Development and evaluation of diagnostics for malaria control

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Dedication

This thesis is for my family in Bougainville, Papua New Guinea.

May the research here contribute in some small way to reducing malaria, so that future generations can live longer and happier lives.

Acknowledgements

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(See individual Chapters for study-related acknowledgements)

Thesis abstract

Introduction

WHO malaria elimination targets require sustained and robust surveillance systems to identify difficult-to-diagnose, low-density malaria infections which threaten control efforts. The WHO 'T3: Test, Treat and Track' initiative further reinforces the importance of these principles (Cox 2010). Early detection and treatment of malaria is especially important during pregnancy given its adverse outcomes. Prior to administration of antimalarials, the WHO recommends confirmation of diagnosis of malaria. The current gold standard for this is still microscopy, with rapid diagnostic tests (RDTs) commonly employed where microscopy services are limited. However, both methods are insufficiently sensitive to detect low-density or "sub-microscopic" infections. Individuals harbouring these infections act as an infectious transmission reservoir contributing to ongoing transmission in low-transmission/near-elimination settings. Unfortunately, only molecular methods such as qPCR- which are largely restricted to use in well-supported laboratories- are currently suited to diagnosing these individuals.

Aims

There is an urgent need for sensitive diagnostic tools for malaria that are better suited to low-resource settings. The overall objective of the thesis is to investigate new diagnostic tests, molecular techniques, and adaptations to enhance the detection of low-density malaria infections in limited-resource settings. This thesis aims to explore new diagnostics suitable for 1) molecular surveillance and 2) improved point-of-care (POC) diagnosis. The specific objectives are to:

1) Assess the suitability of high-throughput molecular assays for differentiating *Plasmodium* species (Chapter 2)
2) Develop a new molecular test for malaria control applications on a portable, field-friendly platform (Chapter 3)
3) Characterise the diagnostic performance of a new RDT for detecting low density malaria

during pregnancy in low and high transmission settings (Chapter 4-6)

Methods and key findings

The differentiation of the main species of *Plasmodium* is important not only for the treatment of liver stages unique to *P. vivax*, but also for accurate surveillance of malaria epidemiology. Chapter 2 explores molecular techniques; namely, High Resolution Melt Curve Analysis (HRMCA) and real-time qPCR, to develop a multiplex qPCR suitable for the detection of the species of malaria most prevalent in the study areas of interest: Indonesia and Kenya. As a result, a new 4-plex qPCR assay was optimised and utilised in a large clinical trial to confirm infection amongst asymptomatic women in Indonesia. The feasibility of using whole blood directly in the 4-plex was also demonstrated, as was the transfer of the qPCR to the more portable Magnetic Induction Cycler (MIC™) device (chapter 3).

Using the multiplex qPCR as a reference standard, the diagnostic performance of a new ultra-sensitive *P. falciparum* RDT (uRDT) was retrospectively assessed in a Stop Malaria in Pregnancy clinical trial in Indonesia (chapter 4). The uRDT's performance was compared to that of the widely used *Pf*/Pan CareStart RDT (csRDT). The overall similar performance of the two RDTs (uRDT 20% sensitivity, 98% specificity and csRDT 23%, 96%) led to an investigation into antigenic mutations that may account for RDT insensitivity (chapter 5). The results from this study suggest a high proportion of infections that were qPCR positive and RDT negative are attributable to HRP2 deletions.

The uRDT performance in comparison to csRDT was further investigated amongst pregnant women in Kenya, to investigate the effects of transmission intensity. The sensitivity of the uRDT (79.9%) was significantly higher than that of the csRDT (74%), although the specificity was significantly lower (uRDT; 90.4% and csRDT; 93.0%). Overall, the performance of the two tests (as indicated by the Diagnostics Odds Ratios, Kappa values and AUROC values) were non-significantly different to each other.

Finally, a novel assay for mosquito insecticide resistance was developed on the portable point-of-care Genedrive® platform, demonstrating its potential for use as a field-friendly diagnostic tool for malaria control.

Conclusions

The uRDT evaluations performed here contribute to critical understanding of how these tests perform in pregnant women in different transmission settings. The results suggest that in low transmission settings this test may not offer an improvement on currently used RDTs, particularly for screening asymptomatic infections during pregnancy. In contrast, the uRDT may be better suited for this role in higher transmission settings, such as in Kenya. Although the uRDT demonstrated lower specificity in the latter scenario, the negative outcomes associated with malaria during pregnancy together with the safety of current antimalarial therapies mitigate the risks of unnecessary treatment.

The poor sensitivity of both HRP2-targetting RDTs do not support their use for screening for malaria during pregnancy, with the caveat that the off-label use of stored samples may have affected their sensitivity. The evidence of HRP2 deletions is the first report of these mutations in Indonesia. Although further confirmatory studies are needed to strengthen these findings, they flag a potential barrier to the current test and treat policies for screening pregnant women in Indonesia. The work highlights the urgent need for wider and more in-depth HRP2 studies in this region to better inform these programs.

Overall, this thesis tackles several pertinent challenges around improving malaria diagnostics. It highlights issues that need to be considered during the product design, development and assessment phases. It also incorporates useful evaluation studies of novel tests with the potential to make malaria diagnostics more accessible. Its translational approach is suited to product developers, as well as those influencing control/ surveillance programs and associated policy.

Contents

Figure Index

Table index

Abbreviations

Active Case Detection: ACD

CareStart™ RDT: csRDT

Controlled Human Malaria Infection (CHMI)

Cycling Threshold: Ct

Deoxyribonucleic Acid: DNA

Diagnostics Odds Ratio: DOR

Duplex assay: refers to Bass *et al*. *Pf/Povm* assay (2008)

Focused Screening and Treating: FTAT

Glutamate-rich protein: GLURP

High Resolution Melt (Curve Analysis): HRM(CA)

Histidine Rich Protein: HRP

Hot populations: Hotpops

In Vitro Diagnostics: IVD

Indoor Residual Spraying: IRS

Intermittent Preventative Treatment during Pregnancy: IPTp

Intermittent Screening and Treatment during pregnancy: ISTp

Isothermal NAAT: iNAAT

Limit Of Detection: LOD

Long lasting Insecticide Treated Nets: LLITNs

Loop mediated isothermal Amplification: LAMP

Magnetic Induction Cycler: MIC™

Malaria in Pregnancy: MiP

Mass Screening and Treating: MSAT

Merozoite Surface Protein: MSP

Multiplicity of Infection: MOI

Negative Predictive Value: NPV

Nested PCR: nPCR

Nucleic Acid Lateral Flow Immunoassay: NALFIA

Nucleic Acid Sequence Based Amplification: NASBA

P. falciparum/ P. ovale/ P. vivax/ P. malariae/ P. knowlesi: *Pf/Po/Pv/Pm/Pk*

Plasmodium Lactate Dehydrogenase: pLDH

(near) Point of Care (Test): (n)POC(T)

Polymerase Chain Reaction: PCR

Positive Predictive Value: PPV

Quantitative PCR: qPCR

Rapid Diagnostic Test: RDT

Reactive Case Detection: RCD

Real-time PCR: rt-PCR

Ribonucleic acid: RNA

rt-PCR: Real-Time PCR

Stop Malaria in Pregnancy trial: STOP-MiP

Target Product Profile: TPP

Transfusion-Transmitted Malaria: TTM

Ultra-sensitive RDT (Alere™): uRDT

World Health Organisation: WHO

4-plex qPCR: refers to multiplex qPCR for the detection of *Pf, Po, Pv* and *Pm*

Chapter 1. Introduction

Contents

1.1. Malaria life cycle and pathogenesis

Malaria is a parasitic disease caused by unicellular eukaryote *Plasmodium.* In humans, *Plasmodium falciparum*, *P. vivax, P. malariae, P. knowlesi* and *P. ovale* (sub-species *ovale curtisi* and *ovale wallikeri*) are the main infective species.

Most *Plasmodium* species have an anthroponotic life cycle, with the exception of *P. knowlesi* which can also infect some macaques (Cox 2010), but also require a mosquito vector for transmission (Nilsson, Childs *et al*. 2015). Humans first acquire infection through the bite of an infected Anopheline mosquito. Only female mosquitoes can transmit the parasite, as they require blood meals to support egg development. Infective sporozoites residing in the mosquito's salivary gland are injected into the skin, where they traverse the dermis, entering the blood. Once in circulation, they sequester to the liver, traversing Kupffer cells before infecting liver cells (~5-6 days post-infection). Here, asexual reproduction 'schizogony' is initiated, or in the case of *P. vivax* and *P*. *ovale* infections, hypnozoites may form lying dormant for long periods of time (months to year) (Nilsson, Childs *et al*. 2015). (Figure 1.1)

During asexual reproduction, a ring stage parasite first develops maturing into trophozoites and then into schizonts. Mature schizonts can develop into multiple infective merozoites or early stage gametocytes. Formation of asexual merozoites causes the cell to rupture, once again releasing these stages back into circulation, where they can re-infect other circulating red blood cells and re-initiate asexual reproduction (24- 72 hour cycle depending on the species). (Figure 1.1)

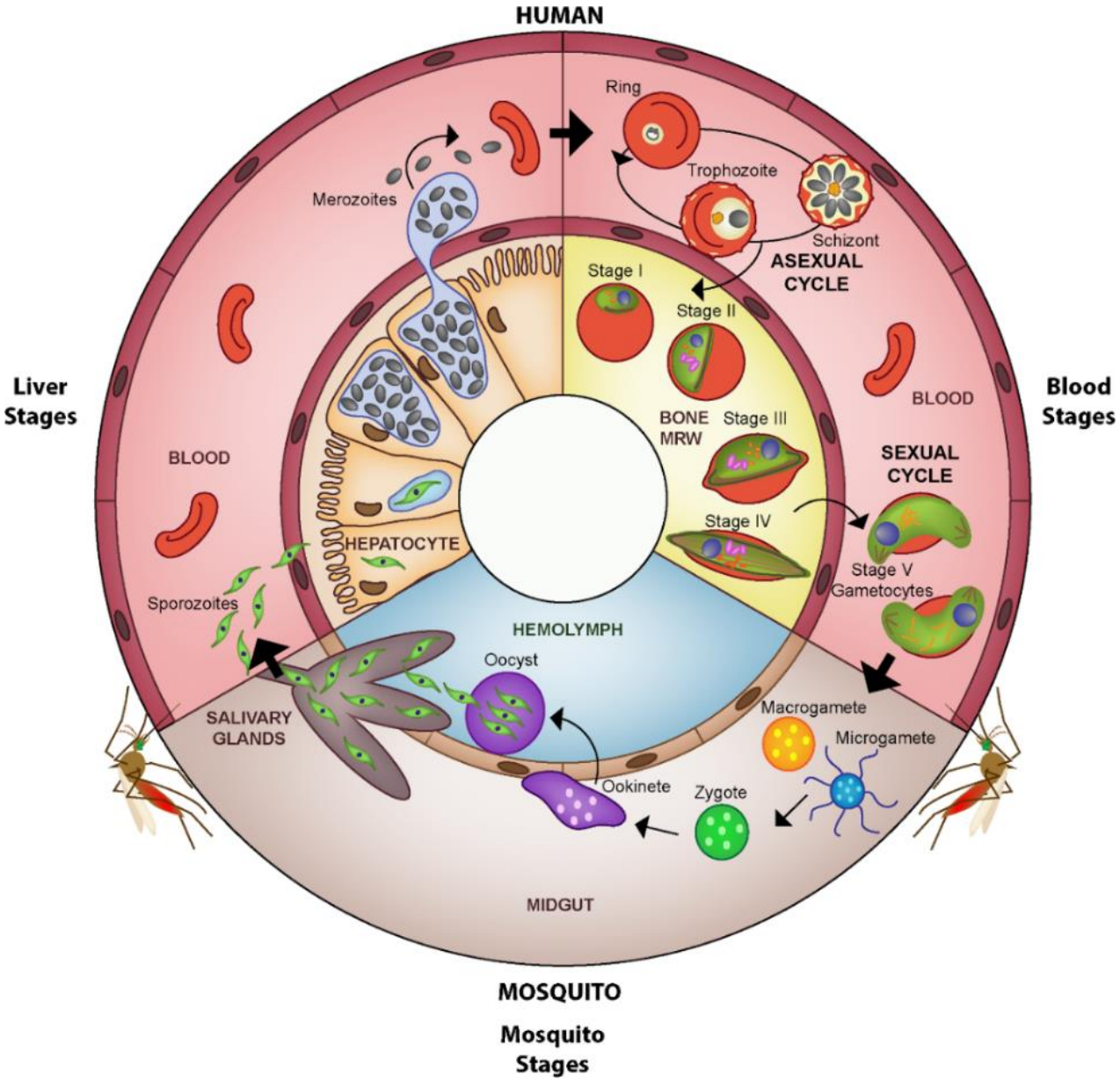
Sexual reproduction, however, comprises seven developmental stages of the gametocytes (8 days). Male and female gametocytes are ingested upon feeding of another

mosquito. Within seconds they fuse in the mid-gut of the mosquito, forming a zygote that continues to develop into an ookinete (~2 weeks). This stage invades the mid-gut lining and matures into an oocyst filled with maturing sporozoites. These are released into the mosquito haemolymph where they are able to sequester to the salivary glands. Upon the mosquito's next blood meal, they are injected alongside the mosquito saliva into the skin of the human host continuing transmission (Nilsson, Childs *et al*. 2015). (Figure 1.1)

Figure 1.1. Schematic of the malaria life cycle.

Development of the *Plasmodium* parasite within the human and mosquito hosts. Image source:

Nilsson *et al.* (Nilsson, Childs *et al*. 2015)



1.1.1. Malaria epidemiology

Malaria is prevalent in 87 countries across South-East Asia, the Eastern Mediterranean, Western Pacific, the Americas and Africa. Despite efforts to control the disease, there are 219 million cases and 405,000 deaths due to malaria every year (WHO 2017). The majority of cases and deaths occur in sub-Saharan Africa, with Nigeria alone accounting for 25% of the global burden of malaria (WHO 2019). *Plasmodium falciparum* remains the most prevalent malaria parasite in the WHO African Region, thus accounting for the majority of global malaria cases (fig 1.2A) (WHO 2019). Although the incidence rate of malaria has declined globally from 71 (2010) to 57 (2018) cases per 1000 population at risk, between 2010 and 2018, concerning the rate of change decelerated drastically, stagnating progress over the past 4 years. Likewise, reduction in malaria deaths has stemmed since 2016 globally. Only the WHO African region and the South-East Asian region showed a reduction in mortality in 2018 compared to 2010 (Ryan, Stoute *et al*. 2006).

Indonesia is co-endemic for both *P. falciparum* (63%) and *P. vivax* (37%), with scarce *P. ovale* and *P. malariae* infections (Mideo, Reece *et al*. 2013). In chapters 2-5, samples from Timika, West Papua in Indonesia were investigated. This region in has the highest prevalence of both malaria species with over 100 cases/ 1000 population (fig 1.2B) (Bartoloni and Zammarchi 2012). In contrast, chapter 6 investigates samples in an African context in Western Kenya, where the *P. falciparum* dominates with only infrequent *P. vivax* infections occurring in some Duffy-negative populations (WHO 2019). The prevalence of *P. falciparum* is highest in this littoral region on the shores of Lake Victoria (fig 1.2C).

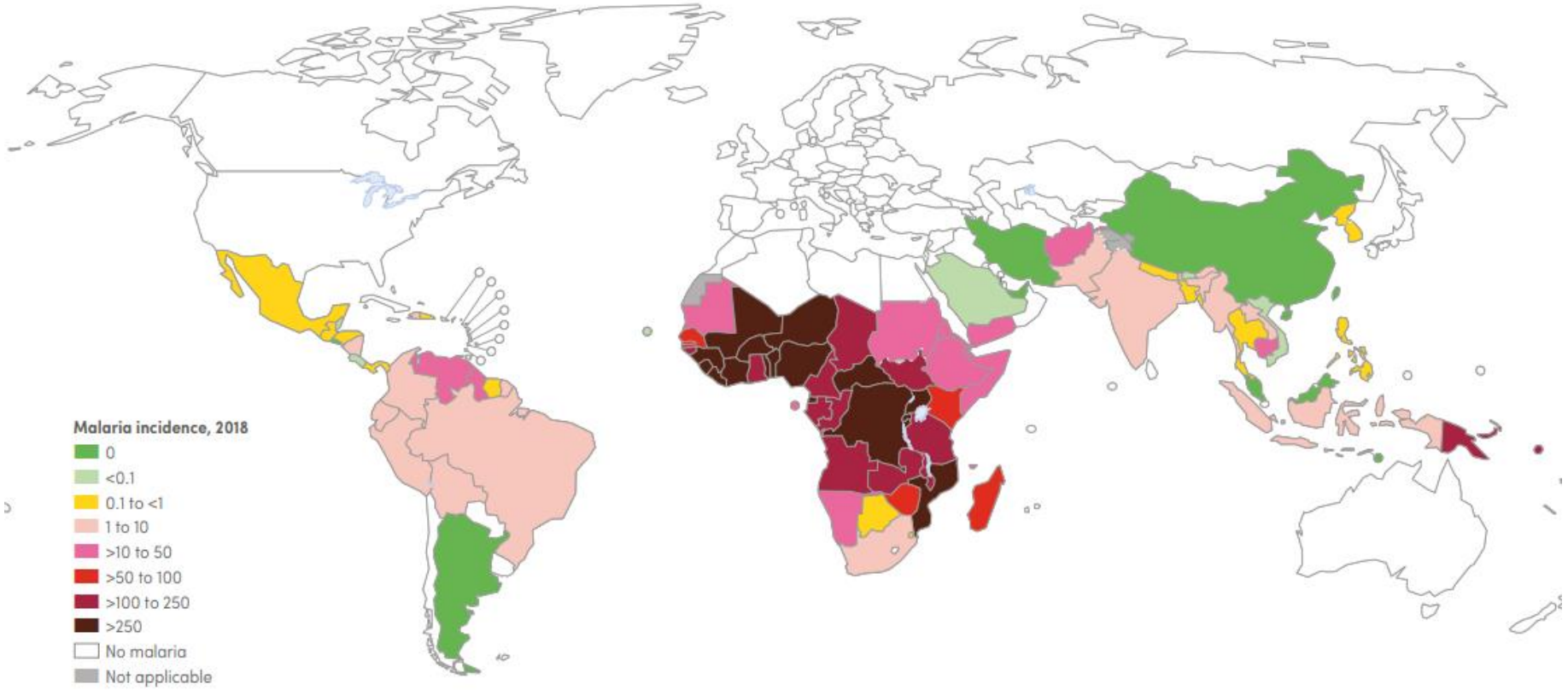
1.1.2. Pathogenesis

Pathogenesis is caused by the parasites' usurpation of red blood cells, sequestration to vital organs and synchronised release of antigenic material, eliciting strong immune responses and resulting in a range of symptoms. (WHO 2019) The most common of these are characterised as 'uncomplicated malaria' and include fever, headache, vomiting, diarrhoea and general malaise. However, the disease can rapidly progress (< 24hrs) to severe malaria, resulting in anaemia, splenomegaly, thrombocytopenia and renal dysfunction, which can eventually lead to coma and death (WHO 2019).

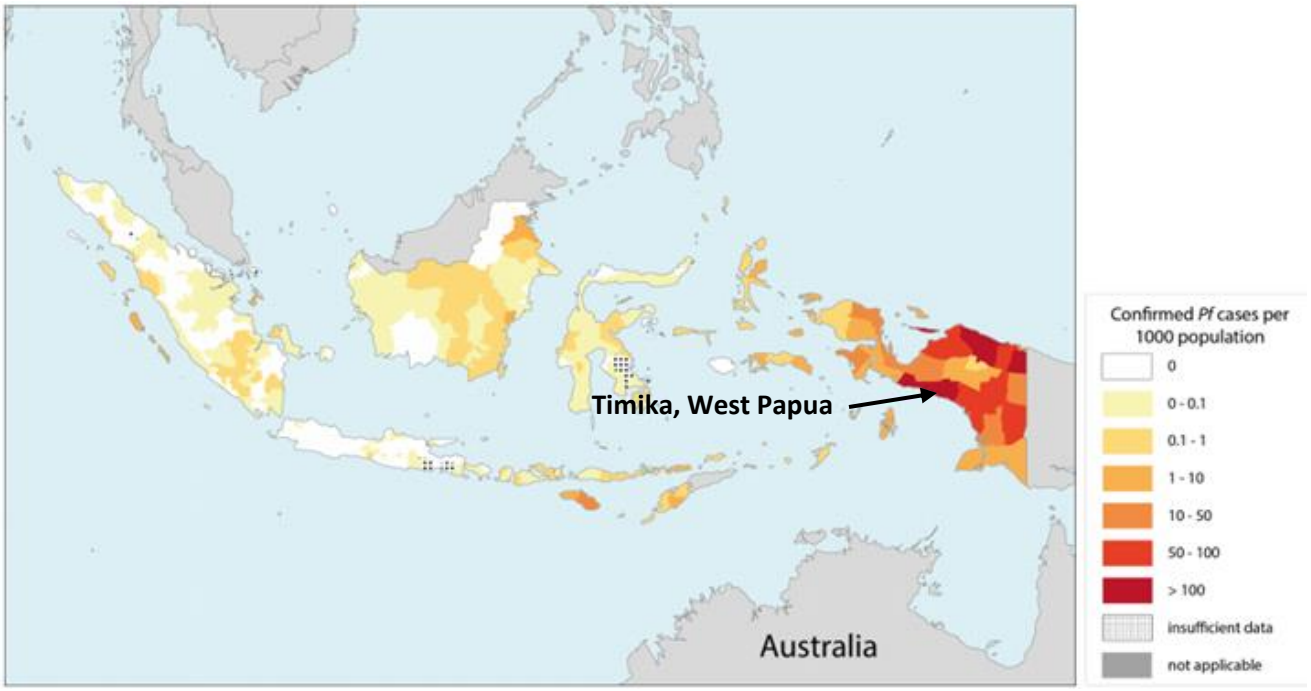
Figure 1.2. Map of malaria of case incidence rate

A. Global incidence of *P. falciparum* malaria shown as cases/ 1000 population at risk, and prevalence of B.i) *P. falciparum* and B.ii) *P. vivax* malaria in Indonesia, and C) *P. falciparum* in Kenya. Source: WHO malaria world report (Desai, ter Kuile *et al*. 2007)

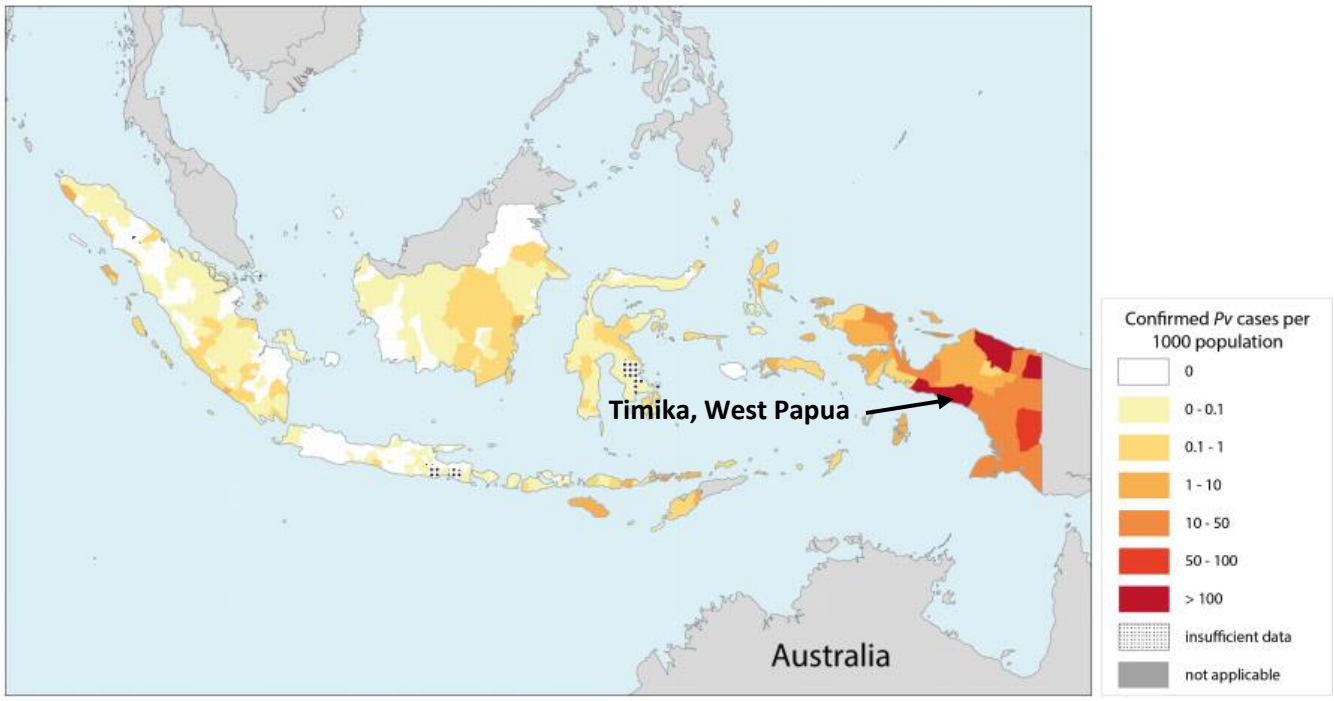
A.



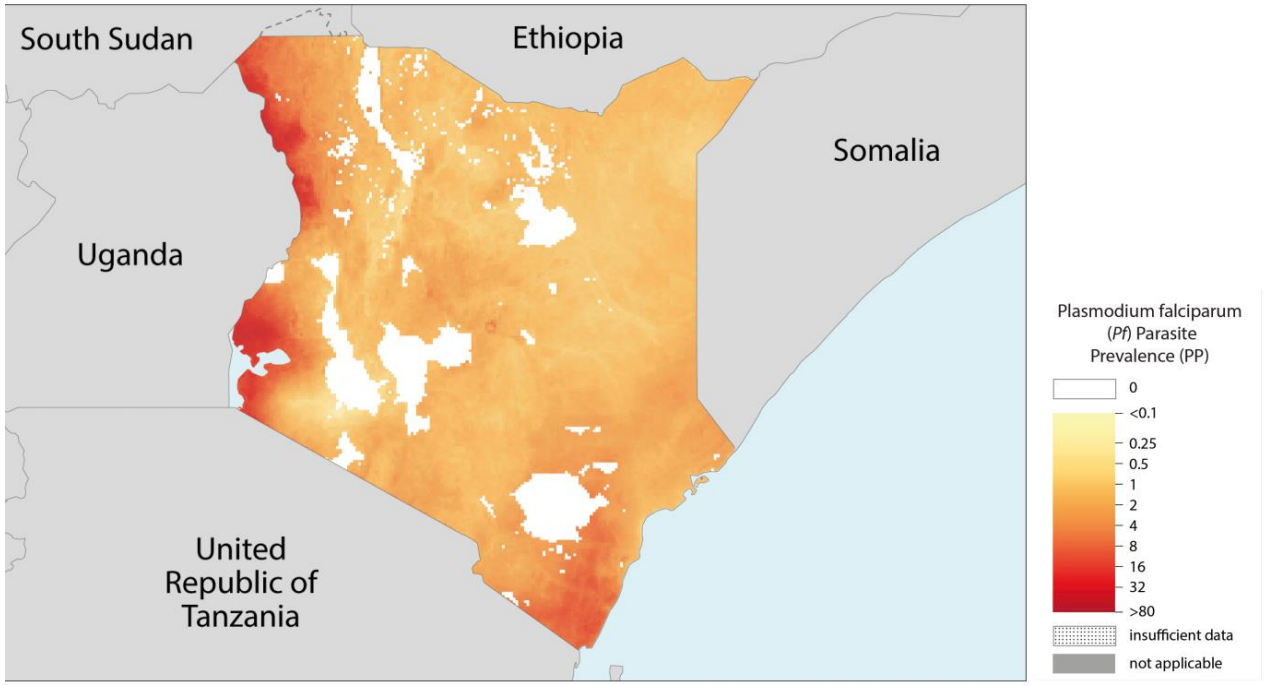
B.i) *Plasmodium falciparum* prevalence in Indonesia (relevant to chapters 2-5).



B.ii) *Plasmodium vivax* prevalence in Indonesia (relevant to chapters 2-5).



C. *Plasmodium falciparum* prevalence in Kenya (relevant to chapter 6).



Disease severity is influenced by many factors including age, immune factors, parasite species/density, host factors such as haemoglobinopathies or co-infection with other diseases. Malaria is most lethal in children under 5 years of age, accounting for 67% (272,000) of malaria death globally (Desai, Hill *et al*. 2018). Although complicated by different immune factors, this is largely due to lack of immunity which is acquired with age.

Pregnant women also represent a particularly vulnerable demographic at risk of malaria, with around 11 million pregnant women exposed to malaria infections (Duffy, G. *et al*. 2006). In addition to the clinical symptoms described above, Malaria in Pregnancy (MiP) is additionally associated with maternal anaemia, preeclampsia, post-partum haemorrhage, and poor birth outcomes including low birth weight, spontaneous abortion, still birth and prematurity, as well

as infant mortality (Bartoloni and Zammarchi 2012, Bhatt, Weiss *et al*. 2015). *Plasmodium falciparum* species can also sequester to the placenta, through interaction between placental chondroitin sulphate and the pfVAR2CSA antigen on the surface of merozoite stages (Sinka, Bangs *et al*.). Immunity against pfVAR2CSA-harbouring parasites is however acquired with parity. Women acquire immunity against these particular strains with increasing exposure during each pregnancy, thus primigravid mother lacking immunity are more susceptible to infection and adverse outcomes (Sinka, Bangs *et al*. 2012).

1.1.2. Disease control

Malaria control is mainly targeted at its two hosts; 1) vector control to reduce interactions between mosquitoes and humans and 2) early detection, prophylactic and curative therapies for the human host.

The global success in reducing malaria over the last 15 years can be largely attributed to vector control interventions (Singh and Daneshvar 2013). As Africa accounts for over 92% of global malaria cases and 93% of malaria deaths, the main vectors of malaria are the Anopheline species most prevalent in this region: *An. gambiae*, *An. stephensi* and *An. funestus (Carrasco, Lefèvre et al. 2019)*. In other regions of the world where *P. vivax*, *P. ovale* and *P. malariae* are more prevalent, the dominant vector species include *An. darlingi (*S America), *An. sundaicus s.l* (SE Asia), *An. koliensis* (Asia-Pacific) and *An. culicifacies* (W Asia) (Ranson and Lissenden 2016). Currently, the main vectors of *P. knowlesi* belong to the *Anopheles leucosphyrus* group, which inhabit forested areas in Southeast Asia where the long-tailed and pig-tailed macaque hosts

reside (Mulamba, Riveron *et al*. 2014).

The use of insecticides in control programs has been central to the decline in malaria, e.g. distribution of long-lasting insecticide-treated nets (LLITNs), Indoor Residual Spraying (IRS) and larval breeding site management. However, with the spread of insecticide resistance threatening this progress, it is important to closely monitor the efficacy of insecticides used in these programs. Insecticide resistance can be broadly categorised into behavioural and physiological resistance. Behavioural resistance includes changes in feeding patterns e.g. feeding times and host preference (Donnelly, Isaacs *et al*. 2016). Physiological changes including cuticular thickening and genetic mutations affecting insecticide metabolism, impair the efficacy of the insecticide (Donnelly, Isaacs *et al*. 2016). Knockdown resistance (kdr) caused by point mutations in the target active-site results in insensitivity to pyrethroids and cross-resistance to DDT (later detailed in chapter 3). Where two kdr mutations were once geographically defined within sub-Saharan Africa the prevalence of both alleles is now widespread, and in some areas in combination to a multiplying mutation which escalates insecticide insensitivity (Neafsey, Juraska *et al*. 2015).

Providing insecticide use remains the mainstay of vector control, fastidious monitoring and rapid response to the changing landscape of insecticide resistance becomes increasingly important (White, Verity *et al*. 2015). The tools used to track resistance should ideally suit the settings they are intended to be used in areas where malaria is prevalent. In this way, the characteristics required of these tools overlap with those required for diagnosing malaria infection (Hoffman, Vekemans *et al*. 2015).

1.1.2.2. Vaccines

The pre-erythocytic stage vaccine Mosquirix (RTS,S/A S01) is the only licensed malaria vaccine to date, having gained approval for use in 2015 (Hodgson, Ewer *et al*. 2015, Ademolue and Awandare 2018, Su, Lane *et al*. 2019). In 2019, pilot vaccination programmes were rolled out in Ghana, Kenya and Malawi, for us in young children and infants in whom the vaccine has previously demonstrated the greatest protective effects. The vaccine is based epitopes in the Circumsporozoite Protein (CSP) of *P. falciparum* and the Hepatitis B virus envelope protein. Other vaccines in Phase I-II stages of the pipeline include those that target different life-stages of the parasite e.g. blood stages, transmission blocking vaccine that target the sexual stages, and combination vaccines that target multiple stages (Sedegah, Hollingdale *et al*. 2015, Kamau, Alemayehu *et al*. 2013, Hoffman, Vekemans *et al*. 2015, Mueller, Shakri *et al*. 2015, Richie, Billingsley *et al*. 2015, Ademolue and Awandare 2018). Diagnostics also play an important role in measuring the efficacy of vaccine candidates (WHO 2015, Su, Lane *et al*. 2019, WHO 2019). In the early phases of development, the sensitivity of the diagnostic is most critical not only to ensure treatment is rapidly administered, but also to most accurately estimate the efficacy of the vaccine. For this nucleic acid detection methods are most accurate, but not appropriate for use in low-resource settings.

1.1.3. Diagnosis and treatment

According to WHO recommendations, patients with suspected malaria should only be provided with treatment following prompt diagnosis of infection (WHO 2019). Given the potential severity of malaria, it is important that the infection is diagnosed as early as possible. Current

methods for diagnosing malaria, as well as new diagnostics in the pipeline are described in depth later in this Chapter (section 1.3).

Artemesinin-based combination therapies (ACTs) are recommended as the first-line treatment of uncomplicated malaria. With no alternative therapies to artemesinin soon to enter the market, the use of artemesinin-based mono-therapies is actively discouraged and efforts are being made towards withdrawing these from the market (WHO 2015).

There are currently 5 ACTs, all manufactured at a fixed dose: Artemether-Lumafantrine, Dihydroartemesinin Piperiquine, Artesunate-Mefloquine, Artesunate- Sulfadoxine-Pyrimethamine, Artesunate- Amodiaquine and Artesunate-Pyronaridine. Alternatively, Quinine in combination with Doxycycline may be used for management of severe malaria (Frampton 2018). For chemoprevention, Chloroquine (for *P. vivax*), Doxycycline, Mefloquine, Proguanil (in combination with Chloroquine) or Artesunate with or without Sulfadoxine- Pyrimethamine, are recommended by the WHO (WHO 2015). The main consideration in treatment choice is determined by the species and drug-resistant strains in circulation. In areas with multiple *Plasmodium species,* it is important to identify *P. vivax* and *P. ovale* species so that anti-hypnozoite therapy can be administered alongside the anti-schizont/gametocyte therapy (WHO 2015). All 5 have gametocidal activity except against stage V gametocytes, which instead require Primaquine or Tafenoquine (Peters and Van Noorden 2009, WHO 2017). These two drugs prevent transmission of the parasite to the mosquito, in addition to clearing *P. vivax* and *P. ovale* hynozoites, and thus prevent relapses. However in patients with G6PD (glucose-6-phosphate dehydrogenase) deficiencies Primaquine can cause haemolysis of red blood cells, and should therefore be tested for this disorder prior to administration of the drug (Cheruiyot,

Auschwitz *et al*. 2016). Impairment of the G6PD enzyme results in in a build of oxidative stress triggered by oxidative drugs such as Primaquine. This results in destruction and elimination of red blood cells in the spleen (Battle, Lucas *et al*. 2019).

This complicates malaria Mass Drug Administration (MDA) programs, whereby populations are treated without prior testing. Due to immense efforts required for MDA programmes, these are currently only recommended for low-transmission settings nearing elimination, or for complex settings where drug-resistance is concerning (e.g. in the Greater Mekong region) or in emergencies where the health system becomes compromised (e.g. during Ebola virus outbreaks) (Rajahram, Cooper *et al*. 2019). In addition to use of monotherapies, drug-resistance in malaria is largely driven by sub-therapeutic dosing whether through unregulated manufacture or counterfeit drugs, and improper administration/ non-adherence. The World Wide Antimalarial Resistance Network (WWARN) provides open access resources relevant to antimalarial resistance. They work towards monitoring the emergence and spread of resistance, as well as international advocacy in these areas (D'Abramo, Gebremeskel Tekle *et al*. 2018).

Monitoring species composition also becomes important in areas nearing *P. falciparum* elimination, as there is evidence that the prevalence of *P*. *vivax* can supersede *P. falciparum* where there is a decline in *P. falciparum* transmission (Rahimi, Thakkinstian *et al*. 2014). There is also mounting evidence that non-*falciparum* and emerging *P. knowlesi* infections may be more severe than originally thought (WHO 2015, WHO 2016, WHO 2017). Together these factors are shifting global attention to include non-*falciparum* species and highlighting the need for diagnostics for multiple species.

The WHO define malaria elimination as a “reduction to zero of the incidence of infection caused by a specified malaria parasite in a defined geographical area as a result of deliberate efforts” (WHO 2018). In 2016 the WHO identified 21 countries for malaria elimination by 2020, as part of the ambitious E-2020 initiative within the wider Global Technical Strategy (GTS) for malaria (WHO 2014, WHO 2017). These countries were chosen based on recent malaria case trends, declared malaria goals and guidance from experts in the field. Data from 2017 showed that 10 out of 21 were on track to reach elimination by 2020, however, nearing the end of 2019, only Algeria and Paraguay are now WHO-certified as having eliminated malaria (WHO 2014). Certification is a voluntary process that requires proof that there is no indigenous malaria transmission within that country for 3 consecutive years. They must also demonstrate that robust surveillance systems are in place to prevent a rebound in transmission (Tietje, Hawkins *et al*. 2014).

As countries near elimination, the feasibility of maintaining elimination is affected by a number of factors, well summarised in figure one (O'Brien, Delage *et al*. 2015). Monitoring the shifting dynamics of malaria epidemiology is paramount to rapidly respond to cases and outbreaks (WHO 2015). For this, more active case detection (ACD) becomes essential- screening outside of health care settings regardless of symptoms- while passive case detection (PCD) approaches must be sustained, i.e. capturing data from those who present to health care providers (O'Brien, Delage *et al*. 2015). (Figure 1.3)

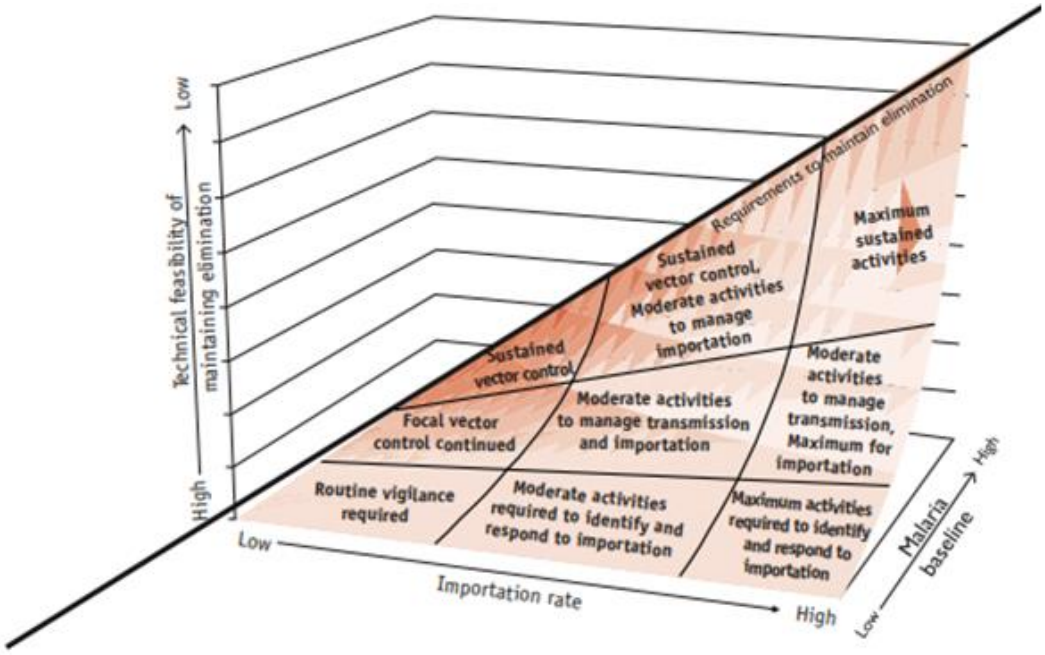
Screening within healthcare settings is also essential with regards to Transfusion-Transmitted

Malaria (TTM), where parasites are unintentionally transmitted to patients of tainted blood transfusions. This can cause severe disease in the recipient, which is particularly problematic in non-endemic countries where individuals lack immunity to malaria (Owusu-Ofori, Gadzo *et al*. 2016). The WHO provides guidelines for hemovigilance (Tietje, Hawkins *et al*. 2014), however the choice of diagnostic used to screen blood donors for malaria differs nationally. Currently, in sub-Saharan Africa microscopy is still the most widely used for screening donor banks, whereas antibody testing followed by an RDT or nucleic acid testing to confirm infection- is more common in higher-resource settings such as in non-endemic countries (WHO 2014). Evaluation of more sensitive RDTs could also offer a practical and affordable diagnostic test for endemic settings where most people would have anti-malarial antibodies (Doolan, Dobaño *et al*. 2009).

To maximise resources, ACD is targeted to populations that meet different criteria depending on the setting e.g. recent travellers to an endemic country. Reactive Case Detection (RCD) is a form of targeted surveillance, where individuals in contact (temporally, spatially, categorically) with a confirmed case (passively or actively identified) are selectively screened. In this fashion, interventions can be better tailored to the local epidemiology (Carneiro, Roca-Feltrer *et al*. 2010). The tools used to identify infections for active surveillance are critical for accurate and timely reporting. It is also important that the correct health system infrastructure is in place to support the use of these.

Figure 1.3. Factors influencing elimination and the likelihood of sustaining elimination.

Figure taken from www.who.int/malaria/publications/atoz/9789241507028/en/ (WHO, 2014)



Modelling has shown that areas with a higher malaria baseline or the 'intrinsic malaria transmission level 'require a higher coverage of more significant intervention efforts to achieve similar outcomes to an area with low malaria baselines. It is therefore important to implement control strategies proportionate to baseline malaria rates (Carneiro, Roca-Feltrer *et al*. 2010). For the estimation of baseline malaria rates, potential contributors to transmission or sources of infection, i.e. the 'infectious reservoir', need to be defined. This will guide interventions to halt transmission.

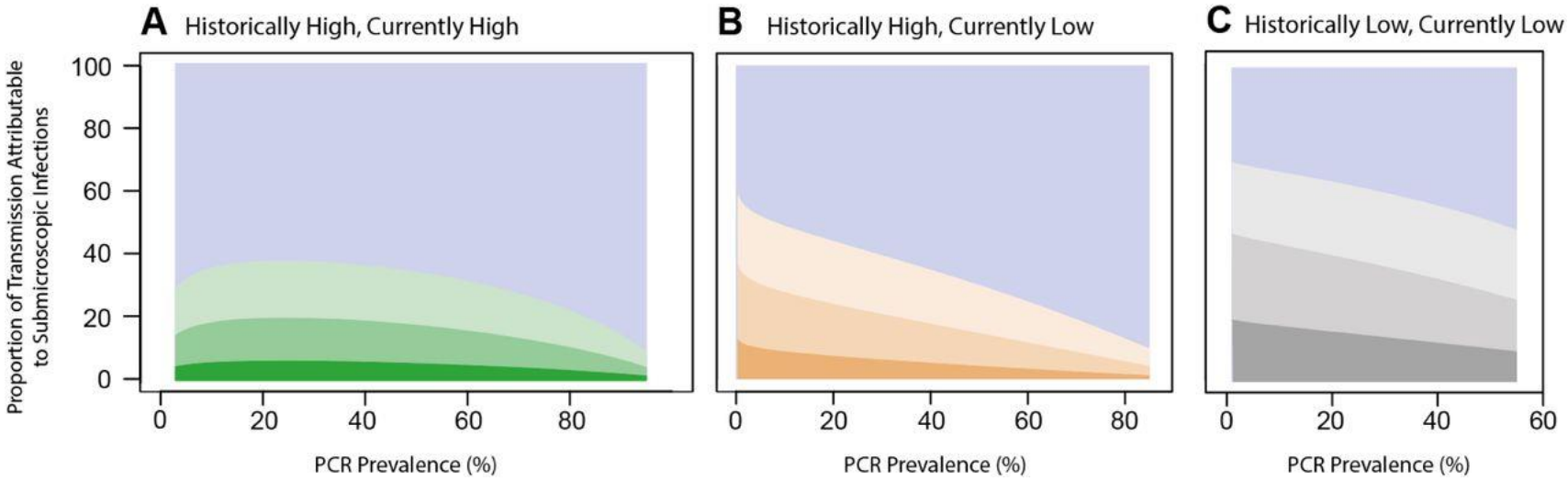
1.1.4. Parasite density

Acquired immunity is central to preventing malaria (van Gool, van Wolfswinkel *et al*. 2011). However, the relationship is not well defined, due to the complexity between repeated exposure, age and acquired immunity in different epidemiological settings (Doolan, Dobaño *et al*. 2009). It was thought that a lack of exposure results in a lack of immunity, explaining the high levels of morbidity and mortality in under 5-year olds. It therefore follows that adults in high-endemic countries can withstand high density infections (>1000 parasites/ µl) without clinical signs. It is also well established that when a source of exposure is removed, e.g. in migrants moving from endemic to non-endemic countries, protective immunity rapidly declines, resulting in clinical malaria at much lower parasite densities in subsequent infections (Whittaker, Slater *et al*. 2019). This effect is also seen in naïve individuals travelling to endemic countries, who can develop disease at much lower parasite densities. Due to the lack of immunity in the individuals, parasitaemia rapidly increases (Whittaker, Slater *et al*. 2019).

It is thought that immunity- although not sterile- can mediate symptoms and severity through repression of parasite reproductivity (i.e. parasite density). Individuals who are not frequently exposed to parasites, such as those in low-transmission settings, would lack the acquired immunity necessary to control parasite reproduction (Ouedraogo, Eckhoff *et al*. 2018). However, it has been consistently shown that low density infections are most prevalent in areas of low transmission where acquired immunity is also low (fig 1.4) (Pett, Goncalves *et al*. 2016, Whittaker, Slater *et al*. 2019).

Figure 1.4. Impact of sub-microscopic infections in different transmission settings.

Image taken from Whittaker *et al*., (Whittaker, Slater *et al*. 2019)



1.1.5. The silent reservoir

Falling below the detection limit of microscopy (50 parasites/ µl blood), low-density or 'submicroscopic' malaria infections have been described as a silent reservoir of infection (see Table 1.3 for diagnostic limits of detections). Such low densities rarely elicit symptoms and so asymptomatic individuals do not seek healthcare, therefore are not treated yet continue to harbour parasites. Despite the low densities of parasites, individuals are gametocytaemic and infectious to mosquitoes (Slater, Ross *et al*. 2019), therefore a source of continued transmission. The presence and density of gametocytes in the human host underpins estimation of transmission potential. Microscopy, as well as rt-PCR for stage-specific RNA are most commonly used to assess gametocytaemia (Okell, Bousema *et al*. 2012).

A recent review showed the ubiquity of gametocytaemic, submicroscopic infections across a range of transmission settings, but that the proportion of these increased in low-transmission settings (Okell, Bousema *et al*. 2012, Mosha, Sturrock *et al*. 2013, Bousema, Okell *et al*. 2014, Nash, Prevots *et al*. 2017, Slater, Ross *et al*. 2019). To prevent a rebound in transmission, it is important to actively identify these individuals, especially in near-elimination settings. Although disease severity is exacerbated with parasite load, it is not clear exactly how this is controlled and what role immunity plays. Longitudinal serology studies show a truly complex relationship between immunity, parasite density and clinical presentation (Ademolue and Awandare 2018, Bjorkman 2018).

In a high transmission setting in Tanzania, children who did not develop infection had fewer anti-parasite antibodies (specifically AMA; apical membrane antigen and MSP1; merozoite surface protein 1) during infancy, but showed a higher IL-1B cytokine (anti-disease)

profile in their cord blood (Hofmann, Karl *et al*. 2017). Anti-disease immunity is acquired quickly and early during childhood, whereas anti-parasite immunity develops with exposure to the parasite and therefore age. Together with innate immune responses, specific antibody responses to liver- and blood-stages might explain the variation in disease outcomes. (Searle, Katowa *et al*. 2017)

Another hypothesis is that strains circulating in these settings are less virulent. Bjorkman *et al*. (2018) suggests the more virulent strains could be being selected against through generation of symptoms that cause health-seeking behaviour (Bjorkman 2018). Thus, these strains will be cleared when the patient is diagnosed and treated. This would also result in a better primed immune system for subsequent infections. (Drakeley 2018) Another factor is infection comprising multiple strains or 'super-infections' that cause symptoms through a sudden increase in parasitaemia. This is supported by studies in Papua New Guinea (WHO 2014) and Zambia (Childs and Buckee 2015), where newly imported strains caused symptomatic malaria. (Mueller, Schoepflin *et al*. 2012) This supports modelling that highlights the importance of importation rate with regards to eliminations strategies (Slater, Ross *et al*. 2019).

1.1.6. Infection chronicity

The timing of sampling is also an important factor of reported parasite density. Cross sectional studies only provide a snapshot of the epidemiology of asymptomatic malaria. It is likely that recent parasite exposure influences the parasite density in subsequent infections.

The handful of longitudinal studies, across a range of transmission settings, tell us that the dynamics are dependent on the locality. Chronic low-density infections (6-9 months) are

known to follow high-density clinical infection, potentially enabling transmission across transmission seasons. This is reflected in the variation in symptoms observed during different seasons, but more work is needed on gametocytaemia persistence to see how infectivity is affected. (Gruenberg, Lerch *et al*. 2019, Roh, Tessema *et al*. 2019.)

Slater *et al.* (2019) found that due to the dynamic nature of parasite density during an infection, asymptomatic episodes were predictive of patent infection, although they could not distinguish between reinfection and recrudescence (Slater, Ross *et al*. 2019). Historically, markers such as Merozoite Surface Protein 1 and 2, and glutamate-rich protein (GLURP) have been used to genotype strains of *Plasmodium* for surveillance and monitoring of drug/ vaccine trials. However, studies have shown preferential amplification of shorter alleles over longer alleles obscuring results in multiclonal infections (Zhong, Koepfli *et al*. 2018). A recent study shows that in-depth sequencing of markers containing many single nucleotide polymorphisms flanked by intergenic tandem repeats improves identification of different genotypes in multiclonal infections.

Currently, there is conflicting evidence around the genetic diversity of infections in low-transmission settings and so it is suggested that Multiplicity of Infection (MOI) may be more important in determining symptoms (Bjorkman 2018, Drakeley 2018).

1.1.7. Impact on transmission

Drakeley *et al*. (2018) discuss four different archetypes of the overall infectious reservoir and their relative contribution in greater depth (Bechtsi and Waters 2017). In summary, the most

important factors seem to be: the length of time that individuals are gametocytaemic, gametocyte density- although less so- and whether the individual develops symptoms and obtains treatment (Slater, Ross *et al*. 2019). More genotyping studies would be advantageous to shed light on these theories, however low amounts of parasite DNA make these investigations difficult. (Mugambi, Peter *et al*. 2018)

Parasite conversion to sexual development is epigenetically controlled (Tietje, Hawkins *et al*. 2014), and demonstrably higher in lower endemicity settings (Peeling, Holmes *et al*. 2006). This impacts on the choice of drugs for elimination programs. Overall, these factors highlight the importance of active identification and treatment of asymptomatic individuals, especially within an elimination context. This needs to include active identification of infections to prevent future disease in the individual, and identification of *hotpops* and *hotspots* at a population level to prevent resurgence.

1.2. The diagnostic pipeline

The diagnostic pipeline is long and costly one. The development of an in-vitro diagnostic (IVD) begins by understanding the problem it is intended to solve and the specific market needs. Product development low-middle-income countries (LMIC) requires additional considerations to those in other countries. Electricity, cold-chain and skilled human resources are often limited in these settings, as is the appropriate infrastructure to support the distribution and use of diagnostic tests.

Understanding the purpose of the IVD as well as the market will help define the 'use-scenario' (who is going to use it and in what health care setting) and other characteristics which come together to form a target product profile (TPP). A TPP describes the minimal and optimal requirements for the final product, including the size, cost, throughput, run-time and storage, as well as details of analytical performance (Land, Boeras *et al*. 2019).

1.2.1. Use-scenarios

There is no one-size-fits-all test for malaria. Point-of-care tests (POCT) have different requirements to those used for surveillance- they need to meet criteria that enable their use near a patient. In Table 1, Tietje *et al*., (2014) outline the different requirements of tests in different use-scenarios, specifically in an elimination context where preventing transmission is most critical (Tietje, Hawkins *et al*. 2014).

In 2006, Peeling *et al*. defined criteria for POCTs for sexually transmitted infections, for consideration in LMICS. The WHO has since adopted the ASSURED criteria for general development of POCTs: *Affordable, Sensitive, Specific, User-friendly, Robust/Rapid, Equipment-Free, Deliverabl*e (Tietje, Hawkins *et al*. 2014).

The first consideration is *affordability*, which is largely dependent on the amount of information gained from that test. For example, a test that accurately identifies malaria, HIV and syphilis simultaneously (the 3 biggest causes of mortality in pregnant women in sub- Saharan Africa) is less burdensome on resources. Even if this test is priced higher than the three separate tests combined, it could still be more cost-effective overall.

Secondly, the POCT must be *user friendly* i.e. as simple to operate as possible, minimizing training and resources. A maximum of 3-6 operator steps is suggested for LRS (project 2014). The desired sensitivity and specificity are determined by the potential impact of the results following testing. Sensitivity is mostly influenced by disease severity and the impact of missing a positive case e.g. if it is highly lethal and/or infectious. The specificity, on the other hand, is affected by the risks associated with unnecessary treatment e.g. highly toxic drugs /severe side effects, as well as impact of misdiagnosis and treatment costs. Differential diagnoses of malaria include a multitude of lethal diseases- most commonly diarrhoeal diseases, sepsis and viral haemorrhagic fevers- and so misdiagnosis could be as dangerous as missing malaria itself. Thus, malaria diagnostics need to be both highly sensitive and highly specific. POCTs must also be *robust*, due to the non-ideal storage conditions (lack of cold-chain and extreme temperatures) common in LMICs. Their patient-facing usage also requires them be *rapid* (<30 min).

Finally, a POCT is ideally *equipment-free* and *deliverable*. These criteria go hand in hand, reducing upfront and maintenance costs of often bulky equipment, as well as minimising the complexity and therefore training requirements. It follows that an equipment-free test is also logistically more deliverable. Although the ASSURED criteria were first designed for POCTs, they provide a good basis for more complex, high-throughput tests.

Table 1.1. Target Product Profile (TPP) framework for malaria diagnostics in an elimination context.

Table is taken from Tietje *et al.* (FIND 2019)

TPP	POC Case Detection	Point of contact ID	High-throughput ID
Requirements	Low cost	Portable	Higher cost
	Easy to use	Rapid results	Standardised
	Rapid results	High sensitivity (low LOD)	High throughput
	High specificity		High sens/spec (low LOD)
Use-scenarios	Passive case detection	Mass/ Focal testing e.g.	Surveillance
	Case follow-up	Networks/ community/ borders	Laboratory confirmation
		Surveys	Parasite quantification

For active screening, test and treat strategies have been explored as a cost-saving strategy, especially for these settings. These programs have the additional benefit of being more publicly acceptable as they avoid treatment of uninfected individuals and reduce the potential to drive drug resistance.

For Mass Test and Treat (MTAT) and surveillance programmes, many samples need to be screened at once, often necessitating batching and storage of samples. This inevitably creates physical and temporal distance between the test operator and the patient. Although Focused Test and Treat strategies (FTAT) can be lower throughput than MTAT, they still require highly sensitive tests with a rapid turnaround. Both strategies are used to screen geographically defined areas dubbed “Hotspots”, which are often related to behavioural patterns, e.g. occupations of the people in an area. Hot populations (*hotpops*) on the other hand are demographically defined, often silent infectious reservoirs in hard to reach locales. Pregnant

women in low-transmission settings could be an example of this, as asymptomatic placental malaria is difficult to diagnose (histological methods). It is important to identify and monitor *hotspots* and *hotpops* to prevent rebounding transmission. In Table 1.1, Tietje *et al.* (2014) categorise different use-scenarios for low-transmission settings describing proposed screening strategies for each (FIND 2019).

Intermittent Screening and Treatment during pregnancy (ISTp) strategies for example could be considered an FTAT strategy, whereby only pregnant women are tested for malaria during crucial antenatal visits, regardless of symptoms and administered treatment if necessary. This controls malaria during pregnancy, but data could also inform epidemiological surveys for monitoring. It is also possible and *preferable* that new diagnostics could be used in several use-scenarios if they meet the required TPP requirements e.g. a highly sensitive molecular test, may be used at point of care if cheap and rapid enough.

1.2.2. TPPs

In collaboration with the WHO, the Foundation for Innovative New Diagnostics (FIND) develops publicly available TPPs for diagnostics needed for poverty-driven diseases such as malaria.

Through engagement and consensus with key stakeholders, these TPPs act as a starting point for product developers. Their most recently published TPPs for malaria include 1) the simultaneous detection of multiple pyrogenic pathogens and 2) drug-resistance assays. Monitoring drug resistance and insecticide resistance, although important, are not discussed extensively in this thesis. However, the phenomenon of 'diagnostic resistance' (parasite

mutations that affect detection) is explored in Chapter 5, whilst insecticide resistance is considered in Chapter 3. For the focus of this thesis, TPPs specific to the detection of the malaria parasites within an elimination context are discussed.

In 2014, a detailed TPP for a POCT for the rapid detection of low-density malaria detection was developed by FIND (FIND 2019). Most notably, they recommend that the POCT should be "performed under zero infrastructure conditions" suitable for "both ACD for control interventions and PCD for case management, as well as for use in surveillance". The other requirements are commonly used for POCT TPPs: sensitivity 97-99%, specificity 90-99%, storage 10-40 °C for >36month etc. They identify Histidine Rich-Protein 2 as a primary target suggesting the inclusion of a second *Pf*-specific analyte and/or a Pan-*Plasmodium* or *P. vivax* analyte. However, since the publication of this TPP they have further defined specific POCT TPPs for 1) acute *P. vivax* infections 'PvA', 2) *P. vivax* sub-patent infections 'PvB1' and 3) a *P. vivax* surveillance tool 'PvB2'. (Okell, Bousema *et al*. 2012)

Unlike for POCTs, it is generally accepted that the molecular surveillance of malaria requires skilled professional operators, as increased capabilities bring increased complexity. This generally translates to a need for infrastructure of at least a Level 1 healthcare facility setting.

This year, Médecins Sans Frontiers (MSF) developed a TPP for the multiple analyte diagnostics platforms (MAPDx), in partnership with FIND and the WHO (Slater, Ross *et al*. 2015). Led by the Febrile Illness Diagnostic group, many of their priorities overlap with the FIND TPP for the detection of multiple pyrogenic pathogens. The breakdown of diagnostic silos through multiple detection technologies is a running theme in the diagnostics landscape. Although the MAPDx

TPP stipulates a platform with dual-detection capabilities for both genetic and immunological targets, its other criteria can guide development of either category. Although they identify several promising platforms that are commercially available/ in late development, they conclude that none of them currently satisfy their TPP (Drakeley 2018).

Although there are no published TPPs for a molecular surveillance tool for identifying malaria in elimination settings, a TPP developed for drug-resistance surveillance shares many of the desirable characteristics and several publications discuss these with particular reference to elimination settings (WHO 2014, FIND 2019). The WHO additionally provide guidance on selecting diagnostics malaria specifically in low transmission settings (Tietje, Hawkins *et al*. 2014). In their 2014 policy brief, they recommend the use of a sensitive NAAT in 4 out of 5 use-scenarios: “Malaria epidemiological surveys, Focus investigations; reactive infection detection after identification of an index case, Mass screening and treatment” and “Screening of special populations (e.g. at border crossings)” (Wongsrichanalai, Barcus *et al*. 2007). The main difference between these is the degree of urgency for results. Considering these resources Table 1.1 briefly outlines a TPP for a rapid malaria NAAT for broad screening purposes in low-transmission settings. Although current POCTs are not suitable for these use-scenarios, but with advances in technology new tests may fill this gap. The following section describes the currently used tests for malaria and those in the pipeline with potential to fulfil the proposed TPP (Table 1.2). Other characteristics e.g. stability/shelf/instrument design life are identical to those described by FIND (Unitaid 2018, WHO 2019).

Table 1.2. General TPP for a malaria surveillance tool suitable for use in an elimination context.

	Characteristics	Optimal requirement
Use scenario	User	Trained lab personnel
	Target	Detection of key malaria species, ideally in additional to parasite specific-resistance markers
	Setting	Level 2 healthcare settings (L1 unlikely to be carrying out epidemiological surveys)
	Scenario	For MTAT or FTAT screening, as well as epidemiological surveys
Assay design	Analytes	Nucleic acids
	Throughput	8-40 samples/8 hr day
	Test kit	Self-contained kit that provides all reagents with minimal additional consumables (e.g. pipette tips)
	Specimen	Minimally: whole blood, preferably other non-invasive sample e.g. saliva and urine
	Sample volume	<5ml whole blood, ideally finger prick (5-50 µl)
	Sample preparation	Ideally integrated in the cartridge, otherwise 3 max steps requiring user operator intervention and 1 precision step
Assay characteristics	Sensitivity/Specificity	As in text for POCT
	LoD	1 parasite per microliter will detect 95% of infectious reservoir, therefore 97% sensitivity in the settings require LoD of 0.1 p/µl
	Test result	Quantitative with qualitative threshold to inform clinical decisions
	Time to result	<90 min
	Controls	Integrated internal control, no need to for positive/negative control every run
	Cost	< USD $5, but this may increase with additional targets
	Safety	No biosafety cabinet required, minimal handling of biohazardous material; easily decontaminated platform
	Disposal	Simple waste disposal route: recyclable or combustible cartridges

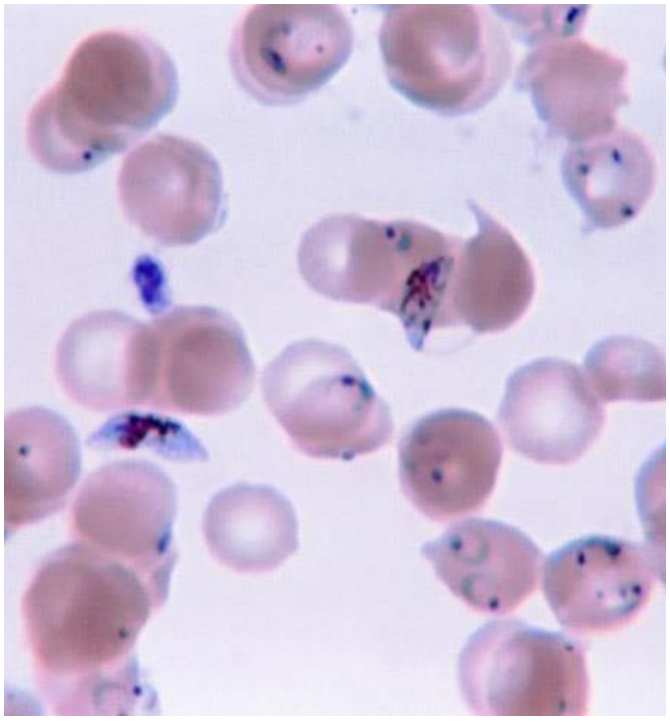
1.3. Malaria diagnostic landscape

1.3.1. The gold standard

The WHO recommends diagnosis of clinical malaria by microscopy or RDT. Despite the obvious pitfalls of microscopy - the need for extensively-trained professionals, subjectivity and a supportive infrastructure to maintain quality control/assurance- it allows species differentiation and quantification (fig 1.5) at a very low cost (WHO 2016). This has kept microscopy as the universal gold standard since the Giemsa staining techniques was developed over 100 years ago.

Figure 1.5. Microscopy slide of Giemsa stained *P. falciparum* in a thin blood smear.

Image source www.cdc.gov/parasites



Malaria rapid diagnostic tests (RDTs) were developed as simple tools, still cheap (< US$ 1) and useful where microscopy was not feasible. The demand for RDTs is rising with sales almost doubled between 2017 and 2018 (412 million RDTs globally), of which 16 countries account for 80% of the demand (Beisel, Umlauf *et al*. 2016, Unitaid 2018). Of this market, 63% is for *P. falciparum* RDTs specifically (WHO 2016, WHO 2018).

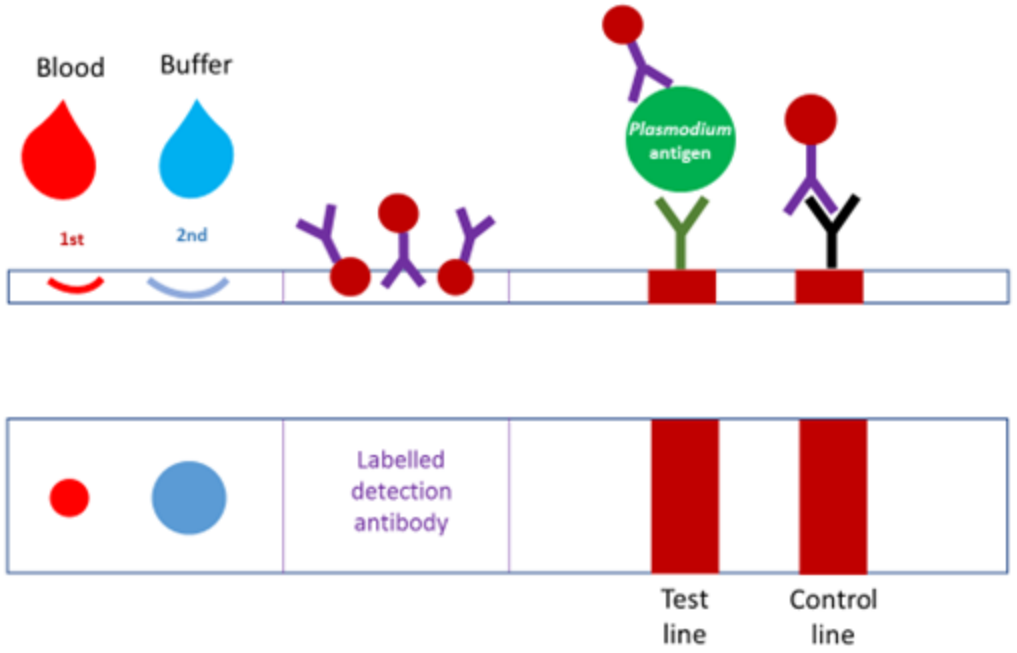
These immunochromatographic lateral flow tests comprise a nitrocellulose paper strip with at least 2 lines (control plus test lines) of immobilised antibodies or antigens to capture target antigens or antibodies respectively. Blood (generally venous, but capillary when patient was declined) is added (a drop, ~ 5- 10 µl) to the first well, followed by a buffer which allows the sample to flow across the strip. The sample flows across a conjugate pad containing detection antibodies (conjugated to a coloured substrate e.g. colloidal gold) which elicits a colour change within 15-20 minutes in the presence of the target. (Figure 1.6)

Although a massive global market, in the field diagnostic performance of malaria RDTs can vary due to non-adherence to the manufacturer's guidelines (timing/ blood volume), poor storage conditions and inherent differences in sensitivity/ specificity (Maltha, Gillet *et al*. 2013). To control for analytical performance and consequently make recommendations, the WHO performs rigorous prequalification testing of malaria RDTs. The WHO- Foundation for Innovative New Diagnostics (FIND) Malaria RDT Evaluation Programme was launched in 2006 to bring transparency to quality assurance of commercial RDTs, the program has helped to accelerate the development and implementation of new malaria RDTs (Wu, van den Hoogen *et*

al. 2015). However, currently prequalification only includes sensitivity testing above of 200 p/ µl (the widely accepted RDT limit of detection; LOD), which is not suitable for the sub-microscopic infections described earlier (Slater, Ross *et al*. 2015).

Figure 1.6. Schematic diagram of an antigen-capturing lateral flow device (LFD).

Parasite-specific antibodies (green) capture parasite antigen, which also bind detection antibodies (purple) conjugated to a coloured substrate (red circle). The control line is produced through binding of the control antibody (black) directly to the detection antibody. Image adapted from Maltha *et al*. 2013 (Slater, Ross *et al*. 2019).



1.3.2. Approaching detection limits

It is generally accepted that microscopy's LOD ranges between 50 parasites/ µl for expert microscopists and 100-200 p/ µl under non-ideal field conditions, whilst the LOD of RDTs are in

the range of 100-200 p/ µl (Ahmed, Levy *et al*. 2015). Modelling shows in a medium-high transmission setting, a diagnostic with an LOD of 100 p/ µl will identify >95% of disease, it will only detect ~50% of infections identified by more sensitive molecular methods such as Polymerase Chain Reaction (PCR [LOD < 1 p/ µl]). This is drastically reduced to just 14% where PCR prevalence is less than 10% (low-transmission settings) (Piper, J. *et al*. 1999, Das, Peck *et al*. 2018).

Despite advances in technology, for POCT only RDTs have been implemented in the field worldwide. However, it is apparent that more sensitive RDTs are needed. It is first important to understand the factors that contribute to the performance of RDTs in order to improve them.

The choice of target analyte or combination of target analytes in an RDT is critical to its performance. Most *P. falciparum* RDTs are based on the detection of Histidine Rich Protein (HRP2), while fewer detect pan-*Plasmodium* aldolase and lactate dehydrogenase (genus specific). RDTs that detect a combination of these antigens show greater sensitivity than a single-antigen RDT (Gamboa, Ho *et al*. 2010). This enables a level of redundancy that compensates for variable antigen concentrations (Jain, Chakma *et al*. 2014) or mutations which affect protein shape and thus the efficacy of capture antibodies (Gascoyne, Satayavivad *et al*. 2004, Katis 2015). Novel biomarkers, including those for specific to gametocyte stages, for new RDTs are in the early stages of discovery and are yet to be commercialised (Soti, Kinoti *et al*. 2015). The dynamic fluctuation of parasite analytes over the course of an infection, coupled with the complexity of immunological responses (described above) has hindered biomarker discovery.

Variation during manufacture is a factor in RDT performance, as is the binding affinity and spacing of monoclonal antibodies on LFDs. A change in the source of any components affecting these can have profound effects. The quality of the nitrocellulose membrane is also crucial for the migration of liquids to the line where capture antibodies are embedded. Thus, methods that focus the path of liquid migration across the paper membranes are also being explored (Shekalaghe, Cancino *et al*. 2013). Precision direction of the flow of sample and subsequent buffers utilises the principles of microfluidics, as in lab-on-a-chip devices (reviewed in Gascoyne *et al.*, 2004) (Oyet, Roh *et al*. 2017).

Finally, operational factors such as adherence to the manufacturing guidelines, storage conditions, accurate data entry and record keeping are also important. These issues highlight the need for better training and call for the use of an automated data entry device, preferably connected to a centralised database.

1.3.3. Reading aids

Automated readers which interpret RDT results are being explored to 1) eliminate the subjectivity of interpreting faint lines, 2) facilitate accurate data-keeping through barcoding and automated uploading to a cloud database. They generally operate in one of two ways: either through an integrated image capture and analysis system, or through analysis of images captured by smartphones or Tablet devices.

Currently the Deki Reader™ (Fio Corporation, Canada) is the only reader to be evaluated for use with malaria RDTs in several countries (Davis, Gibson *et al*. 2014, Herrera, Vallejo *et al*.

2014, Scherr, Gupta *et al*. 2016, Scherr, Gupta *et al*. 2016, Kalinga, Mwanziva *et al*. 2018), with subsequent roll out in Kenya. The utility of automated readers has also enabled movement towards quantifying tests that are designed to be qualitative. Scherr *et al.* (2015) report quantification of parasitaemia down to 20 p/ µl on Paracheck-*Pf*® RDTs (Orchid Biomedical Systems, India) using their iPhone5 mobile app system (Dalrymple, Arambepola *et al*. 2018). Their reader offered a lower LOD than visual readings by inexperienced operators, but not in comparison to more experienced ones (Kamaliddin, Joste *et al*. 2019).

1.3.4. Concentration techniques

Simple, low-cost sample concentration techniques could also offer a quick remedy for improving existing RDTs. Martin *et al.* (Martin, Rajasekariah *et al*. 2009) demonstrated that a simple pre-processing step using their 'low-resource extraction cassette' improved the sensitivity of 5 commercially available RDTs, dropping their detection limits down to 3-13 parasites/ µl. The device is based on magnetic agarose beads and a series of washes, and although requiring some skill to perform, this format of concentrating blood in a pre-RDT step might be more rapidly rolled out whilst newly developed higher sensitivity RDTs progress in the pipeline of validations and evaluation.

Although the specificity of malaria RDTs is generally good (>97%), it is important to consider factors that compromise specificity of LFDs. Aside from the poorer sensitivity of RDTs compared to NAATs, antigen persistence is a critical factor of discrepancy between these test types (Kamaliddin, Joste *et al*. 2019). Following clearance of an infection nucleic acids are degraded relatively quickly, making NAATs better suited for identifying active infection or

monitoring treatment efficacy (Lee, Jang *et al*. 2014, Gatton, Ciketic *et al*. 2018) In comparison, proteins however degrade slower, antigen-tests therefore remain positive for longer and are thus more indicative of *recent* rather than active infection (WHO 2015). False RDT positives as a result of cross-reactivity with human anti-mouse antibody and rheumatoid factors has also been demonstrated (Saiki, Gelfand *et al*. 1988, Singh, Bobogare *et al*. 1999).

1.4. Molecular testing

The high cost of molecular testing, in addition to cold-chain requirements, restricts their use to well-resourced settings. With increasing effort toward 1) improving detection of low-density infections and 2) enhancing accessibility, molecular testing is becoming more feasible in LRS where malaria is most prevalent. However, the reproducibility and therefore robustness of molecular testing has come under question after studies showed significant variation between technical replicates. Together with uncertainties around the impact of treating asymptomatic infections, the WHO currently only recommends molecular testing for research purposes only i.e. genotyping, epidemiological and resistance mapping. For this, they offer an External Quality Assurance (EQA) scheme (Singh, Sung *et al*. 2004) to guide the use of NAATs, in the hope that resultant data will be more reliable and comparable.

1.4.1. NAATs

PCR is a well-established NAAT, used since the 1980s (Rougemont, Van Saanen *et al*. 2004). Nested PCR is a technique used to enhance the sensitivity of conventional PCR, by first

amplifying a larger fragment DNA and then further amplifying species-specific fragments within the amplicon, followed by electrophoresis for visualisation. The “Snounou” nested PCR (nPCR) developed in 1993 was until recently the most commonly used reference standard (Shokoples, Ndao *et al*. 2009). It targets the 18S rRNA gene in Plasmodia allowing differentiation between the 4 main species. In 2005 it was updated to include the *P. knowlesi* species, which had since been recognised as a human pathogen (Kamau, Alemayehu *et al*. 2013). Although a more sensitive assay (10 p/ µl LOD) than the gold standard, the procedure is extremely time-consuming (~6 hrs), prone to contamination due to the transfer of the first amplicon to the second reaction mix and vulnerable to misinterpretation following electrophoresis due to subjective reading of bands on gels.

Fluorescence-based PCR, whether using intercalating dyes that bind double stranded DNA or fluorophore-conjugated probes (sequence-specific oligonucleotides), it still requires - skilled professionals and is more expensive than conventional PCR. On the other hand, these methods are less laborious, less subjective and less prone to contamination than nPCR.

1.4.2. Probe based qPCR

Hydrolysis probes comprise a donor dye and conjugated quencher molecule. The quencher is cleaved upon enzymatic activity, enabling the donor to fluoresce. One of the most popular hydrolysis probes is the TaqMan probe which is cleaved by Thermophilus Aquaticus (Taq) polymerase activity.

Multiplexing of these probes allows the simultaneous detection of multiple *Plasmodium* species, making this one of the most commonly employed assays for the detection of malaria in

blood (Murphy, Prentice *et al*. 2012, Navarro, Serrano-Heras *et al*. 2015, Wu, van den Hoogen *et al*. 2015, Meerstein-Kessel, Andolina *et al*. 2018). However, they are mainly suited to well-equipped laboratories only e.g. reference hospitals in HICs, epidemiological surveys and drug/vaccine development assays e.g. in Controlled Human Malaria Infection models (CHMI) (Unwin, Ahmed *et al*. 2020). These have been especially useful in detecting *P. falciparum* gametocytes for assessing transmission (Schneider, Wolters *et al*. 2005). Advances in probe technology have expanded the molecular toolbox for the development of NAATs (Navarro, Serrano-Heras *et al*. 2015).

Molecular beacons e.g. HyBeacon probes (later discussed in Chapter 3) are sufficiently sensitive fluorescent probes (WHO 2014) (Hodgson, Douglas *et al*. 2015), but have not yet been widely used for malaria, possibly as the cost is similar to existing TaqMan assays. Likewise, integrated primer-probe technologies (e.g. Scorpions, Amplifluor®, LUX™, Cyclicons, Angler®) and nucleic acid analogues (PNA, LNA®, ZNA™) have potential, but are yet to be applied for malaria detection (Imwong, Hanchana *et al*. 2014).

One major downside to fluorescence assays is the requirement for typically cumbersome and expensive (> £20,000) fluorometers or qPCR machines. With advances in technology, these devices are being miniaturised to overcome this (further discussed in Chapter 3). Miniaturisation not only enhances portability but can reduce the volume of clinical sample and expensive reagents required, which also enables more rapid heating and cooling of the smaller volume.

Another issue is that multiple targets normally require multiple fluorophores, incurring additional costs. Melt curve analysis (MCA, discussed in Chapter 2-3) can overcome this to

some extent but is highly dependent on the DNA sequence of the targets.

In order to become ASSURED, molecular tests must be simplified. Whether through elimination of instrumentation, elimination of DNA extraction steps, minimisation of operator-involved steps or use of technology that shortens the runtime, these improvements will also serve to reduce inherent costs. The difficulty arises in maintaining high sensitivity/specificity in a reproducible manner. There are several approaches to enhancing the sensitivity of NAATs for low density infections.

1.4.3. Improving detection

1.4.3.1. Assayed volume

First, the volume of sample examined can be increased to maximise the chance of it containing parasite material. The WHO recommend the use of 5 µl blood minimum for NAATs with a limit of detection of 2 p/ µl max, which corresponds to the detection of 10 parasites in one reaction and thus a log fold improvement to microscopy (Steenkeste, Incardona *et al*. 2009). To maximise the amount of blood assayed, a prior concentration technique is normally needed.

The 'reference standard' for concentrating/purifying DNA is extraction using commercial kits- commonly Qiagen Blood and Tissue DNeasy kits, which are expensive and laborious (further discussed in ch6.1.2). These concentrate DNA ~4-fold (<200 µl blood results in >50 µl DNA) for whole blood samples, or directly (without concentration) extract DNA for dried blood spots (<50 µl blood for 50 µl DNA). Alternatively, a larger volume of venous blood (5 ml) is drawn from the patient and processed en masse (Hofmann, Mwingira *et al*. 2015). However, this is not ideal for the patient, it requires skill to draw blood and incurs costs associated with

storage (EDTA tubes and cold chain). The extraction kits have the additional effect of removing NAAT-inhibitory factors in the whole blood. Although more ASSURED methods exist (explored in Chapter 3), the simplest are direct-blood assays. No single technique has been widely validated and adopted. Labs instead prefer familiar methods that suit project budgets. It would be useful to standardise DNA extraction using an ASSURED method and compare studies globally.

1.4.3.2. Higher copy target

Until very recently, the 18s rRNA gene has been the mainstay of molecular testing for malaria, being highly conserved and having multiple copies within the genome (4- 5 depending on the species and strain). Sequencing of the *P. falciparum* genome in 2002 has since enabled the design of more complex assays, utilising parasite DNA from cultures. Many continuous culture cell lines exist for *P. falciparum* e.g. 3D7, FVO, NF54 and 7G8, facilitating research around this species. Unfortunately, the genome has a low GC content (19%), complicating the design of molecular assays which perform better with high GC% targets for stronger primer/probe interactions.

There are therefore limited suitable multi-copy DNA targets that are also suitable for the design of diagnostic NAAT for *P. falciparum*. Unlike *P. falciparum*, *P*. *vivax* has a relatively high GC content (45%), but the design of NAATS for *P. vivax* is limited by our inability to culture the species. Its elusive life cycle includes hypnozoite stages, complicating the maintenance of *P*. *vivax* in the laboratory and so no continuous cell lines are available. Thus, research on the unculturable species *P. vivax*, *P. ovale* and *P. malariae* is most often based on short-lived

primary cell cultures from clinical isolates. Alternatively, published genomes can be used to create synthetic DNA. Reliance on these methods has made fundamental research on these species very difficult, hindering the development of new drugs and diagnostics for these species.

Mitochondrial DNA targets such as the Cytochrome B gene- a single copy gene per mitochondrion (20-150 mitochondria/ parasite)- offer a higher number of targets (Hofmann, Gruenberg *et al*. 2018), however subtelomeric targets offer higher still (Hofmann, Antunes Moniz *et al*. 2019). The varATS and TARE-2 genes have 59 and ~250 copies/genome and so have been targeted for ultra-sensitive detection of *P. falciparum* (Murphy, Prentice *et al*. 2012). This assay has proven more sensitive than conventional qPCR in clinical trials (Murphy, Prentice *et al*. 2012, Taylor, Howell *et al*. 2014).

1.4.3.3. RNA targets

When conserved high-copy number genes are scarce, an alternative strategy is to target RNA transcripts rather than DNA. The higher number of copies increases the likelihood of detection in smaller sample volumes. Reverse-transcriptase PCR (rt-PCR) assays are currently one of the most sensitive assays for malaria detection (Table 1.3), however they traditionally require the addition of an RNA preservative to the sample, as well as cold chain supply for storage (-80 for long term) and an internal control. The RT-PCR for 18s rRNA developed by Murphy *et al.,* is currently the most sensitive, used to monitor vaccine trials (Taylor, Lanke *et al*. 2017). However, the 1000-fold decrease in limit of detection offered by RNA assays could present a new problem: over-sensitivity. The potential for cross-contamination between samples even at a

low level can produce false positives, and thus compromise specificity. This is a particular issue when extracting dried blood spots, as DNA can be transferred from the cutting device (usually a hole puncher) if not efficiently cleaned between samples (Cheng, Wang *et al*. 2015). To overcome this, Murphy *et al*. (2012) developed a custom-built laser cutting device to minimise handling of spots, only feasibly implemented in well-resourced settings. Although direct-blood lab-on-a-chip methods exist for RNA assays (McNamara, Kasehagen *et al*. 2006, Pett, Gonçalves *et al*. 2016) for high throughput screening the preservation and storage of RNA again becomes an issue.

1.4.3.4. Coupling technologies

Coupling technologies to mitigate the pitfalls of one test with the benefits of another is a common strategy to improve diagnostics. For example, Capture and Ligation probe PCR (CLIP-PCR) offers a high-throughput method for amplifying RNA using an overnight lysate step instead of prior extraction/additional reverse transcription steps. The closed system from sample application to qPCR minimises the risk of cross contamination, also only amplifying the signal rather than the target (Mens, van Amerongen *et al*. 2008).

PCR-Ligase Detection Reaction (PCR-LDR) is another example of coupling technology for accessibility. Originally requiring gel electorophoresis, it has since been adapted to a fluorescent microsphere assay (LDR-FMA) format using sequence specific microspheres (Luminex FlexMap probes). (Roth, de Bes *et al*. 2018) Again, this still requires gel electrophoresis and a fluorescence reader.

Due to the complex relationship of immunity, asymptomatic and sub-microscopic

infection, immunological assays have not been discussed extensively here. However, Enzyme Linked Immunosorbent Assays (ELISA) and Indirect Fluorescent Antibody Tests (IFAT) are widely used to measure antibodies against- or antigens produced by- specific life stages (sporozoite (CSP and SPZ), asexual (HRP2/LDH) and gametocytes (Pfs25) (Abramowitz 1996). Although sensitivity of these assays is better than microscopy/RDTs, they fare poorly in comparison to NAATs, especially with low-density infections. Their use is mainly restricted to research purposes as they are laborious, time-consuming and expensive. Nonetheless, the principles of these techniques can be coupled to NAATs as a simpler method for instrumentation-free reading.

Nucleic acid lateral flow immunoassays (NALFIA) which detect DNA-probe complexes using lateral flow devices (commercially available immunochromatographic cassettes or dipsticks [Abingdon Health, UK]) are a good example of this (Notomi, Okayama *et al*. 2000). In recent field evaluations in a low-transmission setting in Kenya, a direct-blood PCR-NALFIA (db-PCR-NALFIA) for Pan/ *falciparum* achieved good sensitivity and specificity (both ~85 %) in comparison to qPCR (Mallepaddi, Lai *et al*. 2018).

1.4.4 Isothermal NAATs

Loop-mediated isothermal Amplification (LAMP) offers an alternative method for the simple visualisation of amplicon. The amplification reaction produces insoluble magnesium pyrophosphate, which accumulates to the point where it is visible to the naked eye. The isothermal properties of LAMP also eliminate the need for thermocyclers, requiring only an inexpensive heat block. These assays bypass the conventional thermal denaturation step in a

PCR, instead utilising enzymatic initiation followed by cyclical amplification, known as 'auto-cycling displacement'.

This principle was first used in the Self-Sustained Sequence replication (3SR) method (Guatelli *et al.*, 1990), but was quickly applied to other techniques including Nucleic Acid Sequence-Based Amplification (NASBA) and Strand Displacement Amplification (SDA). Although these achieved rapid amplification of DNA, poor specificity was reported due to linear amplification regardless of the presence of template (Polley, Gonzalez *et al*. 2013).

In 2000, Notomi *et al*. developed (LAMP) to improve the specificity of these assays (Hopkins, Gonzalez *et al*. 2013). The new technique involved a complex design of 4 primers detecting 6 distinct targets within the same region of DNA. Several LAMP assays for malaria have since been evaluated in field trials (Schallig, Schoone *et al*. 2003, Poon, Wong *et al*. 2006, Unitaid 2018, Hsiang, Ntshalintshali *et al*. 2019), with several commercially available (Schneider, Wolters *et al*. 2005). The first and most thoroughly evaluated LAMP assay, the Eiken LoopAMP kit, detects the *Plasmodium* genus and, separately, *P. falciparum* species (more detail in Chapter 2). Briefly, studies have reported good sensitivity and specificity of this kit across the 4 main malaria species. This has led to the development of the 'PURE-DNA' extraction kit (Eiken, Japan) to accompany the LoopAMP. Several other LAMP assays use similar heating protocols (Heated blood LAMP) (Pett, Gonçalves *et al*. 2016) or dilution methods (Illumigene, Meridian) in place of expensive DNA extraction kits, which are normally the main bottleneck in molecular testing.

With increased availability of genomic data and advances in technology, quantitative NASBA (QT-NASBA) assays were developed for the detection of DNA from the 4 main species of *Plasmodia* (Mens, Spieker *et al*. 2007, Pett, Gonçalves *et al*. 2016) as well as RNA transcripts for *P. falciparum* gametocytes (Kersting, Rausch *et al*. 2014). These assays will be especially useful for monitoring the dynamics of infective and non-infective stages in response to control strategies or in elimination settings. Although highly sensitive and specific, the intra- and inter-assay variability of QT-NASBA is higher than in qPCR (Li, Kumar *et al*. 2013, Ghindilis, Chesnokov *et al*. 2019).

Recombinase Polymerase Amplification (RPA) (Kersting, Rausch *et al*. 2014), Tandem Oligonucleotide Repeat Cascade Amplification; TORCA (Reboud, Xu *et al*. 2019) and thermophilic Helicase-dependent amplification (tHDA) (Mallepaddi, Lai *et al*. 2018) also offer highly sensitive iNAATs for malaria detection. Most of these however require a colorimeter, fluorometer or LFD coupling for visualisation. Although in the early stages of development/ validation coupling of iNAATs to LFDs e.g. Lateral flow-RPA (Reboud, Xu *et al*. 2019) and LF-LAMP (Crannell, Rohrman *et al*. 2014, Kersting, Rausch *et al*. 2014) has simplified these tests further, and with good sensitivity thus far (98% sensitivity for LF-LAMP in Uganda, and LOD 4-20 parasites/ µl for LF-RPA) (Cordray and Richards-Kortum 2012, Quan, Sauzade *et al*. 2018). RPA is additionally useful as it runs at body temperature (~37 °C) eliminating the requirement for a heat block (Imai, Tarumoto *et al*. 2017). Table 1.3 summarises the characteristics of each of the major molecular techniques.

Table 1.3. Comparison of diagnostic techniques for malaria.

Table source: adapted from Cordray *et al.* (Imai, Tarumoto *et al*. 2018).

Index Test	LOD (p/ µl)	Time	Cost/test ($)	Sens (%)	Spec (%)	Reference test
Microscopy	50	20 min/slide	0.2	97	100	Microscopy
RDT	>100	20 min/slide	0.45- 1.4	100	93	Microscopy
PCR	< 5	1 hr	1.5- 4	100	90	PCR
rt-PCR	0.1- 10	1 hr	4- 5	91	96	Microscopy
PCR-LDA	0.3- 10	1 hr	0.3*	99	94	Microscopy
PCR-ELISA	< 30	6 hr	0.3*	91	96	PCR
LAMP	0.2-5	30 min- 2 hr	0.4- 0.7	76- 99	90-100	PCR/Microscopy
NASBA	0.01-0.1	60 min	5-20	100	86	Microscopy
NALFIA	0.3- 3	1- 1.5 hr	na	98	99	PCR

RDT: rapid diagnostic test; PCR: polymerase chain reaction; RT: reverse transcription; LDA: lactate dehydrogenase assay; ELISA: enzyme-linked immunosorbent assay; LAMP: loop-mediated isothermal amplification; NASBA: nucleic acid sequence–based amplification; NALFIA: nucleic acid lateral flow immunoassay, LOD: Limit of detection, na: not available.

1.4.5. Novel platforms

Digital droplet PCR (ddPCR) is a new quantitative PCR technique that eliminates the need for a standard curve. It achieves sensitive detection through measuring amplification within single droplets (~15,000 droplets total), giving highly accurate and reproducible results. Most laboratories use their in-house standard curves for qPCR, either using synchronised cultures or synthetic plasmid DNA for non-*falciparum* species, rather than a universal reference standard curve. These ddPCR techniques may help to make data more comparable across the globe. Although the instrumentation incurs significant upfront costs (~$100K) compared to qPCR ($20-

30K), the principle of droplet amplification could possibly be applied to the other more ASSURED amplification techniques. (Hayashida, Orba *et al*. 2019)

The capacity to detect and sequence amplicon would be useful for confirming the presence and species, genotyping drug-resistance markers to influence treatment and tracking lineage for monitoring. Currently, sequencing of malaria is largely restricted to HIC due to the costly instrumentation and complexity of operation. Imai *et al.* (2018), have coupled LAMP with the portable MinION sequencer (Nanopore, Oxford) in a bid to track the C580Y mutation associated with artemisinin resistance in *P. falciparum* (Al-Shehri, Power *et al*. 2019, Land, Boeras *et al*. 2019). However, the method currently uses expensive and laborious extraction methods and interpretation of the MinION data requires some skill. Simplification of sample pre-processing in a similar system has been demonstrated for Chikungunya (direct-blood LAMP-MinIon), but not for malaria (Ghayour Najafabadi, Oormazdi *et al*. 2014). With further simplification of the back-end analyses of sequence data, this platform could offer a useful, portable tool for sequencing in the low-resource settings.

1.4.6. Non-invasive samples

The 'holy grail' of malaria diagnostics would ultimately use non-invasive samples for diagnosis. So much so that the ASSURED has been updated to REASSURED to include *real-time connectivity* (e.g. mobile phone readers) and ease of use (non-invasive samples) (Ghayour Najafabadi, Oormazdi *et al*. 2014). This has patient benefits, obviates the need for skilled personnel and reduces costs. Historically, the sensitivity of urine (60-70 %) and saliva (70-83 %) assays have fared poorly in comparison to tests using blood from symptomatic patients

(Ghayour Najafabadi, Oormazdi *et al*. 2014, Ghayour Najafabadi, Oormazdi *et al*. 2014). A recent study reported similar sensitivity (73%) when using stool sample, providing opportunity to screen other parasites e.g. helminths simultaneously (Correa, Coronado *et al*. 2017, Al-Shehri, Power *et al*. 2019). The new approaches to improving NAATs discussed in this Chapter could enhance these non-invasive molecular assays. Initial studies have shown that nPCR targeting a higher copy gene (e.g. CytB) improves the sensitivity of saliva and urine based tests (Berna, McCarthy *et al*. 2015). Conversely targeting higher copy genes in a new LAMP assay (18s rRNA) did not improve sensitivity in comparison to qPCR methods (Kelly, Su *et al*. 2015).

Research on fingerprinting of *Plasmodium* volatiles has been explored primarily with regards to odour attractants for mosquito control, however interest in their use for malaria diagnostics is growing. (Guest, Pinder *et al*. 2019, Heraud, Chatchawal *et al*. 2019, Maia, Kapulu *et al*. 2019). These studies still largely rely on complex mass spectrometry techniques, but detection methods are being simplified. This year it was reported that well-trained dogs have the capability to identify malarial volatiles. It has been proposed that these dogs could assist in rapid screening at border crossings where imported malaria is a significant risk (Mwanga, Minja *et al*. 2019). More portable infra-red devices are being trialled for chemically fingerprinting malaria infections (Karl, Gutiérrez *et al*. 2011, Saha, Karmakar *et al*. 2012, Karl, Laman *et al*. 2015).

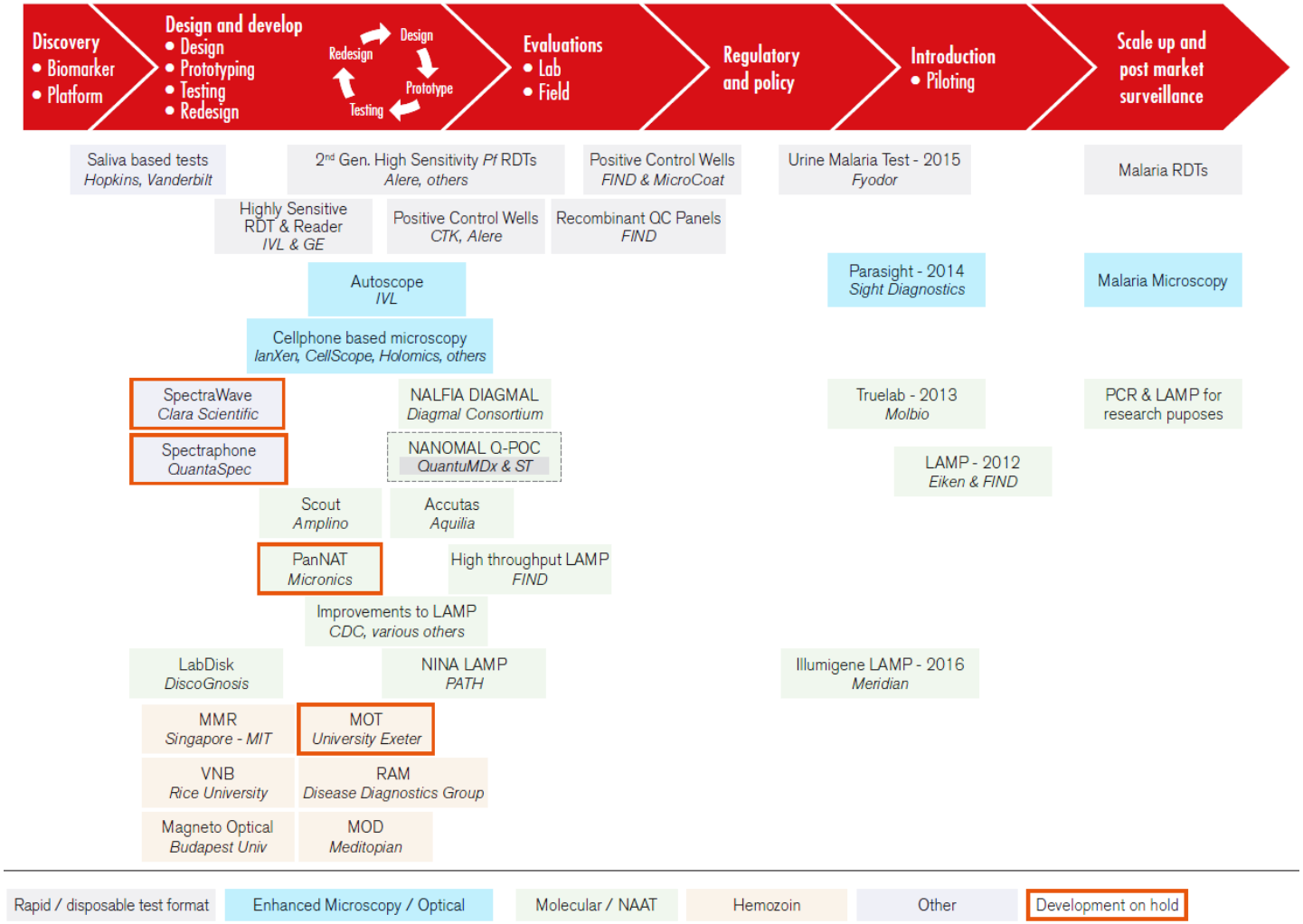
Finally, new techniques exploiting the unique physiology of infected red blood cells (iRBC) also offer an alternative strategy for non-invasive malaria diagnosis that have almost zero running costs. Generally, tests detecting the electromagnetic properties of hemozoin produced by the parasite, have been more successful than detection of deformability of

infected RBCs (Newman, Matelon *et al*. 2010, Fook Kong, Ye *et al*. 2015, Karl, Mueller *et al*. 2015, Kasetsirikul, Buranapong *et al*. 2016, McBirney, Chen *et al*. 2018, Heraud, Chatchawal *et al*. 2019, Thamarath, Xiong *et al*. 2019). It has been demonstrated that the magnetically-induced spin of parasite-derived hemozoin crystals in whole blood, is measurable using inexpensive and portable magnetic resonance devices, although larger clinical evaluation studies are needed (WHO 2016).

Raman spectroscopy of malaria parasites in blood is another emerging field for non-invasive malaria diagnostics. A large clinical trial in Thailand using handheld spectrometers for malaria diagnosis, proved to be highly sensitive (98%) amongst symptomatic patients in Thailand (Chua, Lim *et al*. 2015). These technologies and their stage in the diagnostic pipeline are summarised in figure 1.7 (Cordray and Richards-Kortum 2012).

Figure 1.7. The pipeline of emerging malaria diagnostics.

Image taken from: UnitAid report (Unitaid 2018)



1.5. Aims and objectives

As areas of low malaria transmission approach elimination, low density asymptomatic infections prevail. To end transmission these infections must be identified and treated. There is an urgent need for sensitive diagnostic tools for malaria that are better suited to low-resource settings.

This thesis aims to 1) explore new diagnostics suitable for molecular surveillance and 2) evaluate new POC tests for use-scenarios where molecular testing is simply not feasible.

The specific objectives are to:

1) Assess the suitability of high-throughput molecular assays for differentiating *Plasmodium* species (Chapter 2). This objective addresses the need for robust malaria surveillance in areas nearing elimination. In this scenario, the most sensitive assays (molecular tests) are needed to detect low-density infection as early as possible.

2) Develop a new molecular test for malaria control on a portable, field-friendly platform (Chapter 3). Simplification of complex molecular assays will facilitate the implementation of these more sensitive tests, for use in lower-resource settings where they are critically needed.

3) Characterise the diagnostic performance of a new RDT for detecting low density malaria during pregnancy in low and high transmission settings (Chapter 4-6). Whilst accessibility of molecular testing is improving, RDTs are still a necessary tool for low-cost and rapid diagnosis of malaria. With recent post-market evaluations reporting varying results in uRDT performance- depending on the transmission setting, presence of symptoms and population studied- we evaluate the uRDT in both Asian and African contexts, amongst a particularly vulnerable and understudied population: pregnant women.

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Chapter 2. Molecular detection and differentiation of *Plasmodium* species

Contents

2.0. Declarations

Provision of samples

DNA from clinical samples provided by Dr Jayne Jones (LSTM), previously underwent species confirmation by microscopy carried out by her laboratory. Likewise control DNA obtained from 3D7 cultures was provided by Dr Grazia Carmada (LSTM). DNA controls for malaria species were provided by Dr Debbie Nolder (LSHTM/ PHE).

STOP-MiP blood samples were collected by staff outlined in the STOP-MiP trial protocol. DNA was extracted from these samples by staff at the Eijkman Institute of Molecular Biology (EIMB), who also carried out the LAMP testing at EIMB, Jakarta. DNA samples were transported to the UK for further laboratory testing described below

Laboratory work

I carried out 100% of the HRMCA laboratory testing. I performed 50% of the Duplex testing of the STOP-MiP DNA samples, the other 50% was performed by Mrs Sylvia Sance Marantina from the EIMB whom I trained in the assay during her visit to LSTM.

Building on Ms Rachel Byrne's BSc work (LSTM/ University of Liverpool) optimising a mono-plex qPCR for *P. falciparum*, I further optimised and expanded this to develop the 4-plex qPCR described in this Chapter. Mr Chris Williams (LSTM) provided technical assistance in the determination of the 4-plex LOD and carried out some (33%) of the screening of the STOP-MiP samples by 4-plex qPCR. Mr Chris Willliams and Ms Nadia Kontogianni also provided technical assistance in screening discordant results by nPCR (33%). I carried out the rest of the 4-plex and nPCR screening.

Analyses

I performed 100% of the analyses in this Chapter.

Idea conception, intelligent and experimental design

Dr Emily Adams (ERA) significantly contributed to the intelligent and experimental design of the work in this Chapter. ERA, Professor Feiko Ter Kuile and Dr Rukhsana Ahmed, were also essential in facilitating and coordinating the collaboration between LSTM and the Eijkman Institute of Molecular Biology for this work.

2.1.1. Chapter summary

In this Chapter different molecular methods were investigated for their potential as reference tests for future evaluations of field-ready malaria diagnostics. Following poor performance of two established tests (HRMCA and Duplex assay), a new 4-plex qPCR was developed utilising different prime/probe sets from published assays. The 4-plex qPCR was implemented in a clinical trial, forming a part of the composite reference test for diagnosis of malaria. This resulted in the publication outlined in section 2.8.

2.1.2. Abstract

Background

For case management of malaria, it is important to determine the causative species of a malaria infection as it can influence treatment decisions. Diagnostic tests that differentiate species are also crucial for malaria surveillance and monitoring dynamics of species composition in response to interventions. For this, more sensitive diagnostic tests are needed. This Chapter investigates three molecular tests suited for malaria surveillance and for use as reference standards in diagnostic evaluations.

Methods

Three molecular assays: a high-resolution melt curve analysis assay for five species of malaria (*P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*), a new 4-plex qPCR (excluding *P. knowlesi*) and a reference rt-PCR assay 'Duplex' (*P. falciparum* and *P. vivax/ ovale/ malariae*), were implemented and the limit of detection (LOD) quantified using *P. falciparum* cultures. A

pilot study used clinical samples diagnosed with malaria (by microscopy) to validate the HRMCA. The 4-plex qPCR was validated using samples from a clinical trial in Indonesia. LAMP was used as a reference test, with nested PCR as a gold standard.

Results

The HRMCA and Duplex assays had an LOD of 2-20 parasites/ µl, and the qPCR had an LOD of 0.2-2 p/ µl. The qPCR had poor agreement with the LAMP, potentially due to poor specificity of LAMP, or trace contamination, nPCR confirmed the presence of many LAMP false positives.

Conclusion

This Chapter highlights some of the challenges associated with evaluating new diagnostics that rival the sensitivity of the existing gold standard test. Different strategies could be considered to overcome these challenges, depending on the use-scenario for the diagnostic.

2.2. Introduction

There is a need for more sensitive diagnostics for detecting low-density malaria. In this Chapter we assess the accuracy of molecular assays that offer cost-savings, a simple work flow and/or differentiation of multiple *Plasmodium* species identification to replace the cumbersome nested PCR that is currently the gold standard.

2.2.1. HRMCA

First, a high-resolution melt curve analysis (HRMCA) (Shokoples, Ndao *et al*. 2009) was assessed using control samples of DNA confirmed by microscopy and qPCR (Shokoples, Ndao *et al*. 2009). Dissociation analysis or "Melt curve analysis" (MCA) is a cheaper alternative to probe-based multiplex qPCR making it attractive to low-resource settings. HRM primers are designed to produce amplicons that dissociate from fluorescent probes at distinct temperatures: coined melting temperatures (Tm). The rate of this dissociation is calculated as the change in fluorescence over time, producing a unique melt curve, which peaks in fluorescence at the characteristic Tm.

The Tm is determined by factors such as the sequence of nucleotides as well the base composition and length of the amplicon sequence. A long amplicon with a high ratio of pyrimidines (guanine or cytosine) to purines (adenosine or cytosine) a high "GC" content, requires more energy to denature, thus have a higher Tm.

The shorter the sequence, the fewer bonds to break and therefore a lower Tm is

observed. Likewise, nucleotide stacking plays a role in Tm, a pyrimidine followed by a purine (Y/R) increases the Tm in comparison to a purine/purine R/R.

MCAs can be designed without specific- probes, instead using non-specific intercalating dyes such as SYBR™ Green which bind most dsDNA. This lowers the cost of the assay as sequence-specific probes are expensive to manufacture, however it can compromise specificity if probes bind to primer dimers and any other dsDNA present in the reaction.

Multiplex assays for species ID are also feasible using MCA- if the target gene sequence is sufficiently different between species, yet conserved within the primer binding sites. This enables the use of only one primer set for the identification of multiples species.

Although High-resolution melt (HRM) curve analysis (HRMCA) assays can be designed to work with general intercalating dyes such as SYBR, new assays utilise more specialised HRM dyes that bind dsDNA e.g. EvaGreen or SYTO 9. These dyes are less toxic, functional across a broader range of concentrations and are less selective in the amplicons they bind to. The combination of being able to use a higher concentration and the higher saturation of dsDNA with the dye enhances the resolution of the fluorescence peaks produced. This enables targets with more similar melting temperatures to be included, potentially allowing more targets to be multiplexed within a single assay.

Most recently Chua *et al.* (2015) demonstrated the application of this technology for identification of the five main species of human malaria: *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi.* They designed a single set of primers targeting a highly conserved region of the 18S SSU rRNA gene in all five species, but with a highly variable region between primer binding sites to produce amplicons with distinct melting temperatures. With a lower

LOD of 1-100 copy numbers and runtime of under 2 hrs, their assay showed 100% concordance with PlasmoNex(TM)- a hexaplex PCR system that detects six species. Their method utilises differential MCA (dMCA) to more accurately distinguish between similar melt curves.

This high throughput method, multiple target design and lack of need for specific probes, reduces the cost per sample making it an attractive diagnostic tool for reference laboratories. In this Chapter the aim was to validate this assay, using stored, clinical samples from travellers returning to the UK.

2.2.2. Introduction to RT-PCR

Real-time PCR techniques are currently the most sensitive tests for detecting malaria (Rougemont, Van Saanen *et al*. 2004). The 18Ss rRNA gene (DNA) is most commonly targeted for molecular detection of malaria. Hydrolysis probes utilise the hydrolysing activity of polymerases (e.g. TaqMan- an enzyme derived from *Thermoaquaticus spp.* that is active at high temperature) to release a quencher from the donor dye (fluorophore) on the probe, allowing it to fluoresce. Although RNA-based rt-PCRs (Cheruiyot, Auschwitz *et al*. 2016) offer higher sensitivity than DNA-based ones, they are problematic in other ways (instability of RNA and potential for cross-contamination (see Ch1.4.3.3).

Multiplexing probes has enabled sensitive detection of multiple species of *Plasmodium* (Bass, Nikou *et al*. 2008, Meerstein-Kessel, Andolina *et al*. 2018), as well as identification of gametocytes (Bass, Nikou *et al*. 2010). However, hydrolysis probes are expensive, and these assays require a single probe per species. It is therefore more economical to use as fewer

probes as possible. A commonly used assay for xenomonitoring is published by Bass *et al*. (Ahmed, Levy *et al*. 2015) for the simultaneous detection of *P. falciparum* and *P. ovale/P. vivax/ P. malariae (Povm)* 18S DNA. This assay uses just two primer/ probe sets to make this distinction, reducing the cost of the assay. Given the good reported sensitivity (Chua, Lim *et al*. 2015, HTA 2015) we determined the LOD of this assay in our laboratory to assess its suitability for low-density malaria surveillance.

In addition to this a multiplex qPCR with a lower LOD was optimised for detecting asymptomatic, low density malaria. The qPCR was used in a Stop Malaria in Pregnancy (STOP-MiP) trial as part of a composite molecular test (microscopy, RDT, qPCR, LAMP and nPCR) for detection of malaria (Bass, Nikou *et al*. 2008). In this Chapter I assess the performance of the qPCR using the LoopAMP kit (Eiken, Japan) as a reference test, and nPCR to reconcile discordant results.

2.3. Methods

2.3.1. HRMCA

2.3.1.1. Control DNA

DNA was extracted from *P. falciparum* sorbitol-synchronised 3D7 trophozoite cultures. To estimate the number of 18S rRNA copies present, the DNA was quantified using a Qubit kit (ThermoFisher Scientific, UK) and the concentration was converted to number of genomes (22MB size). DNA from non-*falciparum Plasmodium* species were provided by Public Health England (courtesy of Dr Debbie Nolder, LSHTM). Parasite load was not quantified by in these samples.

2.3.1.2. Clinical samples

Stored DNA samples collected from travellers returning to the UK from abroad between 2002-2011 were used to validate the HRMCA. These samples were DNA extracted (n=12) from whole blood samples was provided by LSTM 's Clinical Parasitology Diagnostics laboratory (courtesy of Dr Jayne Jones). Samples that were positive for any *Plasmodium* species (by microscopy) and conveniently available within the diagnostic archives were selected for investigation.

2.3.1.3. HRMCA index test

The RT-PCR-HRM reaction mixture was identical to those described by Chua *et al.* (Ahmed, Levy *et al*. 2015), except Type-it HRM master mix (Qiagen, Germany) was used in place of MeltDoctor® HRM master mix. Briefly, 20 µl reactions contained 2x Type-it HRM master mix (Qiagen, cat#206542), 0.1 µM of each primer (forward primer 5'-GRAACTSSSAACGGCTCATT-3' and reverse primer 5' -ACTCGATTGATACACACTA-3') (Eurofins) and 1 µl of DNA. Thermal cycling conditions were identical to those published, except we lowered the annealing temperature

after optimisation on the RotorGene-Q 5plex HRM platform (Qiagen, Germany) as follows: initialisation at 95 °C for 10 min, 95 °C for 15 sec and 58°C for 1 minute (40 cycles), followed by a final melt program gradually increasing the temperature (0.1 °C/ sec) from 60°C until 95 °C. DNA controls were performed duplicate, whilst validation test samples were in singlets. Melting curve plots were generated and analysed using the Rotor-Gene Q software.

2.3.1.4. Analytical sensitivity and specificity

Ten-fold serial dilutions of control DNA were used to estimate the limit of detection (LOD) for *P. falciparum* only. This could not be quantified for non-*falciparum* species as they were sourced from clinical isolates (see above).

2.3.1.5. Ethics

Clinical samples were received by the Clinical Parasitology Diagnostic Laboratory (LSTM) for diagnosis of malaria. Following clinical diagnosis, the samples were used to optimise and evaluate alternative malaria diagnostics. It was not feasible nor practical to gain consent for samples stored in a diagnostic archive, however small volumes of unconsented sample may occasionally be used for diagnostic validation for use within the HTA-licensed establishment i.e. the CPDL (Ahmed, Levy *et al*. 2015).

2.3.2 Duplex assay

2.3.2.1. Control DNA

The LOD for detection of P. *falciparum* was estimated using serial dilutions of quantified DNA obtained from control DNA (as in section 2.3.1).

2.3.2.1. Duplex index test

The primers, probes, and cycling conditions used here were identical to those used by Bass *et al.* (Ahmed, Poespoprodjo *et al*. 2019): initial hold at 95°C for 10 min, 40 cycles at 95°C for 10 sec and 60°C for 45 sec. the reaction mix (20 µl total volume) contained: 2x SensiMix (Bioline, QT615-05), 1 µl DNA, 800 nM of each primer and 300 nM of *P. falciparum* probe and 200 nM of the probe for *P. ovale/ vivax/ malaria*. Primers/ probes sequences were as follows: *P. falciparum* probe (5'FAM-TCTGAATACGAATGTC-MGB 3'), *P. ovale/ vivax/ malaria* probe (5'VIC-CTGAATACAAATGCC-MGB 3'), forward primer (5'-GCTTAGTTACGATTAATAGGAGTAGCTTG-3') and reverse (5'- GAAAATCTAAGAATTTCACCTCTGACA-3'), obtained from (ThermoFisher Scientific, UK). Real-time fluorescence was measured using the Mx3000P (Agilent, UK) and the end-point fluorescence was analysed using MxPro QPCR software.

2.3.3. 4-plex qPCR

2.3.3.1. Samples

The control samples used are outlined as above (section 2.3.1).

Clinical samples

Blood samples were collected from pregnant women in Timika and Sumba, Indonesia between 2013-2016 as part of the STOP-MiP (Stop malaria in pregnancy) trial (ISRCTN: 34010937). Briefly, Timika is a coastal region in the Mimika district of West Papua. The area has moderate (15% prevalence (Kamau, Alemayehu *et al*. 2013)) year-round transmission, with all four main species of *Plasmodium* prevalent. The second site Sumba island is co-endemic for *P. vivax* and *P. falciparum* with year-round low transmission (Alemayehu, Melaku *et al*. 2015) (further detail

in Chapter 3 methods). During the trial, venous bloods (4 ml) were obtained at enrolment, antenatal visits and at delivery, where placental and cord bloods were also obtained where possible (Kamau, Alemayehu *et al*. 2013). Dried blood spots were prepared using 50 µl of blood and then stored at room temperature with silica in zip lock bags.

2.3.3.2. DNA extraction

DNA extractions using dried blood spots (DBS) were carried out at the Eijkman Institute of Molecular Biology (EIMB), Jakarta, using the Chelex method. Briefly, 6 mm filter paper (Whatman 3MM) punches were incubated on 0.5 % saponin overnight, before centrifugation and removal of the supernatant. Following rigorous PBS washing, the DBS was heated in 20 % Chelex 100-Ion Exchanger for 10 min (Biorad Laboratories, CA) at 100 °C, and the remaining supernatant stored at -20 °C.

2.3.3.3. LAMP reference test

LAMP testing was also performed at the EIMB throughout the trial. LAMP assays were carried out using the LoopAMP™ Malaria Pan Detection Kit (Eiken Chemical Company, Japan). Briefly, the LAMP reagents were reconstituted in 15 µl water/ tube and left to stand for 2 mins, before adding 15 µl of extracted DNA as recommended by FIND (personal communication with Dr Xavier Ding). Samples were incubated for 40 min at 65 °C and then 5 min at 80 °C. Results were interpreted under a UV light, where a green colour change indicated a positive result. At the end of the trial- and following LAMP testing- DNA was transported to LSTM, UK for subsequent qPCR and nPCR testing for quality control.

2.3.3.4. qPCR

For qPCR, primers and probes published by Kamau *et al.* (2013) for *P. falciparum* and *P. vivax* identification were used (Shokoples, Ndao *et al*. 2009). These were chosen having recently been identified as most sensitive in a review of molecular methods for malaria (Singh, Bobogare *et al*. 1999, Shokoples, Ndao *et al*. 2009). The threshold of this duplex assay was tested in combination with primers and probes for Pan-*Plasmodium* , *P. ovale* and *P. malariae* species (previously published by Shokoples *et al. (Singh, Bobogare et al. 1999)*). This 4-plex qPCR assay was developed for the detection of *Pf*, *Po*, *Pv* and *Pm*. For this, qPCR reactions (10 µl total volume) contained: 2 µl DNA, QuantiFast Pathogen PCR Mix (Qiagen) and primers and probes as outlined in Chapter 3 (Table 4.1). Thermocycling conditions were as follows: 10 min at 95 °C, 15 sec for 95 °C and 60 sec for 60 °C. Fluorescence was acquired using the Rotor-Gene Q 5plex HRM Platform (Qiagen, Germany) and cycling threshold (Ct) values (with a cut-off of 38 cycles) were calculated using the Rotor-Gene Q series software version 1.7 (Qiagen).

2.3.3.5. Nested PCR *(nPCR)*

The nPCR was performed on qPCR/LAMP discordant samples, using primers and cycling conditions described in Singh *et al.*(Ahmed, Levy *et al*. 2015) The nPCR was used as the gold standard and each sample tested in triplicate whereby a single positive result determined the sample as positive for *Plasmodium spp.* The LOD of this assay was ~6 parasites/ µl (Ahmed, Poespoprodjo *et al*. 2019).

All molecular testing was carried out by operators who were blinded to the clinical information of the participants.

2.3.3.6. Statistics

Diagnostic performance indicators (sensitivity, specificity, negative predictive value, positive predictive value, diagnostic odds ratio, kappa value) were estimated using SPSS software and the Varstats Clinical Calculator (Chua, Lim *et al*.). Kappa values <0.2 were considered a poor agreement, 0.21-0.40 fair, 0.41-0.6 moderate, 0.61-0.8 very good (Bousema, Okell *et al*. 2014). The difference in proportions (detection of positives and negatives) was estimated using McNemars fishers exact test in SPSS software v26. The sample size here supports diagnosis of participants in the STOP-MiP trial rather than for diagnostic evaluation purposes, consequently the study in validation described here, consequently the 95% confidence intervals are used here to indicate confidence in data. Post-hoc analyses shows both studies in Timika and Sumba are well powered (P= 80% >) to estimate sensitivity and specificity.

2.3.3.7. Ethics

As above, ethical approval was granted under the STOP-MiP trial (Kamau, Alemayehu *et al*. 2013) by the LSTM, UK, the Eijkman Institute for Molecular Biology, and the National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia. Written informed consent was obtained from all participants.

2.4. Results

2.4.1. HRMCA Results

2.4.1.1. Analytical performance

HRMCA showed single peaks at an average melting temperature of 74.1°C for *P. malariae*, 74.3°C for *P. falciparum* and 75.6°C for *P. knowlesi* control DNA. Two consistent peaks were observed for *P. ovale* at 76.5°C and 77.25 °C. *P. vivax* also produced two peaks at 76.8 °C with a lesser peak at 77.6 °C. One replicate of the P. *vivax* sample produced one peak at 74.3°C and another at 76.8°C. (Fig 2.1.1A) However, the dMCA showed that these *P. vivax* samples were more similar to the other *P. vivax* samples than to the *P. falciparum* samples (fig 2.1.1B). The differential analysis of the other samples also showed clear grouping within species. (Fig 2.1.1)

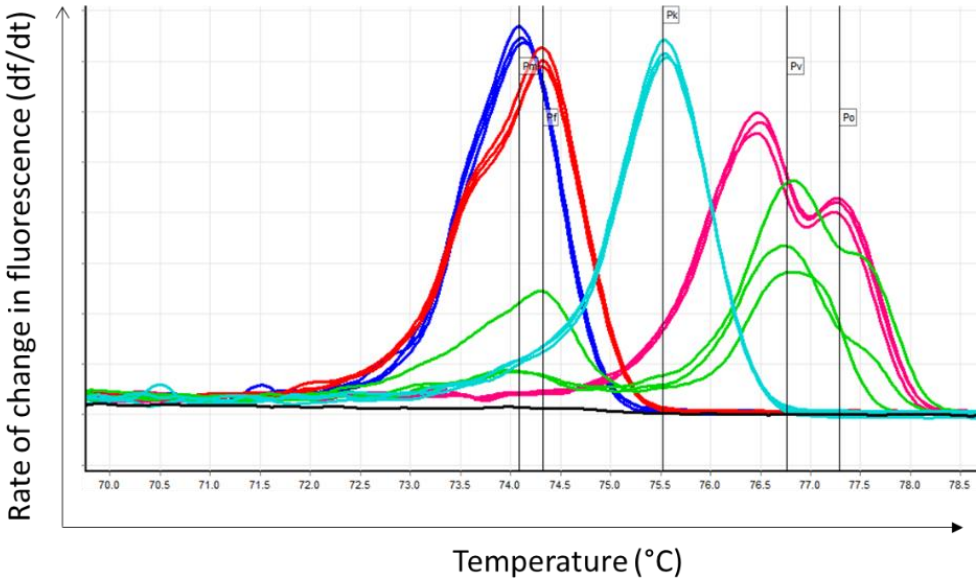
To investigate the effect of DNA concentration, we analysed 10-fold serial dilutions of *P. falciparum* (Table 2.1.1) and non-*falciparum* species (Table 2.1.2). The LOD of the HRMCA for *P. falciparum* was determined as 10 copies/ µl (Table 2.1.1). Generally, the detection of non-*falciparum* species was unreliable below a dilution of 1 in 10, except for *P. knowlesi (only run in* singlet due to low sample volume). (Table 2.1.2)

2.4.1.2. Clinical validation

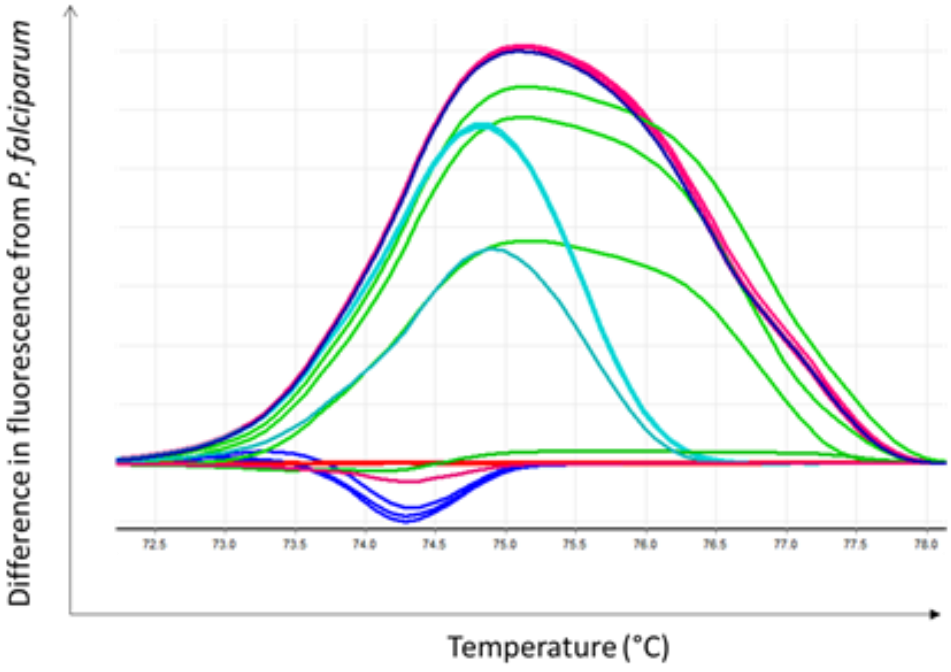
We tested DNA extracted from 12 clinical samples confirmed by microscopy: 5x *P. ovale*, 3x *P. vivax*, 2x *P. malariae* and 2x *P. falciparum*. The dMCA correctly identified 50% of these samples: all 3 *P. falciparum* samples and one *P. vivax* sample. The other samples were too significantly different from any of the controls for a call to be made. (Figure 2.1.2)

Figure 2.1.1. HRMCA of 5 species of *Plasmodium*.

A. MCA and B. dMCA (each line represents one technical replicate) for *P. falciparum* (red), *P. vivax* (green), *P. malariae* (blue), *P. ovale* (pink) and *P. knowlesi* (aqua).



A.



B.

Figure 2.1.2. Differential MCA of clinical samples.

Samples included *P. ovale* (pink, n=5), *P. vivax* (green, n=3), *P. malariae* (blue, n=2) and *P. falciparum* (red, n=2), with a positive *P. falciparum* DNA control (black, n=1) for differential analysis (normalised fluorescence minus normalised fluorescence of the *P. falciparum* control.

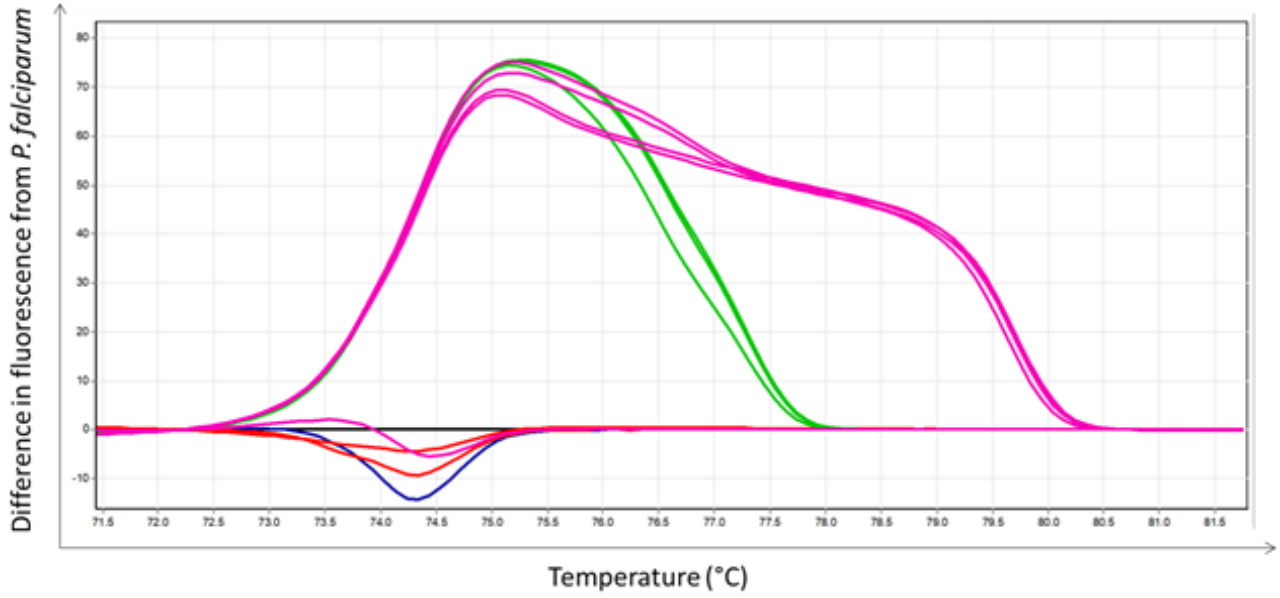


Table 2.1.1. HRMCA copy number limit of detection for *P. falciparum*.

Target Copies	HRMCA call	Mean[Z] Tm °C	Confidence (%)
1000	*P. falciparum*	74.3	98.5
100	*P. falciparum*	74.4	93.8
10	*P. falciparum*	74.3	94.6
1	Unknown	74.4	46.5
0.1	Unknown	74.4	-
0.01	Unknown	74.4	-

[Z] Technical duplicates and *Confidence as calculated by the Rotor- Gene Q HRM software

Table 2.1.2. HRMCA limit of detection for non- *falciparum* species.

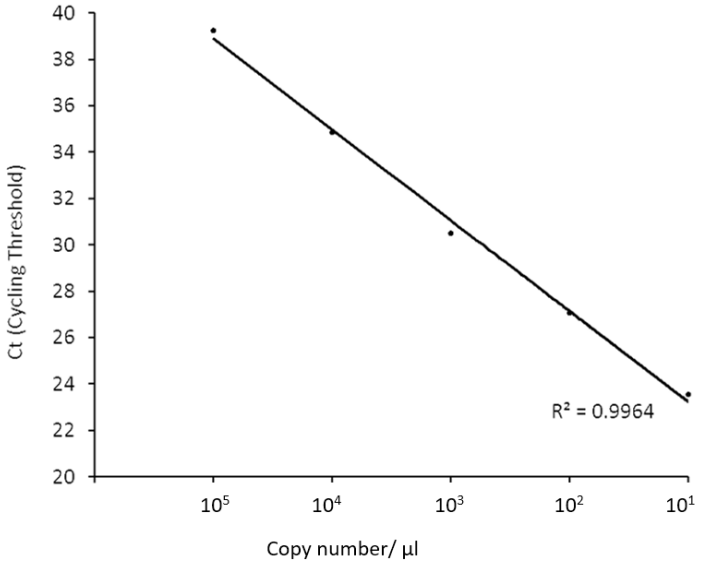
Species	Dilution[ŧ]	HRMCA call	Confidence (%) *
P. vivax	1:10	*P. vivax*	82.8
	1:10	*P. vivax*	82.8
	1:100	Unknown	-
	1:100	Unknown	-
	1:1000	Unknown	-
	1:1000	Unknown	-
P. malariae	1:10	*P. malariae*	91.07
	1:10	*P. malariae*	91.07
	1:100	Unknown	-
	1:100	Unknown	-
	1:1000	Unknown	-
	1:1000	Unknown	-
P. ovale	1:10	*P. ovale*	99.94
	1:10	*P. ovale*	99.94
	1:100	Unknown	-
	1:100	*P. ovale*	-
	1:1000	Unknown	-
	1:1000	Unknown	-
P. knowlesi	1:10	*P. knowlesi*	100
	1:100	*P. knowlesi*	95.51
	1:1000	Unknown	-

[ŧ] All species except *P. knowlesi* were run in technical duplicates and *Confidence as calculated by the Rotor- Gene Q HRM software

2.4.2. Duplex results

The LOD for the detection of P. *falciparum* was estimated using serial dilutions of quantified DNA obtained from cultures. The LOD was determined as 100 copies/ µl (equivalent to approximately 20 parasites/ µl), as only 1 of 2 replicates was detected (Ct 39.23) at 10 copies/ µl (2 p/ µl) and no amplification was observed below this. (Figure 2.2.1)

Figure 2.2.1. Duplex standard curve of *P. falciparum* 3D7 DNA serial dilutions.
Each data point represents the mean of technical duplicates, with a line of best fit.



2.4.3. 4-plex qPCR results

The LOD of *P. falciparum* species in the 4-plex qPCR assay was determined by testing serial dilutions of DNA (10^0- 10^4 target copies/ µl) extracted from 3D7 cultures. There was a strong correlation (R^2=0.997) between copy number and Ct value. (Figure 2.3.1) The highest level of variation was observed at 10^0 copies/ µl (95% CI Ct ± 2.5). The LOD of non-*falciparum* species could not be quantified, however a linear trend of increasing Ct with dilution was observed for all species (fig 2.3.2).

Figure 2.3.1. The 4-plex qPCR limit of detection for P. *falciparum* species.

Ten-fold serial dilutions P. *falciparum* DNA was tested (n= 12 replicates) by 4-plex qPCR. Each dot represents a replicate (n=12), bars represent mean± 95% and dashed line depicts the Ct cut-off (38).

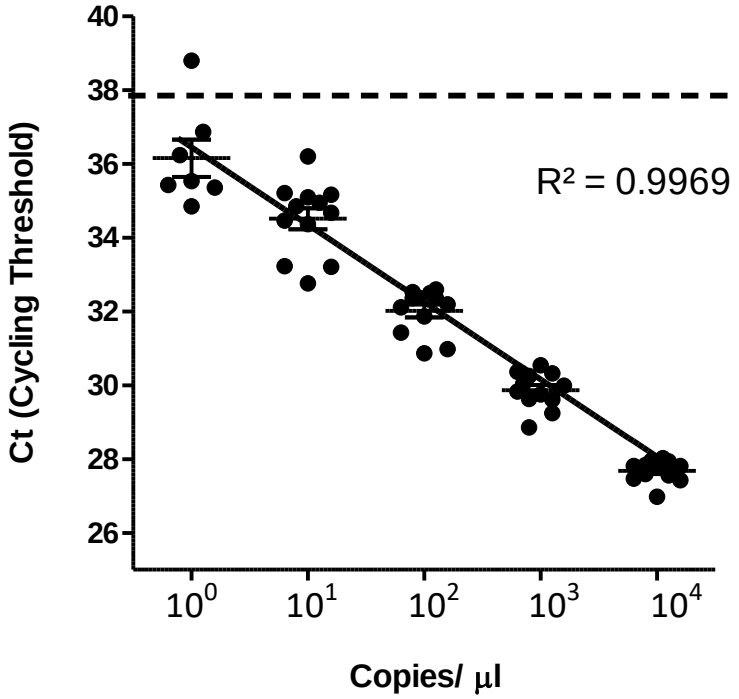
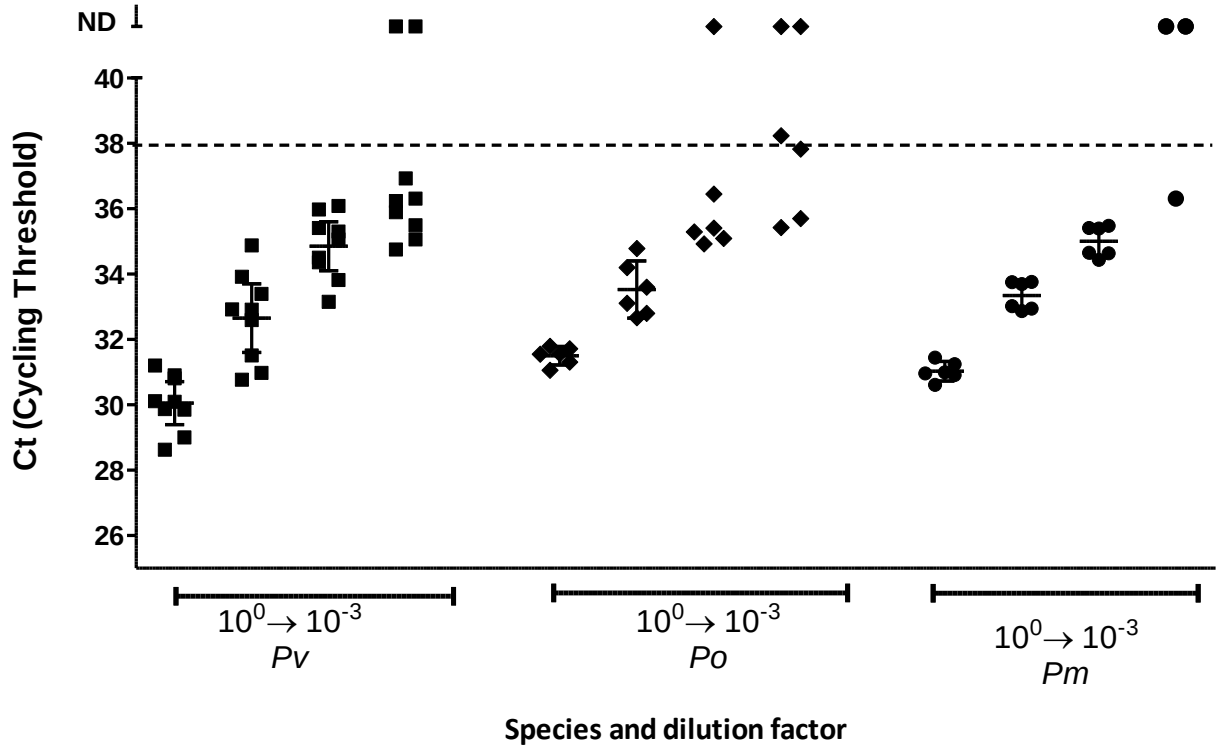


Figure 2.3.2. The 4-plex qPCR limit of detection for species.

Ten-fold serial dilutions of *P. vivax* (squares, n= 10), *P. ovale* (diamonds, n= 6) and *P. malariae* (circles, n= 6) DNA were tested. Each dot represents a technical replicate, bars represent 95% CI around the mean and dashed line depicts the Ct cut-off value (38).

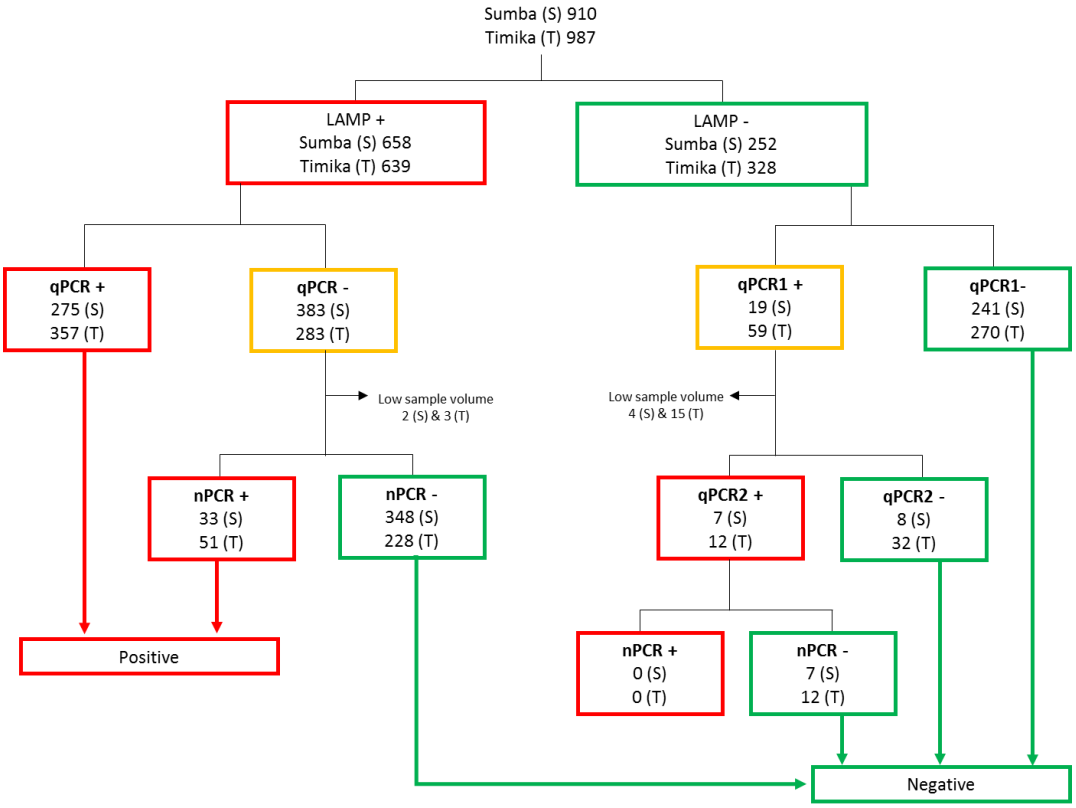


2.4.3.1. Detection of malaria

The diagnostic algorithm in figure 2.3.3 shows the number of samples from each site that was tested by LAMP, qPCR and nPCR. Samples included all LAMP positive samples from both sites and ~5% LAMP negative samples in the STOP-MiP trial. Samples from Timika included 639 LAMP positive samples and 328 negatives (n=967), while Sumba samples (n=910) included 658 LAMP positives and 252 negatives (Table 2.3, appendix table 2.7.1). A description of the frequency of each species as well as the qPCR Ct obtained for these are shown in appendix

table 2.7.1. The distribution of qPCR Cts obtained for different species (confirmed by LAMP) are detailed in appendix 2.7. Interestingly, all *P. falciparum* positive samples (by qPCR) in Sumba were co-infected with *P. vivax* (fig 2.3.4 and appendix 2.7).

Figure 2.3.3. Diagnostic algorithm for sample testing by qPCR, LAMP and nPCR.



2.4.3.2. Diagnostic performance

Using LAMP as a reference test, the qPCR sensitivity was higher in Timika (55.9%; 95% CI 51.9-59.7%) than in Sumba (29.5%; 26.1- 33.2). Specificity, however, was higher in Sumba (95.6%; 92.1- 97.7%) than in Timika (82.3%; 77.7- 86.2%). The level of agreement between LAMP and qPCR is almost double in Sumba (κ= 9.16; 95% CI 4.89-17.15) than in Timika (κ= 5.89; 4.26-8.15), but with overlapping confidence intervals. (Table 2.3) Finally, McNemar's test confirms that

overall the proportion of infections detected by the qPCR is significantly different to that detected by LAMP. (Table 2.3) Figure 2.3.4 shows the breakdown of co-infections determined by qPCR. All *P. ovale* and *P. malariae* samples detected by qPCR were confirmed by LAMP.

Discordant samples (n= 388; Sumba, 291; Timika) underwent nPCR testing (fig 2.3.3).
There were 7 samples in Sumba that were positive by qPCR (two repetitions), but negative by both LAMP and nPCR, whilst in Timika there were 12. There were also 33 and 51 qPCR-negative samples that were positive by both LAMP and nPCR, in Sumba and Timika respectively.

Figure 2.3.4. Positive samples detected by LAMP and qPCR.

The number of mutually detected positive samples in A) Sumba and B) Timika is shown (bold) within the circle segments. (n)= total number of positives detected by that test.

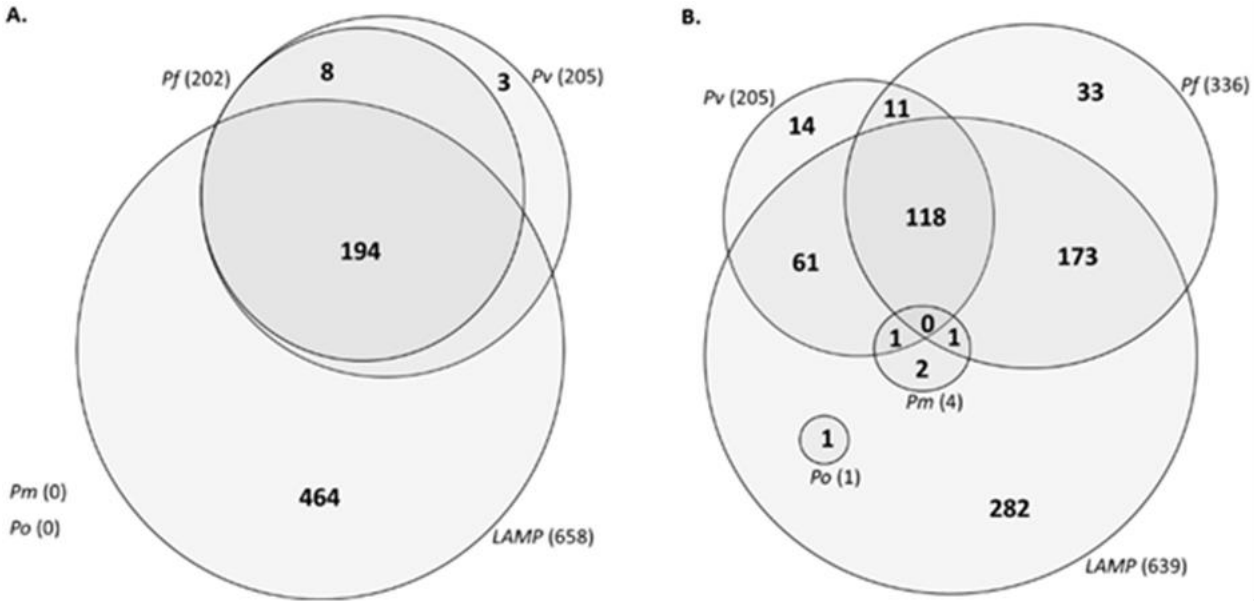


Table 2.3. Diagnostic performance of 4-plex qPCR compared to LAMP reference test in Indonesia

Site	n	TP	FN	TN	FP	Sensitivity %	Specificity %	PPV %	NPV %	DOR	Agreement (k)
Timika	967	357	282	270	58	55.9	82.3	86	48.9	5.6	0.33*
(95% CI)						(51.9-59.7)	(77.7-86.2)	(82.2-89.1)	(44.7-53.2)	(4.3-8.2)	(0.27-0.38)
Sumba	910	194	464	241	11	29.5	95.6	94.6	34.2	9.2	0.16*
(95% CI)						(26.1-33.2)	(92.1-97.7)	(90.3-97.2)	(30.7-38.8)	(4.9-17.2)	(0.13-0.20)

Note- n, number of samples; TP, True Positive; FP, False positive; TN, True Negative; FN, False Negative; Sens, sensitivity; Spec, specificity; (Lower 95% CI- Upper 95% CI); PPV, Positive Predictive Value; NPV, Negative Predictive Value; DOR, Diagnostics odds ratio; k, Kappa value

2.5. Discussion

2.5.1. HRMCA

The HRMCA shows unreliable identification of *non-falciparum* clinical infections, particularly for *P. vivax* and *P. ovale*. Chua *et al.* (2015) report LODs of 0.025, 0.25, 0.027, 0.27 and 0.15 p/ µl for *P. vivax*, *P. knowlesi, P. ovale*, *P. malariae* and *P. falciparum*, respectively. Here we empirically determined the LOD as 2 p/ µl for *P. falciparum*, albeit with DNA extracted from cultures rather than plasmid DNA, which would be more accurate. The average Tm for each species reported here are in keeping with those reported by Chua *et al.* demonstrating its transferability to a different rt-PCR platform (Shokoples, Ndao *et al*. 2009).

We did however experience frequent contamination with *P. falciparum* DNA in our NTCs and non-*falciparum* samples. The data shown here represent the few experiments without contamination. This should be repeated with more biological and technical replicates, to identify the source of contamination.

Another issue we encountered was the two shoulders in the melting peaks for *P. vivax* and *P. ovale,* which led to inaccuracies in the identification of these species. This could be remedied by further optimisation by lowering primer concentrations.

In our study, clinical samples of non-*falciparum* species were not quantifiable and therefore the LOD was not determined for these species. Instead we estimated the maximum dilution factor, which will vary depending on species and parasite density. Ideally synthetic DNA would be used

to quantify LOD for these species. Finally, due to the contamination issues, few replicates were included in the analyses and so it was difficult to characterise this assay.

MCA is generally qualitative and is prone to biased amplification of DNA that is most abundant. This could complicate the analysis of co-infections, whereby the assay may only amplify the DNA of the species with the highest parasite density. Due to the different replication rates, clearance and initial infection load, multiple *Plasmodium* species can be present at very different densities, which could result in amplification bias leading to missed infections and impact on treatment. Future validation of this assay should include clinical samples with confirmed *Plasmodium* co-infections, to guide implementation in different epidemiological settings. In conclusion, the observed LOD excludes its use for infections below 2 p/ µl such as those that are prevalent in low-transmission settings and commonly asymptomatic (Peeling, Holmes *et al*. 2006).

2.5.2. Duplex

The LOD for the Duplex assay determined here as 10-100 copies/ µl (~2-20 parasites/ µl) supports the original reported LOD of this assay. Given asymptomatic infection often falls below 10 parasites/ µl, this assay would not be suited for screening of asymptomatic malaria. A more sensitive assay (4-plex qPCR) was thus pursued for the use in asymptomatic individuals in moderate transmission settings (Indonesia). It is important to consider the LOD of this assay as it is widely utilised for mosquito-infection studies and could result in the underestimation of infection/prevalence amongst vector populations in low-transmission settings.

2.5.3. 4-plex malaria qPCR

The LOD of the 4-plex qPCR (1-10 copies/ µl; 0.2-2 p/ µl) was approximately 10-fold lower than that of the HRMCA or Duplex assays explored in this Chapter. This is in keeping with reported LODs (Slater, Ross *et al*. 2015, WHO 2016) for the primer/probe sets that were used to develop it. A strong correlation was also observed between Ct and copy number for all species, suggesting the assay could be quantitative.

The overall agreement of the 4plex with the LAMP PCR was categorised as *poor* in Sumba and *fair* in Timika. This mostly reflects the very low sensitivity of the qPCR in comparison to LAMP, however most of these were negative when assessed by the gold standard nPCR (n=576). This could indicate poor specificity of LAMP or cross-contamination during sample processing. Given that LAMP gives a binary result positive/ negative, it is impossible to know if trace contamination during the complex chelex DNA extraction step, could have caused such a high number of LAMP false positives. LAMP false positives may have been introduced at two main stages. First, during the hole-punching of the DBS samples where the same hole punch is used for multiple samples. Although the hole-punch is decontaminated between samples, this step is highly reliant on the rigour of the operator. Following DNA extraction, cross-contamination can be introduced during the DNA addition step. This is especially likely as the kit requires 1:1 (15 µl) ratio of DNA: master mix, meaning large volumes of DNA are being pipetted.

In 2010, an Intermittent Screening and Treating during pregnancy (ISTp) policy was introduced in Sumba. Using RDTs, the prevalence of malaria in the area was an estimated 7 % using the gold standard nPCR. Within the STOP-MiP study the prevalence by qPCR in Sumba was 5 %

(assuming no additional infections are detected in the remaining 95% of LAMP negatives), whilst LAMP was 10.3 %. The qPCR prevalence estimated here (2013-2016) is thus more in line with nPCR estimates in 2012, in the same area.

Further characterisation of this 4-plex qPCR- including quantified LOD, robustness, inter-lab/operator variability and analytical performance in field-settings, would support its use in surveillance or as a reference test in diagnostic evaluation studies.

2.6. Conclusions

This Chapter describes the development and validation of a 4plex qPCR able to identify the four main species of *Plasmodium*. The Chapter highlights the issues surrounding diagnostic evaluations when an index test may have superior sensitivity to the gold standard.
Finally, the 4-plex qPCR developed here was utilised in the STOP-MiP trial in combination with LAMP and nPCR data to form a composite molecular reference test. The reference test was used to confirm malaria infection in the trial. The resulting paper is attached in section 2.8 Publication Output.

2.7. Appendix

Table 2.7.1. Distribution of Ct values for infections in Timika (n=967) and Sumba (n=910).

A. Sumba

		Pf	*Pv*	*Po*	*Pm*
N	Ct positive	286	193	0	0
	No Ct	624	717	910	910
Mean		34.9	37.7		
Std. Error of Mean		.3	.2		
Median		34.8	37.5		
Mode		27.6[a]	37.0[a]		
Std. Deviation		5.1	2.6		
Skewness		-.21	.44		
Std. Error of Skewness		.14	.18		
Range		22.9	13.4		
Minimum		22.1	31.6		
Maximum		44.9	44.9		
Percentiles	25	31.7	36.2		
	50	34.8	37.5		
	75	38.6	39.1		

a. Multiple modes exist. The smallest value is shown

B. Timika

		Pf	*Pv*	*Po*	*Pm*
N	Ct positive	446	286	1	5
	No Ct	521	681	966	962
Mean		35.7	35.5	36.6	35.8
Std. Error of Mean		.2	.2		1.1
Median		35.8	35.8	36.6	35.3
Mode		32.7[a]	39.4	36.6	33.0[a]
Std. Deviation		3.4	3.9		2.4
Skewness		-.20	-.36		.19
Std. Error of Skewness		.12	.14		.91
Range		21.3	21.1	.00	5.6
Minimum		23.6	23.7	36.6	33.0
Maximum		44.9	44.8	36.6	38.6
Percentiles	25	33.6	33.1	36.6	33.5
	50	35.8	35.8	36.6	35.3
	75	38.0	38.3	36.6	38.2

a. Multiple modes exist. The smallest value is shown

2.8. Acknowledgments

I would like to thank Dr Thomas Edwards (Centre for drugs and diagnostics, LSTM) for his time training me in HRM and MCA. I am also grateful to Dr David Weetman for his donation of the Duplex reagents, as well as Ms Emily Rippon's time and patience training me in the Duplex assay (Vector Biology department, LSTM). Finally, I would like to thank the huge number of staff and participants in the STOP-MiP trial for their efforts towards the study.

Efficacy and safety of intermittent preventive treatment and intermittent screening and treatment versus single screening and treatment with dihydroartemisinin–piperaquine for the control of malaria in pregnancy in Indonesia: a cluster-randomised, open-label, superiority trial

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Summary

Background *Plasmodium falciparum* and *Plasmodium vivax* infections are important causes of adverse pregnancy outcomes in the Asia-Pacific region. We hypothesised that monthly intermittent preventive treatment (IPT) or intermittent screening and treatment (IST) with dihydroartemisinin–piperaquine is more effective in reducing malaria in pregnancy than the existing single screening and treatment (SST) strategy, which is used to screen women for malaria infections at the first antenatal visit followed by passive case detection, with management of febrile cases.

Methods We did an open-label, three-arm, cluster-randomised, superiority trial in Sumba (low malaria transmission site) and Papua (moderate malaria transmission site), Indonesia. Eligible participants were 16–30 weeks pregnant. Clusters (antenatal clinics with at least ten new pregnancies per year matched by location, size, and malaria risk) were randomly assigned (1:1:1) via computer-generated lists to IPT, IST, or SST clusters. In IPT clusters, participants received the fixed-dose combination of dihydroartemisinin-piperaquine (4 and 18 mg/kg per day). In IST clusters, participants were screened with malaria rapid diagnostic tests once a month, whereas, in SST clusters, they were screened at enrolment only. In all groups, participants with fever were tested for malaria. Any participant who tested positive received dihydroartemisinin–piperaquine regardless of symptoms. The primary outcome was malaria infection in the mother at delivery. Laboratory staff were unaware of group allocation. Analyses included all randomly assigned participants contributing outcome data and were adjusted for clustering at the clinic level. This trial is complete and is registered with ISRCTN, number 34010937.

Findings Between May 16, 2013, and April 21, 2016, 78 clusters (57 in Sumba and 21 in Papua) were randomly assigned to SST, IPT, or IST clusters (26 clusters each). Of 3553 women screened for eligibility, 2279 were enrolled (744 in SST clusters, 681 in IPT clusters, and 854 in IST clusters). At enrolment, malaria prevalence was lower in IST (5·7%) than in SST (12·6%) and IPT (10·6%) clusters. At delivery, malaria prevalence was 20·2% (128 of 633) in SST clusters, compared with 11·6% (61 of 528) in IPT clusters (relative risk [RR] 0·59, 95% CI 0·42–0·83, p=0·0022) and 11·8% (84 of 713) in IST clusters (0·56, 0·40–0·77, p=0·0005). Conditions related to the pregnancy, the puerperium, and the perinatal period were the most common serious adverse events for the mothers, and infections and infestations for the infants. There were no differences between groups in serious adverse events in the mothers or in their infants.

Interpretation IST was associated with a lower prevalence of malaria than SST at delivery, but the prevalence of malaria in this group was also lower at enrolment, making interpretation of the effect of IST challenging. Further studies with highly sensitive malaria rapid diagnostic tests should be considered. Monthly IPT with dihydroartemisinin–piperaquine is a promising alternative to SST in areas in the Asia-Pacific region with moderate or high transmission of malaria.

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See Comment page 919

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Introduction

Approximately 70% of 125·2 million pregnancies in malaria-endemic areas occur in the Asia-Pacific region annually,[1] where antenatal infections with *Plasmodium falciparum* and *Plasmodium vivax* are associated with adverse pregnancy outcomes.[2–4] In the African region, a prevention strategy has been endorsed by WHO, including provision of a long-lasting insecticidal net (LLIN) and intermittent preventive treatment (IPT) in pregnancy, consisting of curative doses of sulfadoxine–pyrimethamine

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Research in context

Evidence before this study

We searched the Malaria in Pregnancy Library and PubMed from their inception to Sept 20, 2018, without language restrictions, for relevant trials of chemoprevention with intermittent preventive treatment (IPT) or intermittent screening and treatment (IST) of malaria in pregnancy. We restricted the search to areas in the Asia-Pacific region, Central and South America, the horn of Africa, and Madagascar, where *Plasmodium falciparum* and *Plasmodium vivax* are co-endemic. The following search terms were used: "(intermittent OR IPT OR prophylaxis OR prevention) AND (malaria OR plasmodium) AND (pregnan* OR trimester OR gestation)". The names of each country in these regions were added as additional search terms. Two IPT trials were identified, one in the Solomon Islands and the other in Papua New Guinea, of which both had used sulfadoxine–pyrimethamine. No IST trials were identified. Our literature search confirmed that few intermittent screening trials or chemoprevention trials in pregnancy have been done outside of Africa. WHO does not have a prevention strategy for malaria in pregnancy in the Asia-Pacific region, where about 70% of the global number of pregnancies in malaria-endemic areas occur. Most countries in this region use a single screening and treatment (SST) strategy for malaria at the first antenatal visit.

Added value of this study

To our knowledge, this study is the first prevention trial to compare monthly IPT or IST with the antimalarial dihydroartemisinin-piperaquine against the existing SST strategy with dihydroartemisinin–piperaquine for the control of malaria in pregnancy in the Asia-Pacific region. The study was designed to support the Indonesian Ministry of Health and WHO in the development of strategies for the control of malaria in pregnancy in Indonesia and the wider Asia-Pacific region.

Implications of all the available evidence

Our results do not support a role for IST with the existing standard malaria rapid diagnostic tests or for IPT with dihydroartemisinin–piperaquine in areas in the Asia-Pacific region with lower malaria transmission. However, our results confirm earlier findings from east Africa and show that IPT with dihydroartemisinin–piperaquine should be considered as a potential strategy to reduce the risk of malaria infection, and the associated adverse consequences in pregnancy in areas in the Asia-Pacific region with moderate to high levels of malaria transmission and high levels of resistance to sulfadoxine–pyrimethamine.

given at every scheduled antenatal visit in the second and third trimesters. However, in the Asia-Pacific region, few countries have chemoprevention strategies for malaria in pregnancy.[4] Most provide LLINs as part of antenatal care and use single screening and treatment (SST) strategies in pregnancy consisting of screening participants for malaria infections at the first antenatal visit followed by passive case detection, with management of febrile cases.[5,6] The paucity of chemoprevention strategies reflects the dearth of prevention trials and widespread parasite resistance in Asia to sulfadoxine–pyrimethamine,[7] the only antimalarial recommended by WHO for IPT.[8]

Three completed trials in areas of high sulfadoxine–pyrimethamine resistance in Kenya and Uganda[9–11] suggest that the fixed-dose, artemisinin-based combination therapy of dihydroartemisinin–piperaquine is a promising candidate to replace sulfadoxine–pyrimethamine for use in IPT. IPT with dihydroartemisinin–piperaquine was associated with much greater reductions in malaria infection and clinical malaria during pregnancy than was IPT with sulfadoxine–pyrimethamine.[10]

In areas with predominantly low malaria transmission, alternative strategies involving regular screening and treatment approaches should also be considered. For example, in refugee camps on the Thai–Myanmar border, the introduction of weekly screening for malaria and treatment of pregnant women who tested positive reduced maternal mortality from malaria substantially.[12] Such intensive screening programmes are unlikely to be feasible under programmatic conditions. However, four trials in sub-Saharan Africa showed good feasibility with less intensive intermittent screening and treatment (IST) strategies involving malaria rapid diagnostic tests done 3–6 times during pregnancy.[13]

Limitations of these screening strategies include the failure to detect *P falciparum* infections that are predominantly sequestered in the placenta, or low-grade infections that are below the limit of detection by standard microscopy or malaria rapid diagnostic tests, which are particularly common with *P vivax*. Furthermore, because of the parasite's dormant forms in the liver, a single *P vivax* infection might cause multiple relapses during pregnancy, when radical cure with primaquine is contraindicated. The SST strategy has the additional limitation of potentially missing re-infections or asymptomatic parasitaemia in later stages of pregnancy.

Here, we report the results of the first trial in the Asia-Pacific region designed to compare the safety and efficacy of monthly IST or IPT with dihydroartemisinin–piperaquine with the standard SST strategy for decreasing the risk of malaria infection in pregnancy.

Methods

Study design and participants

We did an open-label, two-site, three-arm, cluster-randomised, superiority trial in areas in eastern Indonesia that are co-endemic for *P falciparum* and *P vivax*: Sumba Island,[14,15] which has low malaria transmission, and southern Papua, Indonesia,[16–18] which has moderate year-round transmission (appendix p 4).[19,20]

See Online for appendix

Antenatal clinics were eligible for inclusion if they had at least ten new pregnancies per year and were located within 1·5 h drive from the study offices (appendix pp 4–5).

Pregnant women of any gravity attending their first antenatal visit were eligible if they had a viable pregnancy between 16 and 30 weeks' gestation, were residents in the study catchment areas, were willing to complete the study schedule and deliver their baby at the study clinics or hospital, and had not yet been screened for malaria. Exclusion criteria comprised high-risk pregnancies due to pre-existing conditions likely to cause complication in the current pregnancy (e.g. hypertension, diabetes, asthma, renal disease, liver disease, any spinal deformity), severe malaria at presentation, treatment with antimalarials in the previous month, HIV positivity, a family history of sudden death or any known cardiac condition, current use of medication known to prolong the QTc interval, a history of allergy to dihydroartemisinin–piperaquine, and residence outside study area or plans to move within 6 months.

Ethical approval was obtained from the Liverpool School of Tropical Medicine, the Eijkman Institute for Molecular Biology, and the National Institute of Health Research and Development (Litbangkes), Ministry of Health, Jakarta, Indonesia. Written informed consent was obtained from all participants. The trial protocol is provided in the appendix.

Randomisation and masking

The 78 antenatal clinic clusters were matched in triplicate on the basis of location, size, and malaria transmission intensity (appendix p 5). Before the study, the randomisation sequence to allocate clusters to the three intervention arms (1:1:1) was computer generated by the study statistician at the Liverpool School of Tropical Medicine (appendix pp 5–6) and forwarded to Indonesia. The final allocation was achieved during a public ceremony in which local health officials drew one of three identical looking opaque sealed envelopes which assigned their cluster to one of the three study interventions (SST, IPT, or IST; appendix pp 5–6). Study participants, local study nurses and midwives, and the local study coordinators were aware of the treatment allocation. Laboratory staff and off-site study investigators, including the study statistician, remained masked to treatment allocation until after database lock, approval of the statistical analysis plan by the Data Monitoring and Ethical Committee, and completion of the analytical code on the basis of dummy allocation.

Procedures

At enrolment, demographic, socioeconomic, and educational information, and data on ownership and use of LLINs, were collected, and medical and obstetric histories were taken. Gestational age was assessed by fundal height, and fetal viability confirmed by doppler ultrasonography. The pregnant women's axillary temperature, blood pressure, weight, and mid-upper arm circumference were measured, and a blood sample was taken for malaria microscopy, molecular malaria diagnostics (quantitative PCR [qPCR], nested PCR, and loop-mediated isothermal amplification [LAMP]; appendix p 7), immunological analyses, and measurement of haemoglobin concentration (Haemocue, HemoCue AB, Ängelholm, Sweden). In addition, malaria rapid diagnostic tests (First Response Malaria Ag pLDH–HRP2 Combo [I16FRC30]; Premier Medical Corporation, Nani Daman, India) were done at enrolment in all participants in the SST and IST groups, regardless of symptoms, and in symptomatic participants in the IPT group. All participants received an LLIN. Participants were assessed monthly until delivery. At each monthly follow-up visit, clinical, obstetric, and physical examinations were done, and a blood sample taken by fingerprick for malaria microscopy and LAMP–PCR (appendix pp 7–8). In addition, malaria rapid diagnostic tests were done from the same sample as used for microscopy and LAMP-PCR in all participants in the IST group, regardless of symptoms, but only in symptomatic participants in the SST and IPT groups. Participants were encouraged to make unscheduled visits or contact staff if they felt ill or were concerned about their pregnancy. Participants were assessed for adverse events during each scheduled and unscheduled visit.

Participants in the SST clusters were screened with malaria rapid diagnostic tests for malaria infection, regardless of symptoms, at their first antenatal (enrolment) visit only. At subsequent monthly visits, they were tested with malaria rapid diagnostic tests if they were febrile (axillary temperature ≥37·5°C) or had a history of fever in the previous 48 h. The procedures in the IST group were identical to those in the SST group, except that participants were screened with malaria rapid diagnostic tests at each scheduled monthly visit. Participants in the IPT group received 4 mg/kg per day dihydroartemisinin and 18 mg/kg per day piperaquine (in 40 mg/320 mg tablets; Eurartesim, Sigma-Tau, Rome, Italy) at each monthly visit, at which they were not screened for malaria, unless they were febrile or had a history of fever in the past 48 h. The dose was the same throughout pregnancy and consisted of the standard 3-day course of two tablets for participants weighing less than 36 kg, three tablets for participants weighing 36–75 kg, or four tablets for participants weighing 75 kg or more at enrolment. The first dose was provided with a glass of water as directly observed therapy in the clinic. Participants were provided with the remaining two doses to be taken at home. All participants were contacted on day 2 and visited on day 3 to assess adherence and tolerance. In case of vomiting within 30 min, the full dose was repeated. Additionally, all participants who were positive for malaria on rapid diagnostic tests (positive HRP2 or pLDH bands) in all groups were treated with dihydroartemisinin–piperaquine (the same

3-day weight-based treatment as used for the IPT group). Participants with a history of dihydroartemisinin–piperaquine intake in the previous 4 weeks received quinine–clindamycin (10 mg/kg twice daily for 7 days).

At delivery, a maternal blood sample was taken for the same malaria metrics, and placental and umbilical-cord blood samples for histology, malaria rapid diagnostic tests, microscopy, and LAMP and PCR (appendix pp 7–8). Newborns were weighed on a digital scale (±10 g) and their gestational age assessed by means of the modified Ballard score.[21] The presence of jaundice and congenital anomalies detectable by surface examination were assessed at delivery, day 7, and the final visit at 6–8 weeks. In between scheduled visits, infants were followed up passively.

We extracted DNA from dried blood spots using the Chelex method and tested for malaria using LAMP Pan-kits. LAMP-positive samples and 5% of negative samples were then tested by qPCR for verification and species identification, and discordant samples retested with nested-PCR (appendix pp 7–8).

Electrocardiography was done in a subgroup of 33 participants in the IPT group (selected through convenience sampling) to establish whether previously documented transient QTc prolongation associated with piperaquine increased in magnitude with subsequent courses (appendix p 10).

Outcomes

The primary outcome was malaria (any species) at delivery, defined as a composite of maternal malaria (detection of infection in peripheral blood with microscopy, rapid diagnostic test, or LAMP–PCR) or placental malaria (detection of infection in placental blood with microscopy, LAMP–PCR, or histology [active infection]; appendix pp 6–8).

Secondary outcomes at delivery comprised the individual components of the primary composite outcome (maternal or placental malaria), detected by any method and by each method separately. Placental malaria infection detected by histology was classified as active acute, active chronic, active any, past, or any (active or past). In post-hoc analyses, malaria infection in the peripheral blood was stratified further by species. In addition, maternal anaemia (any: haemoglobin level <11 g/dL; moderate: haemoglobin level <9 g/dL) was assessed at delivery.

Secondary outcomes during pregnancy comprised maternal malaria, detected with any method and by each method separately. This outcome was further stratified by patent infection (positive microscopy or malaria rapid diagnostic test) and sub-patent infection (negative microscopy and malaria rapid diagnostic test and positive LAMP–PCR). Morbidity outcomes assessed during pregnancy comprised clinical malaria (documented or history of fever plus positive malaria rapid diagnostic test or microscopy) and unscheduled clinic visits for any reason and for all reasons unrelated to malaria.

Newborn secondary efficacy outcomes included congenital malaria, mean cord haemoglobin concentration, fetal anaemia (haemoglobin <12·5 g/dL), mean birthweight, low birthweight (<2500 g), mean gestational age, preterm delivery (<37 weeks' gestation), mean birthweight for gestational age (Z scores), small for gestational age,[22] fetal loss (spontaneous abortion at <28 weeks' gestation or stillbirth), and the composite outcomes of adverse livebirth (preterm, low birthweight, or small for gestational age) and adverse pregnancy (adverse livebirth or fetal loss). Other secondary efficacy outcomes in the infant included the incidence of clinical malaria and all-cause and non-malaria illness by the end of follow-up (age 6–8 weeks). Mortality outcomes included neonatal, perinatal, and mortality up to age 6–8 weeks.

Safety outcomes included serious adverse events in the mother or infant, overall and by system organ class and preferred Medical Dictionary of Regulatory Affairs term; maternal deaths; congenital anomalies; and QTc prolongation.

Statistical analysis

The trial was initially designed to detect a 50% reduction in malaria at delivery with IPT or with IST relative to SST across both sites pooled. Following recommendations from the ethics committee in Indonesia on June 27, 2014, to stop recruitment in Sumba because of the unexpected low malaria prevalence in the area, a blinded interim re-estimation of sample size was done with the aim to provide the study with 80% power across both sites pooled and 85% power in Papua alone to detect at least a 50% reduction in the primary outcome (two-sided α value of 0·0167, intracluster correlation coefficient of 0·005; appendix p 9). The revised study required 2279 participants (1290 from Papua and 989 from Sumba), accounting for a 13% efficiency loss owing to varying cluster sizes and 20% loss to follow-up.

The modified intention-to-treat (ITT) population included all randomised participants with outcome data. We also assessed all efficacy outcomes in the per-protocol population, which included participants in the modified ITT population who attended every scheduled visit and took all study doses on each occasion. The safety population included participants who received at least one dose of study drug in any of the study arms.

Generalised estimating equation (GEE) models, with treatment group as a predictor and clinic as a cluster effect, were used. Log binomial GEE models were used to obtain risk ratios (RRs) for binary outcomes (including the cumulative risk), and linear GEE models to obtain mean differences for continuous outcomes. The unadjusted analysis, stratified by site, was considered the primary analysis. Because matching was ineffective and the number of triplets small in each site, unmatched analysis of the matched data was done to maximise power as soon as it became clear from the comparison of the baseline data that the matching was not successful.[23,24]

Separate models were run per site (pre-planned), and differences in treatment effects compared by means of the Altman-Bland method.[25]

Secondary, covariate-adjusted analyses of the efficacy endpoints were done with seven prespecified, individual-level covariates and one post-hoc, cluster-level covariate (prevalence of malaria infection at enrolment as a proxy for malaria transmission). The individual-level covariates were study site (overall models only), malaria status at enrolment (binary), season during pregnancy (terciles based on average rainfall during the last 6 months of pregnancy), socioeconomic status (terciles of Socioeconomic Index[27]], calculated with principal component analysis), gestational age at enrolment (binary, based on the median value), gravidity (primigravidae or secundigravidae *vs* multigravidae), and use of insecticide-treated net during pregnancy. Simple imputation was used for missing covariates (<1%); no imputation was used for missing outcome variables. These same covariates, as well as a post-hoc covariate for species on enrolment, were used for subgroup analyses by adding them as interaction terms with treatment group. GEE Poisson regression, with time of follow-up as an offset, was used to obtain incidence rate ratios.

The analysis was done with SAS version 9.3 and Stata version 14. The Data Monitoring and Ethical Committee oversaw the study. A cost-effectiveness analysis will be published elsewhere. The trial was registered with ISRCTN, number ISRCTN34010937.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. RA, CK, FOtK, TC, and DW had full

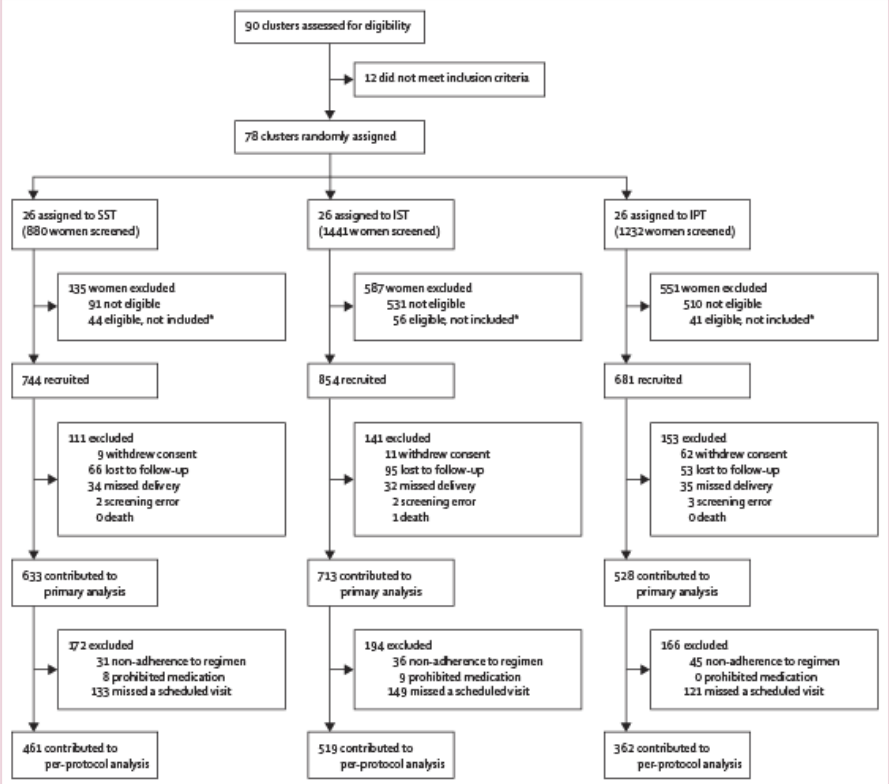

Figure 1: Trial profile

IPT=intermittent preventive treatment. IST=intermittent screening and treatment. SST=single screening and treatment. *The number of recruited participants per cluster was restricted to a maximum of five per day to keep the number needed to follow-up manageable in subsequent visits. On some days, more than five participants were eligible, in which case they were chosen at random by drawing lots among all eligible participants who presented that morning.

access to all the data in the study. The corresponding author had final responsibility for the decision to submit for publication.

Results

Between May 16, 2013, and April 21, 2016 (when the required sample size was reached), 78 clusters (57 in Sumba and 21 in Papua) were randomly assigned to SST, IPT, or IST (26 clusters each). 3553 women were screened for inclusion, of whom 2279 (64·1%) were enrolled (989 from Sumba and 1290 from Papua; figure 1). The last delivery occurred on Oct 9, 2016, and the last infant follow-up on Nov 26, 2016. Overall, 7350 (85·4%) of 8609 scheduled antenatal visits were

	Sumba			Papua			Pooled sites		
	SST (n=337)	IST (n=359)	IPT (n=293)	SST (n=407)	IST (n=495)	IPT (n=388)	SST (n=744)	IST (n=854)	IPT (n=681)
Maternal age, years	28·1 (6·1)	27·8 (5·9)	28 (6·0)	26 (6·1)	25·9 (6·7)	25·8 (6·0)	27 (6·2)	26·7 (6·4)	26·8 (6·1)
Residence, rural vs semi-urban	95·0% (320/337)	91·1% (327/359)	90·1% (264/293)	59·2% (241/407)	67·9% (336/495)	58·0% (225/388)	75·4% (561/744)	77·6% (663/854)	71·8% (489/681)
Marital status, single* vs married	62·3% (210/337)	65·2% (234/359)	63·1% (185/293)	34·6% (141/407)	27·1% (134/495)	35·3% (137/388)	47·2% (351/744)	43·1% (368/854)	47·3% (322/681)
Used a bednet previous night	22·3% (75/337)	25·3% (91/359)	22·5% (66/293)	49·4% (201/407)	45·7% (226/495)	53·1% (206/388)	37·1% (276/744)	37·1% (317/854)	39·9% (272/681)
Attended school	91·1% (307/337)	87·2% (313/359)	88·1% (258/293)	86·0% (350/407)	94·7% (469/495)	89·4% (347/388)	88·3% (657/744)	91·6% (782/854)	88·8% (605/681)
Schooling level†									
Low	22·0% (74/337)	23·4% (84/359)	22·9% (67/293)	22·1% (90/407)	11·7% (58/495)	15·5% (60/388)	22·0% (164/744)	16·6% (142/854)	18·6% (127/681)
Medium	30·9% (104/337)	25·9% (93/359)	26·3% (77/293)	18·2% (74/407)	16·2% (80/495)	13·4% (52/388)	23·9% (178/744)	20·3% (173/854)	18·9% (129/681)
High	21·1% (71/337)	23·1% (83/359)	28·0% (82/293)	22·6% (92/407)	24·2% (120/495)	18·8% (73/388)	21·9% (163/744)	23·8% (203/854)	35·8% (244/681)
Highest	26·1% (88/337)	27·6% (99/359)	22·9% (67/293)	37·1% (151/407)	47·9% (237/495)	52·3% (203/388)	32·1% (239/744)	39·3% (336/854)	39·6% (270/681)
Socioeconomic Index score, terciles									
Low	34·4% (116/337)	32·0% (115/359)	33·4% (98/293)	43·5% (177/407)	30·1% (149/495)	26·8% (104/388)	39·4% (293/744)	30·9% (264/854)	29·7% (202/681)
Medium	32·6% (110/337)	33·4% (120/359)	34·8% (102/293)	32·4% (132/407)	33·1% (164/495)	34·3% (133/388)	32·5% (242/744)	33·3% (284/854)	34·5% (235/681)
High	32·9% (111/337)	34·5% (124/359)	31·7% (93/293)	24·1% (98/407)	36·8% (182/495)	38·9% (151/388)	28·1% (209/744)	35·8% (306/854)	35·8% (244/681)
Pregnancy number, gravidity									
One	28·2% (95/337)	28·7% (103/359)	28·0% (82/293)	26·0% (106/407)	26·5% (131/495)	29·6% (115/388)	27·0% (201/744)	27·4% (234/854)	28·9% (197/681)
Two	22·3% (75/337)	24·0% (86/359)	20·5% (60/293)	29·2% (119/407)	31·5% (156/495)	29·1% (113/388)	26·1% (194/744)	28·3% (242/854)	25·4% (173/681)
Three or more	49·6% (167/337)	47·4% (170/359)	51·5% (151/293)	44·7% (182/407)	42·0% (208/495)	41·2% (160/388)	46·9% (349/744)	44·3% (378/854)	45·7% (311/681)
Gestational age, weeks	24·1 (4·6)	24·1 (4·3)	24·1 (4·6)	23·7 (5·3)	22·8 (5·1)	23·8 (4·5)	23·9 (5)	23·4 (4·8)	23·9 (4·6)
Weight, kg	51·9 (7·0)	51·3 (6·8)	52·0 (8·1)	56·7 (5·3)	56·0 (9·6)	57·8 (9·4)	54·5 (8·6)	54·0 (8·8)	55·3 (9·3)
Height, cm	152·4 (5·4)	152·5 (5·6)	151·8 (6·3)	152·7 (5·3)	152·8 (5·5)	152·8 (5·3)	152·6 (5·3)	152·7 (5·5)	152·3 (5·8)
Mid-upper arm circumference, cm	24·6 (2·8)	24·2 (2·4)	24·7 (2·7)	25·3 (3·1)	25·6 (3·4)	25·8 (3·2)	25·0 (3·0)	25·0 (3·1)	25·3 (3·0)
Haemoglobin, g/dL	11·0 (1·4)	11·0 (1·6)	11·1 (1·5)	11·0 (1·9)	11·7 (1·8)	11·4 (1·9)	11·0 (1·7)	11·4 (1·8)	11·3 (1·7)
Plasmodium infection									
mRDT‡	0·3% (1/337)	0·0% (0/359)	0·0% (0/2)	7·4% (30/407)	3·0% (15/495)	50·0% (3/6)	4·2% (31/744)	1·8% (15/854)	37·5% (3/8)
Microscopy	0·6% (2/337)	0·3% (1/358)	0·7% (2/293)	9·1% (37/407)	3·6% (18/495)	5·9% (23/387)	5·2% (39/744)	2·2% (19/853)	3·7% (25/680)
LAMP-PCR	6·5% (22/337)	2·8% (10/359)	8·6% (25/290)	11·8% (48/407)	5·9% (29/495)	9·0% (35/387)	9·4% (70/744)	4·6% (39/854)	8·9% (60/677)
Any§	6·5% (22/337)	3·1% (11/359)	8·5% (25/293)	17·7% (72/407)	7·7% (38/495)	12·1% (47/388)	12·6% (94/744)	5·7% (49/854)	10·6% (72/681)

(Table 1 continues on next page)

	Sumba			Papua			Pooled sites		
	SST (n=337)	IST (n=359)	IPT (n=293)	SST (n=407)	IST (n=495)	IPT (n=388)	SST (n=744)	IST (n=854)	IPT (n=681)
(Continued from previous page)									
Infecting species¶									
Plasmodium falciparum, mono-infection	3·3% (11/337)	1·1% (4/359)	5·1% (15/292)	8·1% (33/407)	4·0% (20/495)	7·7% (30/388)	5·9% (44/744)	2·8% (24/854)	6·6% (45/680)
Plasmodium vivax, mono-infection	2·4% (8/337)	1·7% (6/359)	2·1% (6/292)	5·2% (21/407)	2·4% (12/495)	1·8% (7/388)	3·9% (29/744)	2·1% (18/854)	1·9% (13/680)
Plasmodium malariae or *Plasmodium ovale*, mono-infection	0·0% (0/337)	0·0% (0/359)	0·0% (0/292)	1·0% (4/407)	0·0% (0/495)	0·0% (0/388)	0·5% (4/744)	0·0% (0/854)	0·0% (0/680)
Mixed infection	0·6% (2/337)	0·3% (1/359)	0·7% (2/292)	3·4% (14/407)	1·2% (6/495)	2·6% (10/388)	2·2% (16/744)	0·8% (7/854)	1·8% (12/680)

Data are mean (SD) or % (n/N). SST=single screen and treatment. IST=intermittent screen and treatment. IPT=intermittent preventive therapy. mRDT=malaria rapid diagnostic test. LAMP=loop-mediated isothermal amplification. *Single includes single unmarried participants only as there were no divorced, separated, or widowed participants. In Sumba, many participants were not legally married, but co-habiting with their partner and considered married within their local communities. †Low was defined as no schooling or primary school not completed, medium as primary school completed, high as junior high school completed, and highest as senior high school or tertiary education completed. ‡Data reflect mRDTs done in symptomatic participants in the IPT group and all participants in the SST and IST groups. §Includes mRDT results from symptomatic participants and microscopy and LAMP-PCR results from all participants. ¶Typing was done by PCR; if PCR was not successful, species was based on microscopy.

Table-1: Baseline characteristics of enrolled participants

attended (appendix p 12). At baseline, 215 (9·4%) of 2279 participants had peripheral parasitaemia; the prevalence was similar in the SST (94 [12·6%] of 744) and IPT (72 [10·6%] of 681) groups, but lower in the IST group (49 [5·7%] of 854; table 1). Overall 418 (90%) of 463 cases of peripheral parasitaemia detected by LAMP–PCR were below the limit of detection for malaria rapid diagnostic tests.

Median follow-up was 3·1 months (IQR 2·1–4·0), with a median number of scheduled follow-up visits of three (range 1–6; appendix p 12). The median number of dihydroartemisinin–piperaquine courses in the IPT group was three (range 0–6). Ultimately, 1874 (82·2%) of 2279 women contributed to the primary endpoint (figure 1). These proportions did not differ significantly overall, but in Papua the proportion of enrolled participants who contributed to the primary endpoint was significantly lower in IPT clusters (70·1%) than in SST (84·3%; p=0·0005) and IST (86·5%) clusters (p<0·0001), whereas in Sumba it was lower in IST clusters (79·4%) than in IPT (87·4%; p=0·017) or SST (86·1%; p=0·070) clusters (appendix pp 14, 31).

The prevalence of malaria at delivery in the modified ITT population was 20·2% (128 of 633) in SST clusters compared with 11·6% (61 of 528) in IPT clusters (RR 0·59, 95% CI 0·42–0·83; p=0·0022) and 11·8% (84 of 713) in IST clusters (0·56, 0·40–0·77; p=0·0005; figures 2, 3). There was no significant difference in the prevalence of malaria at delivery between IPT and IST clusters (1·06, 0·73–1·54, p=0·77; figure 4). Similar results were obtained in covariate-adjusted analyses (figures 2–4), across all subgroups (appendix pp 32–34), in the per-protocol population (appendix pp 35–37), and in a post-hoc sensitivity analysis with matched analysis (appendix p 15). Intracluster correlation coefficient values are shown in appendix p 16.

Analyses of secondary outcomes at delivery showed that relative to SST, IPT was associated with a reduction in patent infections and sub-patent infections in peripheral blood (figure 2; appendix p 38). For IST, the reduction was significant for sub-patent infections only (figure 3; appendix p 39). The prevalence of placental malaria detected by histology (active or past) or other methods was lower in IPT clusters than in SST clusters (appendix p 41). It was similar in IPT and IST clusters in the unadjusted analysis but lower in IST than in IPT clusters in the adjusted analysis (figure 4), owing to reductions in past infections with IPT (appendix p 43). There were no significant differences in malaria detected by placental histology between IST and SST (appendix p 42).

Analyses of secondary outcomes during pregnancy showed that the cumulative risk of incident malaria infection during pregnancy detected by at least one diagnostic method was lower in IPT than in SST clusters (figure 2). Similar results were seen for other definitions of antenatal malaria infection (appendix p 44). The cumulative risk of clinical malaria was also lower with IPT than with SST (figure 2). The cumulative risk of malaria detected by rapid diagnostic test was not significantly different between IPT and SST clusters (appendix p 42). In the IST clusters, by contrast, the antenatal incidence measures of malaria infection were similar to those in SST clusters (figure 3; appendix p 45). The cumulative proportion of participants with malaria infection detected by malaria rapid diagnostic test (including at enrolment) was also similar between IST and SST (34 [4·0%] of 854) and SST (39 [5·2%] of 744) groups (RR 0·91, 95% CI 0·36–2·32, p=0·84), despite the nearly four times higher number of screening events in IST than in SST clusters (2886 *vs* 744). The incidence of clinical malaria with IST

IPT vs SST	IPT n/N (%)	SST, n/N (%)	Crude RR (95% CI)	p value	Adjusted RR (95% CI)*	p value
Overall						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	61/528 (11·6%)	128/633 (20·2%)	0·59 (0·42–0·83)	0·0022	0·63 (0·48–0·83)	0·0011
Maternal plasmodium infection during pregnancy† (cumulative risk)	53/595 (8·9%)	118/680 (17·4%)	0·60 (0·39–0·92)	0·018	0·60 (0·40–0·89)	0·011
Maternal clinical malaria during pregnancy (cumulative risk)	2/595 (0·3%)	13/680 (1·9%)	0·22 (0·05–0·98)	0·048	0·21 (0·05–0·82)	0·025
Maternal peripheral patent plasmodium infection at delivery	1/523 (0·2%)	14/627 (2·2%)	0·09 (0·01–0·60)	0·013	0·09 (0·01–0·58)	0·011
Maternal peripheral sub-patent plasmodium infection at delivery	28/523 (5·4%)	56/627 (8·9%)	0·60 (0·39–0·93)	0·022	0·65 (0·43–0·99)	0·043
Placental plasmodium infection‡	40/500 (8·0%)	95/610 (15·6%)	0·58 (0·36–0·94)	0·026	0·51 (0·35–0·75)	0·0007
Maternal haemoglobin <9 g/dL at delivery	60/524 (11·5%)	93/631 (14·7%)	0·79 (0·61–1·02)	0·070	0·80 (0·63–1·03)	0·080
Fetal anaemia (cord haemoglobin <12·5 g/dL)	85/487 (17·5%)	139/603 (23·1%)	0·75 (0·55–1·01)	0·060	0·79 (0·63–0·99)	0·043
Small for gestational age (<10th percentile[27])	69/512 (13·5%)	71/625 (11·4%)	1·18 (0·86–1·61)	0·32	1·25 (0·98–1·60)	0·080
Low birthweight (<2500 g)	93/512 (18·2%)	86/626 (13·7%)	1·35 (0·97–1·87)	0·070	1·40 (1·05–1·86)	0·021
Preterm birth (<37 weeks)	61/524 (11·6%)	70/635 (11·0%)	1·06 (0·74–1·53)	0·74	1·13 (0·81–1·56)	0·47
Fetal loss (spontaneous abortion or stillbirth)	13/561 (2·3%)	9/662 (1·4%)	1·72 (0·68–4·32)	0·25	1·76 (0·71–4·34)	0·22
Neonatal death (<28 days)	5/524 (1·0%)	7/634 (1·1%)	0·82 (0·17–3·90)	0·80	0·94 (0·21–4·25)	0·93
Adverse pregnancy outcome§	153/561 (27·3%)	151/663 (22·8%)	1·23 (0·97–1·55)	0·090	1·27 (1·06–1·53)	0·011
Sumba						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	29/256 (11·3%)	47/290 (16·2%)	0·70 (0·44–1·09)	0·12	0·72 (0·48–1·09)	0·12
Maternal plasmodium infection during pregnancy† (cumulative risk)	36/276 (13·0%)	40/324 (12·3%)	1·05 (0·62–1·78)	0·85	1·08 (0·70–1·68)	0·72
Maternal clinical malaria during pregnancy (cumulative risk)	0/276	0/324	..	..	..	..
Maternal peripheral patent plasmodium infection at delivery	0/253	1/284 (0·4%)	..	..	..	..
Maternal peripheral sub-patent plasmodium infection at delivery	17/253 (6·7%)	28/284 (9·9%)	0·68 (0·37–1·26)	0·22	0·68 (0·37–1·25)	0·21
Placental plasmodium infection‡	15/249 (6·0%)	20/283 (7·1%)	0·90 (0·49–1·66)	0·74	0·94 (0·52–1·72)	0·84
Maternal haemoglobin <9 g/dL at delivery	34/254 (13·4%)	41/289 (14·2%)	0·91 (0·68–1·23)	0·55	0·88 (0·66–1·17)	0·37
Fetal anaemia (cord haemoglobin <12·5 g/dL)	30/243 (12·3%)	47/278 (16·9%)	0·73 (0·46–1·16)	0·18	0·73 (0·46–1·14)	0·16
Small for gestational age (<10th percentile[27])	40/243 (16·5%)	37/287 (12·9%)	1·32 (0·90–1·95)	0·16	1·38 (0·94–2·02)	0·10
Low birthweight (<2500g)	62/243 (25·5%)	42/287 (14·6%)	1·77 (1·29–2·42)	0·0004	1·84 (1·35–2·51)	<0·0001
Preterm birth (<37 weeks)	37/250 (14·8%)	32/289 (11·1%)	1·33 (0·86–2·07)	0·21	1·38 (0·88–2·16)	0·16
Fetal loss (spontaneous abortion or stillbirth)	8/258 (3·1%)	4/293 (1·4%)	2·25 (0·67–7·57)	0·19	2·29 (0·66–7·96)	0·19
Neonatal death (<28 days)	0/250	0/288	..	..	..	..
Adverse pregnancy outcome§	94/258 (36·4%)	75/293 (25·6%)	1·43 (1·13–1·79)	0·0024	1·45 (1·16–1·83)	0·0014
Papua						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	32/272 (11·8%)	81/343 (23·6%)	0·50 (0·32–0·79)	0·0026	0·55 (0·39–0·77)	0·0006
Maternal plasmodium infection during pregnancy† (cumulative risk)	17/319 (5·3%)	78/356 (21·9%)	0·23 (0·14–0·39)	<0·0001	0·23 (0·14–0·39)	<0·0001
Maternal clinical malaria during pregnancy (cumulative risk)	2/319 (0·6%)	13/356 (3·7%)	0·16 (0·04–0·67)	0·012	0·16 (0·04–0·67)	0·012
Maternal peripheral patent plasmodium infection at delivery	1/270 (0·4%)	13/343 (3·8%)	0·10 (0·02–0·60)	0·012	0·09 (0·02–0·56)	0·0099
Maternal peripheral sub-patent plasmodium infection at delivery	11/270 (4·1%)	28/343 (8·2%)	0·51 (0·32–0·81)	0·0046	0·65 (0·42–1·00)	0·047
Placental plasmodium infection‡	25/251 (10·0%)	75/327 (22·9%)	0·43 (0·26–0·69)	0·0006	0·44 (0·27–0·73)	0·0014
Maternal haemoglobin <9 g/dL at delivery	26/270 (9·6%)	52/342 (15·2%)	0·64 (0·44–0·92)	0·016	0·72 (0·53–0·97)	0·033
Fetal anaemia (cord haemoglobin <12·5 g/dL)	55/244 (22·5%)	92/325 (28·3%)	0·79 (0·59–1·06)	0·11	0·77 (0·56–1·04)	0·09
Small for gestational age (<10th percentile[27])	29/269 (10·8%)	34/338 (10·1%)	1·08 (0·71–1·63)	0·73	1·28 (0·84–1·96)	0·25
Low birthweight (<2500g)	31/269 (11·5%)	44/339 (13·0%)	0·84 (0·53–1·40)	0·50	0·72 (0·40–1·31)	0·28
Preterm birth (<37 weeks)	24/274 (8·8%)	38/346 (11·0%)	0·77 (0·43–1·46)	0·43	0·77 (0·40–1·47)	0·43
Fetal loss (spontaneous abortion or stillbirth)	5/303 (1·7%)	5/369 (1·4%)	1·08 (0·29–3·96)	0·91	1·42 (0·36–5·56)	0·62
Neonatal death (<28 days)	5/274 (1·8%)	7/346 (2·0%)	0·86 (0·22–3·45)	0·84	0·86 (0·21–3·43)	0·82
Adverse pregnancy outcome§	59/303 (19·5%)	76/370 (20·5%)	0·90 (0·60–1·33)	0·58	0·93 (0·58–1·48)	0·75

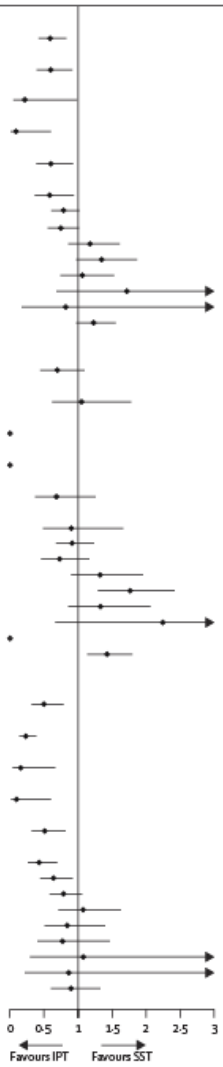

Figure 2: **IPT versus SST for the primary outcome and key secondary outcomes, overall and by site (modified ITT population)**
ITT=intention to treat. LAMP=loop-mediated isothermal amplification. IPT=intermittent preventive treatment. SST=single screening and treatment. RR=relative risk. *Adjusted for site (in the overall models only) and six additional, prespecified, participant-level covariates. †Detected by LAMP, PCR, microscopy, or malaria rapid diagnostic test. ‡Detected by LAMP, PCR, microscopy, malaria rapid diagnostic test, or histology (active and past infection). §Defined as fetal loss, low birthweight, small for gestational age, or preterm birth.

IST vs SST	IST n/N (%)	SST n/N (%)	Crude RR (95% CI)	p value	Adjusted RR (95% CI)*	p value
Overall						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	84/713 (11·8%)	128/633 (20·2%)	0·56 (0·40–0·77)	0·0005	0·72 (0·56–0·93)	0·013
Maternal plasmodium infection during pregnancy† (cumulative risk)	120/786 (15·3%)	118/680 (17·4%)	0·86 (0·59–1·27)	0·45	1·13 (0·74–1·75)	0·57
Maternal clinical malaria during pregnancy (cumulative risk)	8/786 (1·0%)	13/680 (1·9%)	0·56 (0·20–1·58)	0·27	0·82 (0·29–2·30)	0·70
Maternal peripheral patent plasmodium infection at delivery	11/708 (1·6%)	14/627 (2·2%)	0·68 (0·28–1·65)	0·39	0·82 (0·36–1·88)	0·64
Maternal peripheral sub-patent plasmodium infection at delivery	28/708 (4·0%)	56/627 (8·9%)	0·49 (0·35–0·67)	<0·0001	0·63 (0·42–0·96)	0·030
Placental plasmodium infection‡	95/694 (13·7%)	95/610 (15·6%)	0·82 (0·54–1·25)	0·35	0·95 (0·78–1·16)	0·61
Maternal haemoglobin <9 g/dL at delivery	92/710 (13·0%)	93/631 (14·7%)	0·90 (0·67–1·20)	0·46	0·95 (0·73–1·23)	0·69
Fetal anaemia (cord haemoglobin <12·5 gd/L)	130/680 (19·1%)	139/603 (23·1%)	0·81 (0·62–1·07)	0·14	0·81 (0·67–0·99)	0·034
Small for gestational age (<10th percentile[23])	69/695 (9·9%)	71/625 (11·4%)	0·88 (0·63–1·22)	0·44	0·90 (0·67–1·21)	0·48
Low birthweight (<2500 g)	81/698 (11·6%)	86/626 (13·7%)	0·88 (0·63–1·24)	0·47	1·00 (0·75–1·34)	1·00
Preterm birth (<37 weeks)	73/713 (10·2%)	70/635 (11·0%)	1·00 (0·70–1·43)	0·99	0·99 (0·71–1·37)	0·94
Fetal loss (spontaneous abortion or stillbirth)	16/752 (2·1%)	9/662 (1·4%)	1·62 (0·64–4·10)	0·31	1·60 (0·61–4·20)	0·34
Neonatal death (<28 days)	15/712 (2·1%)	7/634 (1·1%)	2·14 (0·78–5·84)	0·14	2·76 (0·97–7·81)	0·06
Adverse pregnancy outcome§	161/752 (21·4%)	151/663 (22·8%)	1·00 (0·77–1·28)	0·98	1·01 (0·83–1·25)	0·89
Sumba						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	20/285 (7·0%)	47/290 (16·2%)	0·43 (0·24–0·78)	0·0049	0·44 (0·23–0·82)	0·010
Maternal plasmodium infection during pregnancy† (cumulative risk)	46/338 (13·6%)	40/324 (12·3%)	1·11 (0·65–1·90)	0·71	1·31 (0·80–2·15)	0·28
Maternal clinical malaria during pregnancy (cumulative risk)	2/338 (0·6%)	0/324	..	-	..	..
Maternal peripheral patent plasmodium infection at delivery	1/282 (0·4%)	1/284 (0·4%)	1·01 (0·07–15·17)	0·99	1·01 (0·07–15·17)	0·99
Maternal peripheral sub-patent plasmodium infection at delivery	6/282 (2·1%)	28/284 (9·9%)	0·21 (0·11–0·43)	<0·0001	0·21 (0·10–0·45)	<0·0001
Placental plasmodium infection‡	16/279 (5·7%)	20/283 (7·1%)	0·87 (0·50–1·51)	0·61	0·85 (0·46–1·55)	0·59
Maternal haemoglobin <9 g/dL at delivery	45/284 (15·8%)	41/289 (14·2%)	1·11 (0·87–1·43)	0·40	1·10 (0·85–1·41)	0·47
Fetal anaemia (cord haemoglobin <12·5 g/dL)	35/274 (12·8%)	47/278 (16·9%)	0·76 (0·53–1·08)	0·13	0·77 (0·52–1·14)	0·19
Small for gestational age (<10th percentile[23])	35/276 (12·7%)	37/287 (12·9%)	1·00 (0·68–1·48)	0·98	0·96 (0·67–1·39)	0·84
Low birthweight (<2500g)	44/278 (15·8%)	42/287 (14·6%)	1·08 (0·76–1·54)	0·67	1·19 (0·85–1·68)	0·31
Preterm birth (<37 weeks)	46/287 (16·0%)	32/289 (11·1%)	1·45 (0·95–2·20)	0·08	1·38 (0·88–2·16)	0·16
Fetal loss (spontaneous abortion or stillbirth)	8/295 (2·7%)	4/293 (1·4%)	1·98 (0·60–6·50)	0·26	1·57 (0·49–5·07)	0·45
Neonatal death (<28 days)	9/286 (3·1%)	0/288	..	-	..	..
Adverse pregnancy outcome§	89/295 (30·2%)	75/293 (25·6%)	1·18 (0·92–1·52)	0·18	1·11 (0·87–1·42)	0·38
Papua						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	64/428 (15·0%)	81/343 (23·6%)	0·63 (0·47–0·85)	0·0025	v0·85 (0·65–1·11)	0·23
Maternal plasmodium infection during pregnancy† (cumulative risk)	74/448 (16·5%)	78/356 (21·9%)	0·67 (0·41–1·09)	0·10	0·67 (0·41–1·09)	0·10
Maternal clinical malaria during pregnancy (cumulative risk)	6/448 (1·3%)	13/356 (3·7%)	0·39 (0·18–0·88)	0·023	0·39 (0·18–0·88)	0·023
Maternal peripheral patent plasmodium infection at delivery	10/426 (2·3%)	13/343 (3·8%)	0·66 (0·33–1·31)	0·23	0·66 (0·33–1·29)	0·22
Maternal peripheral sub-patent plasmodium infection at delivery	22/426 (5·2%)	28/343 (8·2%)	0·66 (0·48–0·89)	0·0076	1·02 (0·67–1·54)	0·94
Placental plasmodium infection‡	79/415 (19·0%)	75/327 (22·9%)	0·84 (0·69–1·02)	0·08	0·90 (0·70–1·14)	0·37
Maternal haemoglobin <9 g/dl at delivery	47/426 (11·0%)	52/342 (15·2%)	0·73 (0·45–1·17)	0·19	0·87 (0·61–1·25)	0·46
Fetal anaemia (cord haemoglobin <12·5 g/dL)	95/406 (23·4%)	92/325 (28·3%)	0·82 (0·62–1·09)	0·17	0·78 (0·61–1·00)	0·06
Small for gestational age (<10th percentile[23])	34/419 (8·1%)	34/338 (10·1%)	0·81 (0·50–1·31)	0·38	0·89 (0·57–1·39)	0·61
Low birthweight (<2500g)	37/420 (8·8%)	44/339 (13·0%)	0·66 (0·37–1·19)	0·17	0·56 (0·28–1·12)	0·10
Preterm birth (<37 weeks)	27/426 (6·3%)	38/346 (11·0%)	0·57 (0·36–0·90)	0·016	0·56 (0·35–0·90)	0·017
Fetal loss (spontaneous abortion or stillbirth)	8/457 (1·8%)	5/369 (1·4%)	1·27 (0·31–5·14)	0·74	2·04 (0·56–7·41)	0·28
Neonatal death (<28 days)	6/426 (1·4%)	7/346 (2·0%)	0·66 (0·21–2·04)	0·47	0·65 (0·21–2·02)	0·46
Adverse pregnancy outcome§	72/457 (15·8%)	76/370 (20·5%)	0·75 (0·49–1·14)	0·17	0·77 (0·44–1·37)	0·38

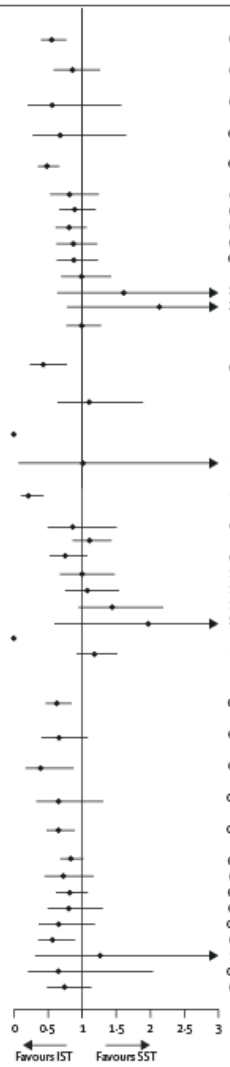

Figure 3: IST versus SST in the primary outcome and key secondary outcomes, overall and by site (modified ITT population)
ITT=intention to treat. LAMP=loop-mediated isothermal amplification. IST=intermittent screening and treatment. SST=single screening and treatment. RR=relative risk. *Adjusted for site (in the overall models only) and six additional, prespecified, participant-level covariates. †Detected by LAMP, PCR, microscopy, or malaria rapid diagnostic test. ‡Detected by LAMP, PCR, microscopy, malaria rapid diagnostic test, or histology (active and past infection). §Defined as fetal loss, low birthweight, small for gestational age, or preterm birth.

IPT *vs* IST	IPT n/N (%)	IST, n/N (%)	Crude RR (95% CI)	p value	Adjusted RR (95% CI)*	p value
Overall						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	61/528 (11·6%)	84/713 (11·8%)	1·06 (0·73, 1·54)	0·77	0·88 (0·65, 1·18)	0·38
Maternal plasmodium infection during pregnancy† (cumulative risk)	53/595 (8·9%)	120/786 (15·3%)	0·69 (0·48, 1·00)	0·045	0·53 (0·34, 0·82)	0·0044
Maternal clinical malaria during pregnancy (cumulative risk)	2/595 (0·3%)	8/786 (1·0%)	0·33 (0·07, 1·50)	0·15	0·26 (0·06, 1·18)	0·08
Maternal peripheral patent plasmodium infection at delivery	1/523 (0·2%)	11/708 (1·6%)	0·13 (0·02, 0·92)	0·042	0·11 (0·02, 0·75)	0·024
Maternal peripheral sub-patent plasmodium infection at delivery	28/523 (5·4%)	28/708 (4·0%)	1·23 (0·81, 1·87)	0·33	1·03 (0·68, 1·56)	0·89
Placental plasmodium infection‡	40/500 (8·0%)	95/694 (13·7%)	0·71 (0·44, 1·16)	0·17	0·54 (0·39, 0·75)	0·0002
Maternal haemoglobin <9 g/dL at delivery	60/524 (11·5%)	92/710 (13·0%)	0·88 (0·65, 1·20)	0·42	0·85 (0·67, 1·07)	0·17
Fetal anaemia (cord haemoglobin <12·5 g/dL)	85/487 (17·5%)	130/680 (19·1%)	0·92 (0·66, 1·27)	0·61	0·97 (0·78, 1·21)	0·78
Small for gestational age (<10th percentile[27])	69/512 (13·5%)	69/695 (9·9%)	1·34 (0·97, 1·86)	0·08	1·39 (1·06, 1·81)	0·015
Low birthweight (<2500 g)	93/512 (18·2%)	81/698 (11·6%)	1·53 (1·09, 2·14)	0·014	1·40 (1·09, 1·80)	0·0093
Preterm birth (<37 weeks)	61/524 (11·6%)	73/713 (10·2%)	1·07 (0·73, 1·56)	0·74	1·14 (0·81, 1·61)	0·45
Fetal loss (spontaneous abortion or stillbirth)	13/561 (2·3%)	16/752 (2·1%)	1·06 (0·51, 2·20)	0·87	1·10 (0·49, 2·47)	0·82
Neonatal death (<28 days)	5/524 (1·0%)	15/712 (2·1%)	0·38 (0·10, 1·51)	0·17	0·34 (0·09, 1·33)	0·12
Adverse pregnancy outcome§	153/561 (27·3%)	161/752 (21·4%)	1·23 (0·95, 1·60)	0·12	1·25 (1·03, 1·53)	0·025
Sumba						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	29/256 (11·3%)	20/285 (7·0%)	1·61 (0·85, 3·07)	0·15	1·66 (0·84, 3·26)	0·15
Maternal plasmodium infection during pregnancy† (cumulative risk)	36/276 (13·0%)	46/338 (13·6%)	0·95 (0·62, 1·45)	0·81	0·83 (0·52, 1·31)	0·42
Maternal clinical malaria during pregnancy (cumulative risk)	0/276	2/338 (0·6%)	..	..	..	..
Maternal peripheral patent plasmodium infection at delivery	0/253	1/282 (0·4%)	..	..	..	..
Maternal peripheral sub-patent plasmodium infection at delivery	17/253 (6·7%)	6/282 (2·1%)	3·19 (1·40, 7·29)	0·0060	3·30 (1·29, 8·43)	0·013
Placental plasmodium infection‡	15/249 (6·0%)	16/279 (5·7%)	1·04 (0·59, 1·83)	0·89	1·11 (0·61, 2·02)	0·72
Maternal haemoglobin <9 g/dL at delivery	34/254 (13·4%)	45/284 (15·8%)	0·82 (0·61, 1·10)	0·18	0·80 (0·60, 1·08)	0·14
Fetal anaemia (cord haemoglobin <12·5 g/dL)	30/243 (12·3%)	35/274 (12·8%)	0·97 (0·57, 1·64)	0·90	0·94 (0·54, 1·65)	0·83
Small for gestational age (<10th percentile[27])	40/243 (16·5%)	35/276 (12·7%)	1·32 (0·90, 1·93)	0·16	1·43 (0·95, 2·16)	0·09
Low birthweight (<2500g)	62/243 (25·5%)	44/278 (15·8%)	1·64 (1·21, 2·21)	0·0014	1·54 (1·17, 2·04)	0·0023
Preterm birth (<37 weeks)	37/250 (14·8%)	46/287 (16·0%)	0·92 (0·65, 1·31)	0·64	1·00 (0·65, 1·55)	0·99
Fetal loss (spontaneous abortion or stillbirth)	8/258 (3·1%)	8/295 (2·7%)	1·14 (0·45, 2·90)	0·79	1·46 (0·47, 4·57)	0·51
Neonatal death (<28 days)	0/250	9/286 (3·1%)	..	..	..	..
Adverse pregnancy outcome§	94/258 (36·4%)	89/295 (30·2%)	1·20 (0·96, 1·51)	0·10	1·30 (1·02, 1·67)	0·035
Papua						
Maternal peripheral or placental plasmodium infection at delivery (primary outcome)	32/272 (11·8%)	64/428 (15·0%)	0·79 (0·52, 1·20)	0·27	0·65 (0·46, 0·91)	0·012
Maternal plasmodium infection during pregnancy† (cumulative risk)	17/319 (5·3%)	74/448 (16·5%)	0·35 (0·22, 0·56)	<0·0001	0·35 (0·22, 0·55)	<0·0001
Maternal clinical malaria during pregnancy (cumulative risk)	2/319 (0·6%)	6/448 (1·3%)	0·43 (0·09, 1·99)	0·28	0·43 (0·09, 1·99)	0·28
Maternal peripheral patent plasmodium infection at delivery	1/270 (0·4%)	10/426 (2·3%)	0·15 (0·02, 0·99)	0·049	0·14 (0·02, 0·93)	0·042
Maternal peripheral sub-patent plasmodium infection at delivery	11/270 (4·1%)	22/426 (5·2%)	0·78 (0·55, 1·11)	0·16	0·64 (0·38, 1·06)	0·08
Placental Plasmodium infection‡	25/251 (10·0%)	79/415 (19·0%)	0·51 (0·31, 0·83)	0·0068	0·49 (0·32, 0·76)	0·0013
Maternal haemoglobin <9 g/dl at delivery	26/270 (9·6%)	47/426 (11·0%)	0·88 (0·55, 1·41)	0·59	0·82 (0·64, 1·06)	0·14
Fetal anaemia (cord haemoglobin <12·5 g/dL)	55/244 (22·5%)	95/406 (23·4%)	0·96 (0·73, 1·26)	0·76	0·98 (0·76, 1·26)	0·85
Small for gestational age (<10th percentile[27])	29/269 (10·8%)	34/419 (8·1%)	1·33 (0·88, 2·01)	0·17	1·44 (1·00, 2·08)	0·05
Low birthweight (<2500g)	31/269 (11·5%)	37/420 (8·8%)	1·28 (0·73, 2·23)	0·39	1·29 (0·71, 2·32)	0·41
Preterm birth (<37 weeks)	24/274 (8·8%)	27/426 (6·3%)	1·36 (0·74, 2·50)	0·32	1·36 (0·73, 2·53)	0·33
Fetal loss (spontaneous abortion or stillbirth)	5/303 (1·7%)	8/457 (1·8%)	0·85 (0·30, 2·37)	0·75	0·69 (0·25, 1·94)	0·49
Neonatal death (<28 days)	5/274 (1·8%)	6/426 (1·4%)	1·32 (0·32, 5·44)	0·70	1·31 (0·32, 5·42)	0·71
Adverse pregnancy outcome§	59/303 (19·5%)	72/457 (15·8%)	1·20 (0·80, 1·81)	0·38	1·20 (0·79, 1·81)	0·39

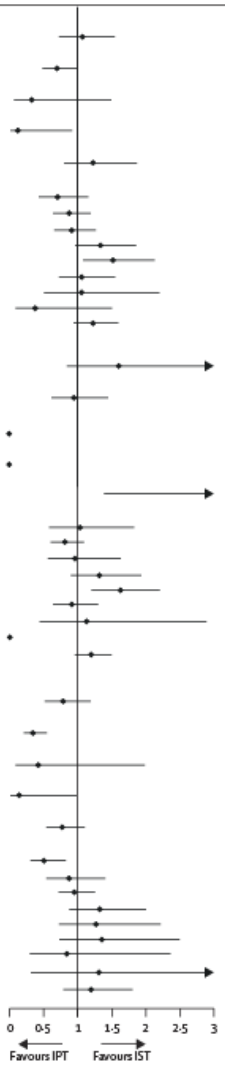

Figure 4: **IPT versus IST in the primary outcome and key secondary outcomes, overall and by site (modified ITT population)**
LAMP=loop-mediated isothermal amplification. IPT=intermittent preventive treatment. IST=intermittent screening and treatment. RR=relative risk. *Adjusted for site (in the overall models only) and six additional, prespecified, participant-level covariates. †Detected by LAMP, PCR, microscopy, or malaria rapid diagnostic test. ‡Detected by LAMP, PCR, microscopy, malaria rapid diagnostic test, or histology (active and past infection). §Defined as fetal loss, low birthweight, small for gestational age, or preterm birth

	IPT (n=681 women, n=524 infants)*			IST (n=854 women, n=712 infants)†			SST (n=744 women, n=634 infants)‡		
	Number of participants with event (%)	Total number of events	Incidence per 100 person-years (95% CI)	Number of participants with event (%)	Total number of events	Incidence per 100 person-years (95% CI)	Number of participants with event (%)	Total number of events	Incidence per 100 person-years (95% CI)
Mothers									
Any serious adverse event	56 (8·2%)	85	54·2 (43·3–67·1)	77 (9·0%)	105	47·2 (38·6–57·2)	71 (9·5%)	113	60·4 (49·8–72·6)
Pregnancy, puerperium and perinatal conditions	47 (6·9%)	62	39·6 (30·3–50·7)	60 (7·0%)	71	31·9 (25–40·3)	51 (6·9%)	65	34·7 (26·8–44·3)
Infections and infestations	8 (1·2%)	8	5·1 (2·2–10·1)	9 (1·1%)	11	4·9 (2·5–8·9)	17 (2·3%)	26	13·9 (9·1–20·4)
Gastrointestinal disorders	2 (0·3%)	3	1·9 (0·4–5·6)	1 (0·1%)	2	0·9 (0·1–3·3)	2 (0·3%)	3	1·6 (0·3–4·7)
Surgical and medical procedures	0	0	0 (0–0)	1 (0·1%)	1	0·4 (0–2·5)	1 (0·1%)	1	0·5 (0–3)
Blood and lymphatic system disorders	7 (1·0%)	7	4·5 (1·8–9·2)	12 (1·4%)	14	6·3 (3·4–10·6)	15 (2·0%)	15	8 (4·5–13·2)
Nervous system disorders	0	0	0 (0–0)	0	0	0 (0–0)	1 (0·1%)	2	1·1 (0·1–3·9)
Reproductive system and breast disorders	1 (0·1%)	1	0·6 (0–3·6)	1 (0·1%)	1	0·4 (0–2·5)	0	0	0 (0–0)
Injury, poisoning and procedural complications	0	0	0 (0–0)	1 (0·1%)	1	0·4 (0–2·5)	0	0	0 (0–0)
Respiratory, thoracic and mediastinal disorders	1 (0·1%)	1	0·6 (0–3·6)	0	0	0 (0–0)	0	0	0 (0–0)
Vascular disorders	1 (0·1%)	1	0·6 (0–3·6)	0	0	0 (0–0)	0	0	0 (0–0)
Metabolism and nutrition disorders	1 (0·1%)	1	0·6 (0–3·6)	2 (0·2%)	2	0·9 (0·1–3·3)	0	0	0 (0–0)
Hepatobiliary disorders	0	0	0 (0–0)	1 (0·1%)	1	0·4 (0–2·5)	0	0	0 (0–0)
Cardiac disorders	0	0	0 (0–0)	1 (0·1%)	1	0·4 (0–2·5)	1 (0·1%)	1	0·5 (0–3)
Neoplasms benign, malignant and unspecified (incl cysts and polyps)	1 (0·1%)	1	0·6 (0–3·6)	0	0	0 (0–0)	0	0	0 (0–0)
Infants									
Any serious adverse event	40 (7·6%)	56	88 (66·5–114·3)	53 (7·4%)	75	89·5 (70·4–112·2)	51 (8·0%)	69	86·5 (67·3–109·5)
Congenital, familial and genetic disorders	11 (2·1%)	11	17·3 (8·6–30·9)	18 (2·5%)	19	22·7 (13·7–35·4)	19 (3·0%)	19	23·8 (14·3–37·2)
Pregnancy, puerperium and perinatal conditions	6 (1·1%)	6	9·4 (3·5–20·5)	14 (2·0%)	14	16·7 (9·1–28)	10 (1·6%)	12	15 (7·8–26·3)
Infections and infestations	18 (3·4%)	19	29·9 (18–46·6)	23 (3·2%)	23	27·5 (17·4–41·2)	24 (3·8%)	25	31·4 (20·3–46·3)
Gastrointestinal disorders	0	0	0 (0–0)	1 (0·1%)	1	1·2 (0–6·7)	2 (0·3%)	2	2·5 (0·3–9·1)
Surgical and medical procedures	0	0	0 (0–0)	1 (0·1%)	1	0 (0–0)	0	0	0 (0–0)
Blood and lymphatic system disorders	1 (0·2%)	1	1·6 (0–8·8)	2 (0·3%)	2	2·4 (0·3–8·6)	0	0	0 (0–0)
Nervous system disorders	1 (0·2%)	1	1·6 (0–8·8)	1 (0·1%)	1	1·2 (0–6·7)	0	0	0 (0–0)
Reproductive system and breast disorders	1 (0·2%)	1	1·6 (0–8·8)	0	0	0 (0–0)	0	0	0 (0–0)
Respiratory, thoracic, and mediastinal disorders	13 (2·5%)	13	20·4 (10·9–34·9)	9 (1·3%)	9	10·7 (4·9–20·4)	10 (1·6%)	10	12·5 (6–23·1)
Metabolism and nutrition disorders	0	0	0 (0–0)	3 (0·4%)	3	3·6 (0·7–10·5)	0	0	0 (0–0)
Hepatobiliary disorders	0	0	0 (0–0)	0	0	0 (0–0)	1 (0·2%)	1	1·3 (0–7)
General disorders and administration site conditions	2 (0·4%)	3	4·7 (1–13·8)	2 (0·3%)	2	2·4 (0·3–8·6)	0	0	0 (0–0)
Neoplasms benign, malignant, and unspecified (including cysts and polyps)	1 (0·2%)	1	1·6 (0–8·8)	0	0	0 (0–0)	0 (0·0%)	0	0 (0–0)

All SAEs were coded using the Medical Dictionary of Regulatory Affairs and are presented here according to their system organ class, the highest level in the dictionary. IPT=intermittent preventive treatment. IST=intermittent screening and treatment. SST=single screening and treatment. *Total follow-up was 222·2 years in women and 83·8 years in infants. †Total follow-up was 156·6 years in women and 63·6 years in infants. ‡Total follow-up was 187·1 years in women and 79·7 years in infants. §Excluding twin births, including liveborn and stillborn infants.

Table 2: Serious adverse events

was lower than with SST, but this difference was not significant (figure 3; appendix p 45). Relative to IST, IPT was significantly more effective in preventing malaria infections during pregnancy (figure 4; appendix p 46). Non-malaria outcomes were similar between the IPT and IST groups (appendix p 46).

There were some significant differences in the effects on the malaria infection outcomes by study site

(figures 2–4; appendix pp 17–25). For the primary endpoint, the p values of the interaction term depicting the difference between sites in the effect of IPT relative to IST were p=0·070 (unadjusted) and p=0·015 (adjusted; appendix p 17) with adjusted analyses showing no significant difference between the two groups in Sumba, but a relative risk reduction of 35% (0·65, 0·46-0·91, p=0·012, figure 2) in Papua. Similarly, the superior effects of IPT relative to IST on malaria infections detected in peripheral blood during pregnancy (figure 4), and in peripheral blood (appendix p 40) and placental blood at delivery (appendix p 43), were also evident only in Papua (appendix pp 19–23). The superior effect of IPT relative to SST on malaria infections in peripheral blood during pregnancy (figure 2) or in placental blood at delivery (appendix p 41) was also evident only in Papua (appendix pp 21–23). There were no significant differences by study site in the effect of IPT versus SST or IPT versus IST on the incidence of non-malaria and all-cause sick visits (appendix p 23).

The analysis of secondary morbidity outcomes showed no significant differences in any anaemia (haemoglobin <11 g/dL) or mean maternal haemoglobin levels (appendix p 27) between groups when both sites were pooled, but in Papua, participants in IPT clusters had a lower prevalence of moderate anaemia (haemoglobin <9 g/dL) than in SST clusters (figure 2). IPT, but not IST, was associated with significantly higher mean haemoglobin concentrations in cord blood (p=0·020; appendix p 25). Relative to SST, IPT and IST did not significantly improve adverse pregnancy outcomes when both sites were pooled (figures 2, 3), but in Sumba, the risk of adverse pregnancy outcomes was significantly higher in the IPT group than in the SST group (figure 2). This difference was not apparent in Papua (figure 2; appendix p 25).

The main adverse events associated with dihydroartemisinin–piperaquine were nausea, headache, and vomiting within 7 days after drug intake. In the IPT group, nausea occurred after 63 (3%) of 2058 courses, headache after 68 (3%) of 2050 courses, and vomiting after 87 (4%) of 2058 courses (appendix p 29). There was no difference between groups in the number of serious adverse events in mothers (table 2). There were three maternal deaths, two in the IST group and one in the SST group; all were considered unrelated to the intervention or malaria (appendix p 30). The prevalence of serious adverse events in infants (table 2), and the risk of congenital malformations, were very similar between groups (appendix pp 47–49).

In total, 33 participants in the IPT group were enrolled in the nested cardiac monitoring. Dihydroartemisinin–piperaquine was associated with a mean QTcF prolongation of 20 ms (SD 19·6) and a mean QTcB prolongation of 14·8 ms (17·6). Neither the mean QTcF nor mean QTcB increased with the total number of dihydroartemisinin–piperaquine courses administered (appendix pp 52–53).

Discussion

This trial highlights that in areas co-endemic for both *P falciparum* and *P vivax* in Indonesia, IPT with dihydroartemisinin–piperaquine compared with the predominantly passive detection afforded by the existing standard SST strategy resulted in a reduction of about 41% in the prevalence of malaria infection at delivery, a similar reduction in its incidence during pregnancy, and a 78% reduction in the incidence of clinical malaria during pregnancy. The effect was evident for both *P falciparum* and *P vivax* infections (appendix p 38), suggesting that monthly dihydroartemisinin–piperaquine was able to successfully delay *P vivax* relapses in the absence of primaquine, which is contraindicated during pregnancy. Of note was the marked difference in the efficacy of IPT between the two study sites. The beneficial effect was evident only in Papua, the higher transmission site, where the prevalence at delivery was reduced by 50% and the antenatal malaria incidence by 77%, similar to the reductions in similar outcomes observed in previous trials with IPT with dihydroartemisinin–piperaquine in western Kenya[9] and Uganda,[10] in which the comparator was IPT with sulfadoxine–pyrimethamine. The greatest reductions were observed for patent parasitaemia (malaria rapid diagnostic test or microscopy positive), which was 91% lower with IPT than with SST at delivery, whereas for subpatent infections, the reduction was 40%. Infants in the IPT cluster had significantly higher mean haemoglobin levels at birth, but otherwise IPT was not associated with improvements in birth outcomes.

Compared with SST, the effect of IST was not consistent. Although IST was associated with a 44% lower prevalence of the primary outcome at delivery, the prevalence of malaria was already 55% lower at enrolment. Furthermore, the effect was only evident at delivery, with no evidence that IST was associated with reductions in the incidence of parasitaemia during pregnancy. Few participants in the SST and IST groups tested positive on malaria rapid diagnostic tests at enrolment or during pregnancy, and contrary to expectations, IST did not detect more infections than SST, despite the four times greater number of screening events. Because the number of participants who tested positive for malaria by malaria rapid diagnostic tests were similar, a similarly low number of participants in the IST and SST groups required treatment and thus benefited from the potential post-treatment prophylactic effect of piperaquine. The observed differences between these two groups in the primary outcome at delivery might thus reflect the lower transmission intensity in the IST clusters that was evident at enrolment or other unknown confounding effects rather than a true intervention effect.

Although, there was no difference between IPT and IST in the composite primary endpoint overall, analyses that adjusted for differences in baseline malaria showed a significant difference in treatment effect between Sumba

and Papua. There was no significant difference between the two groups in Sumba, but a 35% reduction with IPT versus IST in the primary outcome in Papua in the adjusted analysis. IPT was also more effective than IST in reducing malaria infection during pregnancy in Papua. The lack of a difference in the effects of IPT versus IST in Sumba might reflect the lower transmission intensity compared to Papua. It may also reflect the nearly threefold difference between the IPT and IST clusters in malaria risk that was already evident at enrolment in Sumba.

Approximately 90% of infections were below the limit of detection for malaria rapid diagnostic tests. The brand used in the trial did well in the WHO product testing of malaria rapid diagnostic tests and was the best-performing malaria rapid diagnostic test to screen for malaria in asymptomatic pregnant women in our previous diagnostic study in Sumba;[24] although it had an overall sensitivity of 32% to detect PCR-positive infections, and only 13% for *P vivax* mono-infections.[14] The tests were purchased directly from the manufacturer and stored and used according to the manufacturer's instructions.

Dihydroartemisinin–piperaquine was well tolerated, with only one participant in the IPT group vomiting within 30 min after any dose, an adverse event rate that was similar to that reported with sulfadoxine–pyrimethamine in sub-Saharan Africa.[9,10,12] The main adverse events were later vomiting, nausea, and headache within 3 days after drug intake. Overall, among the participants who took dihydroartemisinin–piperaquine, almost 90% complied with the 3-day regimen each time it was administered. The magnitude of QTc prolongation associated with piperaquine was consistent with that seen in other studies with a single course of dihydroartemisinin–piperaquine and similar to previous trials of IPT with dihydroartemisinin–piperaquine in Uganda.[9,10] There was no evidence that QTc prolongation increased with subsequent monthly courses, despite the potential for dose accumulation of piperaquine when dihydroartemisinin–piperaquine is given monthly.[27] In Sumba, there was a higher number of neonatal deaths in the IST clusters than in the other clusters, which was unexplained and could have occurred by chance given the deaths were not related to dihydroartemisinin–piperaquine use and this difference was not observed in Papua. The risk of low birthweight was higher in IPT clusters than in SST or IST clusters, which was probably a chance finding given that this difference was observed only in Sumba.

In a linked feasibility analysis, monthly screening with malaria rapid diagnostic tests was found to be well accepted by asymptomatic participants and providers.[28] By contrast, in this current study, the withdrawal rate was relatively high in the IPT cluster, particularly in Papua, where 14% of participants withdrew, compared with 0% and 2% in the IST and SST clusters, respectively. High rates of withdrawal from IPT were related to concerns about dihydroartemisinin–piperaquine causing potential harm to the mother and baby and being a potential driver of drug resistance.[28] The concept of using dihydroartemisinin–piperaquine for chemoprevention in asymptomatic individuals is new in this region, where to date it has been used only for case management of febrile patients with acute malaria. There is an increasing interest in use of dihydroartemisinin–piperaquine for chemoprevention,[9,10,12] as well as for mass drug administration. In this context it is imperative that careful consideration be given to the optimal use of antimalarials for both treatment and prevention, ideally with drugs that generate opposing selection pressures on the same target.[29] Further feasibility studies with dihydroartemisinin–piperaquine as a monthly IPT are also warranted before its implementation in the region.

The study has several important limitations. First, we used a cluster-randomised design, which, owing to the modest number of assignment units per arm (26 clinic clusters), had a greater potential for bias than trials based on randomisation of individuals. The lower prevalence of malaria at enrolment in the IST arm occurred by chance in both Sumba and Papua, despite our attempt to balance the randomisation by malaria transmission using locally available annual parasite incidence data from the government (appendix p 54). The unequal distribution is a potential cause of bias because of the strong correlation between malaria infection at enrolment and delivery. This is likely to have resulted in overestimation of the effect of IST relative to SST and underestimation of the effect of IPT relative to IST. Second, the study was not powered to detect differences in birth outcomes. Furthermore, in Sumba, the malaria transmission intensity was lower than in previous years, limiting the power to detect differences in infection outcomes, especially for patent infections, which were detected in only 0·5% of participants at enrolment compared with 6·5% in Papua. Patent infections are most likely to be associated with adverse pregnancy outcomes.[2,30–35] Third, only 82% of participants contributed to the primary endpoint, which required collection of maternal and placental blood within a few hours of delivery. Lastly, because HRP2 can remain detectable for up to 1 month after parasite clearance in patients with clinical malaria, malaria rapid diagnostic tests can remain positive for several weeks. This is unlikely to have affected our findings as there were no cases with a positive malaria rapid diagnostic test and a negative qPCR within 1 month.

In conclusion, the effect of IST relative to SST was difficult to ascertain as the 44% difference detected at delivery was already evident at baseline (55% difference) and very few participants tested positive by malaria rapid diagnostic test in this setting, where about 90% of infections were below the limit of detection. Further studies with highly sensitive malaria rapid diagnostic tests should be considered. By contrast, our results suggest that in areas in the Asia-Pacific region with moderate transmission and high-grade sulfadoxine–pyrimethamine and chloroquine

resistance, the strategy of monthly IPT with dihydroartemisinin–piperaquine could be an effective alternative to the existing policy of single screening and treatment.

Contributors

RA and FOtK conceived the study and acquired funding. FOtK was the chief investigator and wrote the first draft of the protocol with RA, who was the co-principal investigator, with further input from DS (co-principal investigator, Indonesia), co-investigators JRP and RNP, and BF, the trial statistician. RA managed the overall trial, supported by DS and FOtK, and coordinated the field work in Sumba. JRP coordinated the field work in Papua. DS liaised with Indonesian Research Ethic Committees, Ministry of Health, and Regulatory Authorities, and was responsible for the laboratory work done at the Eijkman Institute for Molecular Biology, Jakarta, supported by PBSA and SSM. RA supervised the placental histopathology examination. CP was responsible for the safety database and reporting to the oversight committees, ethics committees, and drug manufacturer. MAS-M did the LAMP analysis, supported by ERA. VTU and CTW, supported by ERA, did qPCR and nested PCR at the Liverpool School of Tropical Medicine. JS developed the data management infrastructure and supported TL, who managed the study database and oversaw data entry. CK did the data merging and cleaning and prepared the database for statistical analysis. BF was the trial statistician during the first part of the study and responsible for study design and liaison with statisticians on the Data Monitoring and Ethical Committee and the Trial Steering Committee. CK, RA, FOtK, and BF co-wrote the statistical analysis plan. Following the retirement of BF, DW became the trial statistician and was responsible for the data analysis, supported by TC. FOtK, together with CK, RA, JRP, and RP interpreted the data. RA and FOtK wrote the first draft of the manuscript. All authors reviewed, revised and approved the final version of the manuscript.

Declaration of interests

We declare no competing interests.

Data sharing

All individual-participant data collected during this trial will be available to access, after de-identification. Data and documents, including the study protocol and statistical analysis plan will be available. Data access will be provided to researchers after a proposal has been approved by an independent review committee identified for this purpose. An agreement on how to collaborate will be reached based on any overlap between the proposal and any ongoing efforts. Data will be available beginning at 3 months after publication of this Article. Proposals should be directed to feiko.terkuile@lshtm.ac.uk; to gain access, data requesters will need to sign a data access agreement, and the de-identified database will be transferred by email.

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Chapter 3. Portable diagnostic platforms for molecular testing in low-resource settings

Contents

3.0. Declaration of contribution

Extracted malaria DNA controls used in this Chapter were provided by Dr Debbie Nolder (LSHTM/ PHE). Extracted and genotyped mosquito DNA samples were provided by Dr El-Haji Niang (Université Cheikh Anta Diop, Senegal) and Ms Emily Rippon (LSTM, UK). Mosquitoes reared by Mr Johnathon Thornton (LSTM, UK). I carried out 100% of the MIC™ and Duplex testing, analysis and interpretation, with support from Dr Emily Adams (ERA).

Please see declaration in the publication below for Genedrive® assay development.

Dr Emily Adams (ERA), Dr Gino Miele (genedrive plc, UK) and Prof. Giancarlo Biagini significantly contributed to the intelligent and experimental design of the work in this Chapter, as well as the facilitation of this collaboration.

3.1.1. Chapter summary

This Chapter explores low-cost, portable diagnostic platforms which aim to improve the accuracy and accessibility of molecular malaria diagnostics. First, the feasibility of transferring the 4-plex qPCR (developed in Chapter 2) to the portable MIC™ (Magnetic Induction Cycler, Biomolecular Systems, UK) was investigated. This was followed by a study of simplified DNA extraction techniques using RapiPREP beads to improve its suitability of this assay in low-resource settings. Finally, as part of my MRC CASE studentship funding, I completed a six-month industrial placement with genedrive® plc in Manchester. I spent this time learning about

the process of designing and developing a new diagnostic assay, which lead to the optimisation of an assay for insecticide resistance on the mobile Genedrive platform which was subsequently published. This platform could be used in the future for malaria diagnosis.

3.1.2. Abstract

Background

The miniaturisation of cumbersome and costly instrumentation for molecular analyses offers new promise for sensitive diagnostic testing in field-settings. Here, two portable molecular platforms: the MIC™ and the Genedrive® are investigated for the detection of malaria and insecticide resistance respectively. The use of RapiPREP beads in DNA extraction methods were also investigated in a bid to overcome the common bottle of sample pre-processing.

Methods

First, a melt curve analysis test was designed for use on the Genedrive® for the detection of three genetic mutations responsible for knock-down resistance in mosquitoes. Primer/probe sets were optimised and validated using DNA from field-caught mosquitoes.

Second, the performance of the 4-plex malaria assay (described in Chapter 2) run on the RotorGene-Q (non-portable) was compared to its performance when run on the portable MIC™ platform. The Ct values for each machine were compared using positive control samples for *P. falciparum*, *P. vivax* and *P. ovale*. Finally, the DNA yield obtained using a RapiPREP TB-bead protocol was compared to the use of whole blood directly in the 4-plex qPCR.

Results

Validation of the Genedrive® assay showed 100% agreement with the current gold standard

test for these mutations. The 4-plex qPCR Ct values were comparable when performed on the MIC™ and the RotorGene-Q. The concentration of DNA yielded from the RapiPREP protocol was akin to using diluted whole-blood directly in the 4-plex qPCR assay.

Conclusions

A novel melt curve test was developed for insecticide resistance in mosquitoes. In comparison to the gold standard, the Genedrive assay uses just two probes instead of six, has a simple work flow and is run on a portable rtPCR platform.

Demonstration of the feasibility in transferring the 4-plex qPCR to the portable MIC™ platform, in addition to results showing the assay could be simplified for use with whole blood directly-demonstrates promise for performing this assay in field-settings.

3.2. Introduction

There are a multitude of hurdles that new diagnostics face in reaching their intended market. From validation through to implementation, many promising diagnostics fail to make the jump and succumb to the "diagnostic graveyard". For malaria diagnostics, developers face difficulties in navigating local regulatory and policy requirements, and often lack an understanding of how to support diagnostics in the low-resource settings where malaria is most prevalent. Knowledge transfer between local healthcare professionals, academics and industry can help developers to understand key barriers upfront (FIND 2019). This Chapter includes a collaboration with industrial partners genedrive® plc to tackle two major barriers preventing the use of molecular diagnostics in the field, 1) the prerequisite for laborious DNA extraction, and 2) the requirement for cumbersome and costly instrumentation.

3.2.1. Portable platforms

For clinical management of malaria, microscopy and malaria RDTs are accurate tools to use to detect disease at the point-of-care. However, for surveillance in elimination settings, clinical trials monitoring controlled human malaria infection (CHMI) or treatment efficacy, microscopy and RDTs are not sensitive enough to pick up low-density infections and thus molecular testing is preferable (WHO 2016). Molecular testing is normally restricted to central reference laboratories, however, with advances in technology they are now entering the realms of 'near-POC' (nPOC) diagnostics (WHO 2016). The movement toward miniaturisation of traditionally cumbersome equipment (Nwakanma, D'Alessandro *et al*. 2014), and simplification/ integration

or indeed complete elimination of prerequisite DNA extraction steps, reduces costs and simplifies work flow making molecular testing more accessible.

These field deployable PCR devices are useful for obtaining rapid results that could influence responses at a programmatic level. In low-middle income countries even central reference laboratories often lack the healthcare infrastructure necessary to support molecular testing (Tsai, Liu *et al*. 2019). Adapting PCR assays for the field will enable more accurate diagnoses on the ground in close to real time.

Isothermal molecular techniques e.g. LAMP have largely eliminated the requirement for bulky and costly equipment, and the reagents are often lyophilised, eliminating the need for cold chain (Ng-Nguyen, Stevenson *et al*. 2017). They do still however require a heat block, sometimes a visual analyser (e.g. UV light), are not multiplexed and are still expensive at more than USD $5 per test (personal communication) (O'Sullivan, Linnane *et al*. 2018).

QPCR has the advantage of being quantitative, and with miniaturisation, lyophilisation of reagents and multiplexing, its associated costs could be reduced. Here we investigate the use of two portable PCR platforms as nPOC diagnostic tools for low-resource settings: The MIC™ (Biomolecular Systems, UK) and the Genedrive® (genedrive® plc, UK).

The Genedrive® is currently marketed as a point-of-care, rt-PCR platform that is relatively cheap (~USD $5000 compared to >$20,000 for other qPCR instruments; personal communications) although not yet commercially available, portable (battery operated) and operated by a single button (fig 3.1A). The device uses a single wavelength optical system (400-470nm LEDs, 535nM photodiodes) to read a test cartridge. The device's unique heating and cooling mechanism

generates rapid results (< 1hr) in comparison to most PCR based assays (>2hrs).

Genedrive plc's existing assays include detection of: Hepatitis C virus (HCV), IL-28 SNP mutations to determine HCV treatment (Auburn, Campino *et al*. 2011), *M. tuberculosis* (Walsh, Metzger *et al*. 1991), white-spot shrimp virus and a test for susceptibility to gentamycin-induced deafness (personal communications).

The MIC™ (Biomolecular Systems, UK) is a portable (2kg) PCR machine that uses patented magnetic induction for rapid thermo-cycling (35 cycles in < 25 min for short cDNA amplicons). A spinning rotor mechanism and custom tubes allow for uniform heating across samples to reduce variability. (Figure 3.1B) The Bluetooth-capable device has up to 4 optical channels and can run 48 samples. The provided software is also capable of connecting to and analysing up to ten devices in parallel. Studies using the MIC™ have used it to detect a variety of pathogens including parasites , viruses and bacteria as well as mutations in various organisms (Shenai, Armstrong *et al*. 2016, Duffy, Mottez *et al*. 2017).

Figure 3.1. Portable platforms for molecular detection of malaria.

A. The Genedrive® platform (Kifude, Rajasekariah *et al*. 2008)

B. The MIC™ (source: www.biomolecularsystems.com)

Given its suitability as a POC diagnostic platform, we investigated the feasibility of developing a Genedrive® assay for malaria. Although the qPCR assay was developed (Chapter 2), genedrive® plc prioritised the use of serum over whole-blood, due to their investment in HCV diagnosis. Since the concentration of parasite DNA in serum is lower than in blood (Zainabadi, Nyunt *et al*. 2019) ,and especially in low-density infections, and consequently incompatible with genedrive priorities, other samples were considered. Hence, we developed an insecticide resistance assay to be used on mosquitoes, which was chosen due to simple techniques to extract DNA from mosquitoes and its potential utilisation in malaria control settings. The different characteristics of the two portable platforms are summarised in Table 3.1. The lower throughput of the Genedrive is a major shortcoming of the device, however this could be compensated for by its simplicity, ease of use, kits that are stored at ambient temperatures and rapid run time (Table 3.1).

Table 3.1. Product specifications of the Genedrive® and MIC™.

Product specification	**MIC™**			**Genedrive®**		
Weight	2.1 kg			0.6 kg		
Channels (wavelengths)	Green	Ex 465nm	Em 510nm	Green	Ex 465nm	Em 510nm
	Yellow	Ex 540nm	Em 570nm		-	
	Orange	Ex 585nm	Em 618nm		-	
	Red	Ex 635nm	Em 675nm		-	
Reactions per run	48			3		
Instrumentation cost (USD$)	15,000			5,000		
Thermal ramp rate	Heating	5°C/s		Heating	5°C/s	
	Cooling	4°C/s		Cooling	1.2°C/s	
Power requirements	Mains or battery			Mains or battery		
Closed system	No			Yes		
Disposal	General laboratory waste route			General laboratory waste route		

3.1.2. Simplification of DNA extraction

One of the major bottlenecks in molecular testing is the extraction and purification of DNA. This process is critical for removing the many inhibitors in whole blood (reviewed in Shrader, 2012) and concentrating target DNA, which can be scarce in low-density malaria infections. Although the labour of extraction can be reduced by automation (from 24 samples/run to 96), the process remains costly and prone to cross-contamination. DNA yields can also vary significantly according to the methods used, affecting the sensitivity of downstream diagnostics.

Techniques such as specialised bead extraction (Mens, van Amerongen *et al*. 2008) and boil-and-spin methods (Modak, Barber *et al*. 2016, Roth, de Bes *et al*. 2018) have been explored in a bid to simplify this step and reduce costs/ labour. Alternatively, different direct-blood techniques which eliminate the step altogether have been developed for use in different NAATs e.g. NALFIA (Imai, Tarumoto *et al*. 2017, Choi, Prince *et al*. 2018). Direct-blood methods are becoming more common in isothermal methods (Taylor, Howell *et al*. 2014, Taylor, Lanke *et al*. 2017) which are less prone to inhibition by contaminants. The use of microfluidics has also been explored for DNA extraction to aid simple molecular test e.g. to accompany LAMP (Taylor, Lanke *et al*. 2017). Building on microfluidics, lab-on-chip devices (Unwin, Ainsworth *et al*. 2018) are also available for malaria. These integrate the extraction and analysis steps requiring only whole blood input; however they still require unique instrumentation for reading and are low-throughput (Scherf and Wahlgren 2008).

Currently, expensive but reliable silicon spin-column methods (e.g. Qiagen) are most widely used. In collaboration with genedrive® plc, we investigated the use of RapiPREP-TB beads

(MicroSens Biotech, UK) to concentrate *P. falciparum* DNA in whole blood. Genedrive plc are currently optimising a workflow for their use in concentrating *MTb* DNA, eliminating the need for centrifugation in their commercially available *MTb* tests. Here we use qPCR to analyse DNA yields from processing with the RapiPREP-TB beads in comparison to Qiagen DNeasy extraction kits.

3.1.3. Aims

The work in this Chapter aims to 1) transfer the 4-plex qPCR (optimised in Ch2) to the lower-cost, more portable Magnetic, 2) investigate simplified DNA extraction techniques including RapiPREP TB-beads to facilitate the development of a field-ready, molecular malaria assay and 3) collaborate with genedrive plc to develop a new molecular as part of an MRC CASE studentship.

3.3. Methods

3.3.1. MIC™

The malaria 4-plex assay was tested on the MIC™ using control DNA for *P. falciparum, P. ovale* and *P. vivax* species (provided by Public Health England/LSHTM, courtesy of Dr Debbie Nolder). *P. malariae* was not included in this study as the MIC™ lacks the appropriate filter for the assay's crimson fluorophore tested in Chapter 2. A ten-fold dilution series: neat, 1:10, $1:10^2$ and $1:10^3$ (parasitaemia not quantified due to lack of standards at this time in the study), was prepared for DNA of each species and analysed using qPCR primers and probes identical to those outlined in the Chapter 2 methods (section 2.3.3). Samples were run in parallel on the RotorGene-Q (Qiagen, Germany) and the MIC™ (Biomolecular Systems, UK) in single technical replicates. The resultant qPCR cycle threshold (Ct) values were pooled (n=12) for each platform and compared using a paired t-test.

3.3.2. Genedrive® assay development

The development and validation process for a Genedrive® assay is outlined below. The resulting assay is described in greater detail in the publication by Unwin *et al*. (DiNardo, Hahn *et al*. 2015) (appendix 3.1).

The Genedrive® cartridge consists of three wells (20 µl capacity) enabling either technical triplicates or multiplexing of different assays. During the development phase, assays are optimised

using wet reagents, however as with other Genedrive® tests these could be lyophilised and stored at room temperature.

Primer design for the Genedrive® assays is identical to that of a typical PCR reaction: a conserved sequence around 20 bp in length with a 55-60 °C Tm (melting temperature).

For probe design, Genedrive plc utilise double-fluorophore conjugated HyBeacon™ probes. The target sequence (17-25 bp) should contain two thymidine bases approximately 7 bases apart (one helical rotation), to which the fluorophore is conjugated. This ensures both fluorophores are orientated in the same direction to enhance the signal. Ideally both thymidines are also adjacent to a guanidine, for stable conjugation to the fluorophore.

Probes that exactly match the target sequence result in a higher melting temperature than that of a target with a sequence mismatch. This enables multiplexing of sequence variants (species ID or mutations) using a single primer/probe set, saving time and costs. For multiplex assays, the peak Tm of each target must have a minimum separation of 2 °C to generate distinct melting peaks.

As a point-of-care device, the throughput of the Genedrive® is restricted to 1-3 samples per run depending on the cartridge design. Optimal assay conditions are thus determined through screening on the LightCycler 480 (Roche, Germany), prior to transfer of the assay on to the Genedrive® for analytical testing. Initially 6 sets of primers and 4 probes were designed, which were screened for highest fluorescence peaks and desired melting temperatures. The reaction mix and cycling conditions are described in detail in appendix 3.6 (biotechnologies 2020). Once a primer/probe set was selected, the assay was optimised on the Genedrive® platform. Due to its

rapid heating and cooling capabilities, the initiation, annealing and extension periods are incrementally reduced to minimise total run time whilst still producing good fluorescence peaks. The final Genedrive® conditions were: 50 cycles of amplification at 95 °C for '0' sec (allowed to reach temperature only), annealing at 62 °C for 10 sec, an extension at 72 °C for '0' sec, followed by a melt step increasing the temperature from 42 °C to 80 °C in 0.5 °C increments with continuous fluorescence acquisition, and then cooling to 40 °C for 60 sec, resulting in a total run time of 50 mins.

An integrated algorithm is used to automate interpretation of the Genedrive® results. For this, the minimum peak fluorescence height as well as the melting temperature is defined for positive (presence of target detected) and negative (undetected) results. These parameters are also defined for an internal control to determine test validity.

3.3.3. Simplified DNA extraction

In line with other Genedrive® products, assays must have minimal sample pre-processing and so simplified DNA extraction methods were investigated.

Asexual *P. falciparum* 3D7 parasites were cultured in-vitro to a final parasitaemia of 5% as per standard protocol (Sidstedt, Hedman *et al*. 2018) (provided by Wesam Bakhsh, LSTM). These cultures were used to spike venous human blood (ETDA preserved) directly for "culture-spiked" samples or were used for "DNA-spiked" samples following DNA extraction using a DNeasy Blood and Tissue kit (Qiagen, Germany).

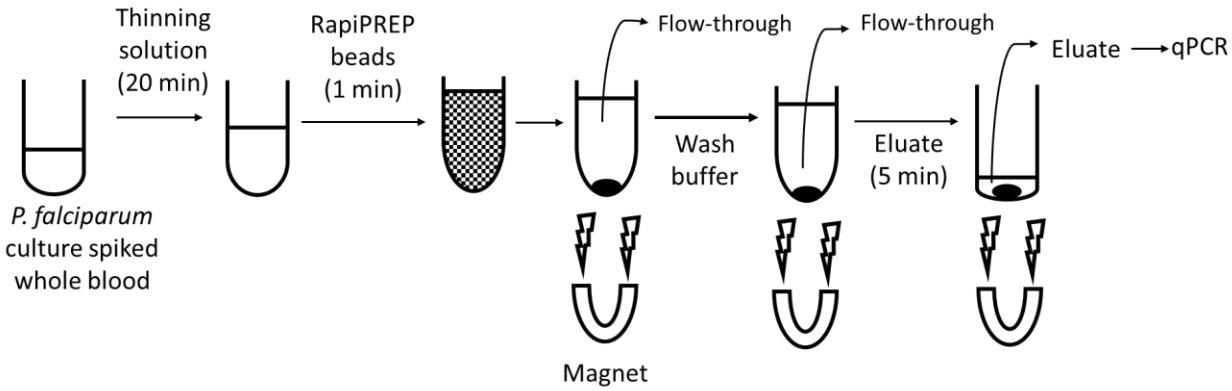
The use of RapiPREP-TB beads (MicroSens Biotech, UK) in concentrating *P. falciparum* DNA was investigated. These magnetic beads, coated in a proprietary polymer, have been previously used for concentrating bacteria such as *Chlamydia*, *Nisseria*, MRSA and *MTb* (Sidstedt, Hedman *et al*. 2018), as well as some viruses (Abu Al-Soud and Rådström 2000). The manufacturer's protocol was adapted as follows: equal volumes of thinning solution (2 % (w/v) NaOH was added to 200 µl of culture-spiked whole blood (1% parasitaemia). This was followed by the addition of 400 µl of TB-Bead solution, inverting the tube multiple times and then incubating at room temperature to capture any DNA present. The tube was transferred to a magnetic separation rack (New England Biosystems, UK) and left for 1 min before the flow-through was removed from the beads. The tube was removed from the rack and 400 µl of wash solution (0.04% sodium hydroxide) was added to resuspend the beads. The tube was immediately placed back in the magnetic rack and the solution removed and stored at 4 °C for later analysis. Finally, 100 µl of elution buffer was added to the beads and incubated for 5 min at room temp to elute the DNA. The stored flow-through (wash buffer) was analysed alongside the final eluate to assess to proportion of DNA lost before elution.

Also, thinning and washing steps were experimentally omitted during the optimisation process. The resultant DNA yield was assessed by qPCR (as described below ('Direct-blood qPCR) in comparison yields obtained using a DNeasy Blood and Tissue kit (Qiagen, 69504). DNA from uninfected blood was also included as a negative control for both methods. (See figure 3.2 for bead extraction procedure)

The inhibitory effects of whole blood in the 4-iplex malaria qPCR (outlined in Chapter 2 methods 2.3.2) were investigated. For this, serial dilutions (in 10^{-1} to 10^{-6}) of DNA-spiked whole

blood and DNA in ddH_2O were compared.

Figure 3.2. DNA extraction work flow using RapiPREP beads



3.3.4. Statistics

The 4-plex malaria qPCR was used to assess both the RapiPREP TB-bead extraction methods and the direct-blood methods, using Ct as a proxy of final DNA yield. A cycling threshold (Ct) cut-off of 38 was used to determine positivity and the Cts between groups were compared using a Mann Whitney test. Correlation coefficients were calculated for the dilution series for spiked-blood and DNA qPCRs.

3.3.5. Ethics

Venous bloods were collected from three healthy, non-endemic volunteers (with no previous travel to endemic countries within the last 3 months) in accordance with the HTA and consent was obtained through signed LSTM participant consent forms. Blood was stored at -4°C and used within 3 hours of collection.

3.4. Results

3.4.1. MIC™

P. falciparum, *P. ovale* and *P. vivax* DNA were all detected on the MIC™, all at lower Ct values than those run on the Rotor-Gene Q (Table 3.1). All dilutions of *P. falciparum* DNA were detected by both platforms, the MIC™ limit of detection for *P. ovale* was 10^{-2}. The RotorGene-Q did not detect the *P. vivax* at 10^{-3} (Table 3.1) which was detected by the MIC™.

Table 3.1. Comparison of qPCR results run on the MIC™ and Rotor-Gene Q (RG).

The Table contains Cycling Threshold (Ct) values obtained from serial dilutions of malaria DNA including *P. falciparum (Pf), P. ovale (Po)* and *P. vivax (Pv).* ND; Not detected using Ct38 cut-off for positivity.

	Dilution	MIC	RG
Pf	Neat	21.46	24.17
	10^{-1}	24.6	28.44
	10^{-2}	28.11	31.68
	10^{-3}	31.17	37.46
Po	Neat	28.5	29
	10^{-1}	32.51	33.7
	10^{-2}	ND	35.29
	10^{-3}	ND	ND
Pv	Neat	25.69	27.99
	10^{-1}	29.05	32.76
	10^{-2}	32.17	36.45
	10^{-3}	34.32	ND

3.4.2. Genedrive®

A multiplex melt-curve assay for insecticide resistance in *Anopheles gambiae* was developed for the Genedrive® as a field-friendly diagnostic for use in low-resource settings (publication

output 3.9).

3.4.3. Simplified DNA extraction

We investigated the effects of adding thinning solution, provided in the RapiPREP TB-bead kit, normally used for sputum samples) on DNA yield following the RapiPREP protocol. The loss of DNA in the discarded flow-through was also assessed.

Using qPCR Ct values as a proxy of DNA yield, figure 3.3 shows more DNA (2-6 Cts or 10- 100 fold) is retained in the eluate compared to the flow-through. The addition of thinning solution results in a lower yield of DNA in the eluate compared to when it is omitted. (Figure 3.3)

Figure 3.4 shows that an additional wash step within the RapiPREP protocol significantly reduces ($P<0.01$) the yield of DNA compared a single wash step. Omission of the beads altogether significantly reduced the DNA yield, showing that the beads do play some role in concentrating the DNA (fig 3.4). Interestingly, significantly lower Cts were obtained when using diluted culture (10^{-1}) directly in the qPCR compared to when neat eluate was added.

Following this observation, DNA-spiked whole blood was directly added to the qPCR to assess its inhibitory effects. Figure 3.4 shows that at a dilution of 10^{-2}, Cts were significantly higher in the direct- culture (mean= 33.5; 95% CI 32.8-34.3) qPCR compared to the spiked-whole blood (mean= 27.8; 95% CI 27.3-28.4) qPCR. Similar Cts were observed for the first dilution of spiked-whole blood (mean= 28.4; 95% CI 28.0-28.9) and the second dilution (mean= 27.8; 95% CI 27.3-28.4), indicating inhibition at this dilution. This is reflected in the higher R^2 value of the direct-blood qPCR when the first dilution is excluded (R^2= 0.98) compared to when it is included (R^2= 0.91).

Figure 3.3. The effect of thinning solution (NaOH) on DNA yields in eluate and flow-through.

Each dot represents a single technical replicate. Statistical analyses revealed no significant difference between groups (P-values all above 0.2).

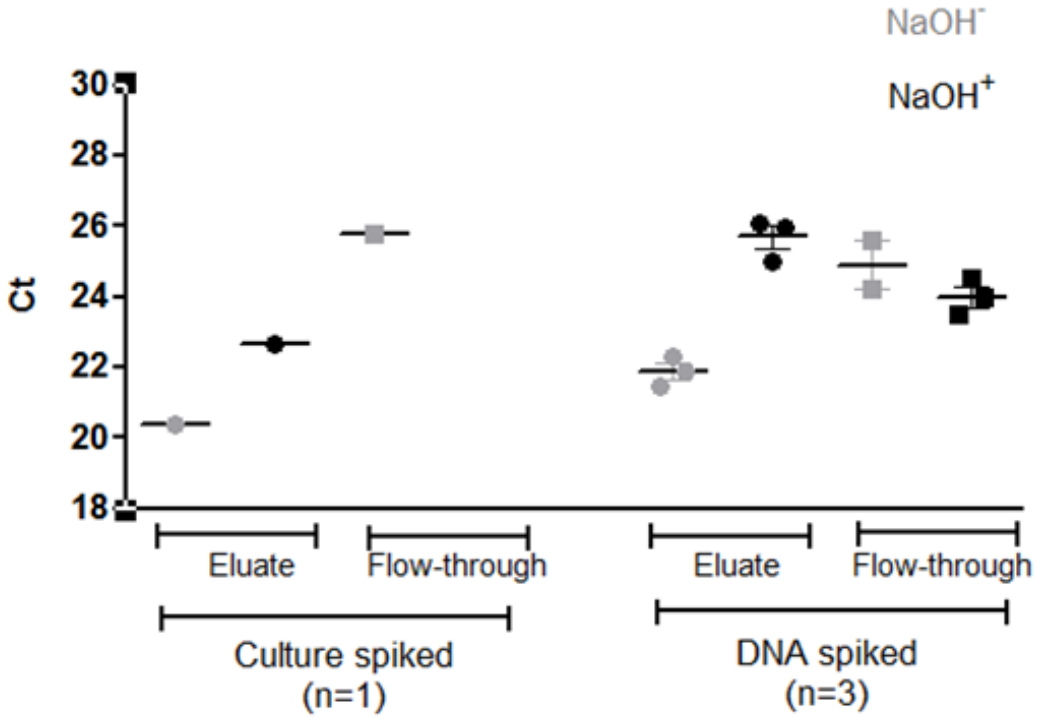
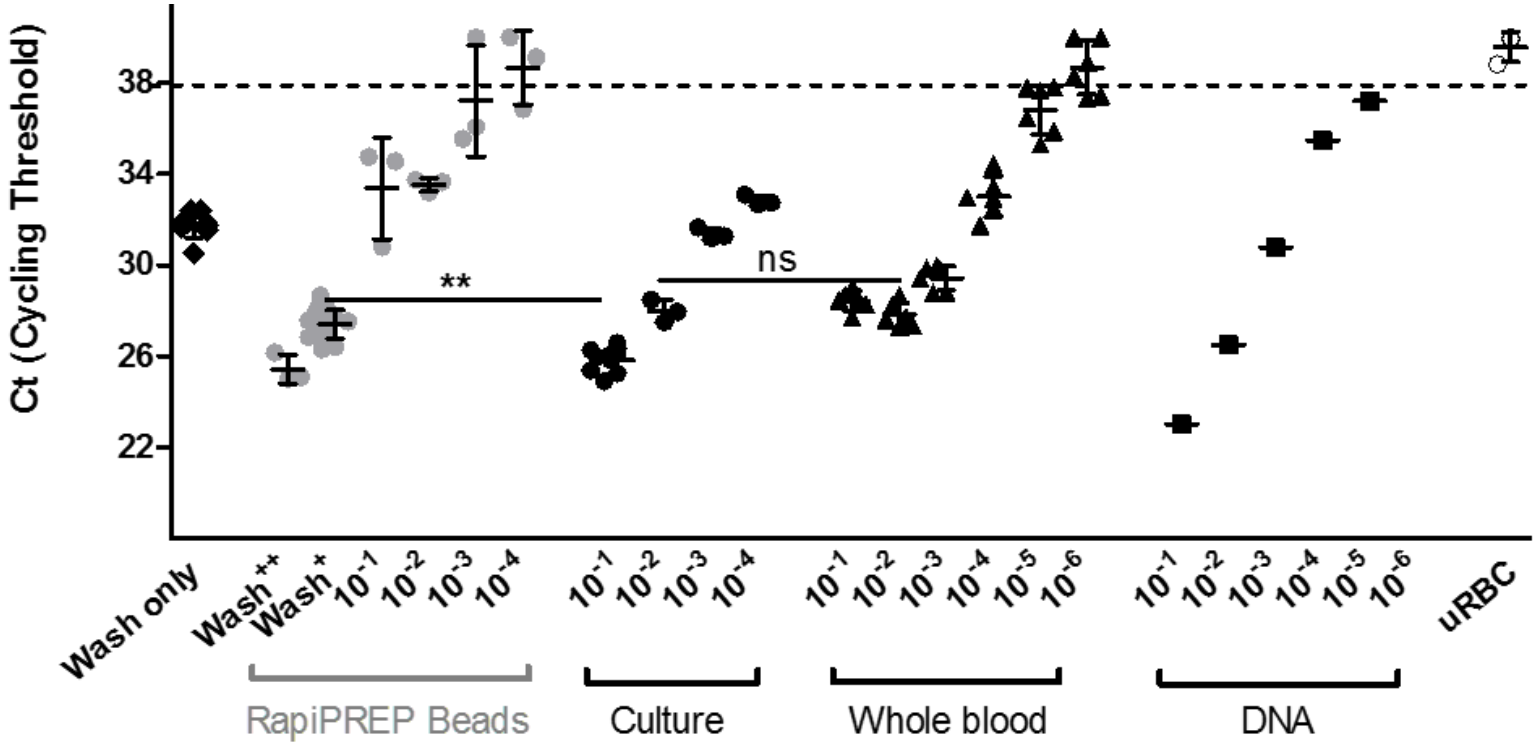


Figure 3.4. Comparison of qPCR results following extraction with different DNA preparatory steps.

Ct values obtained from qPCR of samples following the RapiPREP TB-bead protocol (grey circles): with one wash^{+} (serially diluted ten-fold) or two washes $^{++}$, and omission of the beads (diamonds), or a direct-qPCR of serially diluted (ten-fold) *P. falciparum* culture (black circles), DNA-spiked whole blood (triangles) and DNA extracted by DNeasy kits (squares). Uninfected red blood cells (uRBC) was used as a negative control (clear circles). Each dot represents a single technical replicate (n= 1-6). **P-value <0.01, ns= no significance P-value>0.05



3.5. Discussion

The complexity and associated costs of real-time PCR methods restrict their use to well-supported laboratories. Simplified methods and more portable platforms increase the accessibility of molecular methods for limited-resource settings. In this study, the use of a malaria detection assay on the MIC™ platform was demonstrated, alongside an easy, preparatory method for whole blood that could further simplify the above assay. Alongside this we developed a new molecular assay for insecticide -resistance on the Genedrive® platform, for use in low-resource settings. Having learnt about the process of assay development, the Genedrive® offers a promising platform for the development of a malaria test in the future, in line with an informed genedrive® plc TPP.

3.5.1. Direct-blood qPCR

Historically, whole blood is known to contain an array of inhibitors e.g. haem-derivatives and immunoglobulins, which inhibit PCR reactions (mechanisms investigated by Sidstedt *et al*. (Tsai, Liu *et al*. 2019)). Autofluorescence of red blood cells, as well as denatured proteins from the high cycling temperatures, can also interfere with fluorescence readings (Silva Martins, Silva Pereira *et al*. 2019). Although cheaper alternatives to the reference standard silicone spin-columns are available, they are prone to cross-contamination and still laborious for high-throughput screening. Alternatively DNA extraction can be avoided by using specialist thermostable DNA polymerases that are more resistant to inhibitors or pre-treating the sample with amplification facilitators (Lynd, Oruni *et al*. 2018).

Our preliminary results show the 4-plex malaria qPCR (optimised in Chapter 2) successfully

detects *P. falciparum* DNA directly from diluted, spiked whole blood without prior DNA extraction. Minor inhibition was observed in the least dilute blood samples, but this was improved drastically by diluting in water at a 1:10 ratio (for a final 1:40 in qPCR reaction).

Diluting the sample reduces the volume of blood assayed and therefore the amount of parasite DNA, which affects the sensitivity of the assay. This could also be overcome by amplifying the signal (optimisation of primer/ probe concentrations) or redesigning the assay to target higher copy-number genes e.g. TARE1 and VAR2. The total volume of the reaction could also be increased to boost the volume of blood assayed.

It appears that the polymerase (proprietary) in the Quantifast Pathogen mastermix used here is robust enough to overcome inhibitory factors that might have been historically problematic in whole blood. We also suggest that the centrifugation in the RotorGeneQ's platform is key to the success of this assay, spinning solids to the bottom of the tube away from the laser course. To properly characterise this assay, it is important elucidate exactly which factors are contributing to its success. For this it would be useful to compare other mastermixes with different chemistries, test whole blood from different populations and determine the LOD of the assay. This understanding could lead to the development of a proprietary, potentially lyophilised direct-blood assay, suited to a portable platform.

3.5.2. MIC™

Here it is demonstrated that the 4-plex qPCR (Chapter 2) could be transferred to the portable MIC™ platform with relative ease. The MIC™ detected all *P. falciparum* samples, using the Rotor-Gene Q as a reference standard. Results showed reduced sensitivity of the

MIC™ with regards to *P. ovale*, but potentially increased sensitivity of P. *vivax* in comparison to the Rotor-Gene Q. This suggests the assay needs refining to increase the sensitivity of this assay for these particular species. Again, it would be useful to determine the LOD for this assay on the MIC™ to explore methods that could improve accuracy, and compare its performance to similarly portable open platforms.

It should be noted that this pilot (assay transfer to the MIC) was run in technical singlets, not repeated and the limit of detection (LOD) was not determined. Also, as mentioned above P. *malariae* was not tested in this study. Future work should include more replicates, quantified species-specific DNA for LOD determination and more clinical samples for diagnostic validation. This is the first use of the MIC™ for the detection of malaria but has been utilised other infectious diseases such as dengue, zika and chikungunya (Mulvaney, Fitzgerald *et al*. 2018).

3.5.3. Genedrive®

In collaboration with genedrive® plc we developed an assay for malaria control to run on their mobile platform. Whilst exploring simplification techniques for DNA extraction from whole blood (discussed below), we proceeded to develop a test for insecticide resistance mechanisms in mosquitoes. The resulting assay detected three mutations in the voltage gated sodium channel (VGSC) as well as wild type genotypes. This assay utilised two of the three available wells in a Genedrive® cartridge and had an assay runtime of under an hour. To achieve the same results using standard real-time PCR three separate assays would be required- each with a run time of up to 2 hrs and a total of 6 fluorescent probes rather than 2. Since publication of our assay (2017), two further publications describe the detection of the same tri-allelic gene using 1) Engineered-Tail Allele-Specific-PCR (ETAS-PCR) (Griffin,

Hollingsworth *et al*. 2010) and 2) LNA probes (Slater, Ross *et al*. 2015). Although highly accurate, the first requires visualisation by gel electrophoresis and the second requires three fluorescent probes to date, the simplicity and low cost of our Genedrive® tri-allelic assay (appendix 3.1) is unrivalled.

Handheld PCR devices are widely available (basic thermocycling heat blocks e.g. Ahram Biosystems), however real-time PCR devices that measure fluorescence are less so. One such device is the Biofire Film Array system that contains freeze-dried reagents suited to use in field-settings (WHO 2014). Instead, innovation in post-amplification detectors, e.g. NALFIA are being explored as portable devices that accompany handheld PCR devices.
In terms of costs, the MIC™ retails at £10,000 whereas the Rotor-Gene Q is around £25,000. The cost of the device-specific tubes is comparable, making the main difference the reduced capacity (MIC™: 48 samples vs Rotor-Gene Q: 72). The MIC™ is therefore better suited for a faster turnaround of fewer samples. Furthermore, it uses a similar centrifugal system to the Rotor-Gene Q, so there is also potential to transfer the direct-blood qPCR on the MIC™.

It must be noted that unlike the RotorGene-Q or the MIC™, the Genedrive® is not an open platform and so requires the purchase of assay-specific cartridges. Although cost per sample is comparable to other molecular tests (using their MTb assay as an example), the main limitation of the platform is the low throughput. Designed as a nPOC, the cartridge capacity is either one sample (where each well contains a different assay or where technical triplicates are desired) or three samples run in single replicates. Thus, a malaria Genedrive® assay is better suited for use where a small number of individuals need to be tested quickly and easily. One such use scenario is in focal testing and treatment (FTAT) strategies, where

transmission is low and these strategies are safer and thought to be more cost-effective (Slater, Ross *et al*. 2015, WHO 2015, Unwin, Ahmed *et al*. 2020) than mass drug administration. In these settings, sensitive tools are especially important to detect the low-density infections that are most common. Future market analyses need to be conducted to inform a detailed target product profile for such an assay.

Finally, the Genedrive® does not operate using centrifugal force, which we hypothesise is critical for the success of a direct-blood qPCR. However, its cartridge's unique capillary wells together with the positioning of its laser course could support the use of a direct-blood assay i.e. denatured proteins could sediment at the bottom of the well without affecting fluorescence measurements.

3.6. Conclusions

In conclusion, the findings in this Chapter provide opportunity to further develop a direct-blood qPCR on a portable rt-PCR machine, offering more cost effective, field-friendly, and sensitive testing for malaria.

3.7. Acknowledgments

I would like to thank the technical staff at genedrive® plc for their time training me in their laboratories and guiding me through the developmental process. I would also like to thank the staff at Université Cheikh Anta Diop who collected the original mosquitoes.

3.8. Publication Output

Unwin *et al. Parasites & Vectors* (2018) 11:307
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Parasites & Vectors

RESEARCH Open Access

Development of a rapid field-applicable molecular diagnostic for knockdown resistance (*kdr*) markers in *An. gambiae*

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Abstract

Background: The spread of insecticide resistance (IR) is a major threat to vector control programmes for mosquito-borne diseases. Early detection of IR using diagnostic markers could help inform these programmes, especially in remote locations where gathering reliable bioassay data is challenging. Most current molecular tests for genetic IR markers are only suitable for use in well-equipped laboratory settings. There is an unmet need for field-applicable diagnostics.

Methods: A single-cartridge test was designed to detect key IR mutations in the major African vector of malaria, *Anopheles gambiae*. Developed on the portable, rapid, point-of-care compatible PCR platform - Genedrive® (genedrive® plc), the test comprises two assays which target single nucleotide polymorphisms (SNPs) in the voltage gated sodium channel (VGSC) gene that exert interactive effects on knockdown resistance (*kdr*): L1014F, L1014S and N1575Y.

Results: Distinct melt peaks were observed for each allele at each locus. Preliminary validation of these assays using a test panel of 70 *An. gambiae* samples showed complete agreement of our assays with the widely-used TaqMan assays, achieving a sensitivity and specificity of 100%.

Conclusion: Here we show the development of an insecticide resistance detection assay for use on the Genedrive® platform that has the potential to be the first field-applicable diagnostic for *kdr*.

Background

Control programmes for vector-borne diseases, such as malaria, are heavily reliant on the use of insecticides to reduce vector populations. The use of insecticide-treated nets (ITNs), long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) plays a major role in the overall reduction in global malaria burden. [1]

Until recently, all ITNs and LLINs were formulated with pyrethroid insecticides [2]. The new development of a chlorfenapyr-based LLN offers a potential alternative, since resistance to pyrethroids is now widespread and has been reported in numerous mosquito species [3].

Knockdown resistance mutations (*kdr*) in the para voltage-gated sodium channel (*Vgsc*) of neurones are one of the principle mechanisms of resistance to pyrethroids and dichlorodiphenyltrichloroethane (DDT) in insects, resulting in an increased tolerance to insecticide exposure, compromising vector mortality [4]. Three mutations in the *Vgsc* gene are linked to pyrethroid and DDT resistance in the principal African vectors of malaria: *Anopheles gambiae*, *An. coluzzii* and *An. arabiensis*. Most common are two mutations to the leucine residue at position 1014 in the wild-type Vgsc-1014 [L1014F (TTA > TTT) and L1014S (TTA > TCA)], located in the hydrophobic segment S6 of domain II (IIS6) resulting in a conformational change preventing access of the insecticide to the active site VGSC protein [5]. The L1014F ('F') mutation is the most common *kdr* mutation across diverse insect taxa [6]. L1014S ('S') was previously only found in mosquitoes and has recently been detected in the visceral leishmaniasis vector *Phlebotomus argentipes* [7]. Although first detected in West and East

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Africa, F and S mutations have both been detected across the continent and sometimes co-occur [8]. The mutations do not occur on the same haplotypes, but where both alleles are present, effects on *kdr* are additive [7, 9]. In *An. gambiae* and *An. coluzzii*, a third mutation in the *Vgsc*- Asp1575Tyr (ATA > ATT) has been found in West Africa [10]. The 1575Y ('Y') mutation is only present on the F haplotype and acts as an amplifier of resistance to both pyrethroids and DDT [10, 11].

Existing diagnostics for *kdr* markers, i.e. allele-specific PCR (ASPCR), HOLA, SSOP-ELISA, PCR-Dot Blot, FRET/MCA, TaqMan and HRM, were recently compared to a new SimpleProbe® RT-PCR/melt curve assay in *Culex quinquefasciatus*. The melt curve assay was found to be cheaper, faster and more reliable than alternatives [12]. Melt curve assays are designed on the principle that a sequence-specific probe will dissociate away from a DNA duplex at a characterising melting temperature (Tm). In the presence of a mismatched sequence, probe-binding is less efficient, reducing the Tm, and causing a characteristic Tm shift.

Although melt curve-based techniques have advantages over other PCR-based methods, they still require expensive, mains-powered real-time-PCR platforms and skilled technicians [12]. Furthermore, they have only been validated using purified DNA. There is a need for simpler field-applicable molecular tests that could be used in the resource-limited settings where vector control programmes are commonly employed.

Genedrive® is a molecular diagnostics platform that utilises PCR with detection of a fluorescent reporter dye. The total run time of the system ranges from 45 to 90 min depending on assay, and can be utilised direct from the electricity mains or *via* a portable uninterruptable power supply (UPS) which also functions as a battery. While existing Genedrive® tests target pathogens (HCV [13], MTb [14]) and pharmacogenomic mutations (IL-28B [15]) from such diverse clinical samples as plasma, sputum and buccal swabs, respectively, the technology has not been adopted for use with insects.

Methods

Primer and probe design

Two separate assays were designed to target three individual SNPs within the *Vgsc* gene, the first to discriminate between the two mutations at the L1014 locus and the other at the 1575Y locus.

Dual-labelled fluorescent, HyBeacon-type molecular probes were designed against the L1014F and 1575Y mutation sequences. The probes were designed to dissociate from the amplicon at around 62 °C, whereas mismatched pairing would yield a reduced Tm shift > 2 °C. The Tm of either probe was predicted *in silico* using OligoAnalyzer 3.1 software (https://www.idtdna.com/calc/analyzer) before empirical determination on the LightCycler480 (Roche Applied Science, Penzberg, Germany) (Table 1). Initial screening experiments were carried out on this higher-throughput platform before transfer to the Genedrive® following optimisation. The NCBI-BLAST software was used to design primers and probes specific to the *An. gambiae* complex. Probes were obtained from ATD Bio (Southampton, UK) and primers from Eurofins (Ebersberg, Germany).

Mosquitoes

The following mosquitoes and DNA samples were used for the initial optimisation of the assays:

(i) *Kdr*-susceptible, (S-form) *An. gambiae* (*s.s.*) (wild-type) and *kdr*-resistant, (M-form) *An. coluzzii* (F/F) mosquitoes (Kisumu and VK7 laboratory reference strains, respectively) were provided by the Liverpool Insecticide Testing Establishment (LITE) at the Liverpool School of Tropical Medicine.

(ii) *Kdr*-resistant (S/S) *An. arabiensis* mosquitoes and dual *kdr*- resistant (FY/FY, FF/FY), pre-extracted *An. arabiensis* mosquito DNA came from recent field collections in Senegal, location and sampling details of which will be provided elsewhere.

(iii) A mixed-population panel of *An. gambiae* (*s.s.*) DNA of known genotypes provided from samples archived at LSTM.

TaqMan reference standard

Three separate TaqMan assays are routinely used for genotyping F [16], S [16] and Y [10] mutants. Here, we used these tests as reference standards, performed exactly as previously published [10, 16]. Genotypes of all mosquitoes were confirmed using these established TaqMan genotyping assays.

Lysate preparation

The Genedrive® assay was optimised for use with crude mosquito-leg lysates, although N/Y and Y/Y templates were only available as archived extracted DNA samples.

Table 1 Primer and probe selections following RT-PCR melt curve screening

Primer/probe	Sequence (5'–3')
1014 Forward primer	TCCCCGACCATGATCTGCCAA
1014 Reverse primer	GCACCTGCAAAACAATGTCATGTAA
1014 Probe	**M**GGAAATTTTGTCG**F**AAGTAA**F**GCAA**P**
1575 Forward primer	AAAGAAAGCTGGTGGATCGC
1575 Reverse primer	TGAAAACACTAACCCTTGGACGA
1575 Probe	**M**TATTATGCAA**F**GAAAAAAA**F**GGGT**P**

F, M and P (in bold) denote a fluorescein labelled deoxythymidine base, a trimethoxystilbene and propanol conjugate, respectively.

For all the other genotypes, single mosquito legs were incubated in 100 µl nuclease-free water at 95 °C for 20 min.

DNA extraction and quantification

For comparisons of the limit of detection (LOD) of the assay using purified DNA *versus* crude lysate, total genomic DNA was extracted from L/L and F/F mosquitoes using the Qiagen blood and tissue kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Extracted DNA was quantified using the Qubit dsDNA high sensitivity kit according to the manufacturer's protocol (Thermo Fisher, UK).

The Genedrive® platform

Details of the Genedrive® platform are published in Duffy et al. [15]. In brief, the device uses a single wavelength optical system (400–470 nm LEDs, 535 nM photodiodes) to read a PCR test cartridge comprised of 3 reaction wells. Additional technical specifications are available at https://www. Genedrive.com/Genedrive-system/documentation.php.

Genedrive® assay optimisation

Each reaction contained 10 µl of lysate or extracted DNA template, 0.2 µM of the probe, 0.1 µM of forward primer, 4 µM of reverse primer (Table 1) in a total reaction volume of 20 µl of the following: 1 mM $MgCl_2$, 0.1 mM dNTPs, 12.5 mM Tris (pH 8.5), KCl 62.5 mM, BSA 0.5 mg/ml, GoTaqMDx 0.075 U/µl and 0.25 µl Excipient (GE Healthcare, Amersham, UK). Several primer/probe sets were screened before the final sets were selected (shown in Table 1). Initial optimisations on the LightCycler480 were carried out under the following cycling conditions: 95 °C for 10 min, followed by 50 cycles of amplification at 95 °C for 10 s, annealing at 62 °C for 10 s, an extension at 72 °C for 10 s, and a final extension at 72 °C for 1 min. This was followed by a melt step increasing the temperature from 42 to 95 °C in 0.5 °C increments with continuous fluorescence acquisition. Genedrive® platform heats and cools rapidly allowing thermocycling for less than 1 s and resulting in a short run time of 50 min. Final reaction conditions used were: 50 cycles of amplification at 95 °C for 0 s (allowed to reach 95 °C only) before reducing the temperature to 62 °C for 10 s, and then raising it to 72 °C for 0 s (again reaching that temperature only momentarily), followed by a final melt step increasing the temperature from 42°C to 80 °C in 0.5 °C increments with continuous fluorescence acquisition, followed by a cooling to 40 °C for 60 s.

Analytical accuracy

A randomly-chosen panel of 70 *An. gambiae* DNA samples was compiled from previously-collected and TaqMan-genotyped, mosquito samples, and 10 no-template controls were added. The operator of the Genedrive® assay was blinded to the genotypes of the panel.

Sensitivity of the 1014 assay was assessed using: (i) crude lysate and (ii) normalised DNA, from individual mosquitoes. DNA was tested at 1 ng/µl, 100 pg/µl and 10 pg/µl. Crude lysate on the other hand was tested following dilution factors of: 20, 50 and 100, as DNA concentration could not be estimated in crude lysate owing to interference in absorption by liberated proteins. These dilution series were selected based on the amount of DNA a typical extraction might yield from a single mosquito.

Pooling mosquitoes

Mosquito pools contained a single F/F mosquito with either 3, 5 or 7, L/L mosquitoes. Crude lysates for these pools were obtained as described above following the addition of 100 µl nuclease-free water per mosquito.

Pooling crude lysates

Lysate pools were constructed by mixing 10 µl of crude lysate from individually lysed mosquitoes in the following F/F to L/L ratios: 1:1, 1:3 and 1:4.

Pooling DNA

Extracted DNA samples were first diluted in nuclease-free water to a working concentration of 1 ng/µl. 'DNA pools' were generated in ratios of 1/4, 1/5, 1/6, 1/7 of F/F:L/L DNA.

Results

Primer and probe selection

Candidate primers were screened using WT mosquito lysate as template and the best pair was selected based on peak height fluorescence. Positive control DNA for each genotype was used to screen candidate probes and selection of the final probe was made according to largest observed Tm shifts (°C) between genotypes, in addition to highest peak fluorescence (not shown). Selected primers and probes are shown in Table 1.

Detection of *kdr* alleles

Using the Genedrive® platform, all genotypes were determined based on the presence of Tm specific peaks. Discernible melt peaks were observed for all alleles in both the 1014 or 1575 assays as highlighted in Fig. 1. In each case the peaks between wild-type and mutant were separated by Tm shifts of at least 2 °C. (Fig. 2a-d) In comparison to extracted DNA, average Tm peaks of L and F alleles were slightly higher in lysates (Table 2).

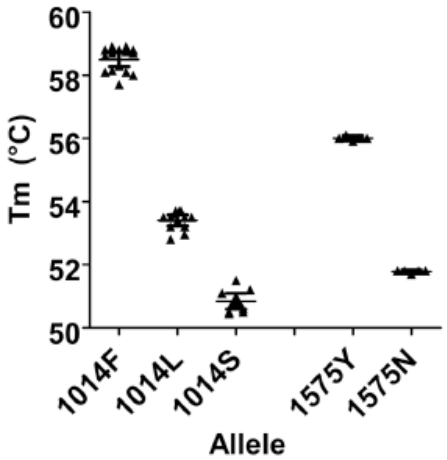

Fig. 1 Detection of *kdr* SNPs by melt curve analysis. Alleles are characterised by the melting temperature at which the probe dissociates from its complementary strand located within the target amplicon, producing a drop in fluorescence, which is then automatically converted into a peak by the Genedrive software using the First Order Derivative (FOD). Mean Tm (°C) for each genotype using extracted DNA ± 95% CI; L/L (53.4 ± 0.17), L/F (58.5 ± 0.22), L/S (50.8 ± 0.25), N/N (51.8 ± 0.06) and N/Y (56 ± 0.09)

Limits of detection

The sensitivity of the 1014 assay when using individual mosquitoes was tested using: (i) extracted DNA at 1 ng/μl, 100 pg/μl and 10 pg/μl and (ii) crude lysate at dilutions 1/20, 1/50 and 1/100. Figure 3 shows distinct Tm peaks (Tm shift between alleles ≥ 2 °C with FOD ≥ 100) detected across all dilutions using DNA template; however, the F-peak starts to drop when using lysates. This suggests pooling of lysates is less sensitive than pooling DNA.

Sensitivity and specificity was determined by screening a panel of *An. gambiae* (*s.s.*) DNA samples of known genotype identified using TaqMan assays as a reference standard. Table 3 shows that both 1014 and 1575 tests had 100% sensitivity and specificity.

Detection of genotypes in mosquito pools

To increase throughput, the sensitivity of the 1014 assay using pools of mosquitoes was investigated. These included: (i) pooling mosquitoes for lysate preparation and (ii) pooling lysates obtained from individual mosquitoes. Mosquito pools contained a single homozygous mutant F/F mosquito with either 3, 5 or 7 homozygous WT L/L mosquitoes. Lysates for these pools were generated as described earlier followed by the addition of 100 μl nuclease-free water per mosquito.

Pooling lysates from individual mosquitoes resulted in poor detection of the F allele when diluted in a background of L alleles; a theoretical pool of 2 mosquitos (a single L/F heterozygote and an L/L homozygote) failed to produce a discernible peak (data not shown). Using extracted and normalised DNA significantly improved pooling results. Using 1 ng/μl of DNA, ratios of 1/4, 1/5, 1/6, 1/7 of homozygous F/F: L/L DNA were tested. Figure 4 shows example melt curves of these pools. Results show two discernible peaks at 58.8 and 53.6 °C, corresponding to the F and L peaks, are observed at a 1/7 ratio (equivalent to a pool of 4 mosquitoes: one F/F and three L/L mosquitoes). Since the Genedrive® cartridge contains 3 wells, there is potential for 12 DNA samples to be screened in one run.

Discussion

Here we have developed a method using the Genedrive® platform for the detection of three mutations that indicate insecticide resistance at the 1014 and 1575 loci in *An. gambiae*. Genedrive® uses end-point melt analysis to give rapid results with limited sample pre-processing, [14, 15] and is shown to be highly accurate when tested on a panel of 80 samples. Although wet reagents were used throughout this method development work, like all other commercially-available Genedrive® tests (HCV [13], MTb [14] and IL-28B [15]), the final optimised formulation will be lyophilised within the cartridges so that only the addition of template is required, and also eliminating the need for any cold chain storage. Additionally, the Genedrive® platform is very easy to use with only a single button required for its operation thereby simplifying the workflow and reducing the need for skilled operators. The Genedrive® platform is portable weighing less than 600 grams [17] and could be used directly in the field for 'point-of-care' monitoring or in decentralised, minimally equipped laboratories.

Several novel diagnostics have been developed for detection of *kdr* in mosquitoes in attempts to simplify assays and reduce costs, yet TaqMan assays, which use expensive fluorescent probes, remain the most commonly used assay [12]. Melt-curve assays have been previously developed to simultaneously detect both F and S alleles, which reduces labour and the reagent costs whilst producing easily interpretable results [18]. However, these methods still require substantial upfront costs for equipment and require skilled expertise to perform.

The 1014 assay has been designed so that only a single probe is required to simultaneously discriminate between the L, F and S alleles, whereas the 1575 assay requires a second probe for the detection of the N and Y alleles, each using their assay-specific primer pairs.

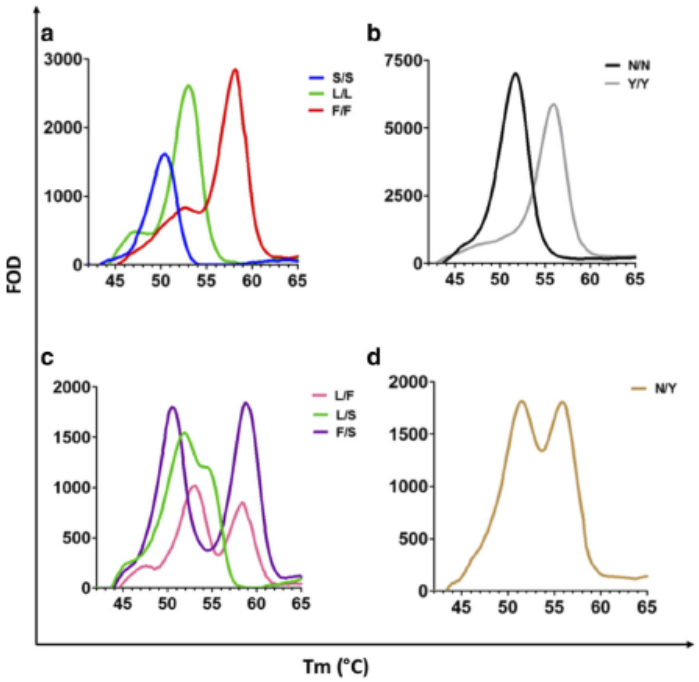

Fig. 2 FOD melt curves of *kdr* genotypes. FOD melt curves of homozygous genotypes at the 1014 locus (**a**): S/S (blue), F/F (red) and L/L (WT, green) and at the 1575 locus (**b**): N/N (WT, black) and N/Y (grey). Heterozygous genotypes are shown in **c** at the 1014 locus: L/F (pink), L/S (green) and F/S (purple), and **d** at the 1575 locus: N/Y (brown)

By comparison, detection of these mutations by conventional TaqMan methods would require 5 different probes: Y, F and S- mutant probes, as well as WT probes for each 1014 and 1575 sequence. The costs of reagents are thus significantly reduced. In addition to the simple workflow and reduced costs on reagents, the cost of the GeneDrive machine itself is estimated to be less than a third of the price of a 48 well rt-PCR machine (US $19,000–20,000 [16]).

It should be noted that we observed an increase in peak Tm across 1014F and 1014L genotypes when comparing sample lysates with purified DNA (Table 3). This is most likely due to the differing background of salt concentrations in the lysates [19]. For automated Genedrive genotype detection, a defined Tm range would require determination through further testing on different lysates to account for intra-individual variability. Although a larger sample size to determine this would result in a broader Tm range, it is important to note that the Tm shift between each of the alleles remains relatively constant. This could be accounted for in the algorithm for automation of the readout.

Table 2 Comparison of melting temperature of lysate and extracted DNA template

	Tm range (°C)		Average Tm ± SD	
Allele	DNA extract (*n*)	Crude lysate (*n*)	DNA extract	Crude lysate
1014F	57.7–58.9 (15)	59–59.55 (12)	58.49 ± 0.39	59.31 ± 0.14
1014L	52.8–53.7 (13)	53.9–54.7 (11)	53.4 ± 0.29	54.29 ± 0.21

The Genedrive cartridge designed here allows for several IR markers to be detected using only two of the three available channels in a cartridge. This allows potential to incorporate a third assay for additional markers or, alternatively the assays could be separated into different cartridges to process multiple samples in one run.

Using pooling strategies, our results show that there is potential to screen DNA from up to 12 mosquitoes for 1014F mutations in a single run (50 min). There is also potential to upscale analysis by increasing numbers of cartridges or machines. Pooling samples for GeneDrive analysis could allow either qualitative detection of the

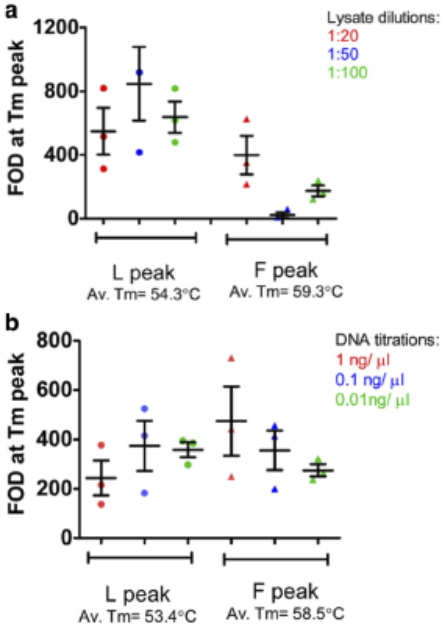

Fig. 3 Titration of crude lysates and extracted DNA to assess functional sensitivity of the 1014 assay. Different titrations of crude lysates (**a**) and DNA extracts (**b**) of F/F and L/L mosquitoes were mixed at a 1:1 ratio. Each dot represents a single peak (one biological replicate) and bars represent SEM

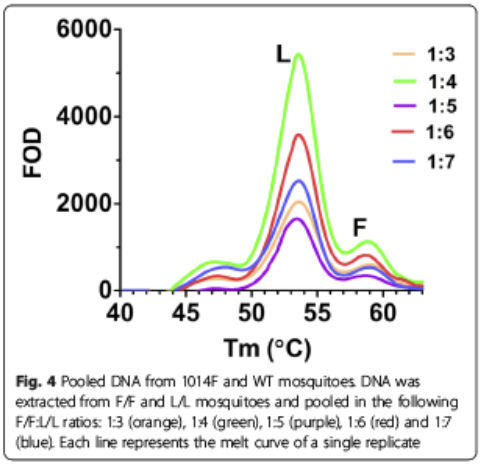

Fig. 4 Pooled DNA from 1014F and WT mosquitoes. DNA was extracted from F/F and L/L mosquitoes and pooled in the following F/F:L/L ratios: 1:3 (orange), 1:4 (green), 1:5 (purple), 1:6 (red) and 1:7 (blue). Each line represents the melt curve of a single replicate

Table 3 Sensitivity and specificity of 1014 and 1575 Genedrive® assays compared to those determined using TaqMan in a test panel of 70 *An. gambiae* DNA samples

			TaqMan								
			1014 loci						1575 loci		
			LL	LF	FF	LS	SS	FS	NN	NY	YY
Genedrive®	1014 loci	LL	1	–	–	–	–	–	–	–	–
		LF	–	3	–	–	–	–	–	–	–
		FF	–	–	58	–	–	–	–	–	–
		LS	–	–	–	–	–	–	–	–	–
		SS	–	–	–	–	1	–	–	–	–
		FS	–	–	–	–	–	9	–	–	–
	1575 loci	NN	–	–	–	–	–	–	52	–	–
		NY	–	–	–	–	–	–	–	14	–
		YY	–	–	–	–	–	–	–	–	4

presence of resistance alleles in a population of mosquitoes or quantitative detection of spatial or temporal variation. Pooling samples has the general limitation of making interactions between alleles within loci (dominance effects) and across loci (epistasis) difficult to detect. For the markers considered here, 1014F and 1014S appear to no more than partially recessive [20, 21] and 1575Y occurs only on a 1014F haplotype [10]. Therefore, useful information can be gained from pooled data, and in terms of vector control management, if a frequency threshold is determined, detection of variation in allele frequencies at resistance phenotype-informative markers from pooled mosquitoes would warrant examination of the insecticide used in a locality. However, for extension to additional markers the importance of dominance level and epistasis must be considered when adopting a pooling strategy.

These assays could be expanded to include different target site mutations and other disease vectors, such as sand flies [22] or triatomine bugs [23], where *kdr* mutations have also been reported. However, although melt analysis-based assays are useful for the detection of DNA substitutions or indels, it is more difficult to detect multiplication mutations, e.g. duplication of detoxifying enzymes, since melt curve analysis is only semi-quantitative.

Conclusions

This study describes the development and validation of two simple molecular assays for *kdr* genotyping in *An. gambiae* mosquitoes. Our results show accurate detection of the L1014F, L1014S and N1575Y *kdr*-associated SNPs in *An. gambiae*. Development on the Genedrive® platform presents a viable methodology for applying these assays as a field-applicable diagnostic in low-resource settings.

Abbreviations
DDT: Dichlorodiphenyltrichloroethane; IRS: Indoor residual spraying; ITN: Insecticide-treated nets; *kdr*: Knockdown resistance; LLIN: Long-lasting insecticide treated nets; RT-PCR: Real-time polymerase chain reaction; SNP: Single nucleotide polymorphism; Tm: Melting temperature; VGSC: Voltage gated sodium channel

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions
VTU, SA and DW designed the assays *in silico*. VU optimized and ran the Genedrive® assays and confirmatory TaqMan assays, as well as drafted the manuscript. EHAN collected, extracted and TaqMan-genotyped the mosquitoes from Senegal that were used for control DNA. ER genotyped the blinded panel of samples using the TaqMan assay. SA, DW, MJIP and ERA facilitated the collaboration between genedrive plc and the Liverpool School of Tropical Medicine, contributed to writing the manuscript and conceived the overall study design. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Competing interests
This study was performed in collaboration with genedrive plc where SA is currently employed. Genedrive plc were involved in the design and optimisation of the assay but played no role in the analysis of the data, the content of this article or the decision to publish.

Publisher's Note

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Chapter 4. Use of a highly-sensitive rapid diagnostic test to screen for malaria in pregnancy in Indonesia

Contents

4.0. Declarations

RA, JRP, TL, DS and FTK were involved in the collection of the original samples and coordination of the STOPMiP trial. VTU, AMP, MASM and RASU carried out the RDTs and molecular testing. VTU, RA, RN, LT, FTK and ERA conceived and coordinated the study.

TL managed the database for this study. VTU, RA, FTK and ERA analysed the data and drafted the manuscript. All authors read and approved the final manuscript.

4.1.1. Chapter summary

The study resulted in the recent Unwin *et al*. (Bousema, Okell *et al*. 2014) publication used here as Chapter 4. This Chapter investigates the use of a new commercially available rapid diagnostic test (RDT) for *P. falciparum* malaria. Using the previously obtained molecular results (Chapter 2) to formulate a composite reference test, the corresponding stored blood samples from Timika, Indonesia were tested for malaria using a new RDT (Alere™), alongside a commonly used brand of RDT (CareStart™). The results showed no significant difference in the performance of the two RDTs in this moderate transmission setting.

Subsequent Chapters in this thesis build on the results reported here- Chapter 5: further investigating Histidine Rich Protein II (HRP2) mutations that could affect RDT outcomes and Chapter 6: expanding evaluations to a prospective study in Kisumu, Kenya, a high transmission setting.

In lines with the UoL PGR Code of Practice (Appendix 7, Annexe 2) the publication has been formatted for the purpose of this thesis.

Use of a highly-sensitive rapid diagnostic test to screen for malaria in pregnancy in Indonesia

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4.1.2. Abstract

Background

The sensitivity of rapid diagnostic tests (RDTs) for malaria is inadequate for detecting low-density, often asymptomatic infections, such as those that can occur when screening pregnant women for malaria. We retrospectively assessed the performance of the Alere™ Ultra-sensitive Malaria Ag *P. falciparum* RDT (uRDT) in pregnant women in Indonesia.

Methods

The diagnostic performance of the uRDT and the CareStart™ Malaria HRP2/pLDH VOM (*P. vivax, P. ovale* and *P. malariae*) Combo RDT (csRDT) were assessed using 270 stored red blood cell pellets and plasma samples from asymptomatic pregnant women. These included 112 *P. falciparum* negative and 158 *P. falciparum* positive samples detected by a composite test (qPCR, LAMP, nPCR) as reference standard. Diagnostic indicators: sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), diagnostic odds ratio (DOR) and the level of agreement (kappa) were calculated for comparison.

Results

Compared with the reference test, the uRDT had a sensitivity of 19.6% (95% CI 13.9-26.8) and specificity of 98.2% (93.1-99.7%). The csRDT was 22.8% (16.7-30.3) sensitive and 95.5 % (89.4-98.3) specific for *P. falciparum* infections. Performance of the uRDT was non-significantly different to the csRDT (p=0.169). RDT outcome was stratified by qPCR cycling threshold (Ct), and performance of the RDTs was found to be comparable across Cts.

Conclusion

The uRDT performed similarly to the currently used csRDTs in detecting *P. falciparum* infections in asymptomatic pregnant women. In these settings, molecular diagnostics are currently the most sensitive for malaria.

4.2. Introduction

Control programs for malaria will need to account for the changing epidemiology of malaria as countries progress toward elimination. The current goal to eliminate malaria in 31 countries by 2030 will require sensitive diagnostic tools (Okell, Bousema *et al*. 2012) to detect the reservoir of low-density and often sub-patent infections (Okell, Ghani *et al*. 2009, Doritchamou, Bertin *et al*. 2012, Okell, Bousema *et al*. 2012, Tadesse, Slater *et al*. 2018). Although the infectivity of these asymptomatic individuals is low, modelling shows that they contribute significantly to onward transmission because they make up the majority of the population in low-transmission areas (Mayor, Moro *et al*. 2012, Mohammed, Salih *et al*. 2013).

Diagnosis of malaria during pregnancy is complicated by the ability of certain *P. falciparum* parasites to sequester in the placenta, which can result in parasite densities in the peripheral blood below the level of detection of existing RDTs and light microscopy (Mockenhaupt, Rong *et al*. 2000, Adegnika, Verweij *et al*. 2006, Desai, ter Kuile *et al*. 2007, Bousema, Okell *et al*. 2014, Desai, Hill *et al*. 2018). Diagnosis and treatment of these infections may improve pregnancy outcomes by preventing them from developing into patent infections and may control transmission by reducing the parasite reservoir (Mayor, Moro *et al*. 2012, Indonesian Ministry of Health 2013, Ahmed, Poespoprodjo *et al*. 2019)). Lateral-flow rapid diagnostic tests (RDTs) detecting circulating malaria antigen are simple, widely used, and the cheapest point-of-care diagnostic tool for malaria. Many countries in the Asia-Pacific region use “Test and Treat” strategies at the first antenatal booking visit to screen all pregnant women for malaria regardless of symptoms (Slater, Ross *et al*. 2015,

Desai, Hill *et al*. 2018), or are exploring intermittent screening and treatment (IST) strategies to test women at each scheduled antenatal visit (Ahmed, Levy *et al*. 2015, Slater, Ross *et al*. 2015). However, the current generation of standard RDTs for malaria has a limit of detection of 100 parasites/µl and is not designed to detect low-density infections in asymptomatic pregnant women (Slater, Ross *et al*. 2015). Low-cost and field-deployable highly sensitive rapid diagnostics may improve the detection of malaria in these populations (Das, Peck *et al*. 2018).

Currently, only nucleic acid amplification tests (NAATs) are sufficiently sensitive to detect these low-density infections (Vasquez, Medina *et al*. 2018). However, these methods are limited to well-equipped laboratory settings due to their inherent complexity and need for laboratory equipment.

Recently, the new Alere™ Ultra-sensitive Malaria Ag *P. falciparum* RDT (uRDT) was developed to bridge the gap between high-sensitivity and field-ready diagnostics (Ahmed, Poespoprodjo *et al*. 2019). To date, studies in Uganda, Ethiopia, Myanmar and Papua New Guinea have shown superior sensitivity of the uRDT in comparison to widely used SD Bioline RDTs in asymptomatic general population. An additional laboratory study reports a log-fold lower limit of detection (LOD) for the HRP2 antigen (80 mg /ml) (Poespoprodjo, Fobia *et al*. 2008). It is not disclosed which new components of the tests proffer enhanced sensitivity, but the additional 5 minute incubation time may be a contributing factor. However, the only study in pregnant women did not observe notable differences in sensitivity between the uRDT and existing SD Bioline RDTs. (Poespoprodjo, Fobia *et al*. 2008)

Here we describe the performance of the uRDT and standard RDT CareStart™ Malaria HRP2/pLDH *Pf*/*VOM* Combo (csRDT) in stored blood specimens of asymptomatic pregnant women in Indonesia compared to a composite molecular tests.

4.3. Methods

4.3.1. Study sites and sample collection

The study was performed using stored samples of red blood cell pellets (pRBC) reconstituted with plasma collected during a recently completed STOPMIP trial (ISRCTN: 34010937) comparing intermittent preventive treatment or intermittent screening and treatment strategies with single screen and treatment in pregnant women in Indonesia (Ahmed, Levy *et al*. 2015). The trial was conducted between 2013 and 2016 in Timika in Mimika District southern Papua-Indonesia, an area with moderate, year-round malaria transmission (Ahmed, Poespoprodjo *et al*. 2019). All four major species of *Plasmodium* are endemic in the area with *P. falciparum* accounting for 57.9 % of infections, *P. vivax*- 33.8 %, *P. malariae*- 4.4 % and *P. ovale*- 0.5 % amongst pregnant women (Ahmed, Poespoprodjo *et al*. 2019). Co-infections of *P. falciparum* and *P. vivax* account for the remaining 3.8 %. Whole blood samples were taken from asymptomatic pregnant women (>16 weeks gestation) at antenatal visits to screen for malaria infection. During the trial this included microscopy, molecular malaria diagnostics (qPCR), nested PCR, and loop-mediated isothermal amplification (LAMP). Malaria RDTs (First Response Malaria Ag pLDH–HRP2 Combo; Premier Medical Corporation, India) were also done at enrolment in the SST and IST groups. The FirstResponse RDT data is not included in our analysis as the number of matched samples was low. The sample processing and diagnostic tests used in this study are described below.

4.3.2. Sample processing

During STOPMIP trial, venous blood (4 ml) was collected from each participant in EDTA tubes at the booking and delivery visits and processed within 24hrs. Samples were

centrifuged for 10 min at 15000 rpm and RBCs and plasma separated and stored at -20°C. Dried blood spots were also prepared at the field laboratory, where 10 µl blood was pipetted on to filter paper (Whatman 3MM) and left to air-dry, stored in zip-lock bags and transported to the Eijkman institute of molecular biology for molecular testing.

4.3.3. RDTs

The RDTs used here recommend using whole blood samples. Due to storage conditions in the trial pellets of frozen RBCs were first reconstituted by adding the corresponding plasma sample to a final haematocrit (HCT) of 30%. This reflects the average haemoglobin level (~11g/dL) observed previously in this population (Ahmed, Poespoprodjo *et al*. 2019) and in STOPMiP participants (Singh, Bobogare *et al*. 1999). The Alere™ Ultra-sensitive Malaria Ag *P. falciparum* RDT (uRDT, Alere, South Korea, Catalogue No: 05FK140, Lot No. 05LDC002A) detects *P. falciparum* infections only. The CareStart RDT (csRDT) (CareStart™ Malaria HRP2/pLDH *Pf/VOM* Combo RDT, Catalogue No: G0171, Lot No. RMR17H111, Access Bio, USA) detects *P. falciparum* and non-*falciparum* parasites (*P. vivax, P. ovale and P. malariae*). With both RDTs, the presence of a line in the 'C' window indicated a valid test. Invalid tests were repeated. A valid uRDT test was determined positive if a line was observed in the *P. falciparum*-line (Pf-line) window, whilst a valid csRDT was determined positive if the *Pf*-line was present regardless of presence or absence of a line in the *Pvom* window.

Both RDTs were performed according to the manufacturer's protocol: briefly, a five µl reconstituted blood sample was added to the RDT cassette, followed by three (csRDT) or four (uRDT) buffer drops. RDTs were read according to the manufacturer's guidelines; after 20mins for uRDTs and 15 mins for csRDTs. RDTs were performed at the Eijkman Institute by an operator who was blinded to the reference test results. Presence of a line on an RDT was

confirmed by two independent operators. A third operator was consulted in case of uncertainty, and the test was declared positive if two operators agreed.

4.3.4. Reference standard: Composite molecular test

Molecular testing was carried out on DNA extracted (Chelex method) in 2016-2017 from DBS samples. Briefly, 6 mm filter paper (Whatman 3MM) punches were incubated on 0.5% saponin overnight, before centrifugation and discard of supernatant. Following rigorous PBS washing, the DBS was heated in 20% Chelex 100-Ion Exchanger for 10 min (Biorad Laboratories, Hercules, CA) at 100 °C, and the remaining supernatant stored at -20 °C (Kamau, Alemayehu *et al*. 2013).

LAMP and qPCR results formed a composite molecular reference. Discordant results underwent nested PCR (nPCR) testing as the gold standard, which was run in triplicate and results were determined positive if a single replicate was positive by nPCR. These composite molecular tests were used as the reference standard (further described by Ahmed *et al.* (Kamau, Alemayehu *et al*. 2013)).

Nested PCR (nPCR) was performed on LAMP-positive/ qPCR negative samples with primers and cycling conditions described in Singh *et al.* (1999). The nPCR was used as the gold standard whereby a single positive result determined the sample as positive for *P. falciparum.* The LOD of this assay is ~6 parasites/ µl (Shokoples, Ndao *et al*. 2009).

LAMP assays were carried out using the Loopamp™ Malaria Pan Detection Kit (Eiken Chemical Company, Japan). Briefly, the LAMP reagents were reconstituted with 15 µl water/

tube and left to stand for 2 mins, before adding 15 µl of extracted DNA. Samples were incubated for 40 min at 65 ºC and then 5 min at 80 ºC. Results were interpreted under a UV light, where a green colour change indicated a positive result.

For qPCR, reactions contained, 2 µl DNA, QuantiFast Pathogen PCR Mix (Qiagen™, Hilden, Germany) and primers and probes published previously for *P. falciparum* (Kamau, Alemayehu *et al*. 2013), *P. vivax* (Shokoples, Ndao *et al*. 2009), and *P. ovale* and *P. malariae*, (Shokoples, Ndao *et al*. 2009). (Table 4.1) Thermocycling conditions (38 cycles) were as follows: 10 min at 95°C, 15 sec for 95°C and 60 sec for 60°C. Fluorescence was acquired using the Rotor-Gene Q 5plex HRM Platform (as above) and cycle threshold (Ct) values were calculated using the RotorGene Q series software version. The LOD of this LAMP and qPCR assays is 2-5 parasite/ µl (Ahmed, Poespoprodjo *et al*. 2019).

All molecular testing was carried out by independent operators blinded to clinical information. The LAMP, qPCR and nPCR were carried out a year prior to testing with the uRDT and csRDT. LAMP, csRDT and uRDT tests were carried out at the Eijkman Institute, Jakarta, Indonesia and qPCR testing was subsequently carried out at the Liverpool School of Tropical Medicine, UK.

Positive DNA controls for each species (provided by the Malaria Reference Laboratory, Public Health England) and negative controls were included in every molecular assay run. All assays were carried out by independent operators blinded to clinical information. Index testing was also carried out without knowledge of the reference standard results.

Table 4.1. Working concentrations and sources are provided for the qPCR primers and probes.

Table adapted from Ahmed et al. (Ahmed, 2019)

	Sequence 5'-3'	Concentration (nM)	Source
Pan reverse (Shokoples, Ndao *et al*. 2009)	AACCCAAAGACTTTGATTTCTCATAA	200	Eurofins
MAL FP (Shokoples, Ndao *et al*. 2009)	CCGACTAGGTGTTGGATGATAGAGTAAA	50	Eurofins
MAL probe (Kamau, Alemayehu *et al*. 2013)	ATTO700-CTATCTAAAAGAAACACTCAT-MGBEDQ	80	Eurogentec
OVA FP (Kamau, Alemayehu *et al*. 2013)	CCGACTAGGTTTTGGATGAAAGATTTTT	50	Eurofins
OVA Probe (Kamau, Alemayehu *et al*. 2013)	Cy5-CGAAAGGAATTTTCTTATT-MGBEDQ	80	Eurogentec
FAL FP (Kamau, Alemayehu *et al*. 2013)	ATTGCTTTTGAGAGGTTTTGTTACTTT	400	Eurofins
FAL RP (Kamau, Alemayehu *et al*. 2013)	GCTGTAGTATTCAAACACAATGAACTCAA	400	Eurofins
FAL probe (Kamau, Alemayehu *et al*. 2013)	FAM-CATAACAGACGGGTAGTCAT-MGBQ	200	Thermo
VIV FP (Das, Jang *et al*. 2017)	GCAACGCTTCTAGCTTAATCCAC	400	Eurofins
VIV RP (Das, Peck *et al*. 2018)	CAAGCCGAAGCAAAGAAAGTCC	400	Eurofins
VIV probe (Das, Jang *et al*. 2017)	VIC-ACTTTGTGCGCATTTTGCTA-MGBQ	200	Thermo

4.3.5. Sample size

The sample size was calculated using G*Power software v3.1.9.2 (University of Dusseldorf, Germany). Estimating a proportion of 0.35 discordant results between the two RDTs (based on previously published data in similar transmission setting (Ahmed, Levy *et al*. 2015)), a sample size of 120 positive samples was estimated to provide sensitivity with 80% statistical power and 99% confidence intervals. Studies have shown both uRDT and csRDT have similarly high specificity (>96%) (Calculator-1 , Calculator-2 , Ahmed, Levy *et al*. 2015). Therefore, the sample size was estimated to be able to detect differences only in sensitivity. The final sample size (n= 270) used for this analysis were those samples with sufficient volumes of both plasma and pRBCs remaining for RDT testing. Of these, 158 were *P. falciparum* positive and 112 negatives by the composite molecular reference test.

4.3.6. Statistics

Diagnostic accuracy was estimated by calculating the total number of true positives (TP), false positives (FP), true negatives (TN), false negatives (FN), sensitivity and specificity (%), positive predictive value (PPV) and negative predictive value (NPV). PPV and NPV calculated according to prevalence within the sample collection. The reference test method to get the TP, FP, TN and FN was the composite molecular test results (LAMP, qPCR and nPCR). Agreement between either RDT and the composite reference test was calculated by determining the kappa value (*k*). Kappa values <0.2 were considered a poor agreement, 0.21-0.40 fair, 0.41-0.6 moderate, 0.61-0.8 very good. (Ahmed, Poespoprodjo *et al*. 2019) The diagnostic odds ratio (DOR) was calculated as the ratio of the odds of positivity in those with malaria (defined by molecular composite reference test) relative to the odds of positivity in those without malaria). The Ct values were used as a proxy of parasite densities.

The significance of the difference observed between the uRDT and csRDT R^2 correlation coefficients was assessed using a Fisher r-to-z transformation. The analysis was conducted using SPSS (version 24.0, Armonk, New York) and an online calculator (Das, Peck *et al*. 2018) which uses the efficient-score method to calculate 95 % confidence intervals.

4.3.7. Ethical approval

Ethical clearance was obtained from the Liverpool School of Tropical Medicine and the Eijkman Institute for Molecular Biology, Jakarta, Indonesia. Written informed consent was obtained (Abdallah, Okoth *et al*. 2015).

4.4. Results

In total, 270 samples were screened. These included 158 *P. falciparum* positive samples (including non-*falciparum Plasmodium* co-infections) and 112 negative samples (by molecular composite). Only 23 positive samples were detected by either uRDT and csRDT (fig 4.1).

4.4.1. Agreement with reference test

The level of agreement of either RDT with the reference test was poor: uRDT; k=0.15 (95% CI 0.09-0.21), csRDT; k=0.16 (0.09-0.23), (Table 4.2.1). The uRDT detected 31 (19.6%) infections confirmed by the reference test, of which 23 (74.0%) were also detected by the csRDT (fig 4.1). The uRDT missed 13 (36%) infections that were detected by the csRDT and confirmed by the reference test. The uRDT detected eight infections that were not detected by csRDT (fig 4.1).

4.4.2. Diagnostic performance

The sensitivities compared with the reference test were 19.6%, (95% CI 13.9-26.8) for the uRDT and 22.8%, (16.7-30.3) for the csRDT. Both tests showed high specificity (>95%). The uRDT DOR was 13.4 (3.1-57.4) and the csRDT 6.3 (2.4-16.7). (Table 4.2.1) When comparing the overall performance of the uRDT and csRDT, the difference in proportions detected was non-significantly different (p=0.169).

4.4.3. Parasite density distribution

The qPCR Ct value was used as a proxy for parasite density. Overall, 36% of qPCR positives fell between Ct 33-35 (reflecting an estimated 1-10 parasites/µl), and nearing the reported uRDT LOD of 3.13 p/ µl (WHO 2020), and 25% fell below Ct 30, the estimated LOD of the csRDT LOD (~100 parasites/ µl) (Singh and Daneshvar 2013). There was no evidence that the relative performance of the RDTs was modified by CT value (p=0.3). (Figure 4.2)

Figure 4.1. Venn diagram of the number of *P. falciparum* positive samples detected by each test.

Positivity by uRDT, csRDT and the composite molecular reference test (Ref. Std) in 270 samples from asymptomatic pregnant women. Numbers within the circles (n) indicate the number of true positives detected by that test.

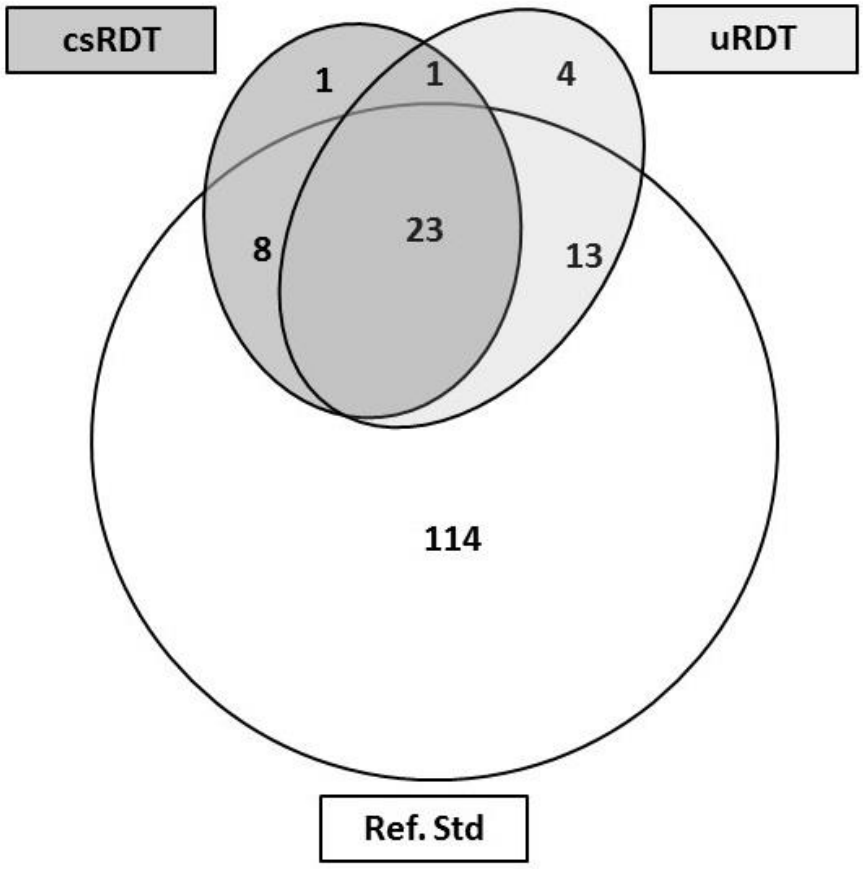


Table 4.2. Detection of P. falciparum and diagnostic performance of the csRDT and the uRDT.

4.2.1. Detection of *P. falciparum* including non-*falciparum* species.

	n	TP	FP	TN	FN	Sensitivity (%)		Specificity %		PPV (%)		NPV (%)		DOR		Agreement (κ)	
uRDT	270	31	2	110	127	19.6	(13.9-26.8)	98.2	(93.1-99.7)	93.9	(78.4-98.9)	46.4	(40.0-53.0)	13.4	(3.1-57.4)	0.15	(0.09-0.21)
csRDT Pf-line	270	36	5	107	122	22.8	(16.7-30.3)	95.5	(89.4-98.3)	87.8	(73-95.4)	46.7	(79.8-88.8)	6.3	(2.4-16.7)	0.16	(0.09-0.23)

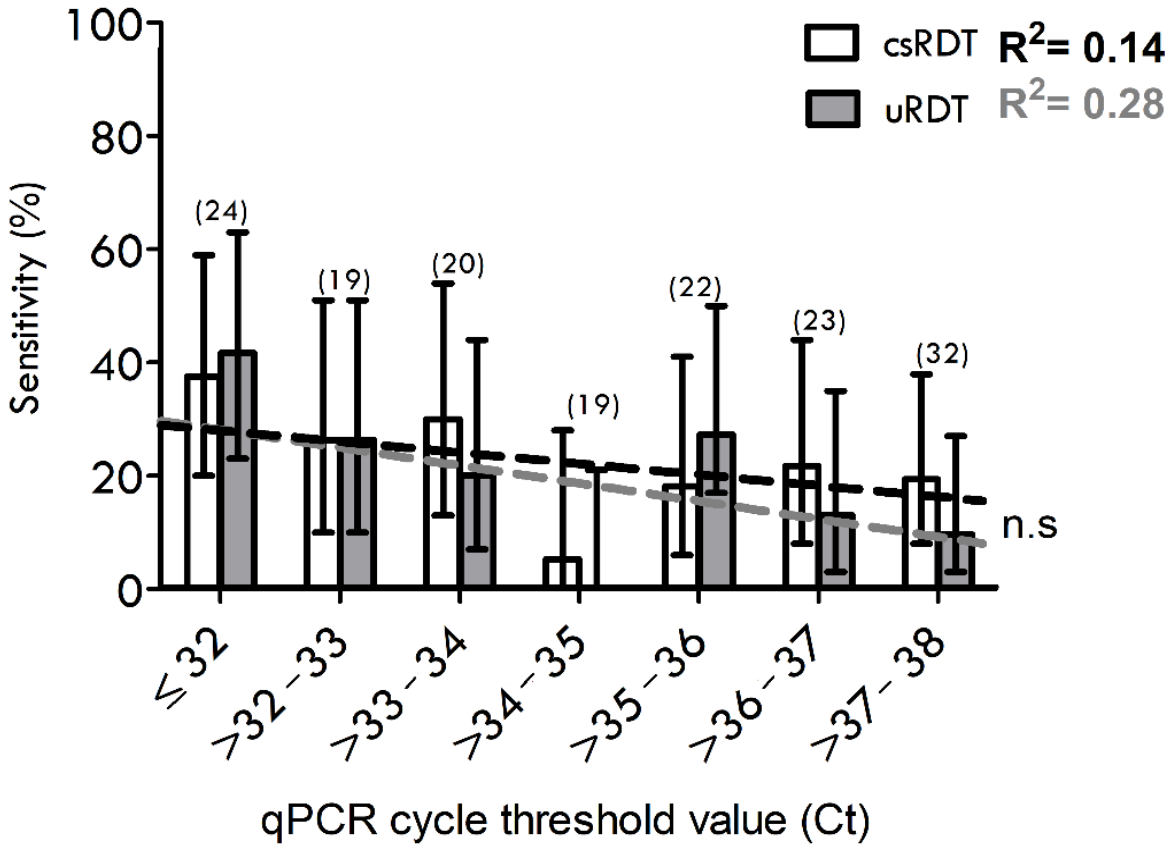
4.2.2. Detection of *P. falciparum* excluding non-*falciparum* species.

	n	TP	FP	TN	FN	Sensitivity (%)		Specificity %		PPV (%)		NPV (%)		DOR		Agreement (κ)	
uRDT	242	31	2	82	127	19.6	(13.9-26.8)	97.6	(90.9-99.6)	93.9	(78.4-98.9)	39.2	(32.6-46.2)	10	(2.3-42.9)	0.13	(0.07-0.13)
csRDT Pf-line	242	36	2	82	122	22.8	(16.7-30.3)	97.6	(90.9-99.6)	94.7	(80.9-99.1)	40.2	(33.5-47.3)	12.1	(2.8-51.6)	0.15	(0.09-0.15)

Note- n, number of samples; TP, True Positive; FP, False positive; TN, True Negative; FN, False Negative; Sens, sensitivity; Spec, specificity; (Lower 95% CI-Upper 95% CI); PPV, Positive Predictive Value; NPV, Negative Predictive Value; DOR, Diagnostics odds ratio; k, Kappa value

Figure 4.2. Diagnostic performance stratified by qPCR Ct values.

The bar chart shows the sensitivity of each test: uRDT (grey) and csRDT (white bar and black trendline) stratified by qPCR cycle-threshold (Ct) values. The number of qPCR- positive samples in each Ct category is given in brackets (n). Trendlines (dashed) are derived from the rolling mean sensitivity calculated for every 10 observations. R squared coefficient values are displayed (uRDT; grey text, csRDT; black text) and difference between R^2 values is non-significant (n.s, p=0.3 one-tailed t-test) (Calculator-2). Error bars represent upper and lower 95% CI.



4.4.4 Exclusion of co-infections

Considering uRDT only detects *P. falciparum* infections, we investigated whether the presence of *P. vivax* altered the performance of the RDTs. When *P. falciparum*-negative/ *P. vivax*-positive samples were excluded from the data set, the number of false positives detected only by the csRDT was reduced (from 5 to 3), thus increasing the specificity and PPV. This resulted in a near doubling of the csRDT DOR (12.1; 2.8-51.6), while the performance of the uRDT remained relatively unchanged. Again, the difference in proportions detected by each test was not different from each other (n=238, p= 0.4, Table 4.2.2).

4.5. Discussion

Our study shows that in a moderate transmission setting in Indonesia, amongst asymptomatic pregnant women, the uRDT performed similarly to the csRDTs, which is a widely used test in the malaria in pregnancy control program in Indonesia.

These results are supported by a study in Colombia, which also used stored samples from pregnant women. Although they note a trend of increased sensitivity by the uRDT, it was not found to be significantly different from the Standard Bioline RDT (sdRDT) (Vasquez, Medina *et al*. 2018). A Tanzanian study in febrile children and adult outpatients also found no difference between uRDT and sdRDT performance, although febrile patients tend to have higher parasitaemia (Hofmann, Antunes Moniz *et al*. 2019). Our results differ from other studies conducted in non-pregnant populations that showed a significant increase in sensitivity of the uRDT compared to current RDTs (Das, Jang *et al*. 2017, Das, Peck *et al*. 2018, Girma, Cheaveau *et al*. 2018, Hofmann, Gruenberg *et al*. 2018). The most apparent difference between the conflicting results is the target population (pregnant vs non-pregnant) and the storage conditions. It would be useful to screen pregnant populations in the same settings where differences in RDT performance were observed. One such study has commenced in Papua New Guinea (personal communications with Dr Leanne Robinson).

The uRDT is an antigen capture test specific to the *P. falciparum* HRP2 antigen, whereas the csRDT captures pLDH specific to *P. vivax, P. ovale* and *P. malariae* (indicated by the VOM window), in addition to PfHRP2 (*Pf* window). Ahmed *et al*. (2015) showed that amongst pregnant women in Indonesia, when using a different CareStart RDT that captures both pan-

LDH and PfHRP2, reading the PfHRP2 window alone was less sensitive than using in combination with the pLDH results (Ahmed, Levy *et al*. 2015). These findings and the low sensitivity of both RDTs demonstrated here, may point towards low HRP2 concentrations that fall below the limit of either RDT (uRDT LOD= 80 pg/ml, csRDT= 200pg/mL). Low concentrations of HRP2 could be due to low parasitaemia, degradation of the protein during storage or deletions in the gene for this protein.

In our sample set we found a total of 8 samples that were *P. falciparum*-positive by the FirstResponse RDT performed during the trial (2014-2016), 6 of which were also positive by microscopy (data not shown). All of these samples were also positive using the uRDT and CaraStart in this study. This suggests minimal degradation over the storage period (1-3 yrs). The non-linear relationship between HRP2 concentration/persistence and parasite density (Das, Jang *et al*. 2017, Das, Peck *et al*. 2018), complicates analyses between RDT positivity and parasitaemia. Although no trend in sensitivity was observed with increasing Ct values (indicative of parasitaemia), however, most samples fell above Ct 30 indicating very low parasitaemias.

Samples were also stored for up to 4 yrs before testing with the uRDT and csRDT, so degradation is possible. Finally, HRP2 polymorphisms have been reported in *P. falciparum* from several countries in South America, Africa and also India (Bharti, Chandel *et al*. 2016, Beshir, Sepulveda *et al*. 2017, Watson, Slater *et al*. 2017), but not yet in SE. Asia. As yet, no full deletions (only polymorphisms (Baker, Ho *et al*. 2010)) of HRP2 have been reported in Indonesia.

These mutations have been implicated in the poor sensitivity of RDTs (Watson, Slater *et al*. 2017). Early laboratory analyses of the uRDT using cultured parasites with HRP

mutations demonstrated that the uRDT performance varies considerably depending on mutations of the HRP protein (Das, Peck *et al*. 2018). Consequently, investigations around HRP2 polymorphisms in this sample set are underway.

A limitation of this study is the use of different sample types used for the molecular and RDT testing. Molecular testing used stored DNA extracted from DBS, whereas RDTs were performed using pRBC and plasma samples reconstituted to an artificial HCT of 30%. Potential degradation of HRP2 in our stored samples could contribute to poor sensitivity of both RDTs since RDT testing was carried out one year after molecular testing. The sensitivity of the molecular testing could also be enhanced through targeting higher copy genes (Hofmann, Mwingira *et al*. 2015) or RNA (Murphy, Prentice *et al*. 2012), or by using improved extraction methods (Zainabadi, Adams *et al*. 2017). We anticipate that the performance of both RDTs could improve using fresh whole blood at point of contact, i.e. for the intended user scenario. However, the low sensitivity observed in our analysis is similar to reported sensitivities using a range of RDTs in pregnant women in Indonesia. (Ahmed, Levy *et al*. 2015) The important finding here is that the uRDT performed similarly to the csRDT.

4.6. Conclusion

We assessed the diagnostic performance of the uRDT in a diagnostically challenging population: asymptomatic, pregnant women in a moderate transmission setting in Indonesia. This is the first study testing the uRDT in pregnant women in Asia and will build evidence to guide policy around the implementation of this test in these populations. In comparison to the composite molecular reference tests, both the uRDT and csRDT showed poor sensitivity/ specificity. Given the negative outcomes associated with malaria in pregnancy, it is crucial to detect and treat these infections rapidly. Further work is needed to assess the diagnostic performance of the uRDT in pregnant women before consideration of this test for implementation where csRDT are already being used- particularly in asymptomatic pregnant women in moderate transmission settings.

4.7. Addendum

1. It must be noted that the uRDT only detects *P. falciparum*, whereas the csRDT has the additional benefit of targeting *P. vivax/ malariae/ ovale* species. Thus, if the uRDT is considered for use in co-endemic transmission settings, it important to offer additional tests for these other species during pregnancy. Furthermore, as mentioned in chapter 1.1.3, the species composition in an area can shift if only one species is being targeted (i.e. diagnosed and treated), making surveillance of non- *falciparum* infections equally as important.

2. In addition to the uRDT evaluation studies outlined in the discussion section of this chapter, studies, the study published by Mesigwa *et al.* should also have been

included. Their cross-sectional study describes the uRDT sensitivity in The Gambia as "modest" and variable depending on the local transmission setting. Their models suggest that the uRDT would be best suited for Mass Test and Treat (MTAT) programs in settings that have very low transmission intensity, rather than for POC (Mwesigwa, Slater *et al*. 2019)

4.8. Acknowledgements

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Chapter 5. First report of *pfhrp2* deletions in Indonesia and impact on RDT outcomes

Contents

5.0. Declarations

For data collection, DNA extraction, molecular testing and RDT testing see previous declarations (in Chapter3). In this study, Dr Grant Kay (GK) provided technical support by carrying out the assays for the optimisation and screening (100%) of samples using MSP1, HRP and β-tubulin assays. I supported the intelligent and experimental design, interpreted and analysed the results of these assays (90%). I carried out 100% of the statistical analyses.

5.1.1. Chapter summary

In the previous Chapter, the sensitivity of the uRDT (Alere™, Korea) and csRDT (CareStart™, USA) was 20% and 23% in a retrospective evaluation of malaria RDTs, among pregnant women in Indonesia. Given the low sensitivity of both RDTs we investigated the target-antigen that both RDTs, Histidine Rich Protein 2 (HRP2). Deletions of the encoding gene for this antigen is known to produce false negative RDT results, however incidence of the mutation it is yet to be reported in Indonesia. Using stored samples from Chapter 2, the status of this antigen-encoding gene was investigated in pregnant women in Timika, Indonesia for the first time.

5.1.2. Abstract

Background

The deletion of genes encoding antigens targeted by malaria rapid diagnostic tests (RDTs) is cause for serious concern. Deletions of the *Plasmodium falciparum* Histidine Rich Protein II (*pfhrp2*) gene impacts on the performance of widely used RDTs. Following WHO recommendations for *pfhrp2* reporting, the incidence and impact of these deletions were investigated in Timika, Indonesia for the first time.

Methods

DNA samples from whole blood collected in a Stop Malaria in Pregnancy (STOP-MiP) trial were tested for deletions in the *pfhrp2* gene and structural homologue *pfhrp3*. Samples were first screened by multi-copy assay, Loop-mediated isothermal Amplification (LAMP) and quantitative polymerase chain reaction (qPCR), for infection with *P. falciparum*. Stored blood samples underwent subsequent testing using two RDTS: uRDT (Alere, Korea) and csRDT (Access Bio inc, USA). DNA yields in these samples were confirmed using single-copy assays, Merozoite Surface Protein-1 (*MSP1*) PCR and β-tubulin real-time PCR (rt-PCR). The presence of *pfhrp2/3* fragments was determined using PCRs for *pfhrp2/3* exon 1/ 2. Sanger sequencing was used to resolve indeterminate samples. The effect of the different assays on RDT outcome was estimated using logistical regression models.

Results

Full deletions of *pfhrp2* (n=8) and *pfhrp3* (n=14) were present in this cohort of pregnant women in Timika. Only complete deletion of *pfhrp2* was identified as a significant predictor for uRDT (β= 2.167, SE± 1.1) and csRDT (β=3.136, SE± 1.3) outcomes.

Conclusion

The evidence for *pfhrp2* deletion, alongside the negative association with RDT outcomes shown here, suggest dual antigen detecting RDTs are preferable to RDTs that only detect PfHRP2. Although further analyses are needed to support these conclusions, the work here flags important considerations for current Intermittent Screening and treating during pregnancy (ISTp) policies in Indonesia.

5.2. Introduction

5.2.1. Diagnosis of malaria

Accurate diagnosis and prompt treatment are central to the Global Technical Strategy for malaria 2016-2030 (WHO 2015). The WHO also advise that antimalarials only be administered following confirmation of a malaria diagnosis, mainly by microscopy or a Rapid Diagnostic Test (RDT) (WHO 2015).

Indonesia is co-endemic for *Plasmodium falciparum* (63%) and *Plasmodium vivax* (37%), resulting in an estimated 1.5 million cases of malaria and 2,700 deaths (2017) (WHO 2018). It accounts for 4% of the global burden of *P. vivax* malaria cases alone. The negative associations of malaria during pregnancy (outlined in Chapter 1, p20) prompted the implementation of Intermittent Screening and Treatment during pregnancy (ISTp) policies in Indonesia in 2012 (Indonesian Ministry of Health 2012, Indonesian Ministry of Health 2013).

However, these strategies have not shown additional benefit in comparison to Intermittent Preventative Treatment during Pregnancy (IPTp) where all women are treated during antenatal visits regardless of symptoms/testing (Desai, Hill *et al*. 2018, Ahmed, Poespoprodjo *et al*. 2019). Due to the inadequate sensitivity of current RDTs and the good safety profile of ACTs during pregnancy (WHO 2015), IPTp methods have proven superior to ISTp (Tagbor, Cairns *et al*. 2015, Awine, Belko *et al*. 2016, Ahmed, Poespoprodjo *et al*. 2019).

Globally, diagnosis is still typically reached through microscopy, although global demand for RDTs almost doubled in 2018 compared to 2017 (WHO 2019). Most of this demand (412 million RDTs in 2018) is for *P. falciparum* only RDTs (64%), with just 16 countries making up the majority of the market (Unitaid 2018, WHO 2019). In Indonesia over 360,0000 diagnoses were reached using RDTs in 2018 (WHO 2019).

5.2.2. Target-antigen mutation

Given the global use of RDTs, mutations in the main RDT target-analyte are cause for concern (WHO 2019, Poti, Sullivan *et al*. 2020). Parasites harbouring full deletions of the *P. falciparum* Histidine Rich Protein II (*pfhrp2*) gene can result in false negative RDTs, whilst RDT sensitivity is maintained in the presence of only partial gene deletions. The latter is thought to be in part due the capture antibodies embedded in the RDT cross-reacting with a structural homologue; *pfhrp3*, which has similar antigenic epitopes (Bharti, Chandel *et al*. 2016, Beshir, Sepulveda *et al*. 2017). This results in a positive RDT test in the presence of *P. falciparum* with wild type *pfhrp3* even when *pfhrp2* is deleted. Similarly, wild-type *pfhrp2* strains can mask the presence of *pfhrp2* deletions in polyclonal infections. The presence of *pfhrp2* antigen in these mixed infections will result in a positive and resulting in misreporting of *pfhrp2* mutations (Beshir, Sepulveda *et al*. 2017). For these reasons, the quantification of the different *pfhrp2/3* genotypes should accompany surveillance of these mutations in order to further our understanding of their dynamics, selection pressures and ultimately- enable rapid programmatic reaction to their emergence (Beshir, Sepulveda *et al*. 2017).

The PfHRP2 antigen is expressed on the surface of infected red blood cells throughout the parasite's life-cycle (within the human host), although at varying levels (Howard, Uni *et al*. 1986, Hayward, Sullivan *et al*. 2000, Baker, Gatton *et al*. 2011). The *pfhrp2* gene spans an intron and two exons, flanked by putative Heat Shock Proteins and exported protein pseudogenes (fig 5.1A). Located within the subtelomeric region of chromosome 8, *pfhrp2* has multiple breakage sites in the flanking genes and within the gene itself (Baker, Ho *et al*. 2010). Re-joining of the gene at breakage sites can result in the excision of portions or the entirety of *pfhrp2*, thus numerous variations of this gene have

been reported (Baker, Ho *et al*. 2010, Dharia, Plouffe *et al*. 2010) (fig 5.1A).

Loss of a structural homologue *pfhrp3* (located on chromosome 13 occurs through a similar mechanism, often accompanied by loss of flanking genes (fig 5.1B) (Dharia, Plouffe *et al*. 2010). The fitness costs associated with deletion of either are not fully understood, they are however known to occur independently of each other. Sequence variation in these genes is now widespread (Baker, Ho *et al*. 2010, Gamboa, Ho *et al*. 2010, Abdallah, Okoth *et al*. 2015, Bharti, Chandel *et al*. 2016, Beshir, Sepulveda *et al*. 2017, Kozycki, Umulisa *et al*. 2017, Watson, Slater *et al*. 2017, Gendrot, Fawaz *et al*. 2019). Mathematical modelling has also shown that low malaria prevalence and high frequency of people seeking treatment contribute to the selection of *pfhrp2* deletion (Watson, Slater *et al*. 2017).

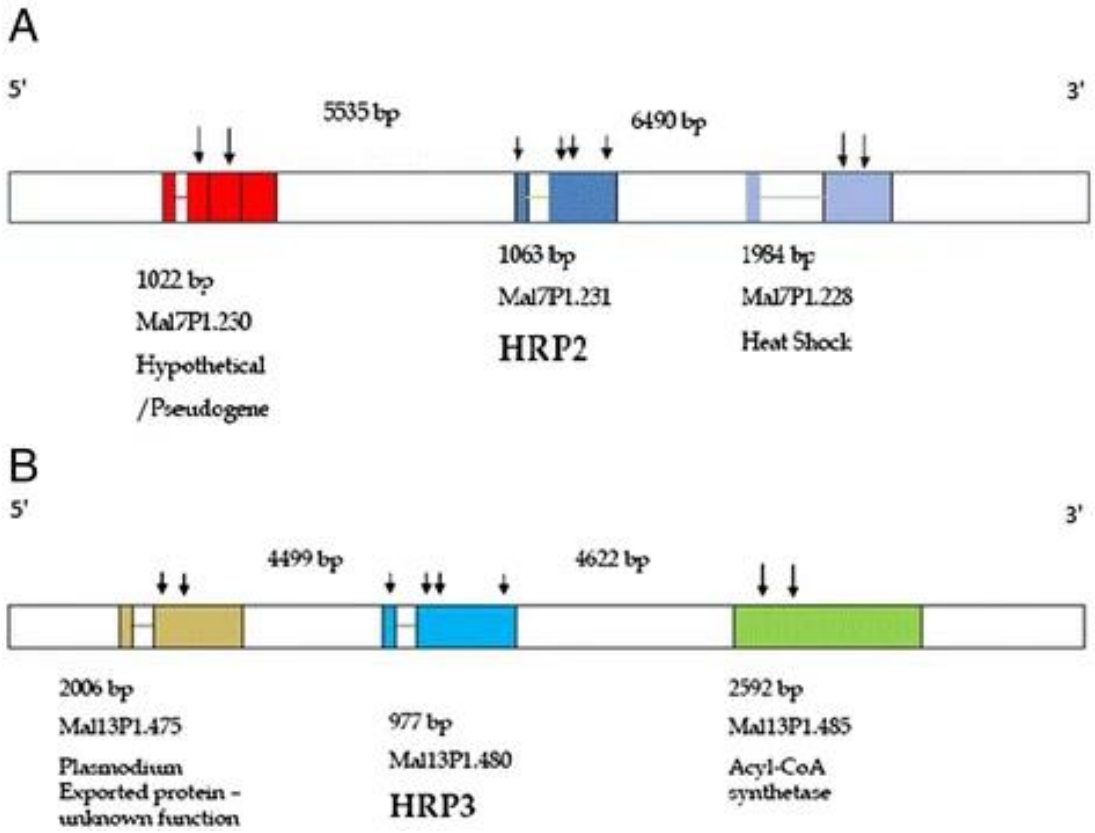
5.2.3. Impact of RDT false negatives

The potential impact of false negative RDTs and missing a malaria diagnosis - particularly during pregnancy- has gained due attention. It is however important to recognise that false negative RDT results can also arise from analytical insensitivities, misreading/misreporting or operational mishaps (WHO 2016). Thus, the WHO has advised caution when reporting *pfhrp2* deletions, recommending specific testing for confirmation of deletions in order to avoid undue panic and costly changes in RDT implementation, as well as mistrust of RDT usage amongst health practitioners (Baker, Ho *et al*. 2010, Cheng, Gatton *et al*. 2014, Parr, Anderson *et al*. 2018). Once confirmed, they recommend baseline reporting of *pfhrp2/3* in these

and neighbouring countries. They suggest that a prevalence of *hrp2/3* deletion above 5% causing high suspected false positivity should warrant a change in RDT (WHO 2019). They have also developed interactive online maps to track *pfhrp2/3* reporting (fig 5.2).

Figure 5.1. The gene structure and organisation of *pfhrp2* and *pfhrp3*.

The *pfhrp2* and *pfhrp3* genes span an intron across exon 1 and 2 on A) chromosome 8 and B) chromosome 13 respectively. Flanking genes are also shown, arrows indicate potential breakage points, bp= base pairs. Image source: Abdallah *et al.*(2015) (Cox 2010)



5.2.4. Aims

To date, full deletions of *pfhrp2* have not yet been reported in Indonesia (fig 5.2). Nevertheless, given the particularly low sensitivity of two HRP2-based RDTs in Chapter 4, here we investigate the gene structure of *pfhrp2* and *pfhrp3* in those samples (Unwin, Ahmed *et al*. 2020). Molecular testing is used here to determine the incidence of *pfhrp2*/3 partial/full deletions and their impact on the outcomes on RDT outcomes in a cohort of pregnant women in Timika, Indonesia.

Figure 5.2. Map of *pfhrp2* studies and confirmed deletions.

Dots represent studies that reported presence (red) and absence (grey) of *pfhrp2/3* deletions (grey). (Map source: WHO Malaria Threats Map: tracking biological challenges to malaria control and elimination www.who.int/malaria/maps/threats/

5.3. Methods

5.3.1. Study Design and samples

This retrospective study uses stored DNA samples from asymptomatic pregnant women in Timika, Indonesia as part of a Stop Malaria in Pregnancy trial (STOPMiP, ISRCTN: 34010937) (Unwin, Ahmed *et al*. 2020). Venous blood samples were collected, DNA extracted and molecular reference testing (LAMP and qPCR) were performed as previously described in Chapter 2 (section 2.3.3). In Chapter 4 (section 4.3.3, two RDTs were also performed: the uRDT (Ultra-sensitive HRP2-based Alere™ Malaria Ag *P.f* RDT) and the csRDT (CareStart™ Malaria HRP2/PLDH (*PF*/PAN) Combo RDT) (Unwin, Ahmed *et al*. 2020). Samples testing positive for *P. falciparum* using the composite molecular reference test were investigated here.

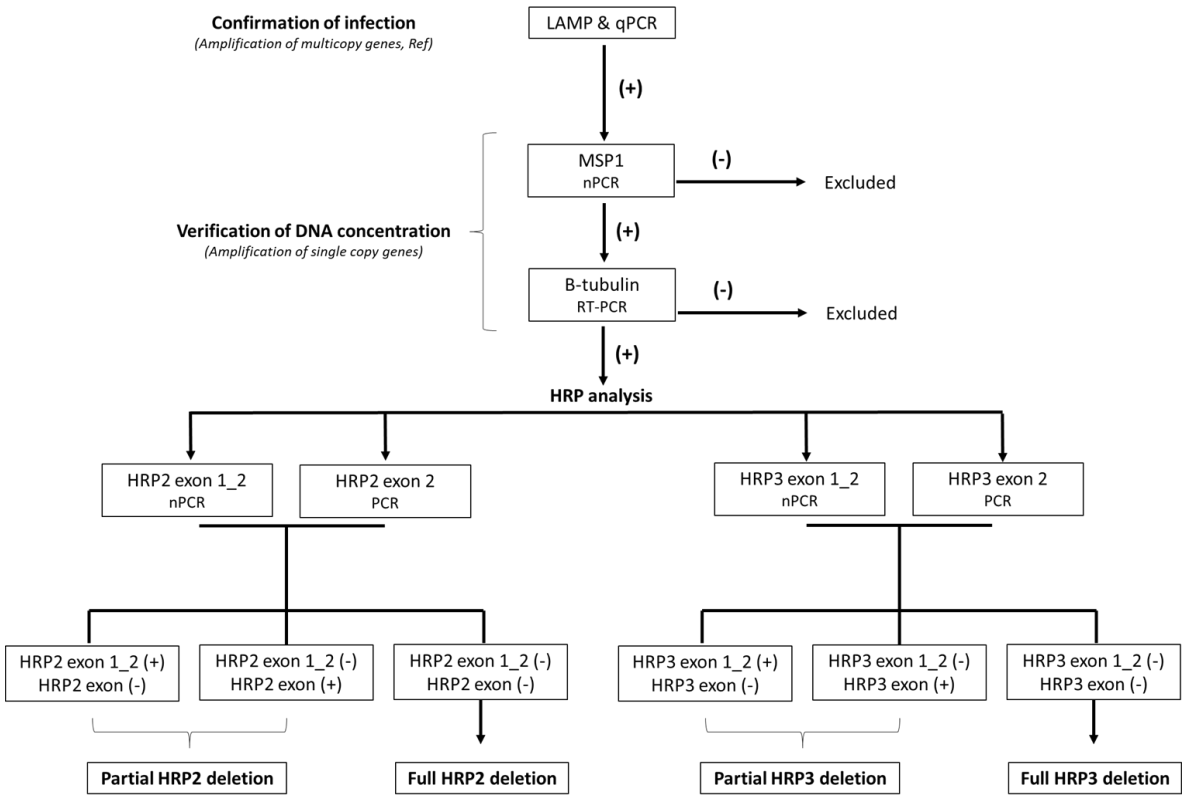
Extracted DNA for positive controls from the following cultured strains of *P. falciparum* were provided by the London School of Hygiene and Tropical Medicine: 3D7 (positive for both *pfhrp2*/*pfhrp3 (Walker-Jonah, Dolan et al. 1992)*), DD2 (*pfhrp2*-deleted (Wellems, Walliker *et al*. 1987)), HB3 (*pfhrp3*-deleted (Su, Ferdig *et al*. 1999)) and 3BD5 (both *pfhrp2* and *pfhrp3* deleted (Walker-Jonah, Dolan *et al*. 1992)).

5.3.2. Sample flow

To determine the status of *pfhrp2* and *pfhrp3,* a diagnostic algorithm (fig 5.3) was formulated according to WHO recommendations for accurate reporting (Cheng, Gatton *et al*. 2014). First, infection with *P. falciparum* was confirmed using two single-copy assays, followed by examination of exons 1 and 2 using four PCR assays. For each *pfhrp2* and *pfhrp3* an ‘exon 1_2’ assay was used for the detection of the region spanning the intron, and

another assay 'exon 2' for the detection of the portion only on exon 2 (fig 5.1). Investigation of flanking genes is recommended for characterisation but optional for reporting, unfortunately this was not feasible in this study given the low remaining sample volumes following HRP analysis (Cheng, Gatton *et al*. 2014).

Figure 5.3. Decision tree for confirmation of *pfhrp2* and *pfhrp3* deletions



5.3.3. Detection of *P. falciparum* species

Two single gene copy assays for *P. falciparum*, Merozoite surface protein-1 (*MSP1*) (Mayengue, Ndounga *et al*. 2011) and β-tubulin (Fennell, Al-shatr *et al*. 2008), were used to confirm sufficient DNA yield for accurate reporting of *pfhrp2/3* deletions. Primers and cycling conditions are outlined in Table 5.1 and for all assays 3 µl of DNA was used. PCR

products were visualised by gel electrophoresis (2% agarose/ Trisacetate-EDTA)) and images analysed using the ImageLab (v6.01, Bio-rad, UK) software. Bands were interpreted by two independent readers blinded to the RDT results, with discordant calls confirmed by a third reader. Expected band sizes for each product are outlined in Table 5.1.

Real-time fluorescence of the β-*t*ubulin qPCR assay was measured using the Qiagen RotorGene-Q (Qiagen, Germany) and analysed using RotorGene-Q Software (Source, version). A Cycling threshold (Ct) of 38 was used as a cut-off for positive *P. falciparum* samples.

Table 5.1. PCR assays for confirmation of *pfhrp2* and *pfhrp3* deletions.
Table source: Grant Kay (LSTM, UK)

Amplicon	Primer sequence	Expected amplicon size	Initial denaturation	Cycling conditions	Final Extension
MSP1	Primary forward: CACAATGTGTAACACATGAAAG	646 bp	95°C x 5 min	40 cycles: 94°C x 1min 55°C x 1 min 72°C x 1 min	72°C x 10 min
	Primary reverse: AGTACGTCTAATTCATTTGCAC				
	Nested forward: TAGAAGCTTTAGAAGATGCAG	555 bp		40 cycles of: 94°C x 1 min 53°C x 1 min 72°C x 1 min	
	Nested reverse: GACAATAATCATTAGCACATAC				
Pfhrp2 exon 1-2	Primary forward: GGTTTCCTTCTCAAAAAATAAAG	308 bp	95°C x 15 min	40 cycles: 94°C x 1 min 50°C x 1 min 72°C x 1 min	72°C x 10 min
	Primary reverse: TCTACATGTGCTTGAGTTTCG				
	Nested forward: GTATTATCCGCTGCCGTTTTTGCC	225 bp		40 cycles of: 94°C x 1 min 55°C x 1 min 72°C x 1 min	
	Nested reverse: CTACACAAGTTATTATTAAATGCGGAA				
Pfhrp2 exon 2	Forward: CAAAAGGACTTAATTTAAATAAGAG	814 bp	94°C x 10 min	40 cycles of: 94°C x 1 min 55°C x 1 min 72°C x 1 min	72°C x 10 min
	Reverse: AATAAATTTAATGGCGTAGGCA				
Pfhrp3 exon 1-2	Primary forward: GGTTTCCTTCTCAAAAAATAAAA	311 bp	94°C x 10 min	40 cycles of: 94°C x 1 min 60°C x 1 min 72°C x 1 min	72°C x 10 min
	Primary reverse: CCTGCATGTGCTTGACTTTA				
	Nested forward: ATATTATCGCTGCCGTTTTTGCT	226 bp	94°C x 10 min	40 cycles of: 94°C x 1 min 62°C x 1 min 72°C x 1 min	72°C x 10 min
	Nested reverse: CTAAACAAGTTATTGTTAAATTCGGAG				
Pfhrp3 exon 2	Forward: AATGCAAAAGGACTTAATTC	719 bp	94°C x 10 min	40 cycles of: 94°C x 1 min 55°C x 1 min 72°C x 1 min	72°C x 10 min
	Reverse: TGGTGTAAGTGATGCGTAGT				
B-tubulin	Forward: AATAAATCATAATGATGTGCGCAAGTGATCC	80 bp	50°C x 2 min 95 x 10 °C	40 cycles of: 95°C x 30 sec 60°C x 1 min Dissociation analysis	
	Reverse: AATAAATCATAATCCTTTTGGACATTCTTCCTC				

5.3.4. Detection of *pfhrp2* and *pfhpr3*

Exons 1 and 2, as well as exon 2 alone were analysed for each *pfhrp2* and *pfhrp3* using the assays outlined in Table 5.1. The concentration of primers (Eurofins Genomics, Germany) and cycling conditions were identical to those published previously, except for the addition of a final extension step for 10 min at 72 °C. *Pfhrp2/3* and *MSP1* reactions contained DreamTaq Green Master Mix (ThermoFisher Scientific, UK), whilst the β-*t*ubulin qPCR contained FastStart SYBR™ mastermix (ThermoFisher Scientific, UK). (Figure 5.3)

5.3.5. Investigating limits of Detection

The reported limits of detection (LOD) for the *pfHRP2* and *pfhrp3* assays are 1 parasite/ µl, whilst single-copy target assays for *MSP1* and β-tubulin are 10 parasites/ µl (Parr, Anderson *et al*. 2018). This difference ensures that a lack of amplification in the HRP assays following confirmation by the single-copy assays is due to mutation of the gene rather than lack of DNA. To support our results, we assessed the relative LOD of each assay empirically. For this, serial dilutions (10^{0}-10^{-3}) of a positive control (provided by Dr Debbie Nolder, PHE/LSHTM) were tested by each assay.

5.3.6. Sequencing

Samples that produced only very faint bands in any *pfhrp2/3* assay underwent Sanger sequencing to determine the presence or absence of amplicon. QIAquick PCR Purification Kit (Qiagen, Germany) was used prior to sequencing by Source BioScience™ (Nottingham, UK). Sequence traces were compared to positive and negative controls using CodonCode Aligner software (version 9.0, USA).

5.3.7. Statistical analyses

The effects of: 18S rRNA qPCR, β-tubulin rt-PCR, presence/absence of each *pfhrp* fragment and complete absence of both exon1_2 and exon 2 on RDT outcome were estimated using

binary and multinomial logistical regression analyses. β- values where P value< 0.05 were used to indicate significant predictors of RDT outcome.

5.4. Results

5.4.1. Confirmation of infection and DNA content

Samples that were positive for *P. falciparum* (LAMP/qPCR) were subsequently tested for DNA content using two single-gene copy assays: *MSP1* PCR and β- tubulin qPCR (n=149). A total of 20 samples were detected by both *MSP1* and β-tubulin tests, with sufficient volume for further testing. (Appendix figure 5.7.1)

5.4.2. Presence of *pfhrp2* and *pfhrp3*

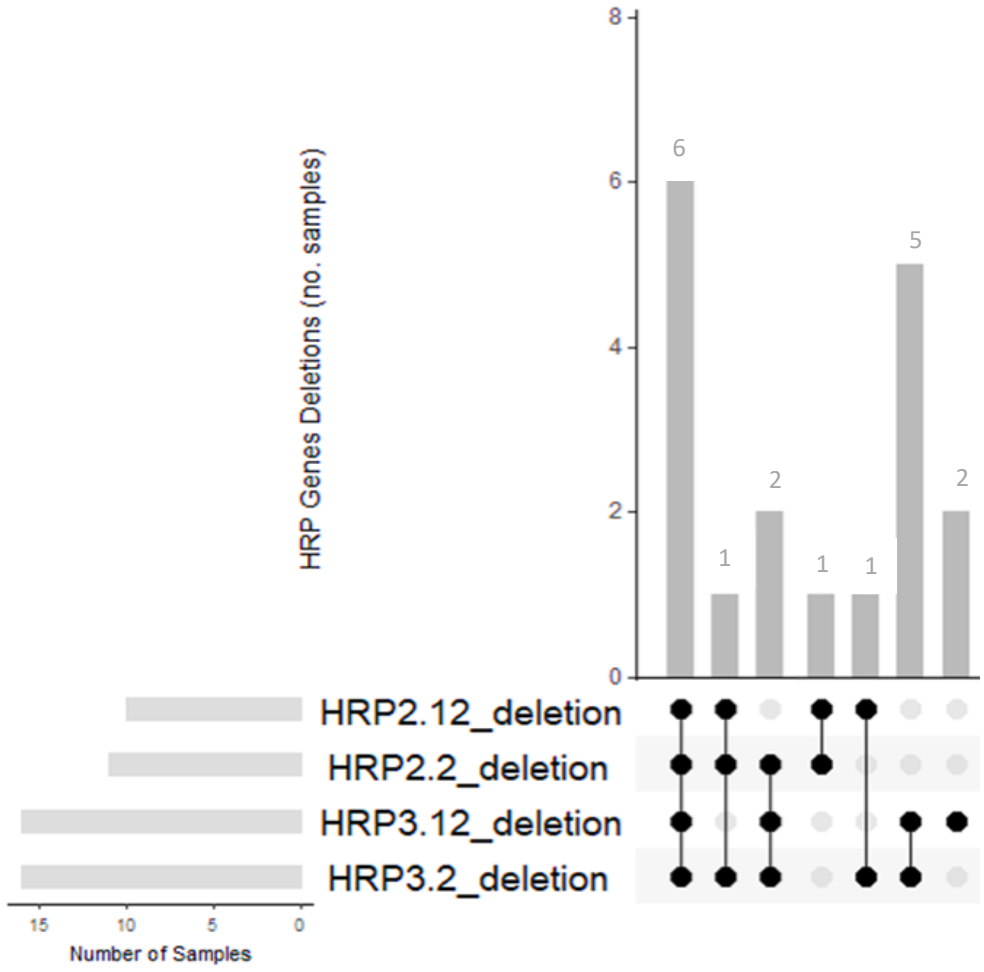
Analysis of the *pfhrp2* exon 1_2 region showed amplification in 10 out of the 20 samples and 10 samples amplified the *pfhrp2* exon 2. Only 4 samples out of 20 were positive for *pfhrp3* exon 1_2, and 4 for *pfhrp3* exon 2. Overall, 6 samples failed to amplify any region of either *pfhrp2* or *pfhrp3*. Eleven out of 20 had at least one suspected deletion in the *pfhrp2* region, 15 in the *pfhrp3* region and 18 with at least one deletion in either region. (Figure 5.4.1)

5.4.3. Impact on RDT outcomes

Of the 6 samples that did not amplify any of the *hrp* gene fragments, five were negative by both csRDT and uRDT and one was negative by csRDT/ positive by uRDT (fig 5.4.3, appendix Table 5.2). Figure 5.4.2 shows the mean and range of the Ct (Cycling Threshold) values observed in the β-tubulin rt-PCR, stratified by presence of *pfhrp* fragments and RDT outcome. Notably there were more suspected *pfhrp2* deletions in samples negative by both RDTs, compared to those positive by both RDTs. (Figure 5.4.2) Additionally, the mean Ct observed for samples positive by both RDTs was 30.4 (95% CI 29,4- 31.4), whilst for RDT (both) negative samples the mean Ct was 34.3 (95% CI 33.0- 35.7).

Figure 5.4.1. Combinatorial frequencies of *pfhrp2* and *pfhrp3* genotypes.

Top bar chart represents the frequency of combinations of deletions and side chart represents the total number of samples with that genotype.



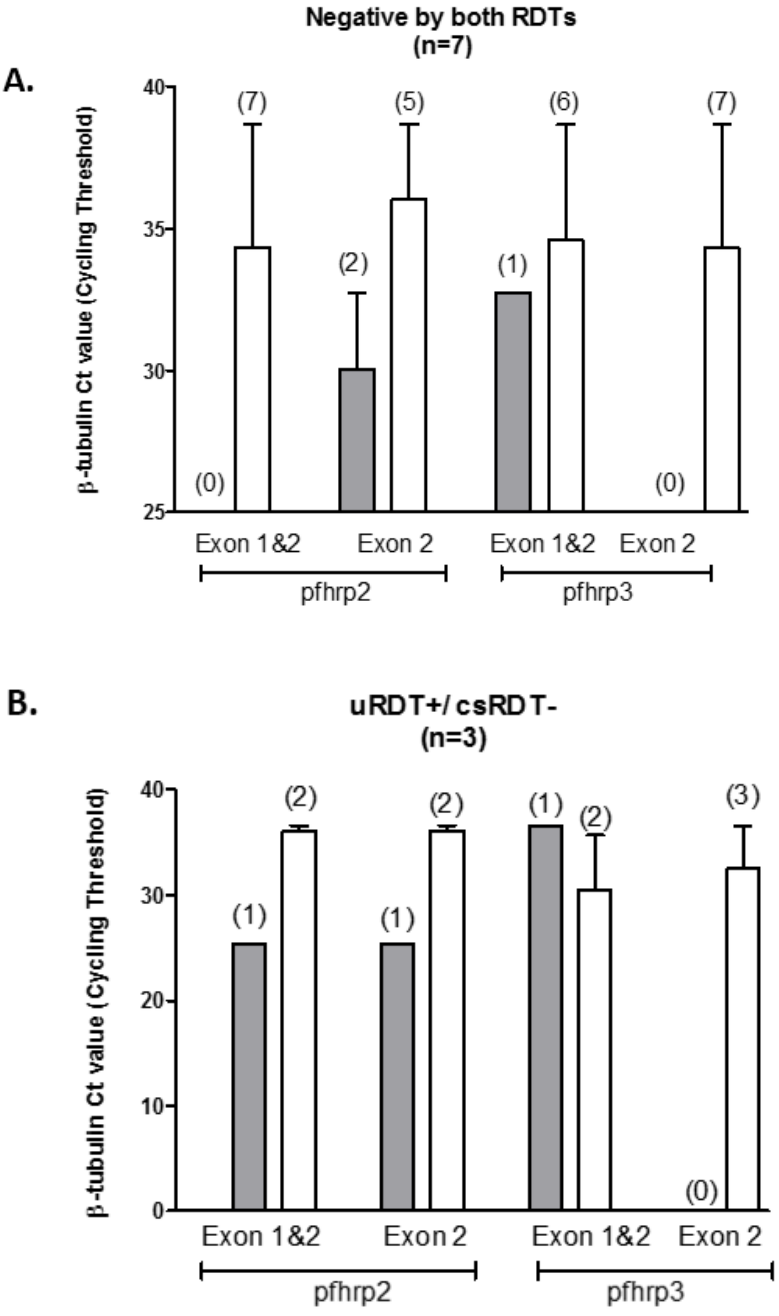
The effects of the 18S rRNA qPCR, the β-tubulin rt-PCR and presence/absence of each of *pfhrp* fragment on RDT outcome was estimated using multinomial logistical regression analyses. Only β- tubulin rt-PCR was found to be a significant predictor (β= -0.452, p<.05, SE 0.23) for csRDT outcomes. The 18s rRNA qPCR, *pfhrp2* exon 1_2/exon2 and *pfhrp3* exon 1_2/exon2, were non-significant predictors of positivity of any RDT (p> 0.05). Binomial logistical regression identified complete absence of *pfhrp2* (negative for exon1_2 and exon 2) as being a significant predictor for both uRDT (β= 2.167, SE 1.1, p= 0.05) and csRDT (β= 3.136, SE 1.3, p=0.02) outcomes.

5.4.4 LOD

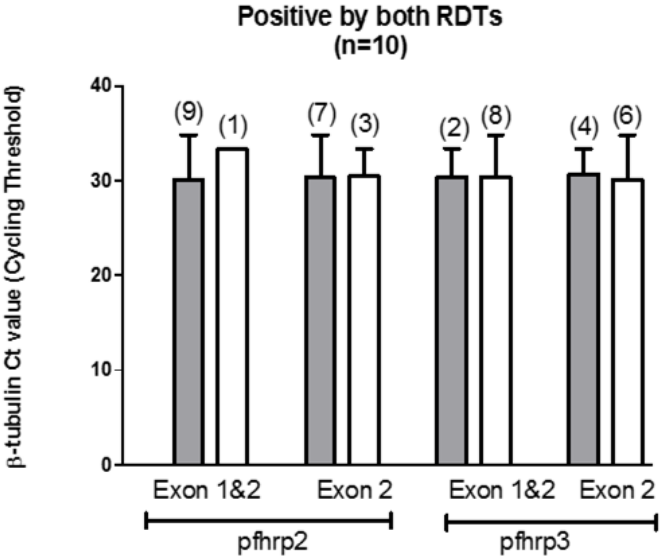
The LODs of all assays were estimated using serial dilutions of the same positive control. Unexpectedly, both *pfhrp2* and *pfhrp3* exon 2 assays failed to detect the most dilute sample, suggesting a higher LOD (lower sensitivity) for these assays in comparison to *MSP1*, exon 1_2 and β-tubulin assays. (Appendix figure 5.7.2)

Figure 5.4.2. Distribution of β-tubulin qPCR Ct values and *pfhrp2/3* deletion by RDT outcome.

Bar chart shows the number (n) of samples that were negative (white bar) and positive (grey bar) for exon 1_2 and exon 2 for each *pfhrp2* and *pfhrp3*. The mean β-tubulin Ct and range of (error bars) are also shown. Data are stratified by RDT outcome: A) Negative by both uRDT and csRDT, B) positive only by uRDT and C) positive by both RDTs.



C.



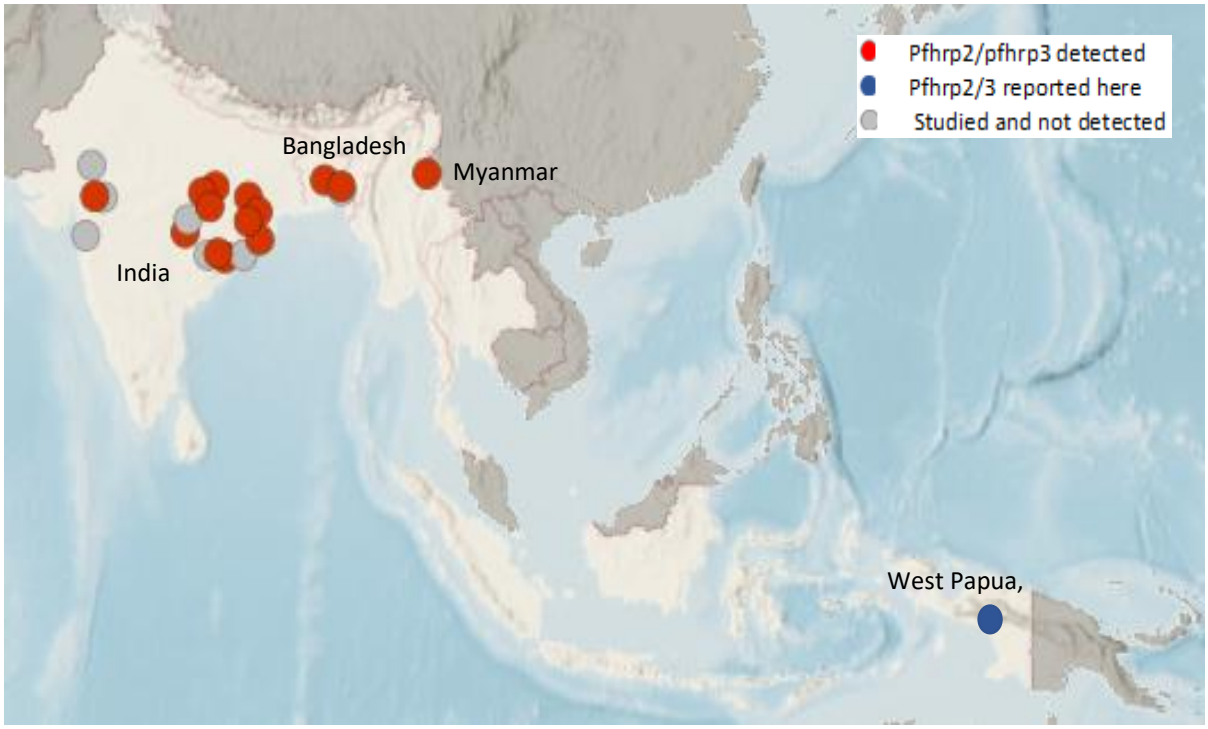
5.5. Discussion

The efficacy of *pfhrp2* RDTs is threatened by deletions in the genes that encode these proteins. In this study amongst asymptomatic pregnant women, the deletion of *pfhrp2* and *pfhrp3* regions was most common amongst RDT negative samples, with 60% showing full deletions (both exon 1_2 and exon 2 regions) of both *pfhrp2* and *pfhrp3* genes. Conversely, 70% of RDT positive samples had both *pfhrp2* and *pfhrp3* (both exon 1_2 and exon 2) intact.

Furthermore, full deletion of *pfhrp2* was found to be a significant predictor of two RDT outcomes (uRDT and csRDT). Partial deletions (absence of either exon 1_2 or exon 2) and absence of *pfhrp3* were not significant predictors of RDT outcome. These results mirror those in a large meta-analytical study by Baker *et al.*(2011) that looked at 16 RDTs and WHO pre-qualification testing (Baker, Gatton *et al*. 2011). This is the first study that provides evidence of complete deletions of *pfhrp2* and *pfhrp3* in malaria infections in Indonesia, and in the southern Asia (Li, Xing *et al*. 2015, Bharti, Chandel *et al*. 2016) (fig 5.5). To date, deletions have only been reported as near as Myanmar, however this is most likely due to

lack of *pfhrp2*/3 surveillance rather than lack of prevalence. As shown in figure 5.2 these mutations are found in bordering countries, so it is very likely that they are also prevalent in neighbouring countries such as Papua New Guinea. This has serious implications for diagnosis and treatment in this region, especially where MTAT or ISTp strategies are being considered.

Figure 5.5 Updated WHO *pfhrp2*/3 threat map to include this study.
Image modified from https://apps.who.int/malaria/maps/threats.



5.5.1. Context

To date, deletions of these genes in Asia have only been reported in India (Bharti, Chandel *et al*. 2016) and the borders of China and Myanmar. Only sequence variation- not deletion- has been reported in SE. Asia (Baker, Ho *et al*. 2010). The high frequency of *pfhrp2* and *pfhrp3* deletions reported here is in line with previous studies in other settings (Bharti,

Chandel *et al*. 2016, Beshir, Sepulveda *et al*. 2017), as are the proportions, with a higher frequency of deletions observed in the *pfhrp3* gene than in *pfhrp2* (Dharia, Plouffe *et al*. 2010, Gamboa, Ho *et al*. 2010, Solano, Okoth *et al*. 2015, Viana, Okoth *et al*. 2017). Additionally, this study echoes findings that *pfhrp3* arises independently of *pfhrp2* deletions (Gamboa, Ho *et al*. 2010, Abdallah, Okoth *et al*. 2015, Viana, Okoth *et al*. 2017, Fontecha, Mejia *et al*. 2018). Rachid Viana *et al.* report *pfhrp3* mutation incidence of 68 % in parts of Brazil and Bolivia (Viana, Okoth *et al*. 2017), where *pfhrp2* mutations were rare.

It is well demonstrated that *pfhrp3* antigens cross-react with *pfhrp2*-RDTs, producing positive RDT results in the absence of *pfhrp2* genes (Baker, McCarthy *et al*. 2005, Baker, Gatton *et al*. 2011, Das, Peck *et al*. 2018). This is also shown here, where one sample that was *pfhrp2*-negative/*pfhrp3*-positive tested positive by both RDTs, whilst another was positive by only the uRDT. This could suggest that *pfhrp3* contribution to RDT-positivity could occur at lower parasitaemia than previously thought (Das, Peck *et al*. 2018).

Curiously, one sample which completely lacked all regions of *pfhrp2* and *pfhrp3* was positive by uRDT (csRDT negative). This could represent a false uRDT result produced by an infection of parasites lacking *pfhrp2*, whereby a line is produced by cross reactivity as has been demonstrated in patients with particularly high levels of Human Anti-Mouse Antibodies (HAMA) and Rheumatoid Factor (RF) (Lee, Jang *et al*. 2014, Gatton, Ciketic *et al*. 2018).

Alternatively, the sample could represent a true positive where infections comprise 1) *pfhrp2*-intact parasites, but at a parasitaemia above the LOD of the *hrp* assays or 2) a mixed infection where the load of *pfhrp2*-deleted parasites exceeds *pfhrp2*-intact parasites (see details on clonal infection below). As infection was confirmed by 18S rRNA, β-tubulin rt-

PCR and MSP1 PCR, a truly positive uRDT result is more likely In this study, although not quantified, the relative LODs of the *pfhrp2* and *pfhrp3* assays were higher than that of the *MSP1* and β -tubulin assays, whereas the opposite was previously reported: 10 parasites/ µl for *MSP1* and β-tubulin, and 1 parasite/ µl for both *pfhrp2*/*pfhrp3* assays. This is reflected in the Ct values for the 18S rRNA and β-tubulin assays, which fell above the highest Ct observed for any *pfhrp2* (and *pfhrp3* exon2) positive sample. However, it was lower than that observed for *pfhrp3* exon1_2.

Further analyses of the flanking genes could help to conclude that the absence of gene amplification here is due to gene deletion rather than a low concentration of DNA.

5.5.2. Limitations

The main limitation of this study is common across studies of asymptomatic malaria- low parasite DNA yields. Whilst it is likely that a number of the false negative RDT results are attributable to *pfhrp2* and *pfhrp3* deletions, it is also possible that many arise due to the poor sensitivity of RDTs at low parasitaemia.

The dynamics of *pfhrp2* and *pfhrp3* remain unclear and the precise drivers of selection for these mutations are not well understood (Akinyi, Hayden *et al*. 2013, Watson, Slater *et al*. 2017). This is especially so in low transmission settings where low parasite DNA yield muddies interpretation of gene absence. This complicates determination of the true prevalence of these deletions, especially in areas where low density infections predominate. Use of more sensitive qPCR methods for infection confirmation as well as detection of gene deletions will improve studies in this field.

It is also important to note that infections can comprise more than one clone and therefore multiple hrp2/3 genotypes (Kun, Missinou *et al*. 2002, Naidoo and Roper 2013, Childs and Buckee 2015, Ayanful-Torgby, Oppong *et al*. 2016). It is plausible that molecular methods selectively amplify an abundance of hrp2-lacking DNA over trace amounts of hrp2-intact DNA, whilst the sensitive RDTs may still detect antigen in these samples. Additionally, the clearance rate of different clones will differ depending on priming of the host immune response (previous exposure, length of infection etc), and since the uRDT detects antigen rather than DNA, it could be truly positive. There is a real need to develop more sensitive assays for accurate HRP2 reporting to further our understanding of the impact of these gene mutations.

The recently developed 'qHRP2/3-del' multiplex qPCR which integrates pfrnr2e2 (single-copy gene) as an internal control is an example of this (Schindler, Deal *et al*. 2019). The qHRP2/3 is quantitative, allowing differentiation of infection with multiple strains of *P. falciparum* and the unmasking of trace genotypes. However, as with all molecular methods, nucleotide variation in primer binding sites may result in failure to amplify targets even in the presence of the gene. Ultimately, sequencing may offer more in-depth insights into the structure, prevalence and dynamics of HRP polymorphism.

5.5. Conclusion

Overall, this study presents evidence of hrp2 deletions that affect RDT outcomes, although supporting studies are needed to solidify these findings. This is deeply concerning given the setting of Indonesia- a country that is heavily reliant on the use of RDTs to prevent malaria

in pregnancy. Here, the introduction of the uRDT in place of currently used RDTs is not advisable. Instead, it remains that RDTs that detect alternative antigens in addition to *pfhrp2* should be used preferentially.

5.7. Appendix

Figure 5.7.1. Flow diagram summary of diagnostic tests.

The total number of samples run in each test and the number of positive (+) and negative (-) results obtained are shown (n).

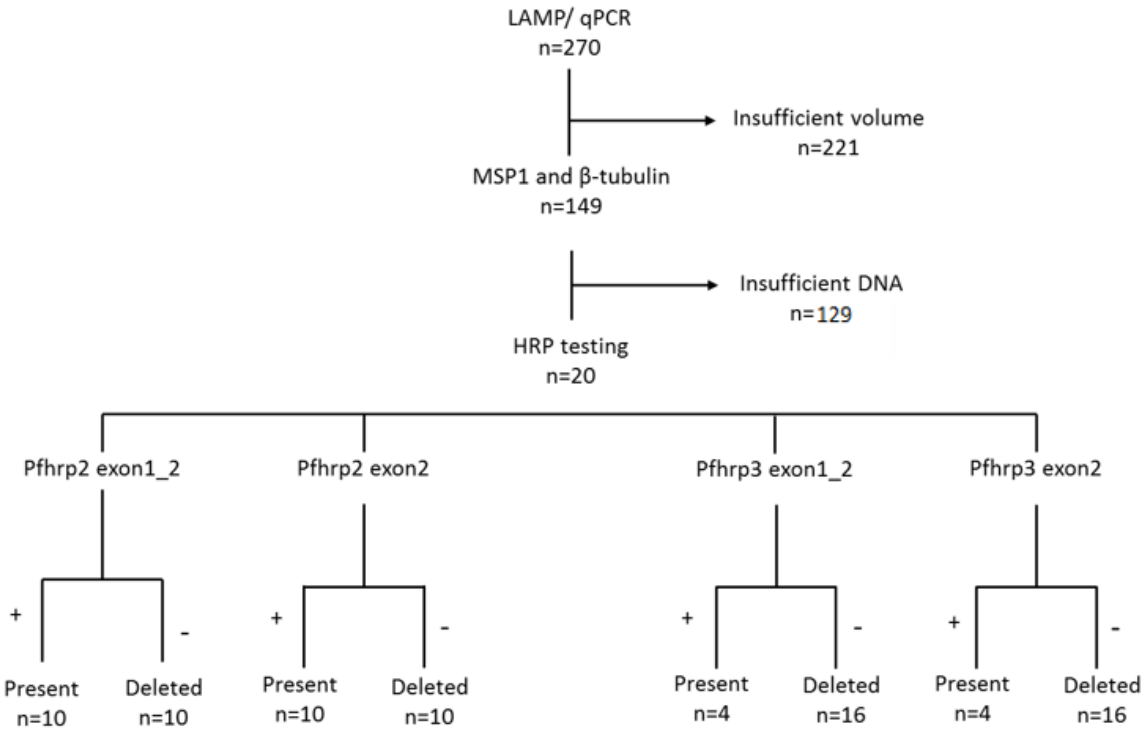
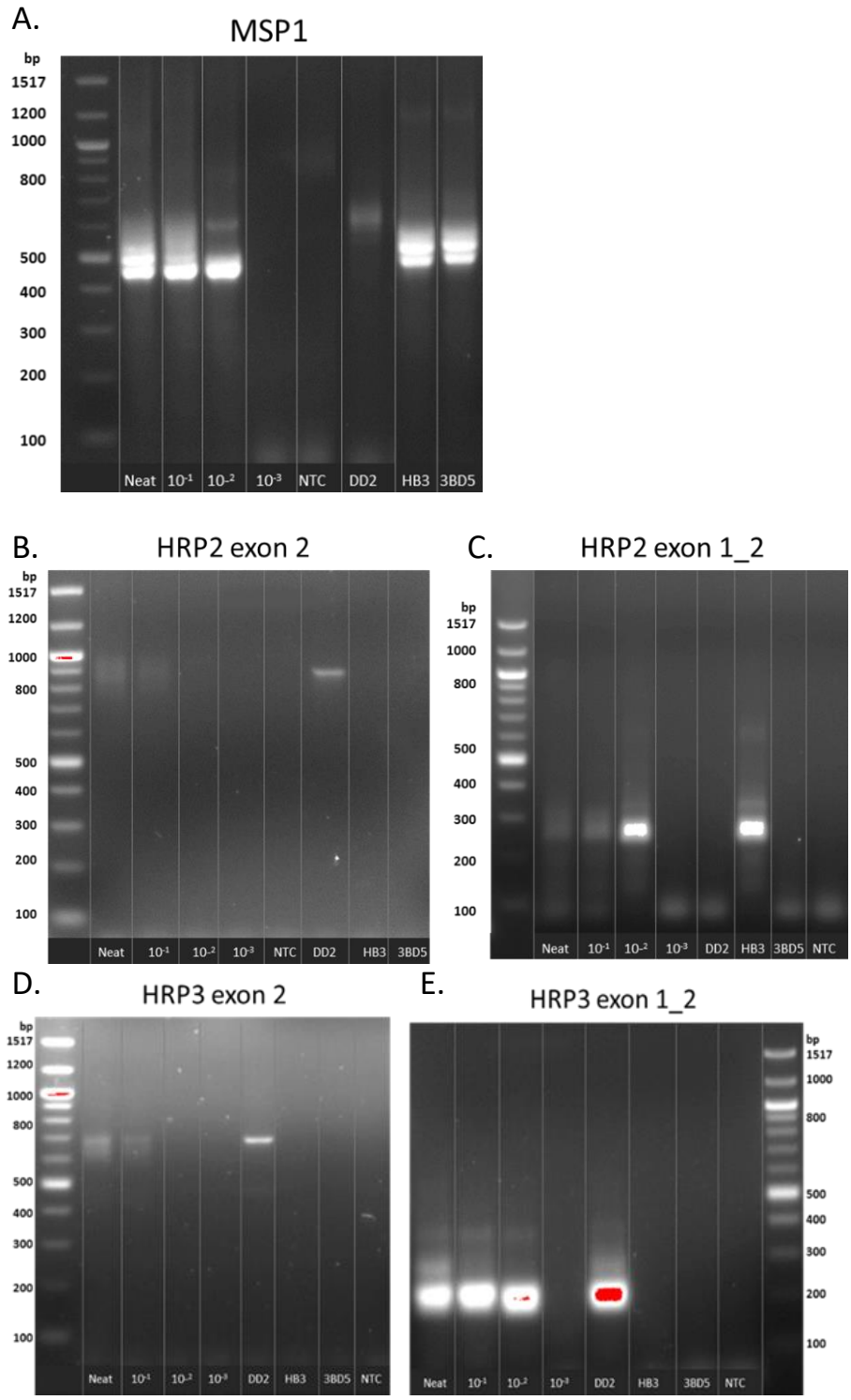


Table 5.2. Full data set of *pfhrp2* and *pfhrp3* presence, RDT outcomes and rt-PCR Cycling threshold values.

	Infection confirmation			RDTs		HRP2		HRP3	
Sample	18S rRNA	β-tub	MSP	uRDT	csRDT	Exon1/2	Exon2	Exon1/2	Exon2
1	31.72	23.48	+	+	+	+	+	-	-
2	26.02	25.36	+	+	-	+	+	-	-
3	25.68	27.37	+	-	-	-	+	-	-
4	29.19	27.43	+	+	+	+	+	+	+
5	31.52	28.08	+	+	+	+	-	-	-
6	33.71	30	+	+	+	+	-	-	-
7	33.79	30.42	+	+	+	+	+	-	+
8	33.93	31.34	+	+	+	+	+	-	-
9	32.98	31.64	+	+	+	+	+	-	+
10	33.29	32.74	+	-	-	-	+	+	-
11	28.1	33.13	+	+	+	+	+	-	-
12	32.74	33.35	+	+	+	-	-	+	+
13	33.20	33.51	+	-	-	-	-	-	-
14	32.85	34.81	+	+	+	+	+	-	-
15	31.57	34.84	+	-	-	-	-	-	-
16	30.74	35.67	+	+	-	-	-	-	-
17	34.25	36.32	+	-	-	-	-	-	-
18	37.08	36.57	+	+	-	-	-	+	-
19	35.66	36.77	+	-	-	-	-	-	-
20	36.53	38.65	+	-	-	-	-	-	-

Figure 5.7.2. Observed limit of detection.

Serial dilutions (10^0-10^{-3}) of 3D7 DNA were tested in each of the assays and compared to positive controls DD2, HB3 and 3BD5. Expected band size for A) *MSP1* is 555 bp, B) *pfhrp2* exon 2 is 814 bp, C) *pfhrp2* exon 1_2 is 225 bp, D) *phfrp3* exon 2 is 719 bp and E) *pfhrp3* exon 1_2 is 226 bp.



5.8. Acknowledgments

As above for data collection, DNA extraction, molecular testing and RDT testing. I am grateful to Dr Grant Kay for his technical assistance in this study and Dr Beshir Khalid (LSHTM) for provision of positive controls.

5.9. References

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Chapter 6. Reading between the lines: An evaluation of diagnostic tools for malaria in pregnancy

Contents

6.0. Declaration of contributions

Samples

Dr Emily Adams (ERA) and Professor Feiko Ter Kuile, Dr Hellen Barsosio (HB), Dr Kephas Otieno and Dr Simon Kariuki facilitated the collaboration between KEMRI, Kisumu and LSTM, UK. ERA and FTK and I contributed to the intelligent design of this study. Experimental design was formulated by ERA and I. Mr Eric Onyango managed the central database for this study. I trained staff at the 3 sites in Kisumu to carry out the uRDTs. Mr Eugene Omanga, Mr Teles Odawo and Mr Geoffrey Niatindo performed the uRDTs, csRDTs. I organised, coordinated and monitored the uRDT sub-study over with support from ERA, FTK and HB.

Lab work

I received technical assistance (25%) with the DNA extractions from Ms Winnie Chebore and several students at KEMRI, Kisumu. I performed the 100% of the qPCR testing and interpretation.

Other

I performed the analyses (100%) with interpretation support form ERA.

6.1.1. Chapter summary

In this Chapter the Alere Ultra-sensitive *Pf* Ag RDT (uRDT) is evaluated prospectively in pregnant women in in W Kenya. The 4-plex qPCR assay developed in Chapter 2 was used as a reference test to compare the performance of the uRDT with the CareStart *Pf/*Povm combo RDT (csRDT). This study, conducted in a high transmission setting, is a large prospective study to mitigate limitations of previous work (Chapter 4).

6.1.2. Abstract

Background

More sensitive rapid diagnostic tests (RDTs) are needed for diagnosis of low density infections. Malaria during pregnancy is associated with adverse outcomes and therefore, more sensitive RDTs are especially important to rapidly diagnose and treat. Here we compare the performance of the Alere ultra-sensitive RDT to the CareStart™ Malaria HRP2/pLDH *Pf*/VOM Combo (csRDT) amongst pregnant women in Western Kenya.

Methods

Samples (n= 493) were collected from pregnant women enrolled in the IMPROVE clinical trial from three sites in Kisumu, Kenya. The two RDTs were performed on whole blood samples, whilst dried blood spots were stored for DNA extraction and qPCR reference testing. Diagnostic indicators (sensitivity, specificity, positive predictive value, negative predictive value, diagnostic odds ratios and kappa values) were calculated to assess the performance of both RDTs.

Results

The uRDT sensitivity (79.9%; 95% CI 72.5- 85.7%) was higher than that of the csRDT (74%; 66.2- 80.6%) in comparison to a qPCR reference test. The proportion of positive infections detected by the two tests was significantly different (p value=0.000), as was the proportion of negatives detected by the two tests (p = 0.000) as the specificity was uRDT; 90.4% (95% CI 86.6- 93.2%) and csRDT 93.0% (95% CI 86.6-95.4). However, the overall performance of the two tests (as indicated by the Diagnostics Odds Ratios, Kappa values and AUROC values) were non-significantly different to each other.

Conclusions

This is the first study to assess the diagnostic accuracy of the uRDT in pregnant women in a high transmission setting. The findings here support the use of the uRDT for treating *P. falciparum* infections during pregnancy due to the urgency of treating MIP.

6.2. Introduction

The importance of robust and sustainable surveillance systems for malaria in elimination settings is outlined in Chapter 1, and specifically for malaria pregnancy in Chapter 4. Briefly, current point of care tests (POCTs), specifically RDTs, are insufficiently sensitive for the detection of low density infections.

6.2.1. The uRDT

In a bid to improve RDT sensitivity, the Alere™ Ultra-sensitive Malaria Ag *P. falciparum* RDT (uRDT) was developed. As described in Chapter 4, the uRDT has been evaluated across a range of transmission settings (Das, Jang *et al*. 2017, Das, Peck *et al*. 2018, Girma, Cheaveau *et al*. 2018, Hofmann, Antunes Moniz *et al*. 2018, Landier, Haohankhunnatham *et al*. 2018, Hofmann, Antunes Moniz *et al*. 2019), but only twice in pregnant women (Vasquez, Medina *et al*. 2018, Unwin, Ahmed *et al*. 2020). The two studies in pregnant women, Vasquez *et al.*(2018) and the study performed in Chapter 4 in this thesis (Table 6.1) show that the uRDT performs similarly to existing RDTs (CareStart RDT or SD Bioline RDT) (Vasquez, Medina *et al*. 2018). The limitations of these studies call for larger prospective evaluation studies of the uRDT, to inform decisions around implementation. Table 6.1 summarises the literature comparing the performance of the uRDT to existing RDTs. The value added to diagnostic performance by the uRDT varies considerably.

6.2.2. MiP prevention

The distribution of insecticide treated nets (ITNs) to pregnant women is at the core of control strategies to prevent MiP, having been proven to cost-effectively reduce many of its adverse outcomes (Cotter, Sturrock *et al*. 2013, Desai, Hill *et al*. 2018). Alongside bednet

use, Intermittent Preventative Therapy during Pregnancy (IPTp) is recommended by the WHO (WHO 2015). For this, a course of 3 doses of Sulfadoxine-Pyrimethamine (SP), spaced at least one month apart, should be given starting in the second trimester. Although prophylaxis is administered during antenatal visits, coverage of IPTp amongst eligible women in 23 African countries is as low as 19% (2016) (WHO 2015, WHO 2015, WHO 2019). MiP in Africa is therefore still one of the major contributors to still-births (WHO 2019).

These strategies are however compromised by the widespread emergence of SP resistance (Chico, Cano *et al*. 2015, Okell, Griffin *et al*. 2017). Alternate drugs for IPTp e.g Dihydroartemesinin- Piperiquine (DP) have also been evaluated for use in areas with high SP resistance. To minimise unnecessary treatment of non-infected women and limit the risk of resistance, alternate strategies such as Intermittent Screen and Treat during Pregnancy (ISTp) are being explored (Tagbor, Cairns *et al*. 2015, Awine, Belko *et al*. 2016, Ahmed, Poespoprodjo *et al*. 2019). This requires diagnostic confirmation prior to treatment, unlike ITPp where all pregnant women are treated without testing and regardless of presence of symptoms. Consequently, the choice of diagnostics used in ISTp strategies is critical to their efficacy (Slater, Ross *et al*. 2015, Slater, Ross *et al*. 2019).

Chapter 4 highlights the need for larger scale and more in-depth investigations into the effects of *pfhrp2* and *pfhrp3* deletions and their impact RDT outcomes. This needs investigation in different transmission settings, where the selection pressures may vary. The development and use of more sensitive and less-subjective diagnostic tools for surveillance of these deletions would also aid our understanding of their impact.

Deletions of *pfhrp2* were previously observed in 19% of PCR-confirmed *P. falciparum* positive samples in Western Kenya (Beshir, Sepulveda *et al*. 2017). Of the hrp2-deleted infections, 10% were both microscopy and RDT positive, with intact *pfhrp3* loci. This suggests that mutated parasites are still detectable by RDT at higher (microscopy-detectable) parasitaemias (Sawa, Shekalaghe *et al*. 2013). It follows that these mutations may have a more detrimental impact among populations harbouring low density infections. Since MiP in Africa rarely results in fever (Desai, ter Kuile *et al*. 2007) it is important to actively identify and treat these more cryptic infections.

Table 6.1. Summary of published uRDT evaluation studies.

Transmission settings are categorised by prevalence: Low (<5%), Moderate (5-20%) and High (≥20%).

Author (ref)	Country (transmission)	Demographic	Study design	Comparator (Ref test)	Significantly different sensitivity
Unwin *et al*. (Unwin, Ahmed *et al*. 2020)	Indonesia (moderate)	Pregnancy	Prospective cohort	csRDT (qPCR/LAMP/nPCR)	No
Vasquez *et al.* (Vasquez, Medina *et al*. 2018)	Colombia (low)	Pregnancy	Retrospective cohort	LM, sdRDT (nPCR)	No
Mesigwa *et al*. (Mwesigwa, Slater *et al*. 2019)	The Gambia (various)	Adults and children	Cross-section	(rtPCR)	No
Hoffman *et al.* (Hofmann, Gruenberg *et al*. 2018)	PNG (moderate)	Adults [b, c]	Cross-section	sdRDT (qPCR)	Yes uRDT>sdRDT
Hoffman *et al.* (Hofmann, Antunes Moniz *et al*. 2018)	Tanzania (moderate)	Febrile outpatients [b]	Prospective cohort	HRP2 ELISA	No

Das *et al*. (Das, Jang *et al*. 2017)	Uganda (high)	Children [a] Adults [b]	Prospective cohort	sdRDT (HRP2 ELISA)	Yes uRDT>sdRDT
Landier *et al*. (Landier, Haohankhunnatham *et al*. 2018)	Myanmar (low)	Adults	Cross-section	sdRDT (HRP2 ELISA, qPCR)	Yes uRDT>sdRDT
Girma *et al*. (Girma, Cheaveau *et al*. 2018)	Ethiopia (low)	Adults and children	Cross-section	csRDT, sdRDT, LAMP (qPCR)	Yes LAMP> uRDT> csRDT> sdRDT

LM; Light Microscopy, usQPCR; Ultra-sensitive qPCR, ELISA; Enzyme Linked Immunosorbent Assay, sdRDT; SD Bioline RDT, NS; Not Significantly different, [a] asymptomatic individuals only, [b] excludes pregnant women, [c] excludes <5 yrs of age

6.2.3. Aims

This study aims to evaluate the performance of the Alere uRDT and CareStart™ Malaria HRP2/pLDH *Pf*/VOM Combo (csRDT) using a qPCR reference test. For this, venous blood was sampled from pregnant women enrolled in the Improving PRegnancy Outcomes With Intermittent preVEntive Treatment in Africa (IMPROVE) clinical trial in Kisumu, Kenya.

6.3. Methods

6.3.1. Study design and participants

This prospective study formed a sub-study under the larger clinical trial IMPROVE (NCT03208179). The IMPROVE trial aims to assess the effectivity of co-administration of an antibiotic alongside antimalarials as intermittent preventive therapy for malaria and STDs in pregnant women. Venous blood samples were collected from pregnant women enrolling in the IMPROVE trial (viable singleton pregnancy, >16 weeks' gestation and willing to deliver in a hospital) in the Kisumu district, Western Kenya. The trial excluded multiple pregnancies (i.e. twin/triplets), HIV-positive status, known heart ailments, severe malformations or nonviable pregnancies observed by ultrasound, histories of receiving SP during this pregnancy, those unable to give consent, known allergies and any other contraindication to any of the study drugs. All enrolled women were tested for malaria using the csRDT for IMPROVE and immediately treated if positive.

Study sites included three of the ten IMPROVE trial sites: Ahero and Rabuor sub-county hospitals, and Akala health Centre (fig 6.1). Venous bloods were collected during October 2018- May 2019 in Rabuor and Ahero, and May 2019 for in Akala (n=493). The two initial sites were chosen for their high initial recruitment rates during the IMPROVE study, whilst Akala was added to improve recruitment rates. Within the IMPROVE trial, these sites were selected for their capacity to process RDTs in addition to those for the IMPROVE study.

Figure 6.1. Map of Kisumu county in Western Kenya and the location of the study sites. The health care facilities are displayed on the map: 1) Rabuor sub-county hospital, Vihiga county 2) Ahero county hospital, Kisumu county and Akala health centre on the border of Siaya and Kisumu county. Map modified from Google Maps.

Malaria transmission is year-round in this area, with two seasonal peaks between June–July and December–January. A recent study reported prevalence of *P. falciparum* malaria in this area to be 33% and 51% by microscopy and PCR respectively (Wanja, Kuya *et al*. 2016).

6.3.2. Detection of malaria

Blood samples were collected by venepuncture (up to 10 mL in EDTA tubes) unless the participant was unwilling, in which case a finger prick (~250 µl) was drawn instead. These were used within 1 hr for RDT testing, whilst dried blood spots (DBS; 50 µl) were also prepared. DBS were stored with silica at room temp for molecular analysis.

Two lateral flow diagnostic tests: the uRDT (Alere™) and the currently used csRDT RDT (CareStart Combo *Pf/Povm* RDT) were performed according to manufacturer guidelines. Both RDTs were performed by the same operator. For visual reading, independent CRFs

were completed for either RDT. Only CareStart RDT results influenced treatment at enrolment as the uRDT has not yet been approved for clinical use by the Ministry of Health in Kenya.

Reference testing was carried out at the end of the 8-month study. Briefly, DNA was extracted from approximately 25 µl of one DBS using the Qiasymphony Blood and tissue DNeasy kit as per manufacturer's guidelines (Qiagen, cat#). One negative and one positive (3D7 culture) extraction control was included per every 72 extractions.

A multiplex qPCR containing 18S rRNA probes for all 4 major species of malaria, *P. falciparum, P. vivax, P. malaria* and *P. ovale*, was used a reference standard for these asymptomatic infections. (STOPMiP, Unwin Refs) For this, qPCR reactions (10 µl total volume) contained: 2 µl DNA, QuantiFast Pathogen PCR Mix (Qiagen™, Hilden, Germany) and primers and probes as outlined in Chapter 2 (section 2.3.3) (Shokoples, Ndao *et al*. 2009). Thermocycling conditions (38 cycles) were as follows: 10 min at 95 °C, 15 sec for 95 °C and 60 sec for 60 °C. Fluorescence was acquired using the Rotor-Gene Q 5plex HRM Platform (Qiagen™, Hilden, Germany) and cycle threshold (Ct) values were calculated using the Rotor-Gene Q series software version 1.7 (Qiagen Inc, Valencia, CA, USA). Negative and positive controls (*P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax* provided by Public Health England) were included in every run.

6.3.3. Statistical analyses

To observe a difference between the sensitivity of the uRDT and csRDT with a power of 0.8 with 95% confidence, a sample size of 233 qPCR-confirmed positive samples was calculated.

Estimating the RDT prevalence to be 25% (Wanja, Kuya *et al*. 2016) and therefore qPCR prevalence ~40%, the sample size was determined to be 580.

The difference in observed proportions of positive tests by uRDT and csRDT were compared using the McNemars proportions test. The Area Under the Receiver Operator Curves (AUROC) was calculated. All statistical analyses were performed in SPPS v26.

6.3.4. Ethics

Under the IMPROVE study protocol and approved ethics, diagnostic testing was done on samples from women enrolling in the IMPROVE study. Informed consent was be obtained before women enrolled in the study. The IMPROVE protocol, the informed consent document and patient information sheets were reviewed and approved by the Research and Ethics Committee of Liverpool School of Tropical Medicine, Liverpool (LSTM), the Research Ethics Committees at KEMRI, Nairobi, Kenya (SERU), the College of Medicine in Malawi (COMREC) or if so requested at national level, by the Malawian National Health Science Research Committee), and by the National Health Research Ethics Committee in Tanzania.

6.4. Results

6.4.1. Incidence of malaria

In this study (n= 493), overall incidence of *P. falciparum* infections was 31.0% by qPCR, 31.5% by uRDT and 28.8% by csRDT. QPCR identified an additional 7 *P. vivax* infections, 2 *P. ovale* and 7 *P. malariae* infections (3% non-*falciparum* infections), whilst the csRDT estimated prevalence of non- *falciparum* infections to be 9.1% (Table 6.2). Table 6.2 shows the proportion of each species detected by each test, at each sampling site. The number of

RDT-positive and qPCR-negative (false positives) was highest in Akala; 4/23 were positive by both RDTs, with 2 additional positive only detected by uRDT. Only one of these samples had any amplification (Ct 39.2) above the cut-off. The csRDT detected more non-*falciparum* infections than the qPCR reference test, across all sites, potentially due to antigen persistence. (Table 6.2)

6.4.2. Diagnostic performance

The performance of the uRDT was compared to that of the csRDT using qPCR as a reference test. Resultant diagnostic indicators are outlined in Table 6.3. The uRDT sensitivity was 79.9% (95% CI; 72.5-85.7) whilst the csRDT was 74.0% (66.2- 80.6) with overlapping confidence intervals. The proportion of infections detected (sensitivity) by the uRDT was significantly higher than that of the csRDT (McNemars p= 0.02, 1-β= 0.8, α= 0.05). The specificities of the two tests were uRDT; 90.4% (95% CI 86.6- 93.2%) and csRDT 93.0% (95% CI 86.6-95.4), however the csRDT specificity was significantly higher (p= 0.01, 1-β= 0.8, α= 0.05). Overall, this is reflected by the similar DOR and Kappa values (Table 6.3) and is echoed in the AUROC values; uRDT 0.87 (95% CI 0.83- 0.91%) and csRDT 0.89 (95% CI 0.85-0.93%). (Table 6.3)

The Venn diagram in figure 6.2 shows that the majority (n= 112) of infections were detected by all three tests (uRDT, csRDT and qPCR). 23 infections were positive by both RDTs and negative by qPCR. The uRDT also detected 11 qPCR-confirmed infections that were missed by the csRDT. The csRDT detected 2 qPCR-confirmed that the uRDT missed. (Figure 6.2)

Table 6.2. The incidence of malaria by index tests (uRDT and csRDT) and reference test (qPCR).

Site	Test	*Pf*		*Po*		*Pv*		*Pm*		*Pan*	
		n	*(%)*	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*
Ahero	uRDT	68	30.8%								
(n= 221)	csRDT *Pf*-line	63	28.5%								
	csRDT *Pan*-line									21	9.5%
	qPCR	63	28.5%	2	0.9%	3	1.4%	3	1.4%	8	3.6%
Rabuor	uRDT	75	29.8%								
(n= 252)	csRDT *Pf*-line	64	25.4%								
	csRDT *Pan*-line									24	9.5%
	qPCR	83	32.9%	0	0.0%	4	1.6%	3	1.2%	7	2.8%
Akala	uRDT	13	56.5%								
(n= 23)	csRDT *Pf*-line	11	47.8%								
	csRDT *Pan*-line									4	17.4%
	qPCR	7	30.4%	0	0.0%	0	0.0%	0	0.0%	0	0.0%

Pf; P. falciparum, *Pv; P. vivax*, *Po; P. ovale* and *Pm; P. malariae*

Table 6.3. Diagnostic performance of csRDT and uRDT using qPCR reference test for *P. falciparum*.

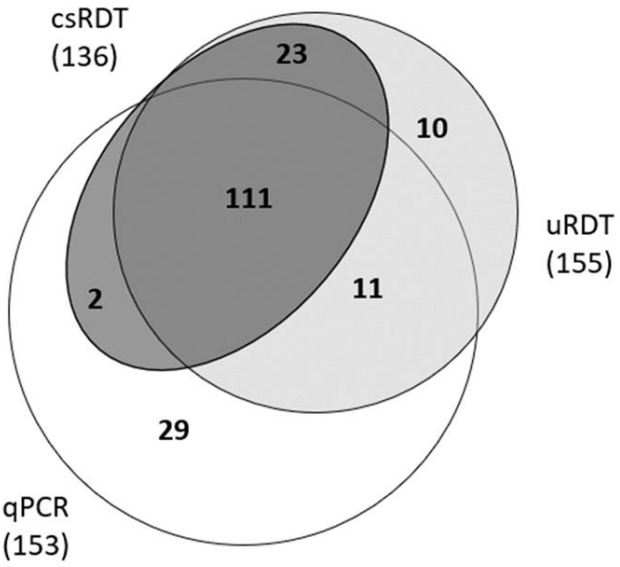
	TP	FP	TN	FN	Sensitivity %	(95% CI)		Specificity %	(95% CI)	
uRDT	122	33	308	31	79.7	(72.3-	85.6)	90.3	(86.5-	93.1)
csRDT	113	23	318	40	73.9	(66.0-	80.4)	93.2	(89.9-	95.6)

	PPV %	(95% CI)		NPV %	(95% CI)		DOR %	(95% CI)		Agreement (κ)	(95% CI)		AUROC	(95% CI)
uRDT	78.7	(71.3-	84.7)	90.8	(87.1-	93.5)	36.6	(21.5-	62.4)	0.7	(0.6-	0.8)	0.87	(0.8- 0.9)
csRDT	83.0	(75.5-	88.8)	88.8	(84.9-	91.8)	38.9	(22.3-	67.9)	0.7	(0.6-	0.8)	0.89	(0.8- 0.9)

TP; True Positive, FP; False Positive, TN; True Negative, FN; False Negative; CI; Confidence Intervals, PPV; Positive Predictive Value, NPV; Negative Predictive Value, DOR; Diagnostic Odds Ratio, Kappa; agreement with qPCR, AUROC; Area Under the Receiver Operator Curve, and n; total number of samples.

Figure 6.2. Venn diagram showing number of positive samples detected by uRDT, csRDT and qPCR.

The number of mutually detected positives is shown within the respective Venn circles. (n)= total number of positives detected by a test.



6.4.3. Parasite density, distribution and sensitivity

Using qPCR Ct value as a proxy of parasite load, Cts were categorised into low (qPCR Ct >32), medium (Ct 26-32) and high (Ct <26) parasitaemia. The distribution of parasite load was skewed toward moderate-low densities (Table 6.4), with only 15 high-density infections (<Ct26). The sensitivity of the uRDT and csRDT was highest, but non-significantly different to each other amongst moderate infections (csRDT 93%, uRDT 96%). In low density samples, the uRDT sensitivity (60%) was significantly higher (p= 0.02) than that of the csRDT (31%). (Table 6.4)

Table 6.4. Diagnostic test positivity stratified by qPCR Ct value as a proxy of parasite load.

		Parasite density			
Test		High < Ct26	Moderate Ct26- 32	Low Ct32 >	Total
qPCR	n	15	75	63	153
	Mean (± 95% CI)	24.9 (0.6)	28.8 (0.4)	34.8 (0.4)	30.9 (0.6)
	LQ-UQ	24.4- 25.6	27.1- 30.3	33.2- 35.9	27.4- 34.3
uRDT	n	12	72	38*	122*
	%	80%	96%	60%	80%
csRDT	n	12	70	31*	113*
	%	80%	93%	49%	74%

Ct; Cycling threshold, LQ; lower quartile and UQ; upper quartile, *p=0.02 (McNemars)

6.5. Discussion

There is still a need for highly sensitive malaria POCTs. Previously (Chapter 3), the uRDT performed similarly to the csRDT amongst pregnant women in a moderate transmission setting. However, the study was retrospective, using frozen red blood cell pellets resuspended in the patient's plasma. Since the manufacturer's guidelines for these tests require whole blood, this prospective study follows the recommended guidelines.

This study investigates the diagnostic accuracy of the uRDT in a high transmission in order to better address limitations outlined previously. First, it was suggested (in Chapter 4) that the similar performance of the uRDT and csRDT could be due to samples falling below the LOD of both RDTs. Second, it was difficult to ascertain whether *pfhrp2* genotypes play a role in RDT outcome due to low parasite DNA yields. Finally, it is important to assess the diagnostic accuracy of new tests in different settings as changing prevalence will affect the PPV and NPV.

The uRDT sensitivity (79.9%) determined here was higher than the csRDT (74%), akin to previous results in high transmission settings in Uganda amongst children and adults (Das, Jang *et al*. 2017). Unlike in Uganda, the uRDT specificity in this study was lower than that of the csRDT. In this study these differences were found to be significant, however the same analyses were not reported in the Ugandan study (Das, Jang *et al*. 2017).

The number of false positives called by only one RDT is in line with the expected specificity of either test. However, the 23 false positive infections detected by both RDTs is more suggestive of antigen persistence following clearance of an infection and therefore no (or low concentrations of parasite DNA. This could be due to recent anti-malarial use or immunity-regulated clearance of parasites. It is well established that due to the more rapid clearance of DNA compared to parasite proteins e.g. HRP2 antigen, PCR methods are more indicative of an *active* infection, whilst antigen detection can also detect a *recently cleared* infection (Dalrymple, Arambepola *et al*. 2018). RDT positivity is thus prolonged beyond PCR positivity following clearance (Martin, Rajasekariah *et al*. 2009, Dalrymple, Arambepola *et al*. 2018).

The high proportion of false positives (by both RDTs) observed at the Akala site is interesting and most likely reflects the recent clearance of an infection (Dalrymple, Arambepola *et al*. 2018) or potentially intrinsic immunological factors (Gatton, Ciketic *et al*. 2018) in the local population. A recent review of factors that could impact RDT outcome showed the false positivity rate of RDTs was elevated in the presence of samples containing Rheumatoid factor (RF) and Human Anti-Mouse Antibody (HAMA) (Lee, Jang *et al*. 2014, Gatton, Ciketic *et al*. 2018). It would be interesting to measure these factors in samples from this site in comparison to the other sites.

Taking sensitivity and specificity into account, the overall diagnostic accuracy of the two tests was similar as indicated by the DOR and AUROC values. This supports findings from the two other publications assessing the accuracy of the uRDT amongst pregnant women. The first, in Colombia (low transmission setting), showed the uRDT offered a slight increase in sensitivity compared to the SD Bioline malaria RDT among febrile pregnant women, however the study was too underpowered to draw concrete conclusions. (Vasquez, 2018).

The second study (Chapter 4) utilised stored samples that had been reconstituted to a uniform haematocrit, which is not recommended by the manufacturer's guidelines. In that moderate transmission context, both the csRDT and uRDT performed similarly poorly (sensitivity ~20%), but still in line with previously estimated prevalence using RDTs in the same area (Ahmed, Levy *et al*. 2015).

6.5.1. Parasite density

When stratified by parasite load, the sensitivity of both RDTs was unexpectedly highest amongst moderate infections (93-96%), with high density infections offering only 80% sensitivity for both RDTs. Previously, impaired sensitivity of malaria RDTs caused by the "prozone" or "Hook" effect has been reported (Gillet, Mori *et al*. 2009, Luchavez, Baker *et al*. 2011). This effect occurs in the presence of samples highly concentrated in the target antigen, which affects the binding capability of the capture antibodies on the RDT. This has

been documented in HRP2-based malaria RDTs previously, and to a lesser extent in pLDH based malaria RDTs (Gillet, Mori *et al*. 2009). It was shown that a prozone-like effect was observed above HRP2 concentrations of 15000 ng/ml (312,000 parasites/ ml) in the laboratory and above 4% (microscopy) in clinical settings (Luchavez, Baker *et al*. 2011). It would be interesting to serially dilute the stored blood samples in our high parasitaemia category (Ct<26) to investigate this effect on these RDTs.

Finally, the methods in section 6.3.2 outline the use of capillary blood where venous blood was not drawn. Although many diagnostic evaluations and clinical trials use these sample types interchangeably, it has been shown that in some settings the use of capillary blood can result in higher diagnostic sensitivity due to sequestration of parasites to these compartments (Mischlinger, Pitzinger *et al*. 2018), whereas in asymptomatic populations there is little difference (Sandeu, Bayibéki *et al*. 2017). Stratifying the data here by blood source would clarify if RDT performance is affected by sample type, this would be influential in developing standard operating procedures for diagnostic screening.

6.5.2. Limitations and future work

The sample size required to power a comparison of the two RDTs was estimated as 580 samples *a priori*. Although we did collect this many samples, verification of the clinical research forms was not complete at the time of DNA extraction, and unmatched/ missing data reduced the total number of samples analysed to 493. However post-hoc analyses

reveal this study is sufficiently powered given the larger than expected differences in the proportions detected.

One limitation of this study is that the same operator carried out both RDTs. Independent uRDT operators were employed for this study, however due to understaffing at the sites operators performed some of the csRDT testing in addition to the uRDTs, which could have introduced some operator bias. The pairing of microscopy data, once available, will help to scrutinise samples that were positive by both RDTs and negative by qPCR. As mentioned previously, additional antigen-based testing (ELISAs) could shed light on prozone effects, antigen persistence and the impact of *pfhrp2* mutations.

It must also be noted that this study occurred over a 9 month period, inclusive of local wet and dry seasons and therefore of different transmissivity periods. Although this will have affected infection density in the study, since the main objective here was to assess the performance of the uRDT in comparison to the csRDT, the inclusion of different transmission seasons better reflects how both RDTs perform overall.

6.5.3. Impact

This study adds to the building evidence guiding implementation of the uRDT. Given the potential severity of MiP, the uRDT's significant improvement in sensitivity could plausibly translate to genuine impact for case management in asymptomatic pregnant women. Although the uRDT specificity was significantly lower than the csRDT, the good safety profile

of ACTs in pregnant women suggests that in asymptomatic infections- it is more important to treat than to risk missing an infection.

For screening strategies as opposed to case management however, more evidence is needed to support implementation. Previously, ISTp strategies have proven inferior to IPTp using current RDTs. (Tagbor, Cairns *et al*. 2015, Awine, Belko *et al*. 2016, Williams, Cairns *et al*. 2016) Evaluation studies (diagnostic accuracy/ cost-effectiveness/ feasibility) of ISTp using more sensitive next-generation RDTs will be needed to support switching from IPTp.

6.6. Conclusions

In this prospective study, the sensitivity of the uRDT was significantly higher than that of the csRDT, in a high transmission setting, amongst pregnant women. However, the overall performance was comparable to that of the csRDT due to the lower specificity of the uRDT. This is the first study to assess the diagnostic accuracy of the uRDT in pregnant women in a high transmission setting.

6.7. Acknowledgements

Special thanks to all the staff at KEMRI Kisumu and the hospitals in Ahero, Rabuor and Akala who facilitated this study, and all the volunteers who participated in the study.

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Chapter 7. Discussion of findings

Contents

7.1. Overarching research challenge

Asymptomatic, low density malaria infections are prevalent across all epidemiological settings, but more so in low to near-elimination settings. Since asymptomatic individuals do not seek treatment, they act as a silent reservoir of infection and transmission. During pregnancy, malaria infection is especially dangerous, and can be low in density due to parasites sequestering to the placenta, making them difficult to detect. These infections must be actively identified to treat individuals and so prevent clinical episodes, as well as prevent continued transmission at a population level. Current diagnostics are central to the case management of malaria, but are inadequately sensitive for low density infections that occur across transmission settings. More sensitive and field-ready diagnostics are urgently required to suit the settings in which malaria is most commonly prevalent.

7.2. Principal findings and impact

This thesis describes the development, validation and evaluation of different diagnostic tests and assesses their suitability for different use-scenarios. It also highlights the various challenges faced when trialling new diagnostics in the field and the complexity of interpreting evidence to guide implementation policy. The research presented here falls broadly into two categories:

1) Development and validation of molecular surveillance tools

2) Assessment of a new commercially available rapid diagnostic test for point of care (POC)

The first includes the development of a multiplex qPCR for the four main species of malaria (Chapter 2), its transfer to the portable rt-PCR MIC™ (Magnetic Induction Cycler) and utilisation of whole-blood directly in the assay (Chapter 3). With further development and characterisation, these additional adaptations (lack of DNA extraction step and performance on a portable platform) could facilitate its use in limited-resource settings.

Alongside this work, a new assay was developed on the handheld rt-PCR Genedrive® platform in collaboration with genedrive plc. Mosquitoes were targeted for this assay due to the simple DNA extraction procedure. The resultant assay accurately detects multiple mutations associated with insecticide resistance simultaneously in the main malarial vector *Anopheles gambiae*. The assay offers a more field-friendly and low-cost alternative to current rt-PCR methods.

Despite simplification, miniaturisation and reduced costs of molecular diagnostics, implementation still requires suitable infrastructure to support their use i.e. skilled personnel, access to instrumentation maintenance, even electricity to charge battery-operated devices.

Thus, RDTs still play a critical role in diagnosing malaria in resource-limited settings. Consequently, the second field of research contributes to the body of evidence around the performance of a commercially available RDT (Alere, uRDT). Already commercialised and meeting the majority of the ASSURED criteria, the uRDT performance was investigated in different transmission settings (Chapters 4-6).

Utilising the 4-plex qPCR (Chapter 2) as a reference test, the diagnostic performance was assessed in a difficult-to-diagnose, yet critical population- pregnant women. For this, the uRDT was tested in 1) a retrospective study in a moderate transmission setting in Indonesia and 2) a prospective trial in a high transmission in Kenya.

The first uRDT study (Chapter 3) utilised stored samples collected in a Stop Malaria in Pregnancy (STOP-MiP) trial in Timika, Indonesia. The sensitivity (20%) and specificity (90%) of the uRDT was non-significantly different to those of the CareStart RDTs (csRDT; 22% sensitivity, 95% specificity). These findings do not support implementation of the uRDT over current RDTs in these settings for use in pregnant women.

The low sensitivity of both RDTs instigated examination of the *pfhrp2* and *pfhrp3* gene structure in these samples (Chapter 4), since mutations have been shown to affect HRP2-RDT performance. Although inconclusive, there was some evidence of double deletions of *pfhrp2* and *pfhrp3* in this cohort. Further work to strengthen this evidence would confirm- for the first time- that these deletions are present in South East Asia *and* that they impact RDT outcomes.

In the following prospective uRDT trial in Kenya, the uRDT was significantly more sensitive (79 %) than the csRDT (70 %), although less specific (90 % uRDT; 93% csRDT). Combining these findings showed the two tests performed similarly overall. However, considering the detrimental outcomes associated with MiP together with the good safety profile of ACT treatment during pregnancy, the potential clinical impact (Ferrante di Ruffano, Hyde *et al*. 2012) may warrant use of the uRDT over current RDTs for screening

pregnant women.

The work here is especially valuable given its focus on pregnant women- an often excluded or under-represented demographic- who are vulnerable to additional detrimental outcomes associated with malaria. This research builds critical evidence required to guide policy decisions around the implementation of this new test, and ultimately strategies for the prevention of malaria during pregnancy.

7.3. Context within existing literature, limitations, and future work

7.3.1. Molecular assays and portable platforms (Ch2-3)

Molecular assays for the detection of multiple species of malaria have been published previously (reviewed most recently by Tedla *et al.*(Tedla 2019)). However, few qPCR assays have been adapted for use in resource limited settings, given the requirements of complex and costly instrumentation, and a DNA extraction step (de Monbrison, Angei *et al*. 2003, Rougemont, Van Saanen *et al*. 2004, Shokoples, Ndao *et al*. 2009, Oddoux, Debourgogne *et al*. 2011, Kamau, Alemayehu *et al*. 2013, Imwong, Hanchana *et al*. 2014). Melt curve analysis (MCA) is well suited for these settings, offering a cheap alternative to qPCR, requiring only one set of primers and an intercalating dye, albeit still requiring DNA extraction.

We consequently explored Chua *et al*'s (2015) (Chua, Lim *et al*. 2015) 5-plex MCA in Chapter 2. Encouragingly we observed a similar limit of detection for *P. falciparum*, however we did frequently observe amplification of *P. falciparum* peaks in our non-template controls

and non-*falciparum* samples. Despite using new stocks, intensive decontamination and even performing the assay in an additional non-malaria laboratory, this could not be remedied and so qPCR methods were explored.

Joste *et al.* (2018) have since developed an MCA assay to distinguish between *P. ovale wallikeri* and *P. ovale curtisi* (Joste, Kamaliddin *et al*. 2018), which has proven useful in the monitoring of treatment efficacy (Kamaliddin, Joste *et al*. 2019). More recently, Murillo *et al.* (2019) developed an MCA assay targeting the 18S rRNA gene (only for *P. falciparum*, *P. vivax* and *P. malariae*) for use in humans and mosquitoes (Murillo, Muskus *et al*. 2019). These tests could offer more robust MCA methods for malaria detection.

The 4-plex qPCR reassuringly achieved similar LODs to the publications in which its probes and primers were first developed, although its agreement with LAMP reference (Chapter 2) was poor. Validation of this qPCR highlighted common challenges in evaluating new diagnostics. First, potential *cross-contamination* issues during DNA extraction were outlined, emphasising the need to minimise sample handling prior to NAAT, preferably in a direct-blood format. Second, the necessity to use a second reference test (nPCR), to confirm discordance between the 4plex and the LAMP reference test, underlines how important choosing the right reference test is. Many studies utilise RDTs and microscopy as gold standards for evaluating new malaria diagnostics, despite their poor sensitivity. Where sensitivity/specificity of the index test may be superior to the reference test, it is useful to

include a comparator for relative performance (e.g. Chapter 5) and/or refer to a composite molecular test (e.g. Chapter 3).

As mentioned previously, it would be useful to fully characterise the 4-plex assay and further validate its performance using 1) lyophilised reagents, 2) whole-blood directly and 3) a portable qPCR platform. Our results show that the MIC™is one possible platform that would suit a use-scenario with high-throughput requirements (48 samples/ run) e.g. in surveillance or for research purposes. Potentially, the unique Genedrive® cartridge shape could also support the transfer of the 4-plex qPCR to this platform, which would be better suited for rapid (< 1 hr) POC diagnosis (1-3 samples/ run) e.g. at antenatal visits (Desai, Hill *et al*.) or for reactive case detection in the community (Hsiang, Ntshalintshali *et al*. 2019).

The additional Genedrive® assay developed here is a proof of principle that a new assay for use in low-resource settings could be developed. To date, it is the only assay that includes a single primer-probe set to distinguish between wild type and 2 SNPs associated with insecticide resistance in mosquitoes. Given its excellent accuracy, the assay developed here could easily be expanded to include 1) other insects that have similar mechanisms of insecticide resistance e.g. sand flies or triatomine bugs, or 2) a wider repertoire of resistance genes. Currently the major limitation of the assay is the low-throughput format of the Genedrive® given its intended use as POC. The pooling studies should be expanded to further assess the feasibility of pooling higher numbers of mosquitoes for cost- and labour-effective screening mosquito populations. In the future, research into the potential market

size, needs and wants for such products would be useful to guide commercialisation and inform target product profiles (TPPs).

7.3.2. A new RDT (Ch4-6)

Previous reports of the uRDT performance have been conflicting (Table 6.1, page 225). Across low, moderate and high transmission settings, in most studies the uRDT demonstrated superior sensitivity in comparison to current RDTs across a range of demographics. On the other hand, as we observed in our study in Indonesia, the only other study carried out in pregnant women also found no significant difference in performance of the uRDT and current RDTs. These findings are supported by the most recent uRDT study, in a cross-sectional screening in low transmission settings in The Gambia (Mwesigwa, Slater *et al*. 2019). Here, the prospective uRDT study in a higher transmission setting also supports this- with regards to overall uRDT performance- the uRDT was demonstrably more sensitive than the current RDT. It must be noted that the uRDT only detects *P. falciparum* malaria, and therefore an alternative test may be needed alongside the uRDT where other species are co-endemic.

It would be useful to further investigate the uRDT "false-positives" in Kenya to determine if they are indeed indicative of recent infection and therefore truly RDT positive, or if cross-contamination, cross-reaction with other blood borne pathogens or mis-interpretation of the RDT by the operator had led to uRDT false positives. The former would mean specificity of the uRDT is actually higher than we report here and therefore would

bolster evidence for implementation of the uRDT. The effects of the latter would call for more cautious interpretation of uRDT positives.

Since *pfhrp2* and *pfhrp3* deletions have previously been reported in this area of Kenya (Beshir, Sepulveda *et al*. 2017), it would also be useful to investigate the RDT false negatives. After this study, a new qPCR was developed by Schindler *et al.*(2019) (Schindler, Deal *et al*. 2019) for the detection of *pfhrp2* and *pfhrp3*. This assay could overcome the insensitivity issues encountered in Chapter 4.

The inverse relationship between RDT positivity and Ct value (a proxy of parasitaemia) in Chapter 5 also needs to be investigated, with particular focus on the Prozone effect. Jang *et al.* have since developed a new immunoassay specifically to aid the evaluation of new malaria RDTs (Jang, Tyler *et al*. 2019). Their assay quantifies *pfhrp2* and pLDH, the most commonly RDT- targeted antigens (Jang, Tyler *et al*. 2019). Quantification of these antigens would help to shed light on the phenomenon of unexpected RDT false negatives in the presence of highly parasitaemic infections (Gillet, Mori *et al*. 2009, Gillet, Scheirlinck *et al*. 2011, Luchavez, Baker *et al*. 2011, Lurdes, Nuno Rocha *et al*. 2015).

7.4. Unanswered questions and future work

The aims in Chapter 1 (page 63) have been met as described below in the thesis summary, however the work also highlights unanswered questions and gaps that still need addressing. Table 1.2 in Chapter 1 briefly summarises key characteristics of a molecular diagnostic for

malaria surveillance; the following section discusses if these have been addressed by the work in this thesis and what further work is needed.

The first set of characteristics define the use scenario of the proposed diagnostic. Although it is widely accepted that molecular surveillance requires trained personnel, the accessibility of the Genedrive® (explored in Ch3) challenges this dogma. Simplified technologies such as LAMP, NALFIA and closed cartridge systems also work towards this accessibility. Increasing accessibility will better support testing at lower level healthcare settings and will enable MTAT/FTAT screening nearer the patient.

The second category describes the optimal design of the assay. Due to the high sensitivity of NAATs the target analyte is suggested to be nucleic acid. In this thesis diagnostics were developed for the detection of DNA rather than RNA. However, it is well established that RNA targets provide far more sensitive detection of malaria, with the bonus of identifying the stage of parasite and even the sex of parasite gametocytes. Historically, DNA analysis has been favoured over RNA due to the added complexity of storing and preserving fragile RNA strands. Advances in chemistry have resulted in RNA preservatives such as Zymo (ZymoResearch 2020) and PrimeStore (Longhorn 2020), which could help mitigate these barriers, improving the accessibility of RNA targeting assays.
The other assay characteristics such as limit of detection and diagnostic performance are discussed extensively in each chapter. However, test result delivery and optimal time to

result (as outlined in Table 1.2) is not really addressed. The 4-plex qPCR developed in chapter 2 has an assay run time of nearly 2 hours, whereas the Genedrive® assay ran in under 1 hr. The optimal time outlined in Table 1.2 assumes a high throughput use-scenario for surveillance. Since the Genedrive® only runs 3 samples, the time per sample is ~ 20 mins, whereas on the RotorGeneQ (72 samples/ run) this equates to less than 2 mins per sample. The usefulness of these measures depends on the use scenario, but it is likely that for surveillance the high throughput format will be more relevant.

Finally, the most influential factor regarding implementation of new tests in limited-resource settings needs to be assessed- the cost. For nucleic acid testing, considerable expense is associated with the isolation and purification steps.

Due to time constraints- and with the goal of global harmonisation between global uRDT evaluation studies- in this thesis we used the most widely used nucleic extraction method (e.g. Qiagen kits) for reference standard testing. Cheaper, simpler and faster alternatives do exist e.g MagMax™ (ThermoFisher Scientific, UK) and Genesig™ Easy extraction (PrimerDesign, UK), however thorough validation studies would be necessary prior to replacement of the standard spin column methods. In chapter 3, we explored the potential of magnetic bead extraction and concluded that using diluted blood directly in the qPCR was as effective as the extraction process. Circumvention of nucleic acid isolation costs ($3-10/ sample) will significantly reduce the cost of molecular screening, again improving accessibility. Aside from the initial cost of instrumentation ($5k+), other expenses associated

with molecular screening relate to the use of plastics (filtered pipette tips), tubes and the associated waste disposal routes.

Sample type also influences the cost and ease of use of the test e.g if additional reagents are needed to stabilise nucleic acids/ degrade nucleases in the sample, or if the sample requires a cold chain or specialised personnel. For both molecular surveillance and RDTs, whole blood is the gold standard for diagnosis of malaria. The use of non-invasive samples (outlined in 1.4.6) could be more cost-effective (by circumventing specialised staff for sample collection) and a more patient-friendly method of diagnosis. It would be useful to validate the use of urine, saliva or faeces in the 4-plex qPCR developed here, as outlined in the TPP described in 1.2.2 (Table 1.2).

The simple RDT format and requirement for only a finger-prick of blood means that there are very minimal associated costs for these tests. Additional costs may instead be incurred when switching RDTs, potentially necessitating staff re-training and changes in procurement logistics. Thus, thorough cost analyses are needed to determine the level of improvement in sensitivity that would warrant switching to a new RDT in different settings with different transmission reduction goals. More insight into the cost-benefits of diagnostics in various use-scenarios will better inform control and elimination strategies, as well as guide TPPs for new diagnostics.

Excitingly, the findings in this thesis also highlight several additional research themes that could be explored/ expanded, contributing to essential translational malaria research.

First, for molecular surveillance it would be useful to genotype locally circulating parasites in addition to monitoring "diagnostic resistance" genes such as *pfhrp2*/3, with particular reference to asymptomatic infection. We need to better understand how low-level transmission is maintained, whether through re-infection of the same strain, recrudescence of dormant parasites or new strains. Some studies have shown that in areas of low-transmission, clinical malaria most often arises from newly imported parasite strains (Kun, Missinou *et al*. 2002, Hofmann, Karl *et al*. 2017, Searle, Katowa *et al*. 2017). Expanding this using more sensitive molecular diagnostics for asymptomatic malaria would be particularly useful for understanding the dynamics of infection, the pathogenesis and general malaria transmission in near-elimination settings. Subsequently, whole genome sequencing may be more cost-effective than detecting multiple genes individually. Promising technological advances in sequencing, mainly the portable Nanopore MinION™ Sequencer (Imai, Tarumoto *et al*. 2017, Imai, Tarumoto *et al*. 2018) may allow this in the near future.

Addressing a major limitation of this thesis- the lack of absolute quantification using synthetic DNA- it would be useful to use standard curves for each species to obtain the limit of detection and identify parasitaemia precisely. Following this, further stratification of parasite load could enable comparison with other studies, helping to further characterise and compare the performance of the uRDT in different settings.

In addition to quantification, as mention in chapter 5 it would be useful to include genotyping of circulating strains in order to gain further understanding of infection dynamics, particularly with regard to emerging mutations that may affect diagnosis or treatment.

It would also be useful to extend the analyses of the uRDT studies to include stratification of parasite density by gravidity or other clinical variables of the participants. Although it is known that immunity to malaria increases with gravidity, there is little work investigating sub-microscopic parasite densities and gravidity (Bouyou-Akotet, Ionete-Collard *et al*. 2003, Ouédraogo, Tiono *et al*. 2012, Haggaz, Elbashir *et al*. 2014, Mayor, Bardají *et al*. 2015). The overall dynamics of low density infection have only recently been investigated (Slater, Ross *et al*. 2019, Whittaker, Slater *et al*. 2019), and not with special consideration for pregnant women.

Okell *et al.*(2012) (Okell, Bousema *et al*. 2012) and Slater *et al.* (2015) (Slater, Ross *et al*. 2015) modelled the effects of using next generation RDTs with a ten-fold lower limit of detection in malaria control programs. Broadly, they estimate that an RDT with an LOD of 10 parasites/ul would detect 80% of the overall infectious reservoir. Given the availability of new data from the uRDT and recent meta-analyses of the dynamics of malaria in low transmission settings, it would be useful to validate and further refine these models- particularly in pregnant women- to help guide the development and implementation of new diagnostics.

7.5. Thesis Summary

This thesis demonstrates:

1) The development of a new molecular test for 4 species of malaria, in which whole blood can be used directly and which has demonstrated transferability to a portable qPCR platform.
2) In a well-designed prospective trial, that the new uRDT is more sensitive than existing RDTs when used in a high transmission setting for screening pregnant women, whereas as in a low transmission it setting it offers no additional sensitivity.
3) The Genedrive® offers a simple, portable platform for molecular detection of insecticide resistance, suitable for use in malaria control settings.

In addition to these, several important considerations for diagnostic development, validation and evaluation are highlighted. The research presented here is useful for product developers, researchers and health policy advisors; it encompasses nuances of designing, validating and evaluating malaria diagnostics, contributing to critical evidence needed to guide malaria control strategies.

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