period by TBS (average and range)	14.5 (12.0-18.0)	16.2 (13.0-18.0)	0.23
% of volunteers with sub-microscopic infections	0.0 (0/4)	37.5 (3/8)	0.17
Peak parasite density in Pf/µL (geometric mean)	2.9 ± 4.8	5.6 ± 77.3	0.94
Parasite multiplication rate	5.7 ± 3.0	8.9 ± 4.2	0.21

Controlled human malaria infection does not affect low and stable HIV viral loads

We assessed potential changes in HIV viral load before, during and after (day 56) CHMI. As shown in Figure 4, two volunteers developed during CHMI HIV viremia that could be measured by GenXpert. On day 56, all volunteers had returned to undetectable HIV viral loads in the serum samples collected. CD4 T cell counts of these volunteers remained also stable, with only volunteer 054-51 displaying during CHMI a drop below 500 cells/microliter (Figure 5).

HIV+ participants experienced malaria re-infection after completion of CHMI

We found that 50% (4/8) HIV+ volunteers were re-infected with *P. falciparum* after completion of the CHMI which included a full course of anti-malarial treatment. Among the HIV- volunteers not a single re-infection was observed (Fisher's exact test, p value = 0.0769) on day 56. These data clearly show that HIV infection is a driver of increased malaria infection as described previously (7).

Discussion:

To our knowledge, this was the first time HIV positive volunteers were immunized using PfSPZ Vaccine with the VE assessed against homologous CHMI. PfSPZ vaccine was well tolerated and safe but less immunogenic and protective in HIV positive Tanzanian volunteers than in HIV negative volunteers. Strikingly, the anti-CSP antibody response did not differ significantly between the two vaccination cohorts, stressing the importance of cell mediated immune mechanisms in PfSPZ vaccine induced protection (14). The PfSPZ vaccine was given five times in a shortened regimen comprising 28 days. This novel shortened regimen has the advantage of providing VE in a short time frame that will foster compliance of vaccinees in the field. During the vaccinations, no vaccine induced safety signal could be observed in all volunteers stressing the safety of PfSPZ vaccine as observed in a number of previous studies (11)(10)(15)(16). During CHMI, all HIV positive volunteers developed asexual blood stage parasitemia that however did not seem to activate the HIV infection as would be reflected by an increase in the HIV viral load. Also, CD4 T cell counts were not affected during the single controlled malaria episode demonstrating that the safety of the volunteers have not been jeopardized during the study. Some Tanzanian volunteers included showed pre-existing immunity against malaria since they contained low parasitemia levels that never reached TBS microscopy threshold. This observation stresses the importance of molecular diagnosis of malaria infections during vaccine efficacy studies in malaria pre-exposed volunteers.

Figure legends:

Figure 1:
Kaplan-Meier curves in volunteers undergoing CHMI as assessed by TBS (A) and by qPCR (B).

Figure 2:
Antibodies to PfCSP by ELISA. IgG antibodies to Pf circumsporozoite protein PfCSP by ELISA two weeks after the 5th dose by HIV status and vaccination group. Antibody responses are reported as the net OD1.0 or the difference between the post and pre-immunization OD 1.0. Filled circles represent volunteers remaining uninfected after CHMI; open circles represent volunteers infected after CHMI. Antibody responses to PfCSP were significantly higher in the PfSPZ Vaccine group than in the Control group by vaccination status (Mann Whitney test,

p=0.02). Antibody response did not differ significantly between HIV positive and HIV negative vaccine recipients groups (Mann Whitney test, p=0.24).

Figure 3:

Parasite density as measured by qPCR given by HIV status and trial group after CHMI. The dashed lines represent the lower limit of qPCR positivity. Parasite densities are plotted with an offset of 0.05 parasite/µL.

Figure 4:

HIV Viral load in HIV positive volunteers before, shortly and long after CHMI. Before: 1-2 days before CHMI; short: 13-20 days after CHMI, long: 56 days after CHMI. Detectability of viral load: 0 = not detected, 1 = detected. Viral loads did not significantly vary before and after CHMI (Wilcoxon matched-pairs signed rank test, p=0.5).

Figure 5:

CD4 count in HIV positive volunteers before, shortly and long after CHMI. Before: 1-2 days before CHMI; short: 13-20 days after CHMI, long: 56 days after CHMI. CD4 count did not significantly vary before and after CHMI (Wilcoxon matched-pairs signed rank test, p=0.5).

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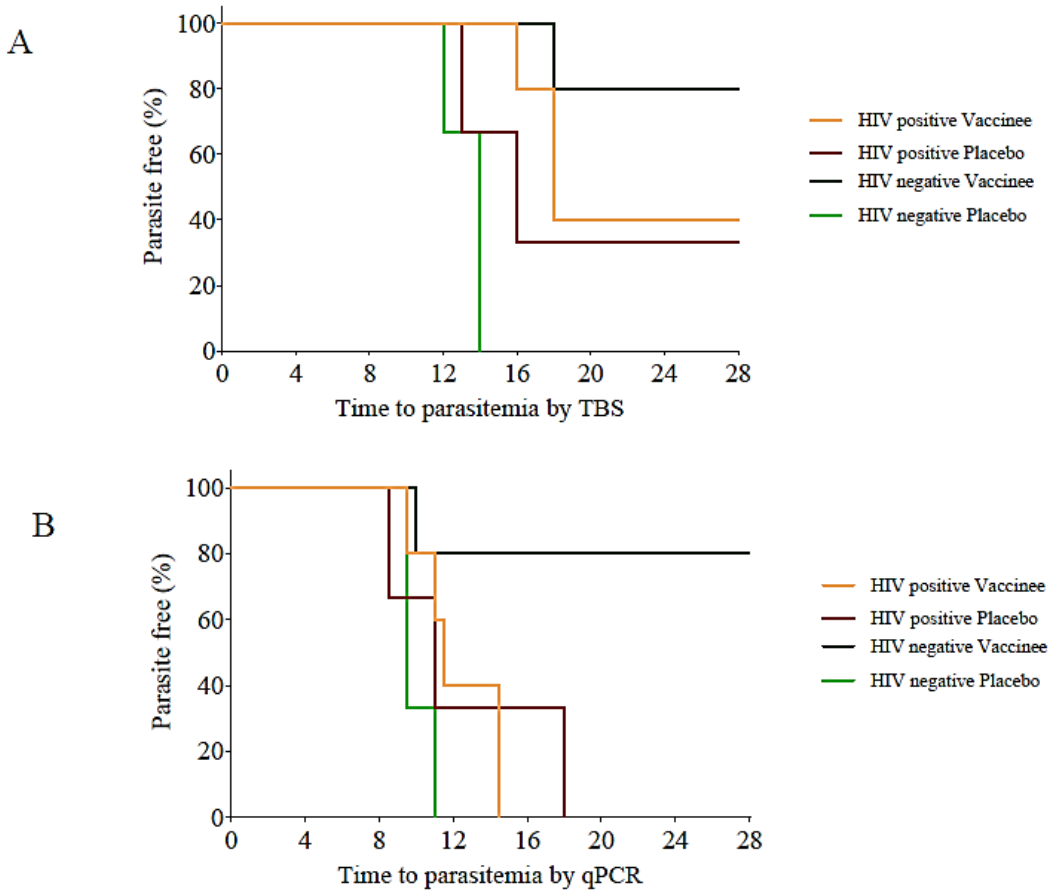
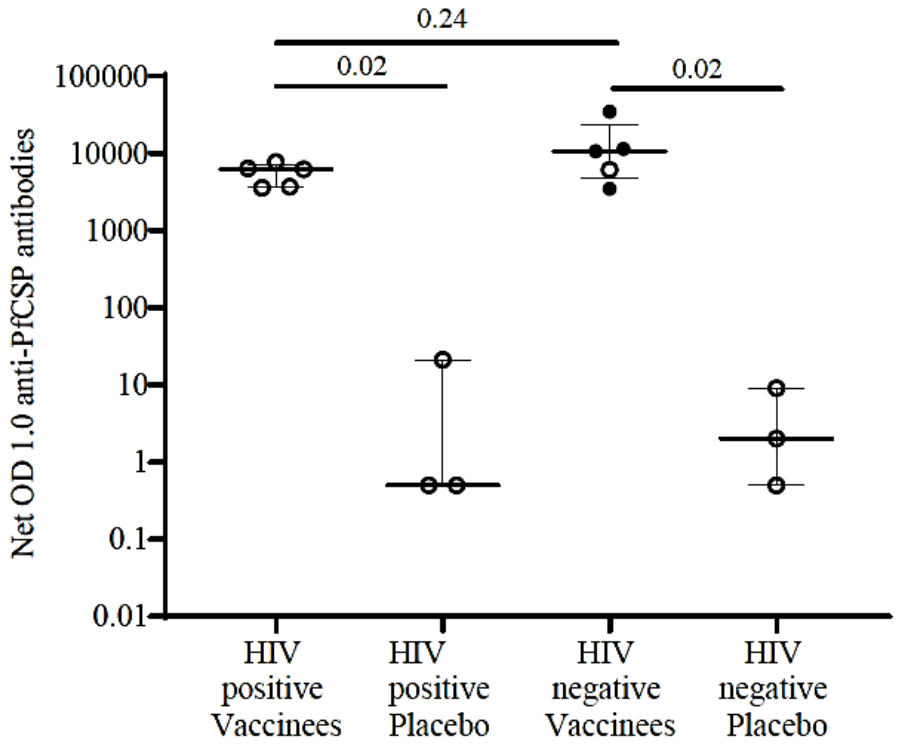


Figure 1: Kaplan-Meier in volunteers undergoing CHMI as assessed by TBS (A) and by qPCR (B).



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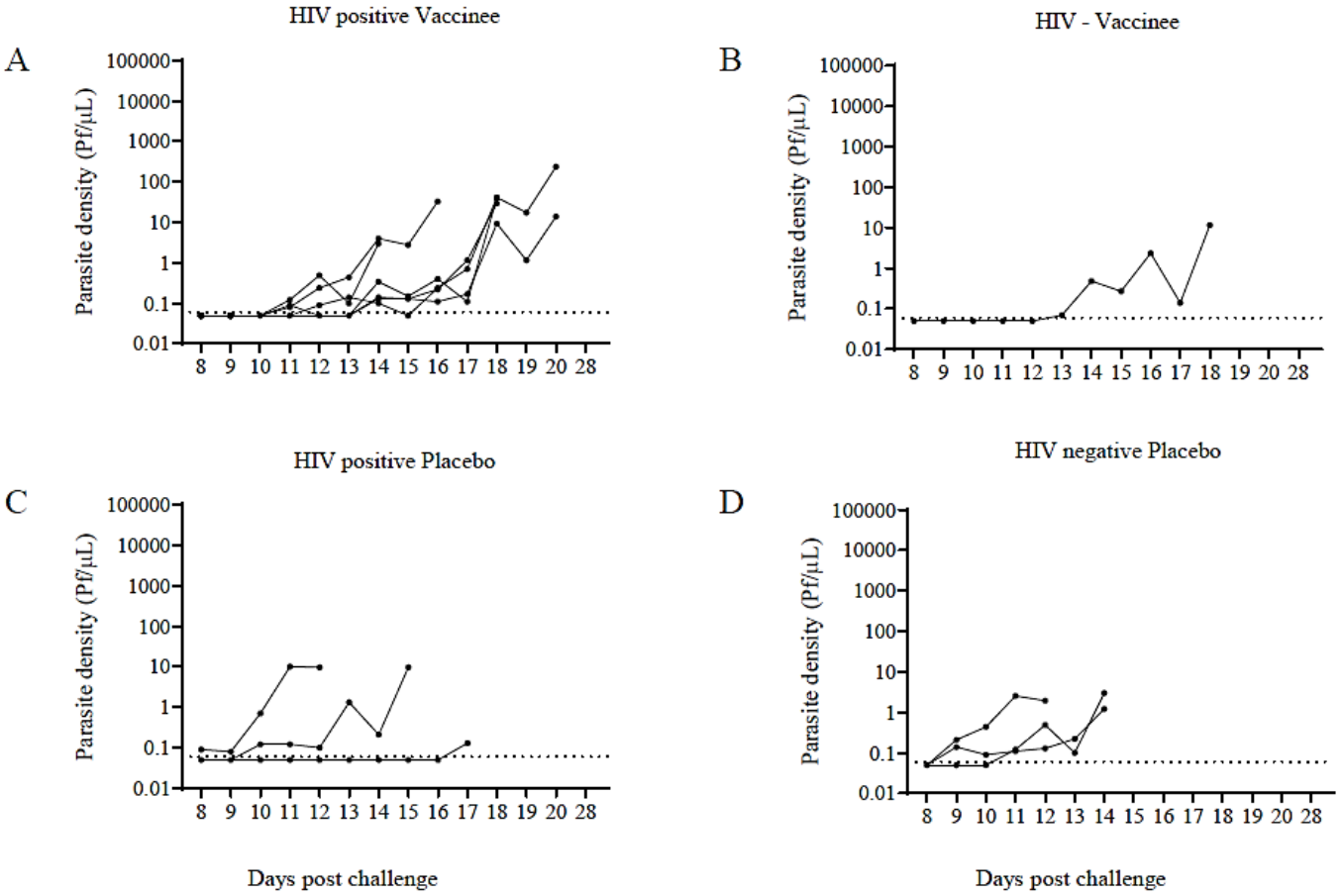

Figure 3: Parasite density as measured by quantitative polymerase chain reaction (qPCR) by HIV status and trial group after CHMI. The dashed lines represent the lower limit of qPCR positivity. Parasite densities are plotted with an offset of 0.05 parasite/µL.

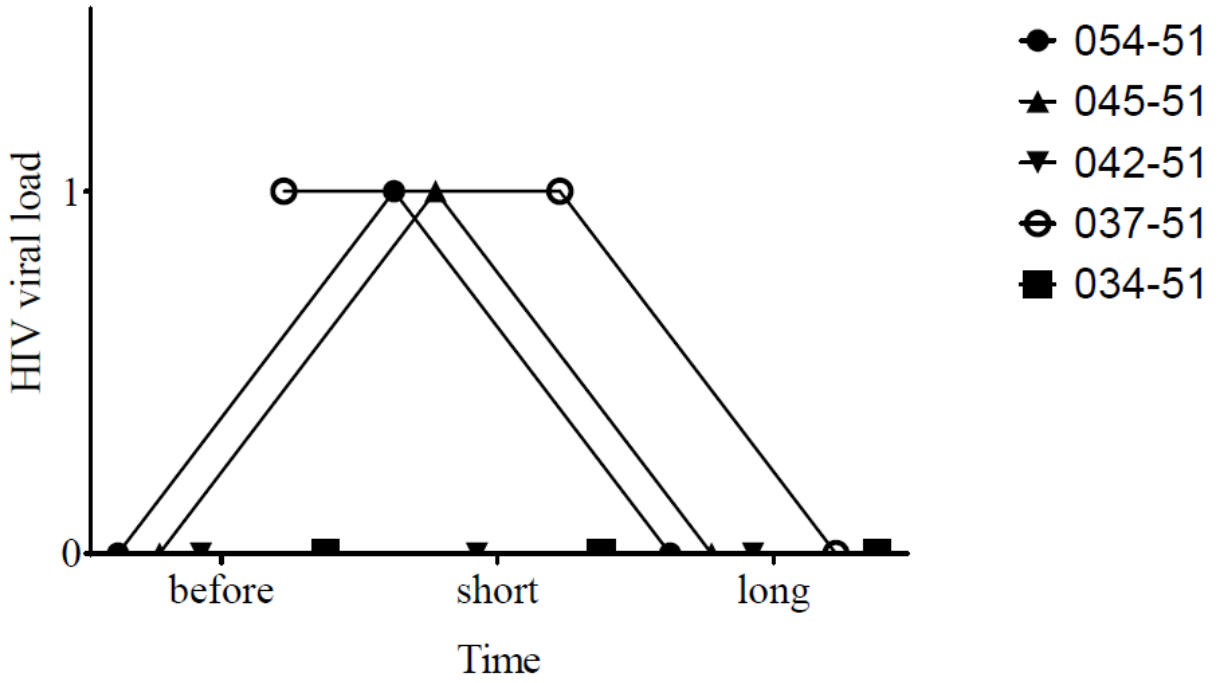

Figure 4: HIV Viral load in HIV positive volunteers before, shortly and long after CHMI. Before: 1-2 days before CHMI; short: 13-20 days after CHMI, long: 56 days after CHMI. Detectability of viral load: 0 = not detected, 1 = detected. Viral loads did not significantly vary before and after CHMI (Wilcoxon matched-pairs signed rank test, p=0.5).

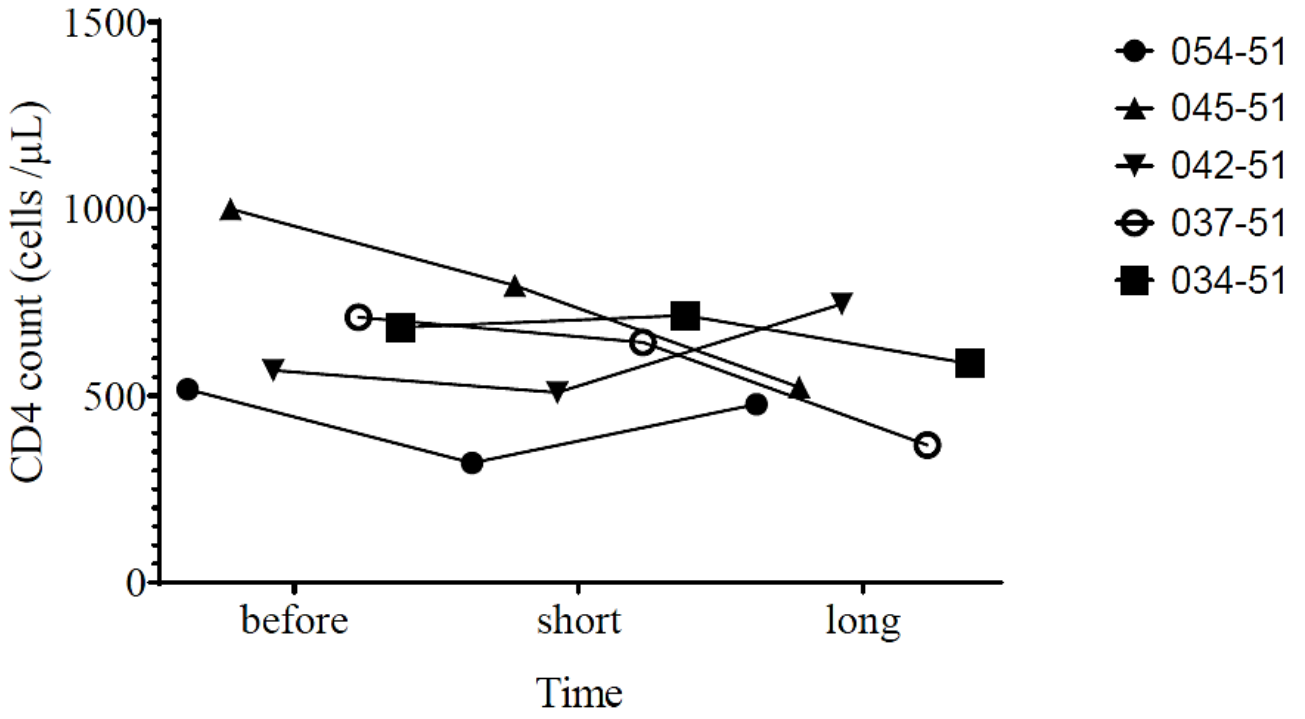

Figure 5: CD4 count in HIV positive volunteers before, shortly and long after CHMI. Before: 1-2 days before CHMI; short: 13-20 days after CHMI, long: 56 days after CHMI. CD4 count did not significantly vary before and after CHMI (Wilcoxon matched-pairs signed rank test, p=0.5).

Chapter 6

General discussion

6.1 The importance of implementing molecular tools in malaria surveillance programs

In almost all endemic countries, malaria surveillance is limited to obtaining the parasite prevalence among sentinel or vulnerable sub-populations, such as schoolchildren, by either microscopy or malaria rapid diagnostic tests (RDTs). This data is being used to evaluate intervention programs and provides the framework for effective allocation of resources (79). With the spread of *P. falciparum* genotypes resistant against artemisinin-based combination therapies (ACT) and strains carrying *pfhrp*2 deletions and therefore escaping detection by widely used malaria RDTs, new serious threats to malaria control and elimination efforts around the world have recently emerged (108). WHO reports that delay in malaria diagnosis and treatment is the leading cause of death. Georeferenced information about the presence, prevalence and risk factors associated with these strains threatening current control approaches of malaria should be included in malaria surveillance programs. Implementing a molecular surveillance approach offers a unique opportunity to support policy makers regarding the choice and change of drugs and tests for malaria diagnosis and treatment before resurgence of malaria occurs.

6.2 Why extract nucleic acids from used malaria rapid diagnostic tests?

Early and accurate diagnosis is essential for effective management of malaria at the individual level for travellers and people living in malaria endemic countries. Microscopic examination of Giemsa-stained thick and thin blood films remained for long time the only available diagnostic tool for parasite identification and is still regarded as the gold standard. This technique that screens essentially 2 to 6 microliters of blood for parasites has shortcomings including low sensitivity (in the range of 50-100 parasites per µL of blood), requirement of

continuous training, maintenance of equipment, dependency on electricity supply and time required for careful preparation of slides and reading (109). As an alternative, lateral-flow based immunochromatographic devices known as RDTs that are point-of-care tests have been developed to overcome these limitations, particularly in resource poor settings. Currently available RDTs are performed using 5 – 10 microliter of blood, easy to perform, provide a result within 20 minutes, achieve a sensitivity of ~100 to ~1000 parasites per µL of blood depending on the target protein used (110) (111) and are widely deployed in malaria endemic countries (112). Apart from point-of-care patient care, RDTs are increasingly deployed for routine malaria surveillance in malaria control programs to monitor malaria prevalence trends either in the general population or particularly vulnerable population like children under five years of age or pregnant women (113). Clear limitations of RDTs are not only the lack of high sensitivity but also the difficulty to diagnose non-falciparum species prevalent in West African countries like *P. malariae* and *P. ovale* spp. lacking HRP2 and HRP3 expression (114). Malaria surveillance is essential to generate information on malaria prevalence data over time that are used for planning, implementing, monitoring and evaluating malaria programs. As transmission decreases, malaria becomes focal, and the intensity and frequency of reporting should increase until final elimination has been achieved (79). Accurate parasitological diagnosis of a malaria case is instrumental in a malaria surveillance system and as countries strive to move towards malaria elimination, more sensitive malaria tests are needed to find the reservoirs of residual transmission (115, 116).

Molecular techniques based on nucleic acid amplification have been developed that are way more sensitive than RDTs or microscopy for malaria detection (117). Molecular techniques can easily be adapted to novel targets for amplification and allow the discrimination of the different human infective *Plasmodium* species (118). One widely used method of blood sample

collection for molecular assays include the collection and storage of around 50 microliter of capillary blood on filter papers (119). If higher blood volumes are needed, several spots on a filter paper can be spotted with one single finger pricked sample, allowing for up to 200 microliters of blood stored. Therefore, when compared to RDTs, around ten to twenty times more blood on a filter paper could be stored increasing the chance of parasites to be collected. The disadvantage of using filter papers is that high-quality papers are costly, are usually distributed by specific research projects, require good storage conditions and do not easily fit into routine work of malaria surveillance since more blood needs to be collected (119). In the laboratory, high-throughput extraction of DNA from filter papers are best performed by the use of a robotic based extraction platforms (120).

In the course of this thesis, we attempted to combine the broad availability of RDTs in the field with the higher sensitivity and quantitative measurement of *Plasmodium spp.* by using the RDT nitrocellulose strip included in the malaria RDTs as carrier for nucleic acids (NA). Previous studies have shown that DNA extracted from RDTs were similar in quality and quantity to those obtained from blood applied onto filter papers in direct side to side comparisons (121, 122). Methods developed over the years for DNA extraction from used RDTs are summarized in Supplementary Table 1 of the manuscript presented in Chapter II (Guirou et al.,). In this work, we have first systematically compared and then adapted different protocols for extraction of NA to arrive at a high throughput method to co-purify DNA and RNA from RDT with limited hands on requirements. We made an important improvement of the currently described extraction method by using the entire nitrocellulose strip directly from the RDT without any manipulation. By removing the manual cutting of the nitrocellulose strip we reduce the processing time, and more importantly, the potential risk of sample cross-contamination by carryover between samples. Our extraction procedure takes around 3 hours, including the 2

hours needed for the incubation period in the extraction buffer. Therefore, following our approach will easily allow to extract 1000 RDTs per week, making it an unprecedented method for molecular malaria surveillance. The possibility of modifying our current method by including a robotic-based extraction platform could further facilitate the use of NA extracted from RDTs in molecular epidemiology studies. Another advantage of combining NA extracted from RDT with molecular assays is the possibility to conduct several experiments using the same sample.

We did not find conclusive evidence that longer-term storage of RDT has a negative impact on the detection rate of *P. falciparum* if initial parasite density spotted on the RDT was above 10 parasites/µL. This is similar to reports in previous studies. This provides the opportunity to conduct retrospective molecular studies over several years using RDTs collected and stored at room temperature (123). After having established a high-throughput extraction method for RNA and DNA from RDT, we were able to extract around 3000 RDTs samples from a malaria indicator survey in 2018 on Bioko Island. The lower limit of parasite detection of our method is 1 parasite/µL of blood applied to the RDT. This represents around 100 times higher sensitivity compared to diagnosis based on RDT only and will certainly help to identify more sub-microscopic malaria infections in the field.

One of the bottlenecks during the process of analysing high numbers of samples using molecular diagnosis is management and analysis of data. This problem has been tackled by the development of software applications described in Chapter II of this thesis. We developed two apps, which support sorting and tracking barcoded RDTs during the extraction process using a smartphone's camera. For high-throughput data analysis the ELIMU-MDx platform was developed during this thesis allowing automatized quality control of qPCR data generated plus the central storage of protocols, reagents and final results (124).

In summary, our study demonstrates the feasibility of extracting NA from thousands of RDTs and to analyze from one single sample not only presence or absence of malaria, but add essential information including (i) parasite quantity, (ii) *P. malariae* and *P. ovale* spp. prevalence, (iii) prevalence and type of synonymous or non-synonymous mutations in the *kelch13* propeller region and (iv) to search for *hrp*2/*hrp*3 gene deletion carrying *P. falciparum*. In combination with the ELIMU-MDx platform we have developed a powerful analysis pipeline for molecular surveillance of malaria that can be still expanded for additional questions such as parasite genotyping or gametocyte detection.

6.3 Monitoring *Plasmodium falciparum* strains lacking histidine-rich protein 2 and 3 genes

Work towards an HRP2-based immunochromatographic RDT began during the early 1990s (125). In 1993, WHO published the Global Plan of Action for Malaria Control stressing the need for improvement of parasitological diagnosis at the peripheral and community level (126). PfHRP2 and PfHRP3 are expressed at high levels during the asexual blood stage cycle, with rapid increase during the ring-stage and slower accumulation in the trophozoite and schizont stages (127). In 2004, less than 1 million malaria RDTs have been used, however by 2013 more than 300 million were being sold yearly. This exponential growth of RDT sales was associated with the development and usage of the new artemisinin combination therapies (ACT) (59). In 2012, WHO launched the T3 initiative: Test. Treat. Track to scale up diagnostic testing, treatment and surveillance for malaria (128). Manufacturers surveyed by WHO for the World malaria report 2018 reported a total of 276 million RDT sales in 2017. Most RDTs (66%) were supplied to sub-Saharan Africa and in 2017 roughly 75% of malaria tests in sub-Saharan Africa

were conducted using RDTs. In areas of declining malaria transmission, parasitaemia is often below the level of detection of microscopy and standard RDT (50 - 200 parasites/microliter). However, it is thought that these sub-clinical infections contribute to malaria transmission, pushing need for better diagnostic tests (129). Ultrasensitive HRP2-based RDTs (uRDTs) with enhanced signal detection methodology have a detection threshold of < 10 parasites/microliter (130). These uRDTs might improve detection of these low-density parasite infections, but will not improve the diagnosis of clinical malaria cases (131, 132). *P. falciparum* strains carrying *pfhrp2/3* gene deletions and therefore evading detection by HRP2/HRP3 based RDTs pose a serious threat to malaria control and elimination programs globally (133). Together with the emergence and spread of artemisinin resistant strains, these parasites are a powerful example that molecular assay based surveillance approaches have to be implemented in malaria control programs to monitor potential increase in prevalence over time due to their selective growth advantages based on either escaping diagnosis or treatment. Studies on *pfhrp2/3* gene deletions in African countries have found a wide range of prevalence reported. A study conducted in Eritrea found a high prevalence of *pfhrp2* gene deletions, many of them also lacked *pfhrp3* (134). HRP2 antigen was absent from these samples, leading to false negative PfHRP2 based RDTs and the need for replacement of the diagnostic tool (134). In the Democratic Republic of the Congo a prevalence of 6.4% *P. falciparum* with *hrp2* gene deletions was reported (135). A study in Mozambique found only 1.4% *pfhrp2* gene deletions and no *pfhrp3* deletion (136). A study conducted in Kenya did not find any *pfhrp2* or *pfhrp3* gene deletions, but reported a high proportion of polyclonal infections (137). A different study in Kenya found 9.0% *pfhrp2* and 1.1% *pfhrp3* gene deletions, respectively, in 89 samples by PCR (138). These deletions were confirmed by analysing the *pfhrp2* and *pfhrp3* genomic loci in greater detail, with the *pfhrp2* and *pfhrp3* gene deleted samples missing either the respective gene or also flanking regions (138). One caveat in all these studies is that many infections are multiple strain

infections in Africa, meaning that a strain carrying a *pfhrp2* gene or *pfhrp3* gene deletion might be masked by the second strain that does not lack these genes.

We have developed during this thesis a novel assay named qHRP2/3-del suitable for high-throughput screening of *P. falciparum* isolates carrying *pfhrp2* and/or *pfhrp3* gene deletions. Major advantages of the novel assay over currently used sequentially performed nested PCR assays include rapidity, ease of performance, and the determination of parasite density. Importantly, the assay allows the detection of *pfhrp2* and *pfhrp3* gene deletions in multiple strain co-infections that are particularly prevalent in Sub-Saharan countries (139). We have started to implement the qHRP2/3-del assay to field samples from Equatorial Guinea. We are currently focusing on RDTs collected from children under five years of age in the malaria indicator survey 2018 that are suffering from anemia and have a negative RDT result. This is intended to identify diagnostic resistant parasites that lack HRP2 and HRP3 production using our newly developed qPCR based read out. With growing availability of qPCR instruments in reference laboratories in Sub-Saharan countries, our novel assay could be used as a surveillance tool to monitor over time the potential expansion of *P. falciparum* strains carrying *pfhrp2/3* deletions.

6.4 Molecular surveillance of malaria in pregnant women on Bioko Island

We used our newly established analysis pipeline to determine the prevalence of *P. falciparum* and non-falciparum species in pregnant and age matched non-pregnant women on Bioko Island in 2018. We were able to utilize the RDTs collected as part of a malaria indicator survey and analysed in total 435 RDTs within less than four weeks of laboratory-based work. Advantages of targeting specifically pregnant women for malaria surveillance include the ease of access

because these women supposedly attend antenatal care facilities several times during pregnancy where blood sampling is possible and their regular inclusion in household malaria surveys (140, 141). However, the true burden of pregnancy-associated malaria is most likely underestimated because women attend antenatal care facilities later during their pregnancies, therefore missing out often women in their first trimester of pregnancy (142, 143). In addition, parasitaemia levels in peripheral blood can be low due to the specific sequestration of *P. falciparum*-infected red blood cells into the placenta through CSA and VAR2CSA interactions (44, 55).

While our sample size in this study is still small, we could demonstrate that the IPTp-SP program implemented on Bioko Island contributes to reduction of *P. falciparum* infections in pregnant women when compared to age matched non-pregnant controls. NA isolated from several malaria positive RDTs did not yield a positive qPCR result for *P. falciparum*. These RDTs could be either false positives RDTs based on the circulation of HRP2 after malaria treatment completion (142) or be real positive based on the fact that the RDT detected HRP2 released from parasites sequestered in the placenta that are undetectable by qPCR (71, 144). Importantly, *Plasmodium*-infected, pregnant women had significantly lower haemoglobin concentrations compared to their uninfected counterparts, confirming that malaria infection in pregnancy - even in the absence of clinical symptoms and at low parasite density - constitutes a cause of maternal anaemia (145). Also, we were able to identify women in the first pregnancy trimester with malaria infections some of them have been missed by RDT based on the low parasitaemia. These women are vulnerable to the effects of malaria infections and should be included into the malaria treatment programs as early as possible (143, 146). We intend to expand this study in 2020 by adding the RDTs collected from pregnant women during the malaria indicator survey conducted in 2019. Since malaria prevalence as defined by RDT

positivity has increased up to 19 % in the general population in 2019 (unpublished result) we would like to understand if pregnant women are affected by this upward trend in malaria prevalence on Bioko Island.

6.5 Following *Plasmodium falciparum* parasitaemia in HIV positive volunteers undergoing CHMI

Chapter 5 of this thesis contains parasitaemia data which was obtained during controlled human malaria infection (CHMI) study conducted in Bagamoyo, Tanzania. The major concern during this study was safety, since this was the first time ever CHMI was tested in HIV positive volunteers. Therefore, real-time parasitaemia during the CHMI was not only monitored by thick blood smear (TBS) microscopy, but also qPCR. This allowed us to study the interaction between the *P. falciparum* parasites and the HIV virus under controlled conditions. No negative short- or long-term impact on HIV viral loads or CD4+ counts was observed upon CHMI. There is also no evidence that *P. falciparum* parasite growth dynamics were different between HIV positive and HIV negative volunteers. We concluded that CHMI was safely conducted in HIV positive volunteers who are under antiretroviral therapy and have stable CD4+ counts and low HIV viral loads. The importance of this study should not be underestimated since it provides additional evidence for the safety of CHMI and the understanding of co-infections between two major infectious diseases prevalent in Sub-Saharan Africa.

Chapter 7

Conclusion and Outlook

Conclusion and Outlook

Point of Care rapid diagnostic tests based on detection of HRP2 secreted by asexual blood stage parasites have become one of the corner stones of malaria control. The switch from the gold standard based on thick blood smear microscopy to RDT has enabled improved diagnosis of malaria in clinical settings since parasitemia is usually higher if a malaria infected person presents with clinical signs and symptoms. Clearly, HRP2 based RDT have limitations resulting in false positive (persistence of the PfHRP2 antigen after the parasite has already been eliminated) and false negative (lower limit of detection at 100 parasites/microliter) test results. Therefore, it is clear that RDTs do not measure all circulating *P. falciparum* infections, a limitation which is particularly important for larger scale malaria surveillance programs in regions with low malaria parasitaemia.

In this thesis, we developed novel approaches and software tools to overcome these inherent limitations of RDTs. We collected used RDTs from malaria indicator surveys in Tanzania and Equatorial Guinea and developed a high-throughput novel approach to extract NA. These RDTs have been collected under field conditions and stored only at RT before being transferred into the laboratory for NA extraction. By running qPCR assays on the extracted NA, we have increased the sensitivity of the malaria detection and improved on the identification of likely false negative tests. Furthermore, we have been able to detect *P. falciparum* parasites carrying *kelch*13 mutations conferring artemisinin resistance and to expand the range of malaria species to *P. malariae* and *P. ovale*.

The limiting hands on time, robustness and ease of conduct are prerequisites to use the ENAR approach also in malaria endemic countries in central reference laboratory facilities where qPCR machines are usually available. We plan to analyse RDTs collected during the MIS 2019 on Bioko Island in our new laboratory facility in Baney resulting in the education of Equatorial

Guinean technical staff that can master molecular testing of malaria. The throughput of our NA extraction method from RDTs could be increased even further by automation using an extraction robot. One of the major limitations of using RDTs for NA extraction is the amount of blood (around 5 – microliter) that can be fixed on the nitrocellulose filters.

In combination with regular malaria indicator surveys, our approach constitutes a convenient way of establishing a biobank that could be stored at room temperature for more than one year. This will not only allow retrospective studies of malaria in different populations of interest like pregnant women, but also the simultaneous monitoring of other blood borne pathogens present in the surveyed populations, including the helminth infections *Loa loa* and *Mansonella perstans*. These pathogens have been successfully detected using the ENAR approach in RDTs collected from Bioko Island (unpublished results). Apart from detection of NA from parasites, we will try as next steps if it is possible to detect NA from viruses such as Dengue virus that seems to be highly prevalent on the island of Bioko. Since human DNA is also fixed on the RDTs, our approach could be used to study the association of selected human genetic traits with pathogens without the need of collecting more blood samples under the umbrella of different study protocols.

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