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MOLECULAR METHODS IN MALARIA CONTROL IN THE ERA OF PRE-ELIMINATION

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COVER PHOTO
The cover photo was taken by Elin Edlund during a LAMP pilot in Zanzibar.

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Molecular methods in malaria control in the era of pre-elimination

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Malaria är en av vår tids viktigaste sjukdomar ur ett globalt perspektiv. Årligen dör över en halv miljon människor i malaria, framförallt drabbas gravida kvinnor och barn under fem år. Över 90 % av dödsfallen sker i Afrika söder om Sahara. Ökad motståndskraft hos malariaparasiten mot nya artemisinin-baserade kombinationsterapier (ACT) hotar de globala framgångar som nyligen skett i kampen mot malaria.

Ett omfattande malariakontrollprogram introducerades på Zanzibar 2003, vilket är ett pionjärprojekt i Afrika avseende hur effektivt enkla kontrollmetoder kan reducera parasitförekomsten i ett geografiskt avgränsat område; så som massdistribution av impregnerade myggnät, gratis tillgång till effektiva ACT läkemedel, införandet av snabbtester för förbättrad diagnostik (Rapid Diagnostic Tests, RDT) och sprejning inomhus med insekticider. Zanzibar har genom sitt effektiva kontrollprogram uppnått ett stadium av pre-elimination och står nu inför utmaningen att utrota malaria. För att uppnå detta mål krävs nya verktyg och strategier för förbättrad identifiering av malariafall och noggrann övervakning av läkemedelsresistens.

I denna avhandling studerades tillämpningen av mycket känsliga molekylära metoder för att säkerställa optimal övervakning och potentiell begränsning av malariatransmissionen, och hur dessa verktyg kan bidra till att uppnå målsättningen att utrota malaria på Zanzibar.

I **Studie I** studerades selektionen av resistensmarkörer hos feberpatienter efter introduktionen av ACT i form av artesunat-amodiakin (ASAQ) på Zanzibar år 2003. Fyra väletablerade mutationer i två gener associerade med läkemedelsresistens analyserades: "*Plasmodium falciparum* chloroquine resistance transporter" (*pfcrt*) position K76T samt "*P. falciparum* multidrug resistance 1" (*pfmdr1*) positioner N86Y, Y184F och D1246Y. Efter sju års användning av ASAQ sågs ingen selektion av mutationer associerade med resistens mot amodiakin.

I S**tudie II** utvärderades tre extraktionsmetoder för att utvinna DNA från använda snabbtester. Möjligheten att utvinna parasit-DNA från snabbtester utgör en intressant möjlighet till förbättrad molekylärepidemiologisk övervakning. DNA-extraktion från prover insamlade i Zanzibar visade att parasit-DNA kan bevaras på snabbtester under fältmässiga förhållanden i Afrika.

I **Studie III** utvecklades och utvärderades en ny mycket känslig metod (cytb-qPCR) för detektion av lågdensitets parasitemier i små mängder blod som har bevarats på filter paper. Denna metod användes i **Studie IV** för påvisande av malariainfektion hos individer som deltagit i tvärsnittsstudier som utfördes år 2005-2013 på Zanzibar. Lågdensitets parasitemier i lågtransmissionsmiljöer utgör en viktig reservoar för fortsatt malaria transmission. Dessa infektioner undgår upptäckt då de ligger under detektionsnivåerna för både RDT och mikroskopi (ca. 100 parasiter/µl blod) vilket, i tillägg till att de ofta är asymptomatiska (dvs ej ger upphov till feber), gör att de undgår läkemedelsbehandling. Vi påvisade en kvarvarade parasitreservoar som bestod av *P. falciparum* och *P. malaria* och som fanns främst hos individer i 5-25 års ålder. Parasitemierna var låga, men det fanns en hög diversitet i parasitpopulationen. Vi såg ingen selektion av mutationer associerade med resistens mot amodiakin, men prevalensen av vissa mutationer var betydligt högre bland asymptomatiska än symptomatiska infektioner.

I **Studie V** rapporterades resultat från den hittills största fältstudien där loop-mediated isothermal amplification (LAMP), en högkänslig, fältanpassad molekylär diagnostisk metod använts för att påvisa förekomst av malariainfektion. Totalt analyserades prover från 3983 asymtomatiska

individer med LAMP vid två laboratorier på Zanzibar. LAMP detekterade 3.4 gånger fler malariainfekterade individer än RDT. Under studiens gång uppstod DNA-kontaminering av proverna som krävde upprepad rengöring av all LAMP utrustning och reagenser. Studien visar att LAMP är ett enkelt och känsligt molekylärt verktyg för användning i fält, men att ett slutet hög-kapacitets system skulle vara optimalt för att reducera risken för kontaminering.

Sammanfattningsvis visar avhandlingen att konventionella metoders möjlighet att identifiera individer med malariainfektion inte är tillräckligt hög för att utrota malaria på Zanzibar. För att nå detta mål krävs nya molekylära metoder för förbättrad identifiering och bekämpning av den kvarvarande parasitpopulationen. Detta är extra betydelsefullt i en tid då både läkemedelsresistens hos malariaparasiten och resistens mot bekämpningsmedel hos myggorna hotar de globala framstegen inom malariakontroll. Den snabba övergången från hög till låg malariatransmission på Zanzibar under de senaste åtta åren utgör en unik forskningsmöjlighet för att studera hur molekylära metoder kan bidra för att uppnå malariaeliminering i en pre-eliminations miljö. I denna avhandling har tillämpningen av molekylära metoder för förbättrad malariaövervakning och kontroll på Zanzibar studerats, och resultaten bidrar därmed till ökad förståelse för användbarheten av dessa verktyg i Afrika söder om Sahara.

ABSTRACT

Increased funding combined with effective malaria control methods for prevention, diagnosis, and treatment, has resulted in a 30% reduction of the global malaria burden over the last decade. As malaria prevalence declines in areas of successful malaria control, the proportion of subpatent infections that fall below the detection level of malaria rapid diagnostic tests (RDTs) and microscopy increases. In these areas new tools and strategies are required for detecting and targeting residual parasite populations. Furthermore, there is an emerging resistance to the artemisinin-based combination therapies (ACTs), which is the recommended first-line treatment for uncomplicated *Plasmodium falciparum* malaria. This resistance is a serious threat to the recent achievements in the reduction of the global malaria burden.

The aim of this thesis was to gain insight into the application of modern molecular methods for enhanced malaria infection detection and surveillance of antimalarial drug resistance in a pre-elimination setting such as Zanzibar.

Study I assessed whether seven years of wide scale use of artesunate-amodiaquine (ASAQ) as first-line treatment selected for *P. falciparum* single nucleotide polymorphisms (SNPs) associated with resistance to the ACT partner drug amodiaquine. No selection of SNPs associated with amodiaquine resistance was observed, indicating sustained efficacy of ASAQ as first-line treatment in Zanzibar.

In **Study II** different methods of DNA extraction from used RDTs were evaluated and it was assessed whether RDTs could preserve *Plasmodium* DNA for the purpose of molecular epidemiological investigations. The Chelex-100 method proved the most sensitive method of DNA extraction in both RDT and filter paper samples. RDTs collected in Zanzibar provided parasite DNA of equal quality as filter papers, suggesting that RDTs are a valuable alternative for DNA storage under field conditions.

In **Study III** a highly sensitive SYBR Green qPCR-RFLP assay was developed for *Plasmodium* detection and species determination in samples collected on filter paper. This method was applied in **Study IV** for characterising asymptomatic *Plasmodium* infections. A declining, albeit persistent, reservoir of parasites present at low-densities was found in asymptomatic individuals, highlighting the need for sensitive molecular methods in malaria pre-elimination settings. The study revealed important characteristics of the remaining parasite populations, including intriguing trends in SNPs associated with antimalarial drug resistance that require further investigation in order to be fully understood.

Study V reports the hitherto largest implementation of a new molecular diagnostic tool based on loop-mediated isothermal amplification (LAMP), for scaled up, centralised mass-screening of asymptomatic malaria in Zanzibar. LAMP detected 3.4 times more *Plasmodium* positive samples than RDT, and was found to be a simple and sensitive molecular tool with potential for use in active malaria surveillance. Contamination is, however, a concern. A higher throughput, affordable closed system would be ideal to avoid DNA contamination when processing larger numbers of samples.

In summary, molecular methods are required for enhanced malaria infection detection and surveillance of antimalarial drug resistance in malaria pre-elimination settings such as Zanzibar. The application of molecular methods may be of particular interest for malaria control/elimination programs, for monitoring progress towards malaria elimination and for optimal orientation of program activities.

LIST OF SCIENTIFIC PAPERS

I. Fröberg G, Jörnhagen L, **Morris U**, Shakely D, Msellem MI, Gil JP, Björkman A and Mårtensson A. Decreased prevalence of *Plasmodium falciparum* resistance markers to amodiaquine despite its wide scale use as ACT partner drug in Zanzibar. Malaria Journal 2012, 11:321.

II. **Morris U**, Aydin-Schmidt B, Shakely D, Mårtensson A, Jörnhagen L, Ali AS, Msellem MI, Petzold M, Gil JP, Ferreira PE, Björkman A. Rapid diagnostic tests for molecular surveillance of *Plasmodium falciparum* malaria -assessment of DNA extraction methods and field applicability. Malaria Journal 2013, 12:106.

III. Xu W, **Morris U**, Aydin-Schmidt B, Msellem MI, Shakely D, Petzold M, Björkman A, Mårtensson A. SYBR Green real-time PCR-RFLP assay targeting the *Plasmodium* cytochrome b gene – a highly sensitive molecular tool for malaria parasite detection and species determination. (*Accepted* for publication in PLoS One).

IV. **Morris U**, Xu W, Msellem MI, Schwartz A, Abass A, Shakely D, Cook J, Bhattarai A, Petzold M, Greenhouse B, Ali AS, Björkman A, Fröberg G, Mårtensson A. Characterising temporal trends in asymptomatic *Plasmodium* infections and transporter polymorphisms during transition from high to low transmission in Zanzibar (*Submitted*).

V. **Morris U**, Khamis M, Aydin-Schmidt B, Abass A, Msellem MI, Nassor MH, González IJ, Mårtensson A, Ali AS, Björkman A, Cook J. Field deployment of loop-mediated isothermal amplification for centralised mass-screening of asymptomatic malaria in Zanzibar, a pre-elimination setting (*Submitted*).

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Björkman A*, Shakely D*, Ali AS, **Morris U**, Bhattarai A, Msellem MI, Abbas AK, Xu W, Cook J, Al-Mafazy A-W, Omar R, Mcha J, Rand A, Elfving K, Bennett A, Petzold M, McElroy P, Drakeley C, Mårtensson A. Pre-elimination achieved but residual malaria transmission calls for new malaria control strategies in Zanzibar (*Submitted*).

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LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapy
AL	Arthemeter-lumefantrine
AMA	Apical membrane antigen
API	Annual parasite incidence
ASAQ	Artesunate-amodiaquine
CI95%	95% confidence intervals
Cq	Quantification cycle
Cytb	Cytochrome b
DEAQ	Desethylamodiaquine
Dhfr	Dihydrofolate reductase
Dhps	Dihydropteroate synthetase
DNA	Deoxyribonucleic acid
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
FIND	Foundation for Innovative New Diagnostics
G6PD	Glucose-6-phospate dehydrogenase
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITN	Insecticide treated net
KAPB	Knowledge, attitude, practice and behaviour towards malaria
LAMP	Loop-mediated isothermal amplification
LDH	Lactate dehydrogenase
LLIN	Long-lasting insecticidal net
MEEDS	Malaria Early Epidemic Detection System
MOI	Multiplicity of infection
MSP	Merozoite surface protein
nPCR	Nested PCR
PCR	Polymerase chain reaction
PDNA	*Plasmodium* Diversity Network Africa
Pfcrt	*P. falciparum* chloroquine resistance transporter
*Pf*EMP1	*P. falciparum* Erythrocyte membrane protein 1
*Pf*HRP2	*P. falciparum* histidine-rich protein 2
*Pf*LDH	*P. falciparum* specific lactate dehydrogenase
Pfmdr1	*P. falciparum* multidrug resistance 1
Pfnhe1	Na+/H+ exchanger 1
PHCC	Primary health care centre
PHCU	Primary health care unit
pLDH	Pan-*Plasmodium* lactate dehydrogenase
PR	Parasite rate
*Pv*LDH	*P. vivax* specific lactate dehydrogenase

qPCR	Quantitative PCR
RBC	Red blood cell
RDT	Rapid diagnostic test
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SE	Southeast
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
SR	Spleen rate
WBC	White blood cell
WHO	World Health Organisation
WWARN	Worldwide Antimalarial Resistance Network
ZAMEP	Zanzibar Malaria Elimination Programme
ZMCP	Zanzibar Malaria Control Programme

1 INTRODUCTION

1.1 THE GLOBAL MALARIA BURDEN

Approximately 3.2 billion people worldwide are at risk of being infected with malaria and developing disease. The World Health Organization (WHO) estimated 198 million cases of malaria and 584 000 deaths globally in 2013 [1], although some suggest that malaria mortality is underestimated [2]. The malaria burden is heaviest in sub-Saharan Africa where 90% of the malaria deaths occur. Pregnant women and children under the age of five, who account for 78% of all deaths, are most susceptible. In 2013, an estimated 437 000 African children died before their fifth birthday due to malaria [1].

Increased funding combined with effective malaria control methods for prevention, diagnosis and treatment has resulted in a 30% reduction in the global malaria incidence and a 47% decrease in the global malaria mortality between 2000 and 2013 [1]. Out of the 97 countries with ongoing malaria transmission, 64 countries have reversed the incidence of malaria and 55 are on track to meet the World Health Assembly and Roll Back Malaria Partnership target of reducing incidence by 75% by 2015 [1]; the long term goal being global malaria eradication.

Despite these tremendous achievements many malaria endemic countries are still far from reaching universal coverage with life-saving malaria interventions [1]. Insecticide resistance in malaria vectors has been observed in 49 of 63 reporting countries. Resistance to artemisinin-based combination therapies (ACTs), the recommended first-line treatment for uncomplicated malaria, has been detected in five countries in Southeast (SE) Asia. Furthermore, the funding provided for malaria control in 2013 (US$ 2.7 billion) reached only half of the estimated costs (US$ 5.1 billion) required to achieve global targets for malaria control and elimination [1, 3]. The Global Malaria Eradication Programme launched in 1955 was first to attempt malaria eradication, but funding collapsed in 1969 resulting in devastating resurgence of malaria in many countries [4]. This reminds us that sustained and sufficient financing is critical for furthering goals of global malaria eradication [5].

1.2 THE *PLASMODIUM* PARASITE

The etiological agents of malaria are single celled apicomplexan parasites of the genus *Plasmodium*. There are over 250 species of *Plasmodium*, of which five infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* [6]. *P. falciparum* is responsible for the majority of deaths due to malaria. It is the predominant species in sub-Saharan Africa and also occurs in SE Asia. *P. vivax* is predominant in South America and also occurs in SE Asia. *P. malaria* may occur in all malarious areas but at a low prevalence [7]. *P. ovale* is principally widespread in tropical Africa; two distinct sub species of *P. ovale* have recently

been described, *P. ovale curtisi* and *P. ovale wallikeri* [8]. *P. knowlesi* causes malaria in long-tailed macaques in parts of SE Asia but has also been shown to infect humans [6]. Much of this thesis focuses on *P. falciparum* malaria due to its severity and predominance in sub-Saharan Africa.

The *Plasmodium* genome encodes approximately 5,300 genes carried on 14 chromosomes. The genome is highly (A + T)-rich, the overall (A + T) composition is 80.6% and rises to ~90% in introns and intergenic regions [9]. Around 60 genes involved in antigenic variation (*var* genes) are located in the subtelomeric regions of the chromosomes [10].

1.2.1 The *Plasmodium* life cycle

The lifecycle of *Plasmodium* species that infect humans is complex and involves a human host and mosquito vector [6]. Female anopheline mosquitoes transmit malaria to humans when taking a blood meal (Figure 1A). Motile sporozoites, which reside in the mosquito salivary glands, are injected into the skin of the host together with the mosquito saliva. The average inoculum measured under laboratory conditions contained 125 sporozoites (range 0-1300), although inoculum in the field are likely to contain less than 100 sporozoites per bite [11].

After deposition in the skin (usually in the dermis) the motile sporozoites must locate and penetrate blood vessels in order to reach the second destination, the liver, where the sporozoites invade hepatocytes (Figure 1B). The process from deposition to hepatocyte invasion is thought to take 2-3 hours. Inside the hepatocytes each sporozoite multiplies producing 10 000-30 000 daughter merozoites during 5.5-8 days [6]. Finally the hepatocyte schizonts burst, liberating merozoites that then invade erythrocytes (red blood cells [RBCs]) (Figure 1C).

Asexual replication occurs in the erythrocytes. The parasites go through several stages, starting as early (ring stage) trophozoites, developing into mature trophozoites, and finally becoming schizonts which rupture the RBC releasing daughter merozoites. Each burst RBC releases between 6 and 30 merozoites, which then infect new RBCs within 30-90 seconds [12]. Symptoms of malaria are associated with this stage of the life cycle, usually referred to as the asexual or blood-stage cycle. Symptoms usually start approximately 6-8 days after emerging from the liver [6].

Some blood-stage parasites develop into longer lived sexual forms known as gametocytes. The sexual forms are taken up by a feeding anopheline mosquito (Figure 1D), where sexual reproduction takes place. A male and female gametocyte forms an ookinete, which develops into an oocyst in the mosquito mid-gut. The oocyst bursts liberating sporozoites that migrate to the salivary glands of the mosquito [6]. It takes 10-21 days development in the mosquito before sporozoites can be injected into a new host during a blood meal.

The *Plasmodium* life cycle is similar between species, with some distinguishing differences. For example, some *P. vivax* and *P. ovale* parasites remain dormant in the liver as hypnozoites, causing relapse of malaria between two weeks to more than one year after the initial infection. The cycle of asexual replication in the RBCs takes roughly 48 hours for *P. falciparum*, *P. vivax* and *P. ovale*, 72 hours for *P. malariae* and only 24 hours for *P. knowlesi* [6]. And finally in *P. falciparum* the gametocytemia is delayed, peaking 7-10 days after the initial peak in asexual stage parasite densities [13].

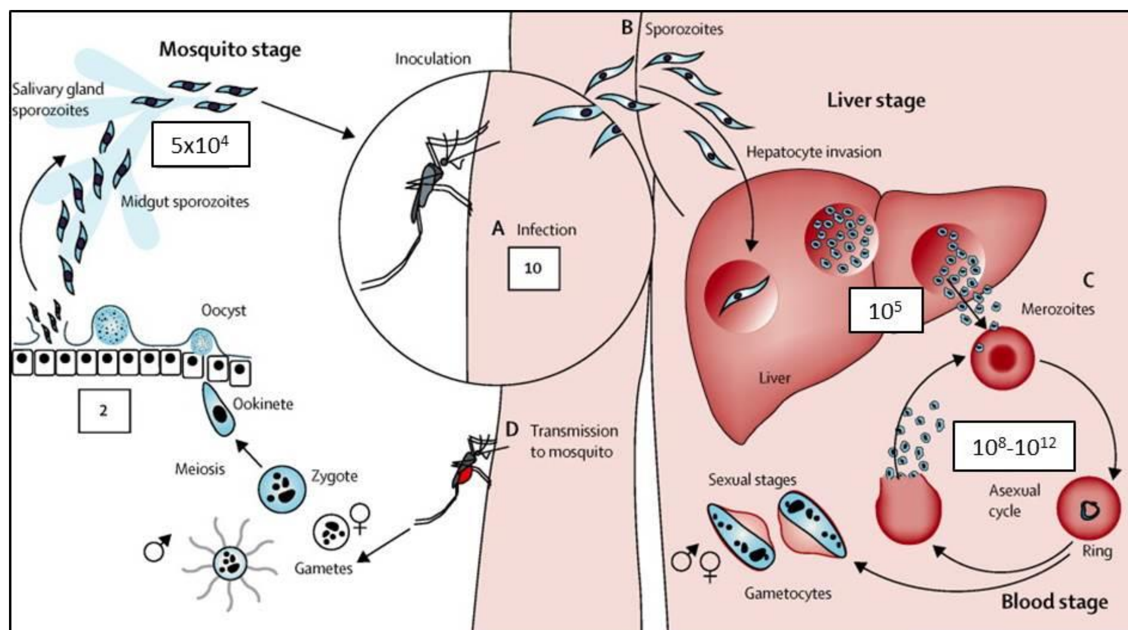

Figure 1: The lifecycle of *Plasmodium* in the human body and the anopheline mosquito. The estimated numbers of parasites for each life cycle stage are shown in boxes. Reprinted from The Lancet, 2013, White NJ *et al*., Malaria, with permission from Elsevier.

1.3 THE HUMAN HOST

1.3.1 Immunity

Individuals residing in malaria endemic regions eventually develop resistance to malaria through repeated exposure. Children born in malaria endemic areas, with moderate transmission intensity, acquire protection against severe malaria by the age of five [6]. Older children and young adults develop complete protection against illness with malaria and are eventually considered partially immune. This immunity protects the host against illness with malaria but does not eliminate the infection. These individuals harbour asymptomatic malaria infections without any signs of disease (Figure 2).

Immunity to malaria is considered to be non-sterile, complex, and not well understood. Immunity develops relatively slowly and is said to wane quickly when adults leave malaria

endemic regions, suggesting that frequent exposure to malaria is needed to maintain immunity [14]. Studies have shown that protective immunity against sporozoite-induced infection requires antigen-specific $CD8^{+}$ T cells, which inhibit development of liver-stage parasites. T-cells are primed early in the lymph nodes draining the skin where some sporozoites are deposited. Dendritic cells in the skin or in lymph nodes are important in the priming of the *Plasmodium* specific $CD8^{+}$ T cells [11]. It is usually assumed that humoral responses are key in blood-stage immunity, where B-cells produce antibodies that block the merozoite invasion of erythrocytes [14]. It is thought that pro-inflammatory cytokines such as interferon-γ and tumor necrosis factor play essential roles in protective immunity against blood-stage *Plasmodium* infections, but are also involved in immunopathology of severe malaria [14, 15]. Antigenic variation in the parasites and short lived nature of malaria immunity in the human host has hampered the development of a malaria vaccine.

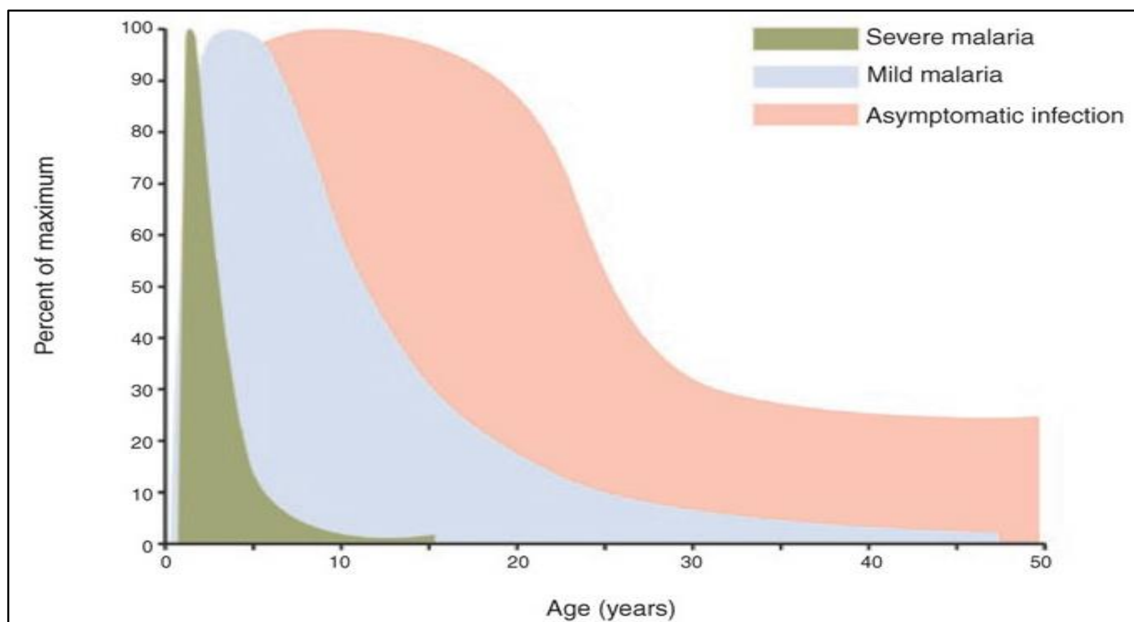

Figure 2: Relation between age and malaria severity in an area of endemic transmission. Reprinted by permission from Macmillan Publishers Ltd: Nature Immunology, 9: 725-732, © 2008.

1.3.2 Human genetics

The genus *Plasmodium* is estimated to have evolved ~150 million years ago, long before the existence of *Homo sapiens* [16]. Humans have evolved in the presence of malaria, and the coevolution has helped shape the human genome [17]. There are a number of polymorphisms that are thought to be protective against severe forms of malaria. The global distributions of sickle cell disease, thalassemias, Glucose-6-phosphate dehydrogenase (G6PD) deficiency and blood group polymorphisms mirrors that of malaria, suggesting that malaria has been a

selective force for these mutations [12, 16]. Additionally, genetic variations in human P450 genes (e.g. *CYP2C8*, *CYP3A4* and *CYP2A6*) result in differential metabolism of antimalarial drugs in humans (i.e. pharmacogenetics) and have important implications in both antimalarial drug effectiveness and tolerability [18, 19].

1.3.2.1 G6PD deficiency

G6PD is a key enzyme in the pentose phosphate pathway and is important in RBCs for protecting against oxidative stress [20]. G6PD deficiency, an X-linked recessive hereditary disease characterised by abnormally low levels of G6PD, is common in many malaria endemic regions [13]. Over 140 mutations lead to different enzyme activity levels predisposing to haemolysis in response to certain triggers such as food, illness or medication. G6PD deficiency has been proposed to modulate disease severity or to be protective against malaria [21]. G6PD deficiency is also important in the choice of antimalarial treatment. Primaquine is used to treat *P. vivax* and *P. ovale* hypnozoites as well as *P. falciparum* gametocytes. However, primaquine causes oxidative stress in the RBC, and people with G6PD deficiency risk having adverse reactions such as severe haemolysis, severe haemolytic anaemia and potentially death if exposed to certain primaquine doses [22].

1.4 THE MOSQUITO VECTOR

Malaria is transmitted through the bites of female Anopheles mosquitoes. There are over 500 recognised species of Anopheles, 70 of which are able to transmit malaria to the human host and 40 of which are responsible for the majority of malaria transmission worldwide [7]. Anopheles mosquitoes breed in still standing water; the dominant vector species are anthropophilic with a preference for human feeding, have longer lifespan, and elevated vectorial capacity. The *Anopheles gambiae* complex, prevalent in sub-Saharan Africa, is the most efficient malaria vector. It contains four principal species: *An. gambiae* sensu stricto, *An. arabiensis*, *An. merus* and *An. melas*. Three other highly anthropophilic vectors in sub-Saharan Africa are *An. funestus*, *An. moucheti* and *An. nili* [7].

Environmental factors such as climate, seasonality, rainfall patterns, temperature and presence of vegetation and surface water play an important role in vector distribution and malaria transmission [7]. Adult female *Anopheles* can live up to one month, but are estimated only to survive 1-2 weeks in nature. The development of the malaria parasite in the mosquito slows as temperatures decline. *P. falciparum* transmission becomes much less likely when temperatures fall below 18ºC and parasites cease development completely at temperatures below 16ºC [23], explaining why malaria caused by *P. falciparum* is mainly confined to the tropics [24].

Insecticides such as pyrethroids are a corner stone in malaria vector control. Widespread deployment of pyrethroids in agriculture and vector control has resulted in the emergence of resistance in mosquitoes. Mechanisms for resistance include changes in the insecticide target

site that reduces binding (knock down resistance) and increases in the rate of insecticide metabolism lowering the amount of insecticide reaching the target site (e.g. over expression of P450 genes) [25]. Resistance may also be cuticular, whereby modifications in the insect cuticle and/or digestive tract lining reduce the uptake of insecticides, or behavioural whereby modifications in the insect behaviour help avoid contact with insecticides [25]. *An. gambiae* sensu stricto and *An. funestus* are typically indoor resting (endophilic) and feed at night when most humans are asleep. These species are therefore susceptible to currently employed vector control methods such as indoor residual spraying (IRS) and insecticide treated nets (ITNs). The effectiveness of these control methods can be reduced by mosquito behavioural changes, such as natural or insecticide-induced avoidance of contact with surfaces, feeding upon humans when they are active and unprotected outdoors, feeding upon animals (many vectors are zoophagic) and outdoor resting. Such changes may arise by altered taxonomic composition of the vector population or altered expression of innately flexible behaviours in the mosquito. *An. arabiensis* is an example of a species that enters a house but then rapidly exits again, even if it has not taken a successful blood meal. *An. arabiensis* has therefore some pre-existing behavioural resilience and is often responsible for persisting residual transmission following successful scale up of ITNs and IRS in sub-Saharan Africa [26].

1.5 MALARIA ENDEMICITY AND TRANSMISSION

Malaria burden is difficult to estimate, especially in low income countries where data collection and reporting quality is poor. The lack of a population denominator makes the real incidence of malaria difficult to assess [7]. Malaria endemicity is a complex indicator of disease prevalence. It is dependent on host exposure, parasite and vector characteristics, environmental factors, and may also fluctuate seasonally. There are several measures of endemicity, for example prevalence of enlarged spleen (spleen rate [SR]), proportion of population with laboratory confirmed malaria infection (parasite rate [PR]), number of infective bites per person per year (entomological inoculation rate [EIR]) and number of microscopically confirmed malaria cases detected during one year per unit population (annual parasite incidence [API]) [7]. Malaria endemicity can be categorised into four groups depending on SR or PR:

- Hypo-endemic areas where prevalences are <11%
- Meso-endemic areas where prevalences are between 11 and 50%
- Hyper-endemic areas where prevalences are between 51 and 75%
- Holo-endemic areas where prevalences are >75%

Malaria endemicity can further be classified into stable and unstable transmission, depending on the average number of feeds that a mosquito takes on a human being during its life [27]. Entomologic parameters are a direct measure of infection rates, but obtaining accurate data is technically complex, highly time consuming, and expensive due to the difficulties of obtaining entomological-based matrices, especially in areas of low transmission. There are also ethical considerations of exposing human beings to malaria infection and measurement error issues [7]. These limitations also apply for the related EIR, which is used to estimate malaria transmission and calculated as the product of the vector biting rate times the proportion of mosquitoes infected with sporozoite-stage malaria parasites. EIRs in sub-Saharan Africa are highly variable ranging from <1 to >1000 infective bites per person per year. It is considered that substantial reductions in malaria prevalence are likely to be achieved when EIRs are reduced to levels of <1 infective bite [28]. In the 2014 World malaria report the WHO classified transmission based on API as high (stable) transmission where >1 case occurred per 1000 population and low (unstable) transmissions where 0-1 cases occurred per 1000 population (Figure 3).

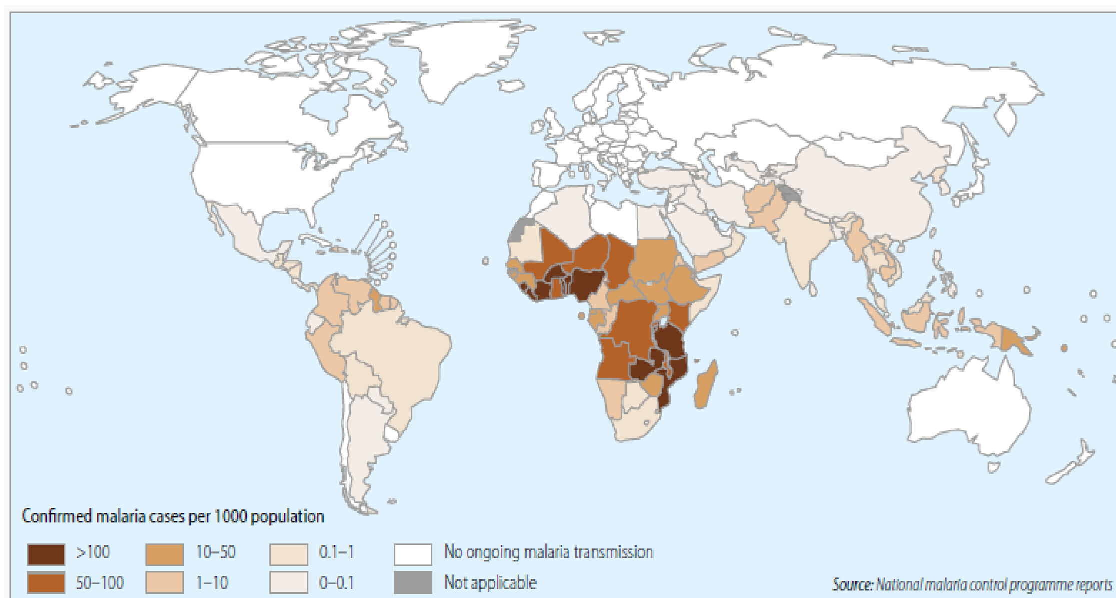

Figure 3: Global malaria endemic situation based on API. Reprinted from WHO world malaria report 2014 [1].

1.5.1 Serology for measuring transmission

An alternative method for measuring transmission involves the serological detection of infection histories [29]. This has several marked advantages over entomological and parasitological parameters, especially in areas of low transmission. PR and EIR are considered insufficiently sensitive to accurately report endemicity and transmission when PR and EIRs have been reduced to $<1\%$ or <1 infective bite per person per year. As antibodies can persist for months or years after infection it is possible to avoid seasonal variation in transmission, and age-specific seroprevalence rates reflect long-term exposure trends [30]. Seroprevalence of IgG antibodies, detected by indirect ELISA, to three recombinant blood-stage malaria antigens (MSP-1_{19}, MSP-2 and AMA-1) correlate with medium and long term trends in malaria transmission [31]. Serological assays allow surveillance of transmission where levels are approaching elimination and sero-conversion rates can provide estimates of recent changes in malaria transmission intensity [32].

1.6 CLINICAL FEATURES OF SYMPTOMATIC MALARIA

Malaria is an acute febrile illness. Symptoms usually appear 10-15 days after an infective mosquito bite. Clinical malaria is classified into uncomplicated (generally non-lethal) and severe (life-threatening) malaria.

1.6.1 Uncomplicated malaria

Symptoms of uncomplicated malaria are non-specific and difficult to distinguish from other typical viral or bacterial infections. Symptoms include fever, headache, fatigue, muscle aches, abdominal discomfort, nausea and vomiting. Cases of uncomplicated malaria may also have mild anaemia and a palpable spleen after a few days. The liver can become enlarged in small children, and mild jaundice may occur in adults. Symptoms are classically associated with irregular fever peaks that occur every 24, 48 or 72 hours depending on the malaria species, although this periodicity is rarely observed. Fever results from schizont rupture during the blood-stage cycle, releasing parasite waste products and cellular material into the blood. This activates monocytes and macrophages and induces the release of proinflammatory cytokines [6]. If uncomplicated malaria is not treated it can quickly develop into severe malaria and result in a fatal outcome.

1.6.2 Severe malaria

Severe malaria is usually caused by *P. falciparum*. The manifestations are dependent on age, and primarily occur in non-immunes and small children. Clinical features of severe malaria may include:

- Hyper parasitaemia (>2% infected RBC)
- Cerebral malaria with seizures or coma
- Metabolic acidosis
- Acute respiratory distress
- Severe anaemia (Haemoglobin <5g/100 mL)
- Hypoglycaemia (<2.2 mmol/L)
- Pulmonary oedema
- Acute kidney injury
- Jaundice

Severe anaemia and hypoglycaemia are more common in children, whereas acute pulmonary oedema, acute kidney injury, and jaundice are more common in adults; cerebral malaria and acidosis occur in all age groups [6].

1.6.2.1 Pathogenesis of P. falciparum malaria

P. falciparum expresses antigenically variant, strain-specific, adhesive proteins which locate to the membrane of the host erythrocyte. The *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) mediates sequestration, a process involving the attachment of the infected erythrocyte to endothelial surface receptors in veins and capillaries (i.e. cytoadherence). The process of sequestration protects *P. falciparum* from the clearance of infected erythrocytes by the spleen, but is also responsible for much of the pathology associated with severe malaria. Receptors such as ICAM1 in the brain, chondroitin sulphate A in the placenta, and CD36 in most other organs bind infected erythrocytes, which in turn bind uninfected erythrocytes (a process known as rosetting), resulting in microvascular obstruction and blockage of the microcirculatory blood flow [12]. Anaemia is thought to mainly result from increased destruction of infected and uninfected RBCs passing through the spleen.

1.7 MALARIA DIAGNOSIS

Early and accurate diagnosis of malaria is essential for effective disease management and malaria surveillance. The WHO recommends prompt parasite-based diagnosis either by microscopy or malaria rapid diagnostic tests (RDTs) in all patients with suspected malaria before treatment is administered. This policy has been adopted in the majority of countries with ongoing malaria transmission [1]. Diagnostic testing improves the management of all patients with febrile illnesses, and may also help to reduce the emergence and spread of antimalarial drug resistance.

1.7.1 Microscopy

Light microscopy remains the gold standard for malaria diagnosis [33]. It involves preparation of thin and thick blood smears, for the detection of parasites in the peripheral blood. Thick films are useful for detecting low-density malaria, whereas thin films provide more accurate data on parasite density and malaria species. Specimens are prepared prior to examination by staining, most commonly with Giemsa stain. Parasite densities (in parasites/µL) are estimated in thick blood smears by counting the number of parasites present against 200 white blood cells (WBC). The numbers of parasites are then multiplied by 40 to give the parasite count per microliter (assuming a standard value of 8000 WBC/µL). In thin blood smears the number of infected RBCs are counted in 10 000 RBCs (or approximately 40 monolayer cell fields using the 100 X oil immersion objective in a standard microscope) [34].

Detection limits of microscopy are highly dependent on the quality of the reagents, the microscope, and on the experience of the microscopy reader. The expected sensitivity that can be achieved by an experienced microscopist in thick blood films is around 50 parasites/µL blood or 0.001% infected RBCs (assuming a total RBC count of 5 x 10^6/µL of blood) [34]. However, under field conditions the sensitivity is likely to be closer to 100 parasites/µL, and in highly optimal conditions it may reach 5-20 parasites/µL [35].

Microscopy is a cheap, well established and informative method, allowing for assessment of species, life cycle stage and quantification of parasite densities. Microscopy is also labour intensive, time consuming (30-60 min) and the quality is highly dependent on the reagents, microscope and microscopist [35, 36]. It is not optimal for the detection of low-density parasitaemias and it is challenging to keep up motivation for careful microscopic examination when more than 95% of slides are negative for malaria.

1.7.2 Rapid diagnostic tests

There are currently over 150 commercially available RDT brands whose performance is assessed by the WHO and the Foundation for Innovative New Diagnostics (FIND). The sensitivity of RDTs (~50-100 parasites/μL for *P. falciparum*, and 200-500 parasites/μL for pan-*Plasmodium*) is similar to that of light microscopy. Results are produced within 15-20 minutes, requiring no additional equipment and minimal expertise [29]. However, RDTs do not provide parasite quantification, and are considered more expensive (0.6-2.5 US$) than light microscopy (0.12-0.40 US$) [36]. Malaria RDTs are lateral-flow devices which use antibody capture to detect soluble malaria antigens by immunochromatography. The principle target antigens for RDTs are *P. falciparum* histidine-rich protein 2 (*Pf*HRP2), produced only by *P. falciparum*, *Plasmodium* and species-specific lactate dehydrogenase (LDH) and aldolase [35]. Figure 4 describes the RDT components and mode of action.

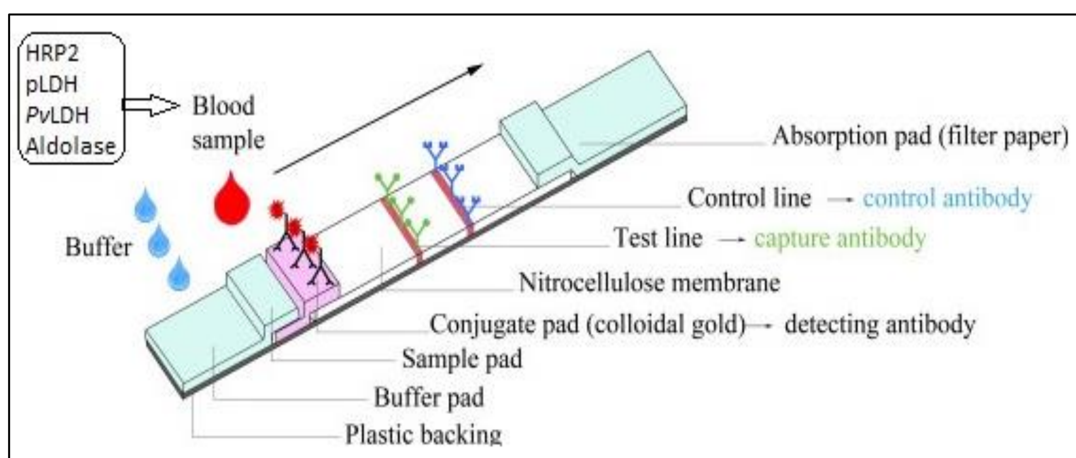

Figure 4: Components and mode of action of malaria rapid diagnostic tests. Adapted from Cnops *et al, Malaria Journal,* 2011, **10**:67**.**

The RDT strip is usually packaged in a plastic case or cardboard folder. The buffer, sample and conjugate pads are mounted on the plastic backing at the proximal end of the strip. The blood sample (5-15μL depending on RDT brand) is added to the sample pad, after which the buffer provided with the kit is added to the buffer pad. The buffer mixes with the blood lysing the RBC. The absorption pad mounted at the distal end of the plastic strip results in migration, driven by capillary forces, of the mixture across the surface of a nitrocellulose membrane. The mixture first passes the conjugation pad where mobile monoclonal antibodies, against a malaria antigen target, bind malaria antigens if present in the sample. The antibodies are labelled (conjugated), commonly with colloidal gold particles. The antigen-antibody-conjugate complex migrates across the nitrocellulose membrane until it reaches a narrow section of immobile capture antibodies. These antibodies capture the antigen-antibody-conjugate creating a cherry red coloured test line. Excess antibody-conjugate migrates further until it reaches control antibodies generating a control line. The absorption pad at the distal end absorbs the residual blood containing mixture [37].

The performance of RDTs may vary. False negative results occur mostly at low parasite densities, but also sometimes at relatively high densities [38]. For *Pf*HRP2-based RDTs possible explanations include deletions of the *Pf*HRP2 gene [39-45], high levels of antigen or anti-*Pf*HRP2 antibodies which block the target antigen detection (prozone effect) [38, 46], and varying manufacturing quality and thermal stability [29]. The HRP2 antigen persists for weeks in the blood after an infection is cleared resulting in false positive results; this limits the usefulness of *Pf*HRP2 RDTs in high-transmission areas [33, 34, 47]. LDH based RDTs detect either all *Plasmodium* species (pLDH), or *P. falciparum* and *P. vivax* specific LDH (*Pf*LDH and *Pv*LDH). LDH does not persist in the bloodstream as does *Pf*HRP2, but LDH-based RDTs are less sensitive than *Pf*HRP2-based RDTs for *P. falciparum* detection and perform less well than in areas of low parasite density [33]. Many RDT brands now combine pan-*Plasmodium* and species-specific detection in two or three line tests.

RDTs may also serve as a source of parasite DNA. Increased deployment of RDTs in health care facilities and cross-sectional surveys may facilitate passive and active collection of biological material for molecular surveillance [37, 48-50].

1.7.3 Molecular methods

The field of molecular biology has witnessed great advances in the development of nucleic acid amplification methods, which provide the, to date, most sensitive and accurate tools to detect and identify pathogens [51].

1.7.3.1 Polymerase chain reaction (PCR)

PCR involves primer-directed amplification of a specific fragment of DNA, under set temperature cycling conditions using thermostable *Taq* polymerase. A number of nested PCR (nPCR) [52-57] and real-time quantitative PCR (qPCR) [58-63] methods have been developed for malaria detection and determination of *Plasmodium* species; qPCR can also be used to estimate parasite densities [60, 61]. The most common targets for malaria specific PCR are the 18S ribosomal RNA (rRNA) genes in genomic DNA, and cytochrome b (*cytb*) gene in mitochondrial DNA. The detection limit of PCR is ~0.7-5 parasite/μL [36] however, RNA-based detection can be even more sensitive [61, 64, 65] and can distinguish between asexual and sexual stages providing a more accurate detection of gametocytes [64, 66, 67]. PCR-based methods are now used frequently for evaluating other diagnostic tools [47, 68-70], in clinical trials for monitoring antimalarial drug and vaccine efficacy *in vivo* [71, 72], and for estimating parasite prevalence in low transmission setting [52, 73-76]. PCR is, however, not yet available in resource-limited settings due to the requirement of complex equipment, reagents and know-how [77]; PCR is also prone to DNA contamination [78].

1.7.3.2 Loop-mediated isothermal amplification (LAMP)

Isothermal amplification techniques, such as LAMP, have potential for field diagnosis of malaria infection [79]. LAMP employs isothermal *Bst* DNA polymerase with strand

displacement activity. LAMP can therefore be performed at a single temperature with a simple heating block or water bath, reducing the need for sensitive and expensive machinery such as PCR thermocyclers. The LAMP reaction is primed by a specific set of four to six primers that identify six distinct regions on the target DNA. The design of the primers result in DNA loop formations and several inverted repeats of the target DNA, creating cauliflower-like structures [80]. This increases the copy number of the amplified product and reduces the time-to-result (30-60 min) [36, 79]. DNA amplification can be detected by the naked eye by a change in turbidity caused by white precipitate of magnesium pyrophosphate formed during the reaction, or under UV fluorescence if a fluorescent indicator such as calcein is added to the reagents [79]. Visual detection avoids the need for opening the reaction tube post-amplification, hence the reaction is conducted in a closed system which reduces the risk of DNA contamination [51]. The *Bst* polymerase is less prone to inhibitors such as haemoglobin than *Taq* polymerase. LAMP can be conducted with DNA extracted by crude extraction methods with a sensitivity comparable to PCR (5-7 parasites/µL) [81-83].

LAMP methodology was first reported in 2000 by Notomi *et al*. [80]. Poon *et al*. (2006) [84] were first to develop a LAMP assay detecting *P. falciparum* 18S rRNA genes, which was shortly followed by a malaria species-specific LAMP published by Han *et al*. (2007) [82]. Polly *et al*. (2010) [81] developed a LAMP method targeting mitochondrial DNA, improving the sensitivity of malaria detection. In 2013 Polly *et al*. clinically evaluated a temperature stable LAMP reaction kit developed by FIND and Eiken Chemical Company, Japan.

The Loopamp™ MALARIA Pan/Pf Detection Kit (Eiken Chemical Company, Japan) is a field-friendly kit, comprising strips of reaction tubes containing vacuum dried and temperature stable reaction components for either genus (Pan)-specific or *P. falciparum* (Pf)-specific malaria detection [85]. The kit has been evaluated both in laboratory and field settings [83, 85-88]. The kit can be used with minimal training [85], and with blood samples collected on filter paper [83]. Although the cost of LAMP is estimated to be a tenth of that of conventional PCR [84], the cost of the field friendly kit is still at 5.3 US$ per reaction [36]. The potential risk of contamination is reduced by using a closed system. However, the risk of contamination is not eliminated due to the high efficiency of the reaction [89, 90].

1.8 TREATMENT OF *P. FALCIPARUM* MALARIA AND ANTIMALARIAL DRUG RESISTANCE

Malaria is a treatable and curable disease. When treated promptly with effective antimalarial drugs, uncomplicated malaria has a mortality of roughly 0.1% [6]. *P. falciparum* malaria has throughout history proven its capacity to develop resistance to virtually all deployed antimalarial drugs. The emergence of artemisinin resistant parasite populations currently poses one of the largest challenges in malaria control and elimination [91-93].

1.8.1 Malaria treatment guidelines

The WHO recommended first-line treatment for uncomplicated *P. falciparum* malaria is ACTs [94]. ACTs are composed of a fast acting artemsinsin derivative together with a more slowly eliminating partner drug, the combination of two drugs is thought to slow the development of resistance [95]. The recommended ACTs include artmether+lumefantrine (AL), artesunate+amodiaquine (ASAQ), artesunate+mefloquine, artesunate+sulfadoxine-pyrimethamine and dihydroartemisinin+piperaquine. The choice of ACT should be based on the level of resistance to the partner drug in the region/country, and should include at least three days treatment with an artemisinin derivative. In order to reduce malaria transmission in areas targeting pre-elimination or elimination, WHO also recommends the addition of a single low dose of primaquine (0.25 mg/kg) for treatment of uncomplicated *P. falciparum* malaria. Severe malaria requires parenteral treatment with either artesunate or quinine [94]. ACTs are effective against all malaria species, although chloroquine may be used to treat *P. vivax* in areas where still effective. Treatment of *P. vivax* and *P. ovale* with either chloroquine or ACT should be combined with a 14-day course of primaquine to eliminate hypnozoites.

1.8.2 Drug Resistance and Tolerance

Antimalarial drug resistance is defined as “the ability of a parasite strain to survive and/or multiply despite the proper administration and absorption of an antimalarial drug in the dose normally recommended” [94]. Whilst drug resistance may lead to treatment failure, not all treatment failures are caused by drug resistance. Treatment failure can also be the result of incorrect dosing, problems of treatment adherence (compliance), poor drug quality and comprised drug absorption. All these factors may however, accelerate the spread of true drug resistance by exposure of the parasites to inadequate drug levels [94].

Development of resistance may be gradual or stepwise, involving a period where drug-tolerant parasites are still killed by the therapeutic doses of the administered drug but withstand higher levels of the drug than fully sensitive parasites. Antimalarial drugs with longer half-lives persist after treatment, providing a post-prophylactic effect. Tolerant parasites can infect individuals after treatment, when there are still residual levels of the drug

that are too high to enable infection by fully sensitive parasites. This may spur development of drug resistance by enabling the tolerant mutation to spread in the population [96, 97].

1.8.3 Mechanisms of drug resistance

Drug resistance generally involves a genetic event, such as introduction of single nucleotide polymorphisms (SNPs), gene copy number variations or microsatellites, which alters the parasite susceptibility to the drug. Some of the key mechanisms of resistance include:

- Reducing or eliminating the drug-target interaction e.g. by:
    - Reduced uptake of the drug (decreased import)
    - Increased export/efflux of the drug
    - Compartmentalising the drug away from its active site
    - Metabolising the drug to an inactive form before it reaches its target
    - Non-activation of a pro-drug
    - Increased target substrate which out competes the drug at the target site
- Alteration of the drug target e.g. by:
    - Changing the affinity of the drug to its target
    - Overproduction of the target
    - Eliminating the need of the target by inducing alternative pathways
- Dormancy, whereby the parasite enters a quiescent, developmentally arrested state, and continues with the normal cell cycle progression once drug concentrations have waned
- Overexpression of systems to handle indirect effects of a drug, e.g. DNA repair mechanisms

1.8.4 Methods to assess antimalarial drug resistance

There are several methods for assessing antimalarial drug resistance, each with inherent advantages and disadvantages. Different methodologies make it difficult to compare inter-study results. The Worldwide Antimalarial Resistance Network (WWARN) was established to coordinate antimalarial resistance monitoring [98]. The *Plasmodium* Diversity Network Africa (PDNA) is an African initiative established across 11 countries in sub-Saharan Africa for the assessment of parasite diversity in malaria-endemic regions. They aim to play a key roal in the global effort for tracking and responding to antimalarial drug resistance [99].

1.8.4.1 In vivo clinical trials

Antimalarial drug efficacy is commonly assessed by monitoring *in vivo* responses to the drug. Accurate estimation of the parasite clearance rate is critical for assessing *in vivo* efficacy, especially in artemisinin derivatives [94]. Patients are usually followed up after treatment for 28 or 42 days. Therapeutic responses are characterised as adequate clinical and

parasitological response, early treatment failure and late treatment failure. Molecular genotyping is required to distinguish between new and recrudescent infections. Studies are usually done in children under the age of five to mitigate the role of immunity in high transmission areas. Clinical trials should optimally be conducted every two years [1], but *in vivo* assessment of therapeutic efficacy is time consuming, expensive and requires expert personnel. Furthermore, clinical trials become impractical in areas of low transmission, where it is difficult to reach a required sample size [100].

1.8.4.2 In vitro/ex vivo assays

Antimalarial drug susceptibility can also be assessed by *in vitro* assays that measure the susceptibility of malaria parasites to drugs in culture [94]. *Ex vivo* refers to studies on fresh parasite isolates, whereas *in vitro* assays are done on parasites that have been maintained for more than two generations. *In vitro* assays usually measure the drug concentration at which 50% of the parasite growth is inhibited (IC_{50}) compared to the unexposed control [100]. Artemsinsin resistance is monitored using the ring-stage survival assay (RSA), which measures the proportion of viable parasites that develop into second-generation rings or trophozoites after a six hour pulse of dihydroartemisinin [101]. *In vitro* assays do not necessary correlate with the *in vivo* outcomes, where host immunity plays a large role [102].

1.8.4.3 Surveillance of molecular markers of drug resistance

Molecular markers of drug resistance are a useful tool to track the spread of resistance alleles in patient samples. It complements the more laborious and expensive *in vivo* and *in vitro* drug efficacy screening. It enables drug policy makers to prepare for first-line antimalarial changes before *in vivo* treatment failures have reached critical levels. It also allows for assessment of resistance levels after a treatment has been withdrawn due to resistance, when it is unethical to conduct efficacy trials. Molecular marker may however, not predict clinical treatment failures but may be a sign of increasing tolerance [10].

1.8.5 Nomenclature of molecular markers

Genes are written in italics (e.g. *pfcrt*), the proteins encoded by the gene in capital letters (e.g. *Pf*CRT). Amino acid changes in proteins are given as the protein name followed by the amino acid residue and change, using the single letter amino acid code (e.g. *Pf*CRT K76T, i.e. lysine substituted with threonine at the 76th amino acid in the *Pf*CRT protein). The SNPs encoding the amino acid changes are written as the gene followed by the amino acid residue and change (e.g. *pfcrt* K76T, i.e. the SNP in the *pfcrt* gene encoding the amino acid change at the 76th residue in the protein).

1.8.6 Historic review of antimalarial drugs and mechanisms of *P. falciparum* resistance

Drugs have been used to treat and prevent malaria for centuries. This chapter will provide a historic overview of some of the most commonly employed antimalarials and how resistance has been achieved in *P. falciparum*.

1.8.6.1 Quinolines and related compounds

Quinine was the first antimalarial drug to become widely available. It was isolated from the bark of the cinchona tree in 1820 and was the drug of choice for treating malaria until World War II [10]. Reduced availability of the drug during the war drove the development of synthetic antimalarial drugs. Chloroquine was developed in the 1930s and was widely used during the 1960s and 1970s [103]. Resistance to chloroquine was first reported along the Thailand-Cambodia border in 1957 from where it spread throughout South and SE Asia [104]. Chloroquine resistance emerged independently in South America in 1959. Resistance was first reported in sub-Saharan Africa in the 1970s and was widespread across the continent by the 1980s [105]. The worldwide spread of chloroquine resistance had devastating effects on malaria mortality, and drove the development of other synthetic compounds to which resistance developed rapidly [103]. Other synthetic derivatives of quinine include amodiaquine, mefloquine, primaquine and piperaquine; lumefantrine is also structurally related to quinine [104].

1.8.6.2 Chloroquine

Chloroquine, a 4-aminoquinoline, acts by interfering with the sequestration of toxic haem, which is produced when haemoglobin is digested by the intra-erythrocytic parasite to obtain amino acids. The parasite crystallises haem into haemozoin in its acidic digestive vacuole. Chloroquine (and other related drugs) binds to haem, preventing the detoxification process [104]. Parasite resistance is thought to be achieved by reduced accumulation of chloroquine in the digestive vacuole.

The *P. falciparum chloroquine resistance transporter* (*pfcrt*), the key gene involved in resistance, was discovered in 2000 [106]. It is located on chromosome 7 and encodes a drug and metabolite transporter protein (*Pf*CRT) located on the membrane of the digestive vacuole. The key mutation *pfcrt* K76T, confirmed by transfection studies, is found in association with other compensatory residue changes at positions *Pf*CRT 72-76, where *Pf*CRT 72-76 CVMNK is the sensitive haplotype and CVIET and SVMNT are most common resistant haplotypes [107]. The observed range of chloroquine resistance is dependent on the genetic background of the parasite line; *pfcrt* K76T increases tolerance to chloroquine so that recrudescence is likely to occur, but does not always lead to clinical failure [104]. A simplified hypothesis of how resistance arises involves the protonation of chloroquine in the acidic conditions of the digestive vacuole. Efflux of positively charged chloroquine (CQ^{2+}) is limited by the charged lysine (*Pf*CRT K76) in the sensitive strain. When lysine is replaced by the neutral threonine (as in *Pf*CRT 76T) then CQ^{2+} can exit down its concentration gradient

via *Pf*CRT, removing the drug from its target site [104]. Verapamil, a calcium (Ca^{2+}) channel blocker, is able to reverse resistance by competing with chloroquine for binding at *Pf*CRT, blocking the efflux of the drug from the digestive vacuole [108].

The *P. falciparum multidrug resistance 1* (*pfmdr1*) gene on chromosome 5 encodes an ATP-binding cassette-type transporter (*Pf*MDR1) [109]. *Pf*MDR1 also sits on the membrane of the digestive vacuole and is thought to import solutes and drugs into the lumen of the digestive vacuole [110]. *Pfmdr1* has been identified as an important secondary locus of chloroquine resistance. *Pf*MDR1 N86Y modulates resistance to chloroquine, perhaps by reducing the transport of chloroquine into the digestive vacuole [104]. Other mutations at *Pfmdr1* Y184F, S1034C, N1042D and D1246Y have been identified in field isolates. These mutations enhance the degree of chloroquine resistance, but do not seem to confer resistance *per se* [111].

1.8.6.3 Quinine

The mode of action of quinine is not fully understood, but as chloroquine it accumulates in the parasite digestive vacuole and inhibits detoxification of haem. Resistance to quinine was first reported in 1910 [10]. Despite widespread use, resistance to quinine remains low (perhaps due to its short half-life of 8 hours) and is limited to SE Asia, Oceania and less frequently in South America [10]. Quinine resistance is thought to be mediated by multiple genes. Both *pfcrt* and *pfmdr1* are involved in resistance; mutations at *pfcrt* K76T, *pfmdr1* N86Y and N1042D have shown to result in the loss of quinine transport. Focus has however, been on the *Na+/H+ exchanger 1* (*pfnhe1*) gene located on chromosome 13 and expressed on the parasite plasma membrane. Specific patterns of microsatellite repeats at locus ms4760 in *pfnhe1* are associated with increased quinine resistance [10, 104].

1.8.6.4 Amodiaquine

Amodiaquine is metabolised *in vivo* to its active form desethylamodiaquine (DEAQ). It is closely related to chloroquine, with a similar mode of action, and there is some cross-resistance between the two drugs [112]. SNPs in *pfcrt* and *pfmdr1* have been associated, both *in vitro* and *in vivo*, with resistance to amodiaquine [113-116]. Strong resistance has been linked to the *Pf*CRT 72-76 SVMNT motif prevalent in South America (the predominant haplotype in Africa and SE Asia is *Pf*CRT 72-76 CVIET) [104, 117]. Selection of *pfcrt* 76T and *pfmdr1* 86Y alleles, as well as *pfmdr1* 1246Y and the *pfmdr1* (a.a.86,184,1246) YYY haplotype has been shown in recurrent infections after treatment with ASAQ or amodiaquine alone [116, 118-123]. In the to-date most extensive report (a pooled analysis of individual patient data), none of the analysed *pfcrt* or *pfmdr1* parasite genotypes were significant risk factors for recrudescent infections (treatment failures), but *pfmdr1* 86Y, 1246Y were selected in re-infections after treatment with ASAQ. Furthermore, in patients treated with ASAQ, parasites carrying *pfmdr1* 86Y, 1246Y, or *pfcrt* 76T appeared earlier during follow-up than those carrying *pfmdr1* N86, D1246, or *pfcrt* K76, indicating that these mutations provide increased tolerance to ASAQ [116].

1.8.6.5 Mefloquine

Mefloquine was introduced in 1977, but resistance was reported from the Thai-Cambodian borders in 1982. The drug remains effective outside of SE Asia, and some regions of South America [10]. Resistance to mefloquine has been highly associated with increased *pfmdr1* copy number (2-5 amplifications of the gene) [115, 124]. *Pfmdr1* amplifications are common in SE Asia and may also contribute to resistance against lumefantrine, quinine and artemisinins [125]. *Pfmdr1* copy number variation is most often associated with *Pfmdr1* N86 in field isolates [116].

1.8.6.6 Lumefantrine

Lumefantrine has shown to select *pfcrt* K76 and *pfmdr1* N86, 184F, D1246, the *pfmdr1* (a.a. 86,184,1246) NFD haplotype, and increased *pfmdr1* copy number [116, 122, 126-130]. Parasites with *pfmdr1* NFD are able to withstand higher lumefantrine concentrations than those with *pfmdr1* YYY [97, 116]. Lumefantrine and amodiaquine select in the opposite directions, suggesting that the mode of action of lumefantrine is not in the digestive vacuole but in the parasite cytoplasm [131]. Presence of *pfmdr1* N86 and *pfmdr1* copy number was shown to be a significant risk factor for recrudescence in patients treated with AL. No association was observed between the *pfmdr1* 184, *pfmdr1* 1246 and *pfcrt* polymorphisms and recrudescent infections in the recent pooled analysis of individual patient data [116].

1.8.6.7 Antifolates

Pyrimethamine was first used as antimalarial in the late 1940s, and resistance was reported shortly after [10]. Sulfadoxine and pyrimethamine were given as a combination drug (sulfadoxine-pyrimethamine [SP]) in the 1960s, to try to overcome resistance to monotherapy. Resistance to SP emerged in SE Asia and the Amazon basin in the mid-1970s, and in Africa in the 1990s [10]. Pyrimethamine targets dihydrofolate reductase (DHFR) activity involved in thymidylate synthesis. Sulfadoxine targets the dihydropteroate synthetase (DHPS) activity involved in *de novo* synthesis of essential folate coenzymes. These drugs act as competitive inhibitors of the natural enzyme substrates [104]. Resistance to sulfadoxine and pyrimethamine arises from the accumulation of mutations in *pfdhps* and *pfdhfr* respectively. In Africa the quintuple mutant *pfdhps* A437G, K540E, *pfdhfr* N51I, C59R and S108N results in highly resistant SP parasites and is a strong predictor of clinical failure [115].

1.8.6.8 Artemisinins

Artemisinin, originally isolated from the herb *Artemisia annua*, has been used in Chinese medicine for centuries and was rediscovered by Chinese biomedical researchers in the 1970s [103]. Only in the 1990s did artemisinin and its derivatives such as artesunate, artemether and dihydroartemisinin become widely available outside of China. Artemisinins have a half-life of around 1-2 hours, are very fast acting, and reduce the parasite load quickly [10, 132]. Artemisinin resistance is suspected when an increase in parasite clearance time, defined as

parasites detectable on day 3 after ACT treatment, is observed in ≥10% of cases. Confirmed resistance is defined as treatment failure after oral artemisinin-based monotherapy with adequate antimalarial blood concentrations, evidenced by the persistence of parasites for 7 days, or the presence of parasites at day 3 and recrudescence within 28/42 days [133]. To prevent development of resistance the WHO has recommended the use of artemisinins in combination with other antimalarials for treatment of uncomplicated malaria.

Artemisinin is a sesquiterpene lactone endoperoxide. The mode of action of artemsinsin is not yet fully established. The endoperoxide bridge seems to be critical for its function [10] and is believed to produce the active compound upon interaction with reduced iron in haem moieties (deriving from haemoglobin digestion) [134] and free intracellular reduced iron [135]. The mechanism of action is thought to be either oxidative damage to parasite membranes or inactivation of parasite proteins by free radicals produced by the drug [10, 136]. Early studies report the alkylation of specific proteins including transporters [104], iron sulphur proteins and the malaria translationally controlled tumor protein (TCTP) [137]. The *Pf*ATP6, a sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) orthologue has also been suggested as a target [138-140].

Several studies showed that *P. falciparum* employed a quiescence mechanism *in vitro* that allowed it to survive artemisinin treatment. Drug pressure induced a state of developmental arrest in sub-populations of ring-stage parasites, whereby a small number of parasites remained dormant for several days to weeks after the removal of the drug, before resuming normal growth [141-144]. An artemisinin resistant strain, obtained by long term *in vitro* culture of parasites in the presence of the drug, showed transcriptomic modifications associated with the resistance phenotype including overexpression of heat shock and erythrocyte surface proteins, and down expression of a cell cycle regulator and a DNA biosynthesis protein [141]. A recent *in vivo* population transcriptomics study showed that resistant parasites remained in a state of decelerated development at a young ring stage, while responses to mitigate protein damage caused by artemsinsin were upregulated [145].

Mutations in *pfatp6*, *pfcrt*, *pfmdr1* and *pfmdr1* amplification were initially found to modulate sensitivity to artesunate *in vitro* [146-149]. Cheeseman *et al*. (2012) identified, by cross-population genomic comparisons, a region on chromosome 13 that was significantly associated with parasite clearance times [150]. Similarly, Takala-Harrison *et al*. (2013) identified the same region on chromosome 13, as well as two other regions on chromosome 10 and 14 [151]. Ariey *et al*. (2014) conducted whole genome sequencing of an *in vitro* cultivated artemsinsin-resistant parasite line originating from Africa and clinical isolates from Cambodia. Non-synonymous mutations in the kelch propeller domain (*pfkelch13*), encoded on chromosome 13, were associated with clearance times both *in vitro* and *in vivo* [152]. The kelch protein is thought to mediate diverse cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses [153]. Ariey *et al*. reported that artemisinin tolerance *in vitro* was conferred by the *pfkelch13* M476I mutation, prolonged parasite survival *ex vivo* was conferred by the Y493H, I543T, R539T, and C580Y mutations, and *in*

vivo parasite half-life was increased by Y493H, R539T, and the most prevalent C580Y mutation [152]. The involvement of these SNPs in artemsinsin resistance has been confirmed by genome editing studies [153, 154]. Straimer *et al*. (2014) showed that the level of resistance varied depending on the genetic background of the parasites, indicating the role of additional genetic factors [153]. Resistance was likely to arise on a particular genetic background common in SE Asia, with several background markers. These background markers may be compensatory, reducing fitness costs associated with *pfkelch*13 mutations, or enhance phenotypic effects of artemisinin resistance [155].

The first evidence of *in vivo* artemsinsin resistant malaria was observed in Western Cambodia in 2008, with reports of delayed parasite clearance times and treatment failures [91]. Treatment failures were confirmed in 2009 in Pailin, Western Cambodia, where median clearance times (84 hours) were significantly higher than clearance times (48 hours) in North-western Thailand. Mutations in *pfmdr1* and *pfatp6* were absent in both studies and the slow parasite clearance *in vivo* did not correspond with reductions in conventional *in vitro* susceptibility testing [92]. A population genetics study discovered three distinct subpopulations of *P. falciparum* that were associated with clinical resistance to artemsinsin in the same location in Cambodia [156]. These subpopulations showed evidence of clonal expansion, suggesting that resistance may have arisen independently at least three times[157]. A longitudinal study conducted along the North-western border of Thailand showed that the proportion of patients with slow clearance rates (≥6.2 hours) increased from 0.6-20% from 2001-2010, proving that resistance had emerged or spread westward from Cambodia. [158]. Recent studies have shown that the *pfkelch*13 mutations have originated independently in multiple locations [155, 159]. Ashly *et al*. (2014) showed that slow clearing infections (parasite clearance >5 hours) were strongly correlated with point mutations in *pfkelch*13, and were detected throughout mainland SE Asia from southern Vietnam to central Myanmar. Slow clearing parasites had a higher level of gametocytemia, suggesting greater potential for transmission. Importantly a six day course of ACT had high cure rates in western Cambodia [93].

ACTs remain efficacious in sub-Saharan Africa [160-165]. Molecular surveillance has shown the absence of the SE Asian *pfkelch13* alleles. Other *pfkelch13* mutations have been reported [166-168], although the frequency of mutations does not seem to have increased over time [166]. It may however, be harder to quantify the amount of resistance in sub-Saharan Africa where host immunity may mask the loss of drug efficacy [169]. Borrmann *et al*. (2011) reported a small decline in parasitological response rates to ACTs in Kenya, perhaps due to reduced drug sensitivity or a reduction in population-level clinical immunity [170]. There have been a couple of reports of treatment failure with AL in returning travellers [171, 172], and Betson *et al*. (2014) reported persistent day 7 parasitaemia in chronic multi-species malaria infections in Ugandan children after AL treatment [173].

*Pfkelch*13 has been identified as a key determinant of delayed parasitological clearance after artemisinin treatment in SE Asia [93, 152, 159]. So far resistance has been documented in

five countries in the Greater Mekong sub region (Cambodia, Laos, Myanmar, Thailand and Vietnam) [1]. Molecular markers in *Pfkelch*13 may provide the means for large scale surveillance of resistance in SE Asia, aiding the WHO plan for artemisinins resistance containment. In sub-Saharan Africa it is important to maintain surveillance of resistance to ACT partner drugs. Resistance to partner drugs has historically manifested before that of artemisinins [116], and may spur the development of resistance to artemisinin derivatives and to ACTs [174, 175]. Table 1 summarises the key established molecular markers in antimalarial resistant *P. falciparum* that have been discussed above.

Table 1: Summary table of molecular markers of antimalarial drug resistance.

Drug	**Gene**		
	Pfcrt	***Pfmdr1***	**Other**
Chloroquine	76T	86Y, Y184, 1034C, 1042D, 1246Y	
Quinine	76T	86Y, 1042D	*pfnhe1* ms4760
Amodiaquine	76T. 72-76 SVMNT	86Y, Y184, 1246Y	
Mefloquine		N86, Amplification	
Lumefantrine	K76	N86, 184F, D1246, Amplification	
Sulphadoxine-pyrimethamine			*Pfdhps* 437G, 540E *Pfdhr* 51I, 59R, 108N
Artemisinins	K76	N86, D1246, Amplification	*K13 propeller* C580Y, M476I, Y439H , R539T, I543T

1.8.7 Fitness cost of mutations

Fitness describes the ability of a particular genotype to survive and reproduce in a given environment. Genetic polymorphisms conferring resistance are frequently accompanied by fitness costs. The spread of drug resistance may be limited by fitness costs, although compensatory mutations that increase virulence or transmission may accumulate after resistance has developed [176].

Fitness costs can be measured *in vitro* by comparing growth rates and in competitive growth experiments. Mutations in *pfcrt* and *pfmdr1* as well as increased *pfmdr1* copy number have all been associated with fitness costs [176-178]. Parasites may benefit from carrying these mutations when under direct pressure, but are outcompeted by sensitive strains that lack the mutation when drug pressure is absent [177]. A typical example is the return of the chloroquine sensitive form (*pfcrt* K76) in areas where chloroquine use has been discontinued due to high levels of resistance [179-183].

1.9 MALARIA CONTROL AND MALARIA ELIMINATION

The WHO uses the following terminology to refer to malaria endemic status [7]:

- Malaria control: reducing malaria disease burden to a locally manageable level where it is no longer a public health problem.
- Malaria elimination: interruption of local transmission; reduction to zero of the incidence of infection in a defined geographical area; continued measures to prevent re-establishment of transmission.
- Malaria eradication: permanent reduction to zero of the worldwide incidence of infection; intervention measures are no longer needed.

Successful malaria control has resulted in marked reductions in malaria prevalence over the past decade, re-inviting the possibility of malaria elimination in some areas.

1.9.1 Corner stones in malaria control

1.9.1.1 Vector control

The coverage of vector control interventions in sub-Saharan Africa has increased substantially during the last 10 years [1]. ITNs and long lasting insecticide treated nets (LLINs) protect individuals by diverting or killing mosquitoes. LLINs have shown to reduce the number of malaria cases by 50% [184] and represent an important corner stone in malaria control. In 2013 almost half the population at risk had access to a ITN in their household, and in 2014 a total of 214 million nets were projected to be delivered to sub-Saharan Africa [1]. Replacing worn out nets is crucial for maintaining universal coverage [184].

IRS involves the application of insecticides to the interior walls and surfaces of houses which serve as resting places for mosquitoes. The use of IRS has declined since 2010 due to withdrawal or downsizing of cumbersome spraying programmes. In sub-Saharan Africa, 7% of the population at risk were protected by this method of vector control [1]. Target vector control strategies to foci of transmission can scale up the type and intensity of interventions in a cost effective manner, and should be complemented with robust monitoring and evaluation [185]. Resistance to pyrethroids, the most frequently used insecticide accounting for 75% of total IRS coverage and used in all treated bed nets poses a threat to malaria control [3].

Environmental management including larval control with biological means (fish or bacteria) or insecticides might be cost effective in areas of high-density human populations, such as urban areas [27]. The WHO reported 38 countries using larval control to complement core vector control methods [1]. Ivermectin, an antihelminthic drug which can be given to both humans and animals, has been found to kill mosquitoes when taking a blood meal, and may thus also provide an interesting opportunity to reduce residual malaria transmission [186].

1.9.1.2 Improved diagnostics

The WHO recommends diagnosis either by microscopy or RDTs in all patients with suspected malaria before treatment is administered. RDTs are recommended by the WHO in areas where good quality microscopy is not available; 160 million RDTs were distributed in 2013 which for the first time exceeded the number of distributed ACTs [1]. The scaled-up use of RDTs poses some challenges such as access to RDTs on the scale needed to achieve universal coverage, policy changes required for implementation at a community level, and targeted information and training required for proper adherence to test results [187]. Furthermore, missed diagnosis of malaria and new infections that become apparent soon after a consultation with a malaria negative RDT may undermine confidence in results [188].

1.9.1.3 Access to effective treatment

Access to effective treatment in order to decrease morbidity and mortality and to interrupt transmission is another corner stone in malaria control. Access to antimalarials is dependent on supply demands being met, in order to avoid stock-outs. Subsidised antimalarials are currently available through public facilities in most malaria endemic areas, but only a proportion of those at risk have access to these sources. ACTs available through the private sector are priced according to market demands [131] and may be deemed as too expensive [189]. The T3: Test. Treat. Track. initiative was launched by the WHO in 2012 to urge the global malaria community to achieve universal coverage with diagnostic testing and antimalarial treatment, and to enhance case reporting in order to improve malaria surveillance systems [190]. It is also important to monitor the efficacy of ACTs, especially in the light of artemisinin resistance [191].

1.9.1.4 Chemoprophylaxis

Intermittent administration of antimalarials at regular time points is used in areas of high transmission for preventing infection. Intermittent preventive treatment (IPT) is recommended for pregnant women (IPTp) and infants (IPTi) as well as in children under the age of five [3]. SP still plays an important role in IPT, although SP resistance decreases the treatment and prophylactic efficacy. There is an urgent need to identify a replacement drug with a long half-life, which is well tolerated and safe to administer during infancy and pregnancy [131]. The WHO also recommends seasonal malaria chemotherapy (SMC) with amodiaquine + SP for children aged 3-59 months in areas of highly seasonal malaria transmission [1].

1.9.1.5 Malaria vaccines

The complexity of the malaria parasite makes development of a vaccine very difficult, and despite decades of intense research efforts there is currently no commercially available malaria vaccine [1]. There are several vaccine candidates undergoing field trial, the most advanced is the RTS,S/AS01 [1]. RTS,S is a pre-erythrocyte vaccine based on *P. falciparum* circumsporozoite surface protein [192]. Vaccine recipients that received all planned doses of

RTS,S/AS01 showed a 46% reduction of severe malaria in children and 27% reduction in infants [1]. This falls short of the traditional goals of a vaccine, but might give infants and small children a better chance of surviving when most vulnerable [193]. The WHO decision regarding a policy recommendation for the use of RTS,S is expected in 2015 [1].

It has long been known that inoculation of *P. falciparum* sporozoites by mosquito bites induces high-level protection against human malaria [194]. Another vaccine candidate is the PfSPZ vaccine, composed of radiation attenuated *P. falciparum* sporozoites. Seder *et al.* (2013) report that five intravenous inoculations with the PfSPZ vaccine provided 100% efficacy in a Phase I safety trial in six individuals [195]. There are however, several limitations and logistical issues with this potential vaccine candidate such as producing irradiated sporozoites on an industrial scale, the need for multiple intravenous administrations, and the need for a cold chain during vaccine delivery.

1.9.2 Malaria elimination

WHO classifies countries according to their malaria programme phase as follows [1]:

- Pre-elimination phase: Test positivity rate <5% among suspected malaria cases throughout the year and API <5 (i.e. fewer than 5 cases/1000 population).
- Elimination phase: API <1; malaria is a notifiable disease with a manageable number of reported malaria cases nationwide (e.g. <1000 cases, local and imported).
- Prevention of reintroduction: Recently endemic countries with zero local transmission for at least 3 years; case management of imported malaria and maintaining the capacity to detect malaria infection and manage clinical disease is required.
- Malaria-free: Certified malaria free within last five years.

The transition from malaria control to malaria elimination requires an already operating effective malaria control programme, with additional components such as surveillance and response systems that are able to detect and eliminate residual reservoirs of symptomatic and asymptomatic infection [196]. This requires enhanced laboratory support that embraces sensitive techniques, such as PCR, genotyping and serology. Research and cross-border collaborations are required within and between malaria eliminating countries. Modelling and predicting costs of achieving malaria elimination and preventing re-introduction, compared with the costs of maintaining controlled low-endemic malaria is a must [197, 198].

1.9.2.1 Malaria hotspots and hotpops

A considerable shift in the malaria epidemiology occurs in areas approaching malaria elimination due to heterogenic malaria transmission [199]. Malaria infections tend to cluster in geographically defined areas (hotspots), typically smaller than 1km^2, where malaria transmission exceeds the average level. Factors determining hotspots are not fully defined, but include the proximity to mosquito breeding sites, household structural features, and

human behaviour and genetic factors [200]. Clustering also occurs in certain demographic high-risk groups (hot populations or hotpops) which are often related to occupation or travel [201]. Hotspots and hotpops serve as a source of parasites that fuel subsequent transmission. Individual infections are often asymptomatic and subpatent, with parasite densities falling beneath the detection level of both microscopy and RDTs, and occurring in hard to reach areas. Clustering of asymptomatic parasite carriage determined by PCR and malaria-specific immune response determined by serology are robust indicators of hotpots [202, 203]. Identifying hotpots and hotpops can be used to target parasite reservoirs effectively and achieve maximum impact of control/elimination programmes [200, 204].

1.9.2.2 Identifying asymptomatic and subpatent infections

There are inherent challenges with locating asymptomatically infected individuals as these do not seek medical treatment and the infections may often be subpatent especially in low-transmission settings. However, clearing this parasite reservoir is critical for malaria elimination as these individuals still remain infectious to mosquitoes [199, 204, 205]. The proportion of subpatent infections appears to increase in areas with recent declines in transmission. A meta-analysis showed that microscopy detects as little as 12% of PCR-detectable infections in areas where PCR prevalence is <10% [206]. Detecting subpatent infections requires sensitive molecular diagnostic tools such as PCR or LAMP [65], and still very low-density infections may be missed [201, 207]. Synchronised sequestration in *P. falciparum* [208, 209] and hypnozoites in *P. vivax* and *P. ovale* also contribute to sub-microscopic carriage [210].

1.9.2.3 Transmissibility of asymptomatic malaria and targeting gametocyte reservoirs

Some asymptomatic infections may become symptomatic within days or weeks of initial detection, but most infections can persist asymptomatically for many months. Single *P. falciparum* clones have shown to circulate for ~200 days before eventually being cleared from the bloodstream [65], although the maximum reported duration of a *P. falciparum* infection is 13 years [211]. Sensitive molecular methods have shown that gametocytes are detected in most asymptomatic and subpatent infections. Gametocytes comprise <5% of the total parasite biomass and are often present in sub-microscopic densities. The transmissibility of these infections has been discussed [210]. At least one male and one female gametocyte must be taken up in a 2-3μL mosquito blood meal for transmission to occur; transmissibility is positively associated with gametocyte density [65]. Human to mosquito transmission rates have been estimated to be two to 16 times lower in subpatent compared to patent carriers [212], but the high prevalence of subpatent carriers suggest that these are still important contributors to the human infective reservoir [65]. Subpatent infections in very low transmission setting are estimated to account for 20 to 50% of all human to mosquito transmission [205] and can play a significant role in sustaining malaria transmission [213].

Although artemisinins possess some gametocytocidal properties which are thought to contribute to reduced transmission [214], primaquine is the only currently available drug that

actively clears mature *P. falciparum* gametocytes and prevents malaria transmission. In areas of *P. falciparum* drug resistance or areas targeting malaria elimination, WHO recommend a single low-dose (0.25 mg/kg) of primaquine given on the first day of ACT treatment [1]. Primaquine induced haemolysis is dose dependent, and the low dose is suggested to be safe for G6PD-deficient patients, but high enough to have a gametocytocidal effect and block transmission. Before primaquine can be implemented, countries will need to register its use and preferably assess the local prevalence and types of G6PD deficiency [215], even within the context of low doses which may avoid the need to screen for G6PD deficiency [22].

1.9.2.4 Passive versus active surveillance

Passive case detection relies on health-seeking behaviour of symptomatic individuals to reporting health facilities and high test rates. Strong reporting systems are essential in areas of malaria control [196, 199], but passive detection alone is unlikely to be sufficient for malaria elimination [198].

Targeting the asymptomatic reservoir of infections requires active surveillance at a household level. Active infection detection can be categorised into reactive and proactive methods. Reactive infection detection is triggered when a case is identified by passive case detection [198]. It involves visiting the household of the reported case and screening neighbours within a defined radius, although there are no standardized guidelines on the size of the screening radius. Reactive infection detection is better suited for low transmission settings, especially if high risk groups are undefined [202]. Proactive infection detection involves screening of high risk populations, without the trigger of a passively identified case, and is more suitable in moderate transmission settings. To be successful, active surveillance requires accurate and sensitive diagnostic tests. RDTs are often used, but may not be sensitive enough to detect asymptomatic carriers limiting the impact of these screening techniques on parasite prevalence [202, 216]; LAMP could be a more useful tool in such programmes. High coverage of the target population is important for these methods to be successful. To increase coverage, whole communities should be screened; those not at home should be recorded and revisited where possible [201].

1.9.2.5 Mass and focal screening and treatment

Mass screening and treatment (MSAT) involves screening of all individuals in a given geographical area and treatment of those found positive for malaria [190]. Focused screening and treatment (FSAT), employs active detection in a smaller geographical area or hotspot, and may be triggered by an index case which is followed up by screening contacts of the infected individual. FSAT may be more feasible to conduct due to the reduced scope compared to MSAT, although FSAT is still labour intensive and requires that an effective surveillance system is in place. MSAT and FSAT programmes have had varying results [216, 217], and have been evaluated as a tactic to contain the spread of artemisinin resistance [218]. Both may provide valuable epidemiological information [218], although with a high price tag

and logistical difficulties [191]. More research is necessary to determine the long-term and cost effectiveness of MSAT and FSAT in a wide range of settings [212].

1.9.2.6 Mass drug administration

Mass drug administration (MDA) is a strategy whereby everybody in a defined geographical area or high risk group receives treatment, irrespective of the presence of symptoms and without malaria diagnosis [102]. MDA overcomes the issues of missed infections due to insensitive diagnostic tools [65, 202] however, the optimum combination of drugs, timing, number of rounds per year and total duration of MDA need to be defined [199]. The impact of MDA on transmission maybe short lived, especially in high transmission areas. MDA may also increase the likelihood of the selection of drug resistance genotypes, and is therefore not currently recommended by the WHO. To reduce the risk of resistance emerging, one idea is to use different classes of antimalarials for MDA and individual case management [190] combined with a transmission limiting treatment such as primaquine [199]. Targeted MDA to households or groups identified by passive or active surveillance may be more efficient, and high local coverage is more operationally feasible [200]. High degree of community participation was identified as a key factor for the successful MDA campaign and elimination of *P. falciparum* and *P. vivax* on Aneityum island (718 inhabitants), Vanuatu in the 1990's [219].

1.9.2.7 Detecting and preventing importation of malaria

In malaria eliminating settings, imported malaria cases are a critical threat to achieving and maintaining elimination [199]. Countries considering elimination should aim to prevent importation of infections through proactive case detection at borders. This is however, costly, labour intensive and misses subjects crossing unofficial borders and infections which are still in the incubation period [201].

1.10 THE CURRENT SITUATION IN ZANZIBAR

Malaria was the leading cause of death in Zanzibar before scale-up of malaria control activities, accounting for more than 50% of in-patient cases and deaths in hospitals. Endemicity was moderate to high, with malaria prevalences ranging from 9-50%. In year 2000, chloroquine had a treatment failure of 60% at 14 days follow-up [220].

Zanzibar was one of the first regions in sub-Saharan Africa to deploy ACTs to malaria patients free of charge through public health facilities. ASAQ and AL were introduced in September 2003 as first and second-line treatment of uncomplicated malaria [221]; quinine replaced AL as second-line treatment in 2009. LLINs were mass distributed in 2006 to high-risk groups. The combination of ACT and LLIN deployment resulted in a substantial decline in *P. falciparum* malaria among febrile children, from approximately 30% to 1-2%, and a reduction of crude child mortality of approximately 50% between 2003 and 2006 [221]. The Zanzibar Ministry of Health implemented malaria control interventions through the Zanzibar Malaria Control Programme (ZMCP); the introduction of malaria control interventions are presented in Table 2.

Table 2: Introduction of malaria control strategies in Zanzibar [Bjorkman and Shakely *et al.*, submitted].

Year	**Interventions**
Nov 2002	Policy decision on introduction of ACTs: 1st line ASAQ, 2nd line ALU
Sep 2003	ACT deployment in all public health facilities.
2004	Limited ITN distribution Introduction of intermittent preventive treatment in pregnancy
Sep 2005	LLIN distribution to all children <5 y and pregnant women
July 2006	IRS (pyrethroid) rounds aiming at annual universal coverage
2006	RDT provision to all primary public health facilities.
2008	LLIN distribution to all (two nets per household)
2009	New antimalarial treatment policy: 1st line ASAQ, 2nd line Quinine
2012	LLIN distribution (two nets per household) IRS (carbamate) change – from universal to focal targeting hotspots Introduction of malaria case notification and household RDT screening

In 2006, 90% of children under the age of five were reported to sleep under an LLIN [221]. Vector control was further strengthened by mass-distribution of two LLINs per household in 2008-2009, and annual rounds of IRS targeting all households in Zanzibar (excluding Stone Town) between 2006 and 2009. Coverage of vector control interventions remained high in 2009 despite reduced perceived threat of malaria among caretakers, with 70% of under-five children reported to sleep under a bed net and 94% living in a house targeted with IRS.

Combined, 98% of children were covered by at least one of the vector control methods [222]. A study conducted in 2010-2011 reported that 90% of the *Anopheles gambiae* complex was *An. arabiensis* with a small number of *An. gambiae s.s.* and *An. merus* present. *An. arabiensis* in Pemba, but not Unguja, were found to be resistant to Lambda-cyhalothrin, the pyrethroid used in LLINs and IRS. Two thirds of LLINs examined were damaged after three years of use and insecticide levels were significantly reduced. The study concluded that resistance to pyrethroids and the short lived efficacy of LLINs could seriously threaten the sustainability of the gains achieved in malaria control in Zanzibar [223]. In 2012, Zanzibar introduced Bendiocarb 0.1%, a carbamate insecticide for IRS with focal targeting of hotspots instead of universal coverage.

RDTs were introduced in 2006 for improved case detection, and targeting of treatment to patients with confirmed malaria infections. RDTs were shown to improve management of fever patients, providing adequate treatment and health outcomes without increased cost per patient. Adherence to the test results was high, and resulted in a significantly reduced amount of prescribed ACTs as supposed to clinical diagnosis of malaria. [224]. The usefulness of RDTs was again assessed in 2010 in the new context of low malaria transmission. This study found a test positivity rate of 3.1%; the adherence to test results remained high, but the sensitivity of the tests against PCR and microscopy was relatively low (76.5 and 78.8%, respectively) [70]. Mass screening and treatment employing RDTs for parasite detection in five malaria hotspots in Zanzibar did not reduce subsequent malaria incidence compared with controlled areas. These results were most likely due to the low sensitivity of RDTs, and low population coverage achieved in the study [216].

The Malaria Early Epidemic Detection System (MEEDS) was established in Zanzibar in 2008. MEEDS is a malaria case notification system that involves weekly reporting of laboratory confirmed malaria cases using mobile phones. It enables district malaria officers to follow-up all malaria cases by reactive case detection, involving testing and treatment of index case contacts. MEEDS also enables the tracking of malaria hotspots and resurgences, the identifying risk groups, and provides means for procurement assessments and reduces the risk of stock outs of RDTs and ACTs in health facilities [220, 225].

Zanzibar is presently in a state of malaria pre-elimination. The test positivity rate was 1.3% in 2010, with 1549 confirmed malaria cases and an approximate API of 1-2 infections per 1000 individuals [226]. Compiled malariometric indices from two sentinel districts in Zanzibar indicate that the major decline in malaria transmission occurred between 2004 and 2007, after which there appears to be a steady state of persistent low-level malaria transmission [Bjorkman and Shakely *et al.*, submitted]. Zanzibar was first in conducting a malaria elimination feasibility assessment in 2009, with the core conclusion that elimination is feasible but will be very challenging in Zanzibar [227]. The Zanzibar Ministry of Health has declared the goal of malaria elimination and ZMCP officially converted its name to the Zanzibar Malaria Elimination Programme (ZAMEP) in August 2013.

2 RATIONALE FOR THESIS

The ZMCP (now ZAMEP) has been unique in its success in malaria control. High intervention coverage was rapidly achieved after introduction. This achievement was probably influenced by comprehensive information to the public and health care staff and a strong commitment of the Zanzibar government to rapidly ensure free intervention coverage. Furthermore, Zanzibar residents have, in general, easy access to public health facilities located within 5km from any community and served by good public transport [222]. However, as presented in this comprehensive literature review, the transition from malaria control to malaria elimination will require additional components such as surveillance and response systems that are able to detect and eliminate residual reservoirs of symptomatic and asymptomatic infection. Such systems will need more sensitive diagnostic tools than those that are already available, such as PCR and genotyping for molecular surveillance of parasite prevalence and genetic markers of drug resistance.

The rapid transition from high malaria transmission to pre-elimination in Zanzibar during the last decade provides a unique research opportunity to study how molecular methods may assist malaria elimination efforts in a pre-elimination setting. Karolinska Institutet has since 2001 acted as the main scientific partner of ZMCP/ZAMEP with two main study sites in Zanzibar: North A (Unguja Island) and Micheweni (Pemba Island) districts (Figure 5). Longitudinal samples collected in these study sites, as well as samples collected throughout Zanzibar, have been used in this PhD-programme to gain insight into the application of molecular methods for improved malaria surveillance and control.

4 AIMS AND OBJECTIVES

4.1 OVERALL AIM OF THE THESIS

To gain insight into the application of modern molecular methods for enhanced malaria infection detection and surveillance of antimalarial drug resistance in a pre-elimination setting such as Zanzibar.

4.2 SPECIFIC OBJECTIVES

Study I: To assess whether prolonged use of ASAQ as first-line antimalarial treatment selects for *P. falciparum* SNPs associated with resistance to the ACT partner drug amodiaquine.

Study II: To evaluate different methods of DNA extraction from RDTs and to assess whether RDTs collected under field conditions could preserve parasite DNA for the purpose of molecular epidemiological investigations.

Study III: To evaluate the performance of a new, highly sensitive SYBR Green qPCR-RFLP assay for *Plasmodium* detection and species determination.

Study IV: To characterise temporal trends and transporter polymorphisms in asymptomatic *Plasmodium* infections during the transition from high to low transmission in Zanzibar.

Study V: To report results from the hitherto largest implementation of LAMP in the field, for scaled up, centralised mass-screening of asymptomatic malaria in Zanzibar.

5 MATERIAL AND METHODS

5.1 STUDY LOCATION AND POPULATION

All studies included in this thesis were conducted in Zanzibar, an archipelago located approximately 35 km off the coast of Tanzania in East Africa (Figure 5A). Zanzibar consists of two main islands, Unjuga and Pemba, with respective populations of approximately 900,000 and 400,000. Subsistence farming and fishing are the main occupations; ~40% of the population live below the basic needs poverty line [228]. Malaria transmission is perennial with two peaks associated with seasonal rainfalls in March-June and October-November. *P. falciparum* is the predominant malaria species; *Anopheles gambiae* and *An funestus* are considered the main vectors. ASAQ was introduced as the first-line malaria treatment in September 2003 [229].

Samples for Studies I-IV were collected in two sentinel districts in Zanzibar, North A (Unguja Island) and Micheweni (Pemba Island) (Figure 5B). Both districts are rural with a population of approximately 100,000 people each. Both districts have one Primary Health Care Centre (PHCC), which provides in- and outpatient care, and 12 public Primary Health Care Units (PHCUs), where basic medical management is provided. Two private health facilities have been established in each district in recent years [70]. Study V was conducted in 10 districts throughout Zanzibar (6 in Unguja and 4 in Pemba).

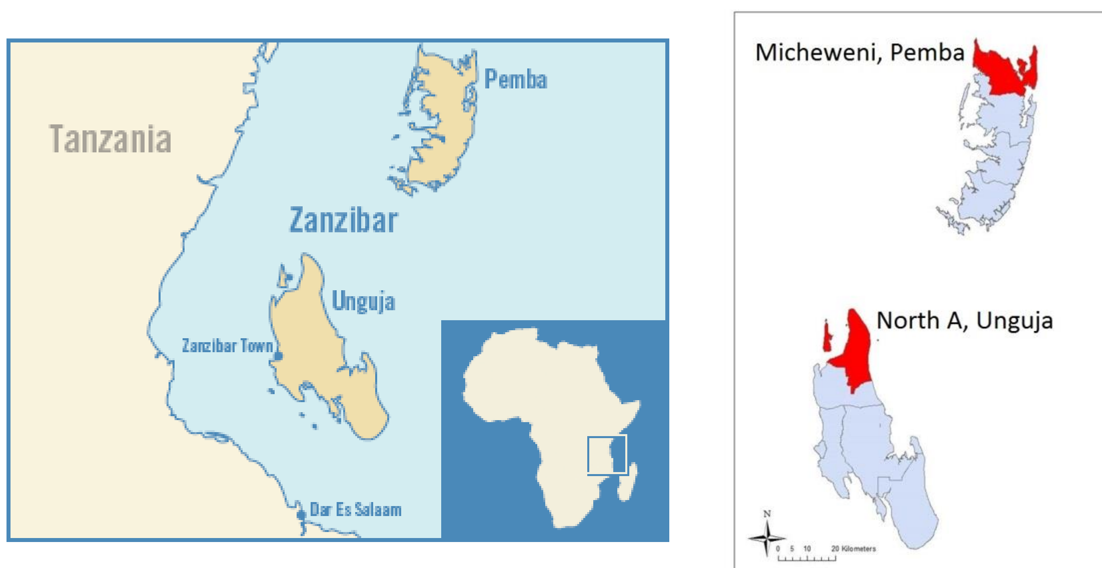

Figure 5: A) Map of Zanzibar, East Africa. B) Map of North A and Micheweni districts (highlighted in red).

5.2 SAMPLE COLLECTION

5.2.1 Clinical studies

ACO I: A clinical trial comparing the efficacy of ASAQ and AL, before the implementation of ASAQ and AL as first and second-line treatments in Zanzibar, respectively. The trial was conducted in children under the age of five, during November 2002 and February 2003, in the two PHCCs in North A and Micheweni district [230]. Inclusion criteria were age 6-59 months, parasitaemia levels of 2000-200,000 asexual parasites/μL blood and axillary temperatures of ≥37.5ºC at the time of enrolment or history of fever during the preceding 24 hours. Exclusion criteria were symptoms or signs of severe disease. Blood samples collected on filter paper on day 0 of the study (prior to treatment with antimalarial drugs) were used in Study I.

RDT-ACT: A study aimed to evaluate the usefulness of an *Pf*HRP2 based RDT in the hands of primary health care workers, for detection of *P. falciparum* in fever patients of all ages [70]. The study was conducted during May-July 2010, in 12 public health facilities, six each in North A and Micheweni districts. Inclusion criteria were all ages ≥2 months, presenting at the study sites with fever (≥37.5ºC at the time of enrolment or history of fever during the preceding 24 hours). Exclusion criteria were symptoms or signs of severe disease. The study is registered on ClinicalTrials.gov with study identifier "NCT01002066". Blood samples collected on filter paper and RDTs were used in Study I and II.

5.2.2 Cross-sectional household surveys

Cross-sectional household surveys have been conducted during the period May-July on an annual or biannual bases in North A and Micheweni districts since 2003 to monitor community prevalence of malaria and uptake of malaria control interventions [221], [Bjorkman and Shakely *et al.*, submitted]. Shehias (the smallest administrative units in Zanzibar) were randomly selected from the two districts in 2003; households in each shehia were randomly selected for each survey. Sample size was based on the estimated proportion of children <5 years with malaria parasitaemia in 2003; sample sizes remained similar in subsequent surveys.

Household visits were conducted by trained health personnel. Finger prick blood samples for malaria screening by microscopy or RDT were collected from healthy individuals, of all ages, present during the surveys. In addition, blood samples were collected (from the same finger prick) on filter paper for molecular analyses. Epidemiological data in forms of demographic and clinical information were also collected at each survey. Microscopy/RDT data and blood samples collected on filter paper from 2005, 2009, 2011 and 2013 were used in Study III and IV (where referred to as asymptomatic samples).

5.2.3 Collection of RDTs at public health facilities

Primary health care facilities in North A and Micheweni district were asked to passively collect positive RDTs from fever patients attending the clinic. They were asked to record age, date and health facility on the back of the RDT device. RDTs collected during Jan-July 2013 were used in Study IV (where referred to as symptomatic samples).

5.2.4 Deployment of LAMP in the field and KAPB survey

LAMP was deployed as part of a larger study regarding knowledge, attitude, practice and behaviour (KAPB) towards malaria [Cook *et al.*, unpublished data], a cross-sectional household survey conducted in ten districts throughout Zanzibar (six in Unguja and four in Pemba) in April-May 2014. Household visits were carried out in 60 villages. Tablet computers were used to conduct questionnaires in each household. All individuals present and willing to participate in the randomly selected households were screened for malaria by RDT. In every other household, participants were asked to provide 60μL of finger prick blood for LAMP screening. RDT results and whole blood samples were used in Study V.

5.2.5 *In vitro* parasite culture and generation of dilution series

For the purpose of evaluating molecular methods, dilutions series of known parasite densities were generated from *in vitro* cultured 3D7 *P. falciparum* parasites and *P. vivax*, *P. malariae* and *P. ovale* blood samples from patients diagnosed and treated at the Karolinska University Hospital, Stockholm, Sweden. Tenfold dilution series were prepared, in fresh uninfected whole blood. Dilution series were used in Study II and III, and in Study IV for determining parasite densities.

5.2.6 Collection and storage of blood samples on filter paper and RDTs

Dried blood spots on filter paper have been widely used in field studies as the DNA is relatively well preserved, and the samples are easy to transport to laboratory settings without the need for a cold chain [231]. Finger prick samples (approximately 50-100μL) were collected on filter papers (3MM®, Whatman, United Kingdom), air dried and individually packaged in plastic envelopes with desiccants.

Several studies have shown that RDTs are a reliable source for parasite DNA preservation [37, 48, 50]. RDTs were performed according to the manufacturer's instructions and allowed to air dry before storing without desiccants.

All samples were stored at room temperature (<25ºC) and shipped to Karolinska Institutet, Stockholm, Sweden, for molecular analyses.

5.2.7 Data entry

Data collected in clinical trials and cross-sectional surveys (apart from in 2013) were double-entered in Microsoft Access or CSPro and validated using Excel. Data collected in the cross-sectional survey in 2013 and the field deployment of LAMP in 2014 were entered using Nexus 7 tablet computers as the studies were conducted.

5.2.8 Ethical considerations

All field studies were performed in accordance with the Declaration of Helsinki [232] and Good Clinical Practice [233]. Informed consent was obtained from all study participants, or from parent/guardians of children, prior to study enrolment. Ethical approvals were obtained from the ethical committees in Zanzibar at the time of the trials, the Regional Ethics Committee in Stockholm, Sweden and the Medical Ethics Committee at Karolinska Institutet, Stockholm, Sweden.

5.3 DIAGNOSTIC TOOLS

5.3.1 Blood slide microscopy

Examination of thick blood smears was conducted by experienced microscopists in Zanzibar according to standard WHO procedures [221], [Bjorkman and Shakely *et al.*, submitted]. Thick blood films were stained with 5% Giemsa for 30 minutes and parasite densities were calculated against 200 white blood cells. If fewer than ten parasites were detected, examinations were extended to 500 white blood cells. Blood slides were considered negative if no asexual parasites were found in 200 high-power fields. Quality control (independent second reading) was conducted for all positive slides as well as 10% of the negative slides. Blood slide microscopy was used in the cross-sectional surveys conducted in 2005 and 2009 (Study IV).

5.3.2 RDT devices

Three RDT devices have been used in this theses: Paracheck-Pf® (Orchid Biomedical Systems, India), SD-Bioline Malaria Ag P.f/Pan® (Standard Diagnostic, Republic of Korea), and *First Response* Malaria Ag Combo (pLDH/HRP2) (Premier Medical Corporation Ltd., India).

Paracheck-Pf, a *Pf*HRP2 based RDT, has been widely used in sub-Saharan Africa, and was the first RDT to be implemented in Zanzibar in 2006. Zanzibar recently changed to SD-Bioline P.f/Pan, as this test also detects species other than *P. falciparum*. Both SD-Bioline P.f/Pan and *First Response* Malaria Ag Combo (pLDH/HRP2) are combo RDTs detecting

both *Pf*HRP2 and pLDH. RDTs were conducted according to the manufactures' instructions in Study II, IV and V.

5.4 DNA EXTRACTION METHODS

5.4.1 DNA extraction from dried blood spots on filter paper

5.4.1.1 Sample preparation

Filter paper disc/discs of Ø 3 mm with blood saturated on both sides (≈ 3-5 µL blood/disc) was/were cut into 1.5 ml safe-lock tubes with manual paper punchers rinsed in water and ethanol in between samples. Extracted genomic DNA was stored at -20°C until use (Studies I-IV).

5.4.1.2 ABI PRISM 6100 extraction

ABI PRISM 6100 Nucleic Acid PrepStation™ with NucPrep reagents is a system that uses 96-well filtration plates containing a glass fibre membrane, and a small benchtop vacuum manifold (Applied Biosystems, USA). Samples were lysed with Proteinase K. Cell lysates were passed through the glass fibre membrane, one sample per well. The membrane was washed, and high quality DNA eluted in a two-step elution buffer system. The yield of DNA is 70-90% on a per cell basis and is optimal for 150µL fresh or frozen blood. DNA was extracted from three 3mm filter paper discs (≈ 15µL blood) following the protocol for "isolation of DNA from fresh or frozen whole blood" [234] with some minor modifications [235]. The final DNA containing elution volume was 200µL (Study I and II).

5.4.1.3 Chelex-100 extraction

Chelex-100 is a boil and spin method with the addition of Chelex-100, a chelating resin that binds cations such as Mg^{2+} protecting the DNA from degradation by DNAases. Samples were lysed in Saponin. DNA was extracted from one 3mm filter paper disc as described by Wooden *et al.* (1993) [236] with minor modifications. The final DNA containing elution volume was ~90µL (after deduction of 10% Chelex-100 from a total volume of 100µL) (Study III and IV).

5.4.1.4 QIAmp DNA mini kit

The QIAamp DNA mini kit (Qiagen Gmbh, Germany) provides silica membrane-based nucleic acid purification with a spin-column procedure. Recovery of DNA from older samples has been shown to be somewhat improved by the use of commercial DNA purification columns [231]. Samples were lysed with Proteinase K. DNA was extracted from three 3 mm filter paper discs according to the protocol "DNA purification from dried blood spots" [237]. The final DNA containing elution volume was 150µL (Study IV, for re-extraction of samples collected in 2005).

5.4.2 DNA extraction from RDT

5.4.2.1 Sample preparation

RDT cassettes were opened using a thin metal spatula. The nitrocellulose strip, containing the equivalent of ~5µL blood, was held at the buffer pad with forceps and cut into 3 x 3 mm pieces using scissors. The forceps and scissors were washed in 70% ethanol and dried on clean tissue paper in between each sample to minimise cross-contamination during sample preparation. RDT preparation was in accordance with WWARN guidelines [238]. Extracted genomic DNA was stored at -20°C until use (Study II and IV).

5.4.2.2 Simple elution

The simple elution method for DNA extraction from RDTs involves boiling the proximal part of the nitrocellulose strip in molecular grade water followed by centrifugation [37]. Samples are lysed in the buffer provided with the kit during the performance of the RDT. DNA is captured on the proximal end of the nitrocellulose membrane separating the DNA from the cell lysate that contains PCR inhibitors such as haem. This crude method of DNA extraction works better for some RDTs than others; a plastic film covering the nitrocellulose strip seems to hamper the release of DNA during boiling. The final DNA containing elution volume was 50µL (Study II).

5.4.2.3 ABI PRISM 6100 extraction

ABI PRISM 6100 extraction from RDTs is based on the same protocol as for filter papers, but with some further modifications involving lyses of the biological samples in three-fold volume of NucPrep reagents, and separating the solid material from the lysate by passing the content through a 5 mL syringe. The final DNA containing elution volume was 200µL (Study II).

5.4.2.4 Chelex-100 extraction

Chelex-100 extraction from RDTs is based on the same principle as for filter papers, involving an overnight incubation in saponin followed by boil and spin with the addition of Chelex-100 [239]. The final DNA containing elution volume was ~190µL (after deduction of 5% Chelex-100 from a total volume of 200µL) (Study II and IV).

5.4.3 DNA extraction from whole blood

5.4.3.1 Simple boil and spin

The simple boil and spin method was used for extraction of DNA from 60mL whole blood in conjunction with LAMP according to protocol [240]. This crude method of extraction works well with LAMP, as LAMP is less prone to inhibition than PCR [79] (Study V).

5.5 MOLECULAR METHODS

5.5.1 PCR for detection of *Plasmodium* species

5.5.1.1 Nested PCRS

18S rRNA nested PCR (18S-nPCR): Snounou *et al.* (1993) [54, 55] established one of the earliest nested PCR methods targeting the 18S rRNA sequences of *Plasmodium* species. This method allows for *Plasmodium* species detection using pan-*Plasmodium* primers, and *Plasmodium* species determination (in pan-*Plasmodium* PCR positive samples) by four additional PCRs using species-specific nested primers (Study II and III).

Cytb nested PCR (cytb-nPCR): Steenkeste *et al.* (2009) [57] established a nested PCR targeting the *cytb* gene of the four major human *Plasmodium* species. This method was further modified by Hsiang *et al.* (2010) [52] for use of restriction fragment length polymorphism (RFLP) analysis with AluI enzyme for species determination (Study II and III).

5.5.1.2 Gel electrophoresis

Nested PCR products were visualised under UV transillumination after gel electrophoresis on 1.5-2.5% agarose stained with ethidium-bromide or GelRed, and documented with a GelDoc™ system (Study I-IV).

5.5.1.3 Real-time quantitative PCR

18S rRNA probe-based real-time PCR (18S-qPCR-R): Rougemont *et al.* (2004) [62] developed a probe-based qPCR targeting the 18S rRNA sequences of *Plasmodium* species. *Plasmodium* species were detected using pan-*Plasmodium* primers and probe. PCR positivity was defined as quantification cycle (Cq) values below 40 (Study II and III). The method also allows for species determination by additional qPCRs using species-specific probes, and was adapted for multiplexed detection of single and mixed-species infections by Shokoples *et al.* (2009) [63] (Study II).

18S rRNA probe-based real-time PCR (18S-qPCR-K): Kamau *et al.* (2011) [61] also developed a genus-specific probe-based qPCR targeting the 18S rRNA sequences of *Plasmodium* species. PCR positivity was defined as Cq values below 40. This method is designed for pan-*Plasmodium* detection only, and does not allow for species determination (Study II and III).

Cytb-qPCR assay (cytb-qPCR): We developed a SYBR Green real-time PCR restriction fragment length polymorphism assay targeting the *cytb* gene of the four major human *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) [Xu *et al.*, in press]. Positivity was confirmed by gel-electrophoresis on a 1.5% agarose gel stained with GelRed. *Plasmodium* species was determined by RFLP on positive cytb-qPCR products (Study III and IV).

5.5.2 Quantitative PCR

5.5.2.1 Parasite densities estimated by qPCR

Parasite densities were estimated by a modified version of the 18S-qPCR-K (described above) [61]. In brief, positive samples were subjected to the 18S-qPCR-K using published protocol, primers and probe. Parasite densities were determined against a standard curve generated by a Chelex-100 extracted, 10-fold *P. falciparum* and *P. malariae* dilution series (10,000-1 p/µL) spotted on filter paper (Study IV).

5.5.2.2 Determining Pfmdr1 copy number

Pfmdr1 copy number has been associated with resistance to mefloquine, lumefantrine and artemisinins [116]. *Pfmdr1* copy number was determined by the comparative ΔΔCq method following a TaqMan® probe-based real-time multiplex qPCR, with a Cq cut-off of 35 [124]. The multiplex qPCR amplified the target gene (*pfmdr1*) and a reference gene (*β tubulin*) which is present in only one copy. A strain of *P. falciparum* with a single copy of *pfmdr1* (3D7) was used as a calibrator and a strain with more than one copy of *pfmdr1* (Dd2) was used as a multicopy control. *P. falciparum* with multiple copies of *pfmdr1* are rare in East Africa [241]; the method was used in Study II as an assessment of the quality of the extracted DNA.

5.5.3 Nested PCR-RFLP for genotyping of SNPs

Samples containing *P. falciparum* were genotyped for SNPs associated with amodiaquine resistance at positions *pfcrt* K76T, *pfmdr1* N86Y, Y184F and D1246Y by previously described nested PCR-RFLP methods [242-244]. Lab clones 3D7, Dd2 and 7G8 were used as positive and negative restriction controls (Study I, II and IV).

5.5.3.1 Mixed infections

An infection was defined as mixed if both alleles were present at a particular locus during RFLP. Mixed infections were dealt with differently in each study. In Study I a mixed infection was considered to contain two *P. falciparum* strains, each contributing one allele to the wild type and mutant allele frequency. This method may be biased since the multiplicity of infection (MOI) was unknown. In Study II the mixed infections were analysed as a separate group. In Study IV SNP frequencies were defined as the proportion of isolates containing alleles associated with antimalarial drug resistance (including mixed infections).

5.5.4 Other molecular methods

5.5.4.1 Nested PCR and sequencing for screening pfkelch13 SNPs

A nested PCR protocol published by Ariey *et al*. (2014) [152] was used to amplify a 850bp fragment of the kelch propeller domain with Q5 high-fidelity polymerase (New England Biolabs, UK) [152]. Direct Sanger sequencing of the PCR product was performed by Macrogen in the forward direction only (Additional results).

5.5.4.2 Microsatellite analysis

Microsatellite analysis was used to estimate the MOI, i.e. the number of different *P. falciparum* strains present in a single infection, and to estimate the heterozygosity as a measure of genetic diversity. Seven previously published microsatellites were analysed by capillary electrophoresis [245, 246] (Study IV).

5.5.4.3 Loop-mediated isothermal amplification (LAMP)

LAMP was conducted by technicians in Zanzibar with limited or no experience of molecular methods. The technicians were provided with three and a half days of training. DNA was extracted by the boil and spin method [240]. The LAMP assay was performed with Loopamp™ MALARIA Pan/Pf Detection Kit (Eiken Chemical Company, Japan) as per protocol [240]. Samples positive for Pan-LAMP were retested with Pf-LAMP (Study V).

5.6 BIOINFORMATICS

The web-based software Primer3 v.0.4.0 (http://frodo.wi.mit.edu/) was used for designing primers targeting a conserved area in the *cytb* gene of the four major human *Plasmodium* species for the cytb-qPCR assay. The NCBI primer-blast function (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to assess primer specificity (Study III). PlasmoDB version 9.3 (http://plasmodb.org/plasmo/) and the NCBI database (http://www.ncbi.nlm.nih.gov/) were used to obtain gene sequences. Sequencer version 5.0 (Gene Codes Co, Arbor, MI) and ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were used to align DNA sequences, primers and probes (Study II and Additional results).

5.7 STATISTICAL ANALYSES

Statistical analyses were conducted using SigmaPlot® 11.0 (Systat Software Inc, USA) or Stata/SE 12.0 (StataCorp LP, USA). Statistical significance was determined as $p<0.05$ in all studies.

Table 3: Summary of methods used in each study.

	Study I	Study II	Study III	Study IV	Study V
Sample collection	Aco I and RDT-ACT studies	Dilution series and RDT-ACT study	Dilution series and 2011 cross-sectional survey	2005, 2009, 2011 and 2013 cross-sectional surveys; RDTs collected at health facilities	LAMP deployment study
Sample material	Filter paper	Filter paper and RDTs	Filter paper	Filter paper and RDTs	Whole blood
DNA extraction	ABI PRISM	Simple elution; Chelex-100; ABI PRISM	Chelex-100	Chelex-100; Qiamp DNA mini kit	Simple boil and spin
PCR for detection of *Plasmodium*		18S-nPCR; cytb-nPCR; 18S-qPCR-K; 18S-qPCR-R	18S-nPCR; cytb-nPCR; 18S-qPCR-R; 18S-qPCR-K; Cytb-qPCR	Cytb-qPCR	
PCR for genotyping resistance markers	Nested PCR-RFLP for genotyping SNPs	Nested PCR-RFLP for genotyping SNPs; qPCR for *pfmdr1* copy number		Nested PCR-RFLP for genotyping SNPs	
Other methods		RDT	Bioinformatics	Microscopy; RDT; qPCR for parasite densities; microsatellite analysis	RDT and LAMP

6 RESULTS AND DISCUSSION

6.1 STUDY I

Decreased prevalence of *Plasmodium falciparum* resistance markers to amodiaquine despite its wide scale use as ACT partner drug in Zanzibar

Gabrielle Fröberg, Louise Jornhagen, Ulrika Morris, Delér Shakely, Mwinyi I. Msellem, José P. Gil, Anders Björkman and Andreas Mårtensson

The aim of this study was to assess whether prolonged use of ASAQ as first-line antimalarial treatment selects for *P. falciparum* SNPs associated with resistance to the ACT partner drug amodiaquine. This was assessed by comparing the prevalence of *pfcrt* 76T, *pfmdr1* 86Y, Y184 and 1246Y SNPs and the *pfcrt* K76T / *pfmdr1* N86Y, Y184F, D1246Y haplotypes in 2002-2003, before the implementation of ASAQ as first-line treatment, and seven years after wide scale use of ASAQ in 2010. Prevalences were compared by chi-square test.

The main findings from this paper include:

- A significant decrease in the prevalence of *pfcrt* 76T (96–63%; p<0.001), *pfmdr1* 86Y (75–52%; p<0.001), Y184 (83–72%; p=0.024), 1246Y (28–16%; p=0.020), despite wide scale use of ASAQ (Figure 6).
- A significant decrease in the most common *pfcrt* K76T / *pfmdr1* N86Y, Y184F, D1246Y haplotypes *pfcrt/pfmdr1* TYYD (46–26%; p<0.001) and TYYY (17–8%; p=0.017).
- A significant increase of *pfcrt/pfmdr1* KNFD (0.4–14%, p<0.001) and KNYD (1–12%; p<0.001) haplotypes.

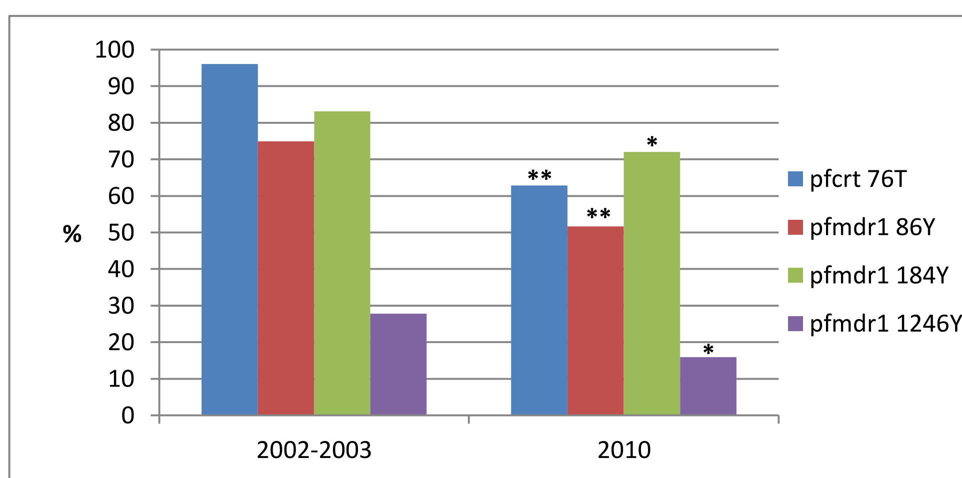

Figure 6: SNP frequencies in Zanzibar before (2002-2003) and seven years after (2010) ASAQ implementation. Asterisk (*) and (**) indicate p values below 0.05 and 0.001, respectively.

Four hypotheses were put forward as potential contributing factors to these results:

- Genetic dilution by imported *P. falciparum* parasites from mainland Tanzania [247]. The current first-line treatment in Tanzania is AL, which has shown to select the opposite alleles as ASAQ, i.e. *pfcrt* K76, *pfmdr1* N86, 184F and D1246 [116].
- Selection by artesunate *per se*. *Pfcrt* K76, *pfmdr1* N86 and D1246 have been associated with decreased susceptibility to artemisinins *in vitro*, but not *in vivo* [146-149].
- Fitness costs of SNPs associated with amodiaquine resistance. Malaria transmission was higher in 2002-2003 when chloroquine was the first-line treatment. The higher frequency of malaria treatments and higher re-infection rates resulted in a greater drug pressure for selection of SNPS. In 2010 the malaria prevalence was lower, with lower treatment frequency, and reduced risk of re-infections after ASAQ treatment. The fitness cost of the SNPs and TYYD/TYYY haplotypes would have been greater in the new epidemiological context.
- Finally, the association between these SNPs and amodiaquine resistance may not be strong enough for selection of mutations after wide scale use of ASAQ. The rapid reduction in parasite load and gametocytocidal properties of artesunate may protect against the selection of SNPs by reducing the exposure to amodiaquine and by reducing transmission of resistant parasites [214]. In a later study, a pooled analysis of individual patient data, none of the analysed *pfcrt* or *pfmdr1* parasite genotypes were significant risk factors for recrudescent infections (treatment failures), but *pfmdr1* 86Y and 1246Y were selected in re-infections after treatment with ASAQ [116], confirming this hypothesis.

A decrease in the prevalence of SNPs previously associated with amodiaquine resistance was observed. ASAQ as first-line treatment did not selected for SNPs associated with amodiaquine resistance in febrile patients, despite seven years of wide scale use. These unexpected results may indicate sustained efficacy of this ACT [248] as first-line treatment in Zanzibar, but require more elaborate studies of temporal trends of molecular markers associated with ASAQ resistance both among symptomatic and asymptomatic *P. falciparum* infections, to improve the understanding of the observation.

A limitation of this study includes the way mixed infections were dealt with in the analysis of SNPs. Mixed infections were considered to contain two *P. falciparum* strains, contributing with one of each SNP alleles during PCR-RFLP. However, since the true MOI was unknown, this could potentially introduce a bias, especially since the MOI was likely to be higher in 2002-2003 due to higher transmission than in 2010. Ideally the analyses should have been repeated after removing the mixed infections to ensure that these were not biasing the results.

6.2 STUDY II

Rapid diagnostic tests for molecular surveillance of *Plasmodium falciparum* malaria - assessment of DNA extraction methods and field applicability

Ulrika Morris, Berit Aydin-Schmidt, Delér Shakely, Andreas Mårtensson, Louise Jörnhagen, Abdullah S. Ali, Mwinyi I. Msellem, Max Petzold, José P. Gil, Pedro Ferreira and Anders Björkman

The aim of this study was to compare and evaluate different methods of DNA extraction from used RDTs and to assess whether RDTs collected under field conditions could preserve *Plasmodium* DNA for the purpose of molecular malaria surveillance. Three methods of DNA extraction were compared under laboratory conditions in two RDT devices (Paracheck-Pf® and SD Bioline Malaria Pf/Pan®). Paired RDT (Paracheck-Pf) and filter paper (Whatman® 3MM) blood samples (734 RDT negative and 121 RDT positive samples) collected from febrile patients in Zanzibar were used to assess the field applicability. RDT positive samples were genotyped at single nucleotide polymorphisms (SNPs) in *pfmdr1* and *pfcrt* as well as for *pfmdr1* copy number. Genotyping outcomes were compared by kappa (κ) analysis and qPCR Cq values were compared by Wilcoxon rank-sum test.

The main findings from this paper include:

- The DNA detection limit varied with RDT device and extraction method. Chelex-100 extraction performed best for all extraction matrixes (table 4). Increasing the size of the fragment of the nitrocellulose strip did not generally improve the level of detection. DNA extraction from RDTs was equal to or better than an equal volume (5µL) of blood spotted on filter paper.
- There was no significant difference in PCR detection rates in DNA extracted from RDT and filter paper. In total 118 (13.8%; CI95% 11.4-16.2) field samples were PCR positive in both RDTs and filter paper (κ=0.94).
- There were no significant differences in the PCR success rates or the genotyping outcomes for the respective SNPs in the 121 RDT positive samples (Figure 7). There was no significant difference in the ability to detect mixed infections or in the Cq values (when assessing *pfmrd1* copy number) in the RDT and filter paper samples.

Table 4: Summary of RDT-DNA extraction sensitivity (parasites/µL) under laboratory conditions. § = not determined due to negative results.

	Paracheck-Pf	SD Bioline Pf/Pan	Filter paper
Simple elution	§	2	200
Chelex-100	2	2	2
ABI 6100	20	20	200

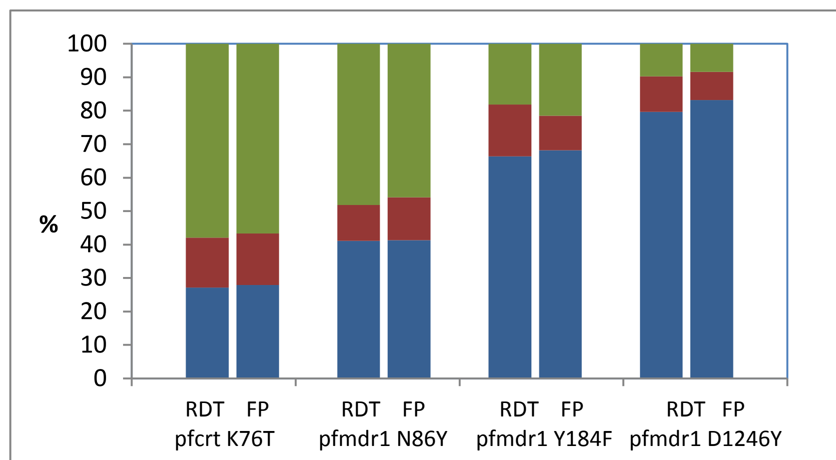

Figure 7: Genotyping outcomes for RDT and filter paper extracted DNA at SNPs in *pfmdr1* and *pfcrt*. Blue colour represents *pfcrt* K76, *pfmdr1* N86, Y184 and D1246; red represents mixed infections; green represents *pfcrt* 76T, *pfmdr1* 86Y, 184F and 1246Y.

We provided a comprehensive comparative study of three DNA extraction methods and found that the method, the design of the RDT, and the choice of PCR, all affected the sensitivity of extraction:

- Simple elution, although cheap, did not work well for DNA extraction from Paracheck-Pf and its use is maybe limited by RDT design and choice of PCR.
- Chelex-100 extraction, a relatively inexpensive but moderately labour-intensive method, preformed best in all extraction matrixes and could be particularly suitable for low-density infections. One concern is the storage capacity of Chelex-100 extracted DNA which may be more susceptible to DNA degradation during freeze-thawing.
- The ABI 6100 extraction method had higher sensitivity, but provided high quality DNA although at a substantial cost and requiring specialised equipment. The ABI 6100 method provided adequate results for the analysis of molecular markers of drug resistance in RDT positive fever patients.

RDTs delivered DNA of equal quality as filter papers suggesting that RDTs are a valuable alternative for DNA storage under field conditions. RDTs offer multiple logistical advantages during sample collection, such as providing diagnosis and blood sample from the same finger prick and providing protection from cross-contamination by its plastic case. However, RDTs provide limited amount of biological material (usually 5µL of blood compared to 50-100µL on filter paper) and no possibility for re-extraction. Wide scale collection of RDTs is being implemented as an integral part of molecular surveillance of malaria in Zanzibar.

A limitation with this study is the use of detection limits as a surrogate for DNA concentration when comparing the DNA extraction methods under laboratory conditions, and that the different extraction volumes were not taken into consideration. The Cq values provided by the 18S-qPCR-K method showed inconclusive results when comparing the Chelex-100 and ABI extracted samples. Ideally the *in vitro* experiments should have been repeated and analysed by qPCR in triplicate.

6.3 STUDY III

SYBR Green real-time PCR-RFLP assay targeting the *Plasmodium* cytochrome b gene – a highly sensitive molecular tool for malaria parasite detection and species determination

Weiping Xu, Ulrika Morris, Berit Aydin-Schmidt, Mwinyi Msellem, Delér Shakely, Max Petzold, Anders Björkman and Andreas Mårtensson

The aim of this study was to evaluate the performance of a new, highly sensitive SYBR Green qPCR targeting the *cytb* gene of the four major human *Plasmodium* species, and RFLP assay for species determination (cytb-qPCR). The cytb-qPCR was primarily developed for detection of low-density malaria infections, in DNA extracted with Chelex-100 from samples collected on filter paper. The performance of the cytb-qPCR was evaluated against four reference PCR methods [56, 57, 61, 62] under laboratory conditions, and in 2977 samples collected in a cross-sectional survey conducted 2011 in Zanzibar. Field samples were defined as 'final positive' if positive in at least two of the five PCR methods. The individual PCRs were compared against the 'final positive' samples by McNemar's exact test and kappa analysis; sensitivity and specificity were also calculated.

The main findings from this study include:

- The cytb-qPCR performed equal to, or better than the reference PCR methods, with a parasite detection limit of 1-2 parasites/µL, robust PCR amplification efficiency (98.2-110.4%), a sensitivity of 100% (CI95% 94.5-100%) and specificity of 99.9% (CI95%, 99.7-100%), and could reliably determine *Plasmodium* species by RFLP.
- The cytb-qPCR was proven specific by gel electrophoresis, primer-blast analysis of the cytb-qPCR primers against the NCBI nucleotide and chromosome database, and in malaria negative samples.
- The cytb-qPCR had 100% reproducibility in parasite densities ≥10 parasites/µL. For parasite densities of 1 or 2 parasites/µL, the cytb-qPCR was reproducible in four to six out of eight replicates.
- Comparable detection limits to those previously reported with the 18S-qPCR-K and cytb-nPCR (0.05 and 0.075 parasites/µL, respectively) were obtained with the cytb-qPCR with one fortieth of the biological material (3-5µL blood as opposed to 200µL in extraction from whole blood).
- There was high level of agreement (κ=0.86-0.98), high sensitivity (89.2-100%) and high specificity (99.6-100%) between the 18S-qPCR-K, cytb-nPCR, cytb-qPCR and the 'final positive' samples. The 18S-nPCR found a significantly lower number of PCR positives ($p<0.01$) resulting in a lower sensitivity (49.2%; CI95% 36.6-61.9). The 18S-qPCR-R had a significantly higher number of PCR positives ($p<0.01$) resulting in a lower specificity (86.6%; CI95% 85.3-87.8).

- The discrepancies between the PCR methods may be explained by variations in PCR product size, target gene copy number and PCR primer and probe binding sites. The 18S-nPCR has a substantially larger first PCR product which may reduce the PCR efficiency. The *cytb* gene (present in mitochondrial DNA) is likely to have a higher copy number than 18S rRNA genes, and mitochondrial DNA may be better preserved than genomic DNA. The low specificity of the 18S-qPCR-R may be due to unspecific amplification and/or probe degradation at high quantification cycle numbers. And finally, it was observed in the sequence alignments of the 18S rRNA genes that the species-specific 18S-nPCR and 18S-qPCR-R only targeted two to three copies of 18S rRNA genes, potentially reducing the sensitivity of these species-specific PCRs, whilst the cytb PCRs targeted conserved regions of the four major human *Plasmodium* species equally.

Although the cytb-qPCR performed well, there are several limitations with this method and study:

- Importantly the cytb-qPCR requires gel-electrophoresis for determining positive samples and RFLP for species determination. An ideal method would both detect and determine species in a single qPCR. This was not possible for the cytb-qPCR due to the high AT content of the amplified product, precluding the use of melting temperatures to determine positive samples and *Plasmodium* species.
- There is no optimal statistical tool for comparing multiple methods. In the absence of an appropriate gold standard a conservative approach was used; the individual PCR methods were compared against ‘final positive’ samples defined as being positive in at least two of the five PCRs.
- Finally, the detection limits were based on dilution series of blood samples with parasite densities determined by microscopy. Although rigorous methods were applied to obtain as accurate parasite densities as possible, the accuracy of assessing the detection limits may be doubtful. Perhaps the use of standard dilution series with defined parasite densities [249], or exact copy numbers of plasmid DNA could have circumvented this. However, the comparative analytical sensitivity between the methods still stands for DNA extracted from filter paper.

6.4 STUDY IV

Characterising temporal trends in asymptomatic *Plasmodium* infections and transporter polymorphisms during transition from high to low transmission in Zanzibar

Ulrika Morris, Weiping Xu, Mwinyi I. Msellem, Alanna Schwartz, Ali Abass, Delér Shakely, Jackie Cook, Achuyt Bhattarai, Max Petzold, Bryan Greenhouse, Abdullah S. Ali, Anders Björkman, Gabrielle Fröberg, Andreas Mårtensson

The aim of this study was to describe temporal trends in the asymptomatic *Plasmodium* reservoir during the transition from high to low transmission in Zanzibar. Healthy individuals participating in cross-sectional surveys conducted in 2005, 2009, 2011 and 2013 were screened for asymptomatic malaria by cytb-qPCR. Infections were characterised with regards to *Plasmodium* species, geographic- and age distribution, qPCR-determined parasite densities, complexity and diversity of infection, and temporal trends in *P. falciparum* SNPs associated with amodiaquine resistance. See manuscript for detailed description of the statistical analyses.

The main findings from this study include:

- PCR-determined parasite prevalence declined from 21.1% (CI95%17.4-24.9) to 2.3% (CI95%1.7-2.9) from 2005-2013. As the prevalence of malaria declined, the proportion of subpatent infections increased (64.3%-91.2%).
- *P. falciparum* remained the predominant species (78.5-100% of infections) followed by *P. malariae* (12.2-43.2%). No cases of *P. ovale* or *P. vivax* were identified.
- The prevalence of asymptomatic malaria was higher in Micheweni than in North A for all years, although the difference was not significant in 2013.
- In 2005 children aged 5-15 were most likely to have malaria (OR 3.8; CI95% 1.9-7.4), whereas in 2011 and 2013 the burden was highest in young adults aged 15-25 (OR 3.4; CI95% 1.3-8.8 and OR 3.7; CI95% 1.8-7.5, respectively).
- The majority of asymptomatic infections had parasite densities lower than 10 parasites/µL. Densities ranged from <1-28918 parasites/µL in *P. falciparum* monoinfections and 1-8 parasites/µL in *P. malariae* monoinfections. The median parasite density was significantly higher in 2005 than in the subsequent years ($p<0.01$).
- There was a significant decrease in the MOI from 2.8 in 2005 to 1.7 in 2009 ($p=0.01$). Heterozygosity in the analysed loci was high. F_{ST} analysis did not detect any changes in the population of parasites from one year to another.
- A significant decrease occurred in the asymptomatic samples in the *pfcrt* 76T allele frequency between 2005 and 2013 (99.2-64.7%, $p<0.001$) and in the *pfmdr1* 86Y allele frequency between 2009 and 2013 (89.4%-66.7%, $p=0.03$) (Figure 8).

▪ The *pfmdr1* YYY and YYD haplotypes were significantly more frequent in the asymptomatic samples ($p<0.001$), whereas the *pfmdr1* NFD was more frequent in the symptomatic samples ($p<0.001$) in 2013.

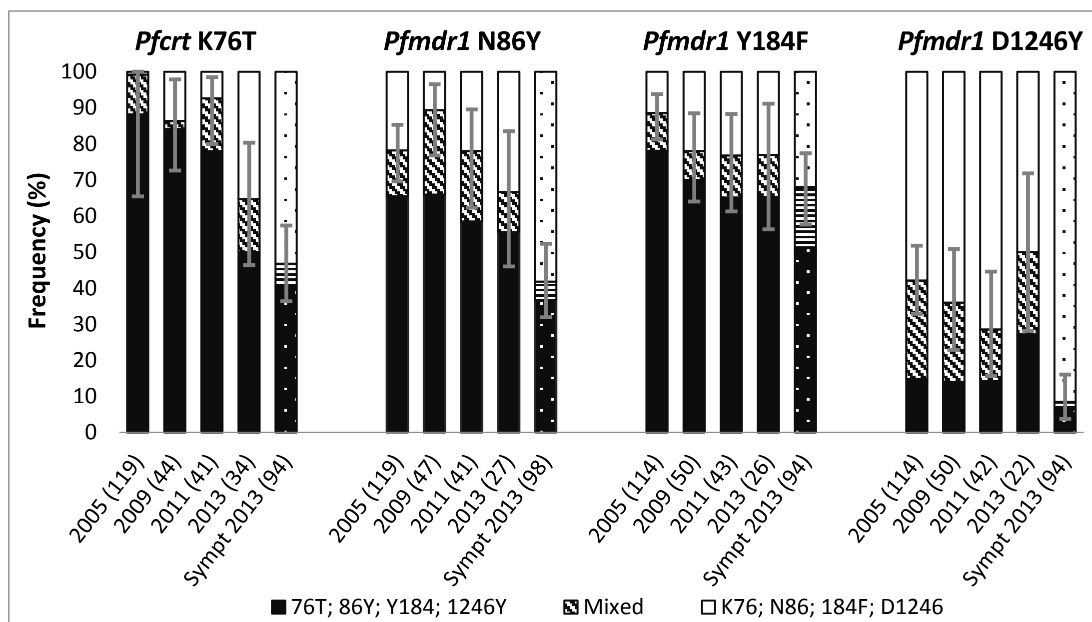

Figure 8: Molecular genotyping of SNPs in *P. falciparum* infections. Clustered, stacked bar chart showing the frequency of polymorphisms associated with amodiaquine resistance. Error bars represent 95% confidence intervals of the proportion of isolates containing resistance alleles (either alone or in mixed infections).

There is a declining, albeit persistent, reservoir of parasites present at low-densities in asymptomatic individuals in Zanzibar. As malaria prevalence declines, the epidemiology shifts resulting in temporal and spatial heterogeneity of malaria with concentration in particular localities and demographic groups. The majority of infections being subpatent shows the importance of implementing sensitive molecular methods for elimination efforts in Zanzibar. The decline in the prevalence of SNPs associated with amodiaquine resistance is similar and equally as intriguing as the results observed in Study I. The higher prevalence of SNPs in asymptomatic than symptomatic infections suggests that asymptomatic infections could be an important reservoir for resistance genes that confer a fitness disadvantage relative to wild type alleles [250]. However, little is known regarding the association between fitness and virulence, and the association is most likely dependent on a complex interplay between parasite fitness, host immunity, transmission intensity and drug pressure.

Some limitations of this study include:

- The cross-sectional design limits the understanding of the history, progression and dynamics of the infections.
- The lack of reliable information on fever, treatment histories and gametocyte carriage provides little insight of the contribution of these infections to malaria transmission.
- Clinical trials are required to assess the true efficacy of ASAQ in Zanzibar.

6.5 STUDY V

Field deployment of loop-mediated isothermal amplification (LAMP) for centralised mass-screening of asymptomatic malaria in Zanzibar, a pre-elimination setting

Ulrika Morris, Mwinyi Khamis, Berit Aydin-Schmidt, Ali Abass, Mwinyi I. Msellem, Majida Hassan Nassor, Iveth J. González, Andreas Mårtensson, Abdullah S. Ali, Anders Björkman, Jackie Cook

The aim of this study was to report results from the hitherto largest implementation of LAMP (N=3983) in the field, for scaled up, centralised mass-screening of asymptomatic malaria. Samples were collected in 60 villages in ten districts throughout Zanzibar. All individuals present and willing to participate in randomly selected households were screened for malaria by RDT. Blood samples for LAMP were collected in every other household. LAMP was conducted in two centralised laboratories in Zanzibar, by trained technicians with limited or no previous experience of molecular methods. The LAMP assay was performed with Loopamp™ MALARIA Pan/Pf Detection Kit (Eiken Chemical Company, Japan). Samples positive for Pan-LAMP were retested using Pf-LAMP specific kits. McNemar's test was used to compare RDT and Pan-LAMP results.

The main findings from this study include:

- This is the largest hitherto reported implementation of LAMP for detection of asymptomatic malaria in a field setting.
- The LAMP testing was centralised in order to scale up the breadth of sampling, meaning samples could be collected from all over the islands with fewer resources with a 24 hour time-to-result.
- Sample collection was conducted during 19 days with an average of 220 samples processed per day in the two laboratories combined. The participation rate was high (96.9%) among subjects present at the time of the survey (Figure 9).
- Pan-LAMP detected 3.4 (CI95% 2.2-5.2) times more *Plasmodium* positive samples than RDT ($p<0.001$). Out of the Pan-LAMP, samples 64.6% (42/65) were also positive by Pf-LAMP.
- Repeatability of LAMP after freezing of diluted DNA was low (63.7%), suggesting that DNA extracted by simple methods such as the boil and spin may not be suitable for long term storage and should be amplified by LAMP within a short period of time.
- During the study DNA contamination of LAMP arose in the central laboratory in Pemba. The contamination was resolved by repetitive decontamination of all equipment and reagents with 5% hypochlorite over 3 days, and moving away from the epicentre of the contamination to a laboratory space available in another building.

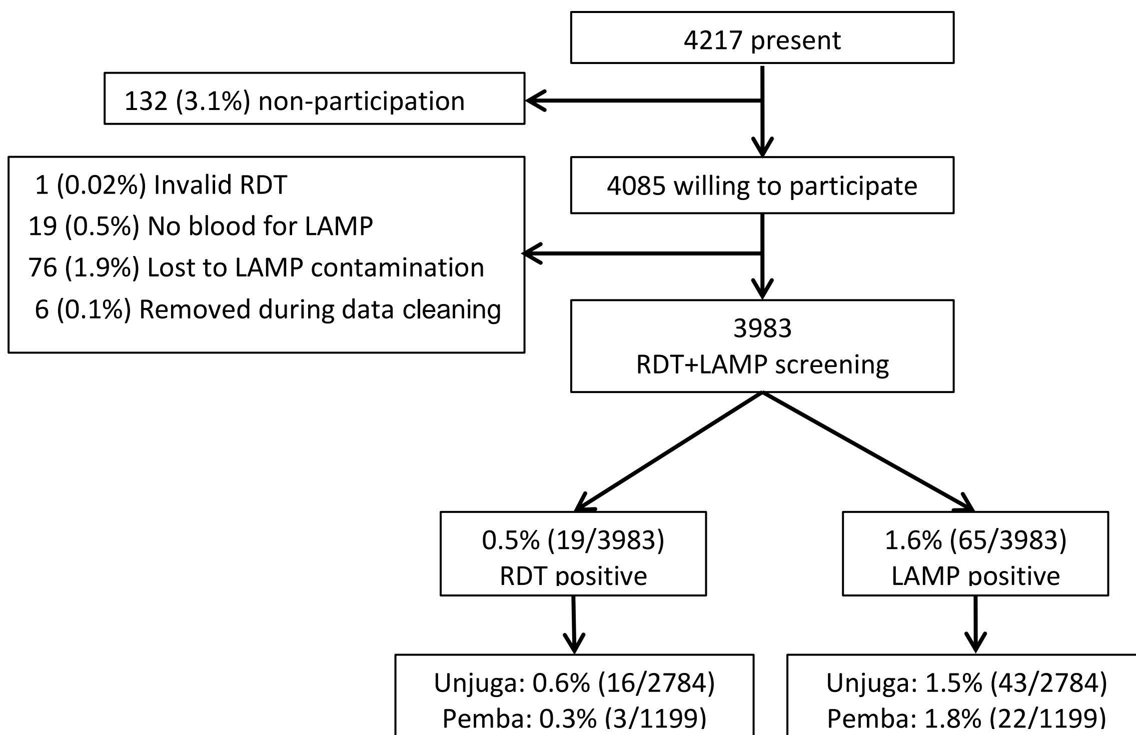

Figure 9: Flow chart of study.

LAMP is a useful and sensitive tool for detection of low-density asymptomatic malaria infections in field settings. The potential risk of contamination with LAMP is high due to the high efficiency of the reaction, although the risk is reduced when using a closed system. Contamination issues have also been experienced in other research settings, but are not well reported; a higher throughput, affordable closed system would be ideal to avoid contamination issues.

Some limitations of this study include:

- A recent study in Zanzibar showed a larger presence of species other than *P. falciparum* than previously reported [216]. A LAMP pilot conducted in Zanzibar in 2013 found that several *P. falciparum* infections positive in Pan-LAMP were missed by Pf-LAMP [88]. Although LAMP is a sensitive tool for detection of low-density infections, it does not provide a very detailed and/or accurate description of the *Plasmodium* species composition.
- DNA extracted from whole blood by the boil and spin method is most likely not suitable for PCR, due to contamination with inhibitors of *Taq* polymerase such as haemoglobin [36]. LAMP can also be used on filter paper samples [83] which may be a preferable matrix for sample collection. Biological material from LAMP positive samples could then be stored and re-extracted for more detailed analysis of, for example, *Plasmodium* species, parasite density, and molecular markers of drug resistance by PCR.

6.6 ADDITIONAL RESULTS

6.6.1 Summary of genotyping data

Figure 10 shows a summary of all genotyping data presented in Study I and Study IV. The initial idea in Study IV was to compare all the available data, but due to the differences in study design between the clinical and cross-sectional surveys (e.g. year and age groups) we opted to only include the comparison between the 2013 asymptomatic and symptomatic samples in the manuscript. I hereby present the additional comparisons which are not presented in the manuscripts, bearing in mind the limitations regarding the differences in study design. SNP frequencies were defined, as in Study IV, as the proportion of isolates containing alleles associated with antimalarial drug resistance (including mixed infections), shown as the combined proportion of the black and striped stacked bars in Figure 10. SNP frequencies were compared by Fisher's exact test.

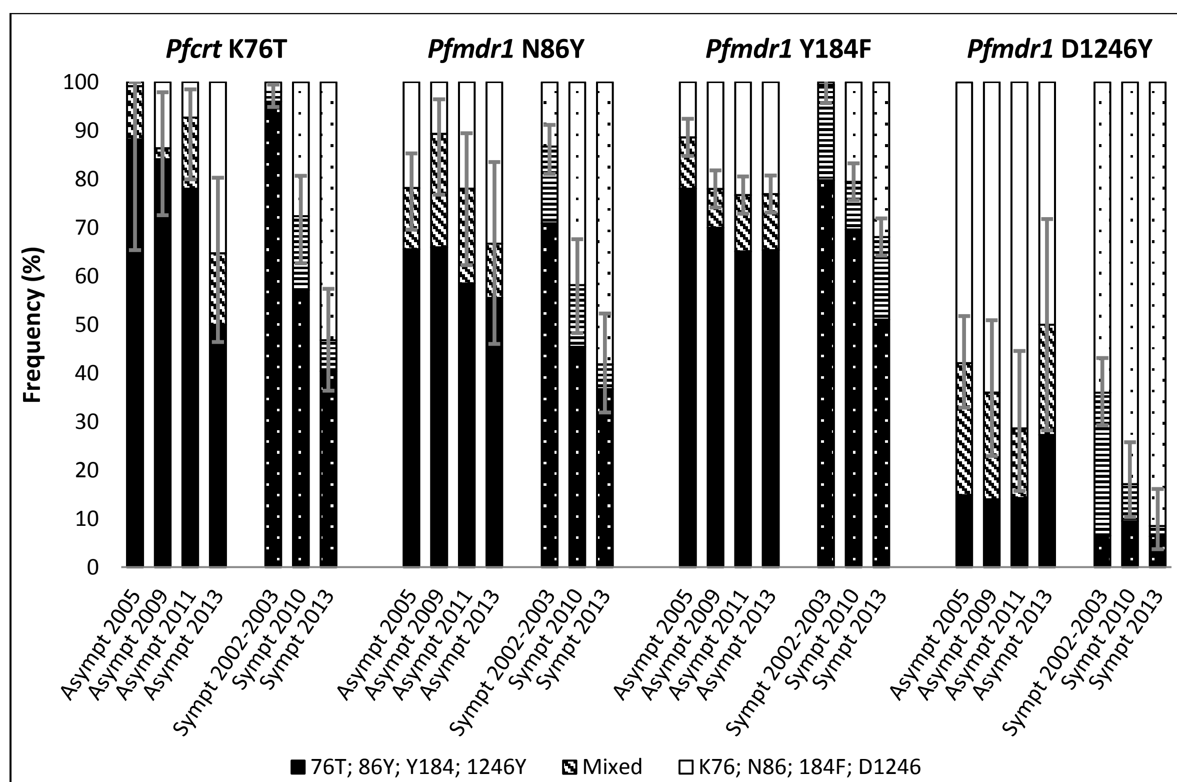

Figure 10: Molecular genotyping of SNPs in asymptomatic and symptomatic *P. falciparum* infections. Clustered, stacked bar chart showing the frequency of polymorphisms associated with amodiaquine resistance. Solid and spotted bars represent asymptomatic and symptomatic infections, respectively. Error bars represent 95% confidence intervals of the proportion of isolates containing resistance alleles (either alone or in mixed infections).

When comparing the baseline data in 2005 (asymptomatic) and 2002-2003 (symptomatic), the only significant difference was a higher prevalence of *pfmdr1* Y184 in the symptomatic samples (99%) compared to the asymptomatic samples (89%) (p<0.001).

In Study I a significant decline in the prevalence of resistance markers was observed in symptomatic fever patients between 2002-2003 and 2010. As described in the limitations of Study I, these results were based on the assumption that mixed infections contained two *P. falciparum* strains each contributing one SNP allele. These reductions remain significant (p<0.001 for all SNPs) when the calculations are repeated with frequencies defined as the proportion of isolates containing alleles associated with antimalarial drug resistance (including mixed infections). However, when mixed infections were removed completely, the significance was lost in *pfmdr1* 1246Y (p=0.8).

The addition of the symptomatic data from 2013 showed a continuation of the trends described in Study I. There was a significant further decrease in the prevalences between 2010 and 2013 in *pfcrt* 76T (72-47%, p<0.001) and *pfmdr1* 86Y (58-42%, p=0.03). There was a decline in the prevalence of *pfmdr1* Y184 and 1246Y but the difference was not significant (79-68% p=0.08 and 17.1-8.5% p=0.09, respectively). The overall decrease from the baseline in the symptomatic samples was stronger (p<0.001 for all SNPs) than in the asymptomatic samples, where a significant decrease was only described in *pfcrt* 76T (p<0.001) since baseline and in *pfmdr1* 86Y after 2009 (p=0.03) (Study IV).

In Study IV the prevalence of SNPs in the asymptomatic and symptomatic samples in 2013 were compared, finding a significantly higher prevalence of *pfmdr1* 86Y and 1246Y in the asymptomatic samples (p=0.03 and p<0.01, respectively). The combined prevalence of the asymptomatic samples collected in 2009 and 2011 (N=117) were compared with the symptomatic samples collected in 2010 (N=122). The frequencies of *pfcrt* 76T, *pfmdr1* 86Y and 1246Y were significantly higher in the asymptomatic samples when compared with the symptomatic samples (p=0.004, p<0.001 and p=0.01, respectively).

In conclusion, it seems that the baseline prevalence was similar between the asymptomatic and symptomatic samples. There has been a decline in the prevalence of SNPs in both the asymptomatic and symptomatic samples over the period of eight or ten years but the decline seems to have been more pronounced in the symptomatic samples. Differences in the fitness cost/benefits associated with these SNPs may affect the rate of reversion of these SNPs to wild type, in the presence of wide scale use of ASAQ. As mentioned in the discussion of Study IV, the differences between the asymptomatic and symptomatic samples are likely to depend on a complex interplay between parasite fitness, host immunity, transmission intensity, and drug pressure, and require further investigations in order to be fully understood.

6.6.2 Screening for mutations in *pfkelch13*

A subset (N=70) of the samples collected on RDTs in health facilities in 2013 in Zanzibar (Study IV) were screened for mutations in the kelch propeller domain. Out of the 70 samples, 34 (49%) were positive in the nested PCR published by Ariey *et al*. (2014) [152]. The 850bp PCR product was sequenced by Sanger sequencing in the forward direction only. Two SNPs were identified in the sequencing data in the initial round of screening (A582D in one sample and Y630stop in one sample). These SNPs were not present after repeating the PCR and sequencing of these samples (unpublished data).

Bearing in mind the small sample size, these results show little evidence of *pfkelch13* polymorphisms in samples collected in Zanzibar. These results are consistent with other studies [166-168] which have shown absence in sub-Saharan Africa of the putative artemisinin resistance mutations in the *pfkelch13* gene which have been described in SE Asia [93, 152, 159].

Although a high fidelity polymerase was used, two potentially false SNPs were observed in the sequencing data. It is important to note that unique SNPs should be validated in order to minimize the risk of reporting false results. Both PCR and Sanger sequencing present a small but inherent risk of introducing single nucleotide errors.

7 CONCLUSIONS

7.1 OVERALL CONCLUSION OF THE THESIS

This thesis shows that highly sensitive molecular methods are required for enhanced malaria infection detection and surveillance of antimalarial drug resistance in malaria pre-elimination settings such as Zanzibar. The application of molecular methods may be of particular interest for malaria control/elimination programs, for monitoring progress towards malaria elimination and for optimal orientation of program activities. Furthermore, intriguing trends in SNPs associated with antimalarial drug resistance, during a decade of wide scale use of ASAQ as the first-line treatment of uncomplicated malaria, were observed in Zanzibar. These trends require further investigation both *in vivo*, with clinical trials, and *in vitro* with further molecular methods.

7.2 SPECIFIC CONCLUSIONS

Study I:

- Seven years of wide scale use of ASAQ as first-line treatment for uncomplicated malaria did not select for SNPs associated with resistance to the ACT partner drug amodiaquine. On the contrary, this study presented a decreased prevalence of *pfcrt* 76T, *pfmdr1* 86Y, Y184 and 1246Y.
- These results indicate sustained efficacy of ASAQ as first-line treatment in Zanzibar. However, the results were unexpected and call for more elaborated studies to improve the understanding of this observation.

Study II:

- Out of the three methods evaluated for DNA extraction from RDTs, the Chelex-100 method proved most sensitive in both RDT and filter paper samples.
- RDTs collected under field conditions were found to be a valuable source of parasite DNA and could reliably preserve parasite DNA for the purpose of molecular epidemiological investigations.

Study III:

- The cytb-qPCR assay performed equal to or better than four reference PCR methods for the detection of low-density malaria infections, in DNA extracted by the Chelex-100 method from samples collected on filter paper.
- The results indicate that the cytb-qPCR may represent an opportunity for improved molecular surveillance of low-density *Plasmodium* infections in malaria pre-elimination settings.

Study IV:

- There is a declining, albeit persistent, reservoir of parasites present at low-densities in asymptomatic individuals in Zanzibar.
- The study revealed important characteristics of the remaining parasite populations, including intriguing temporal trends in molecular markers associated with antimalarial drug resistance.
- The results highlight the need for sensitive molecular methods for identifying and characterising the residual parasite reservoir in malaria pre-elimination settings.

Study V:

- LAMP was found to be a simple and sensitive molecular tool, with potential for use in active surveillance and mass-screening programmes for the detection of low-density asymptomatic malaria in pre-elimination settings.
- LAMP protocols may need to be adapted for processing large numbers of samples; a higher throughput, affordable closed system would be the ideal solution to avoid DNA contamination.

8 PERSONAL REFLECTIONS AND FUTURE PERSPECTIVES

This thesis provides an insight into the application of modern molecular methods in a pre-elimination setting such as Zanzibar. We developed and/or evaluated highly sensitive tools for enhanced malaria infection detection (Study III and Study V) and surveillance of antimalarial drug resistance (Study II). We observed a decrease in molecular markers previously associated with amodiaquine resistance, both in symptomatic (Study I) and asymptomatic individuals (Study IV) in Zanzibar, despite wide scale use of ASAQ over the last decade. I believe these insights are valuable for other pre-elimination settings in sub-Saharan Africa, showing the need for sensitive molecular methods for improved malaria surveillance and control.

8.1 IS MALARIA ELIMINATION FEASIBLE IN ZANZIBAR?

Effective implementation and high uptake of malaria control interventions, such as access to parasitology based diagnosis for all age groups (primarily by RDTs), availability of efficacious malaria treatment free of charge (ASAQ), and universal coverage of LLINs and IRS, has resulted in a rapid transition in Zanzibar from high malaria transmission to pre-elimination during the last decade. These malaria control interventions do not however, address the changes in malaria epidemiology that occur in areas transitioning from malaria control to elimination [199], where both geographically and demographically heterogeneous transmission and higher proportions of asymptomatic and subpatent infections are typical characteristics [205]. These characteristics make finding and targeting remaining parasite populations increasingly more difficult, perhaps explaining why Zanzibar, after an initial rapid decline in parasite prevalence, appears to be in a steady state of persistent low-level malaria transmission [Bjorkman and Shakely *et al.*, submitted]. Zanzibar will require new tools and strategies, including surveillance and response systems that are able to detect and eliminate residual reservoirs of symptomatic and asymptomatic infections, in order to maintain progress towards malaria elimination. Such systems will need more sensitive molecular tools such as nucleic acid amplification based methods for monitoring parasite prevalence, genotyping for surveillance of genetic markers of drug resistance, and methods such as serology for accurate estimations of malaria transmission [198].

The Zanzibar malaria elimination feasibility assessment concluded that malaria elimination is technically feasible but will be operationally complex, with financing being one of the most critical challenges. It was estimated that 2020 was the earliest that elimination could reasonably be achieved, although interventions would need to be sustained for several decades regardless whether the programme aimed for malaria control or elimination [227].

Treatment of all malaria infections with antimalarials reduces malaria transmission, especially if combined with gametocytocidal primaquine. However, asymptomatic malaria in semi-immune populations poses a difficult hurdle in obtaining 100% treatment coverage. In areas like Zanzibar, where immunity still exists, malaria control interventions must be sustained through a transition period long enough for clinical immunity to wane. In settings where there is no or little clinical immunity to malaria, most people who get malaria also get sick and are therefore rapidly treated and elimination becomes more stable [4]. Achieving and sustaining malaria elimination will require adequate and consistent funding. In the 1970's Zanzibar attained very low levels of malaria by IRS with DDT, only to result in explosive resurgence of malaria when partner funds decreased and IRS was stopped [220]. Today 90% of Zanzibar's malaria funding relies on international donors; perhaps increases in domestic financing, endowment funds and earmarked taxes could provide a more secure financing base for ZAMEP [227].

One of the largest threats to sustained malaria elimination is importation of malaria. The technical feasibility assessment suggested that elimination in Zanzibar will require prevention of malaria reintroduction either by reduction of malaria in mainland Tanzania or through border screening of travellers and/or high risk groups [227]. Mobile phone usage data and ferry traffic between Zanzibar and mainland Tanzania, together with mathematical modelling of importation and transmission rates, were used to evaluate malaria importation rates [247]. Local transmission in Zanzibar was estimated too low to sustain transmission in most places, whereas the malaria importation rate was estimated to be 1.6 incoming infections per 1000 inhabitants per year. The study concluded that malaria infections in Zanzibar largely resulted from imported malaria, and that Zanzibar residents travelling to malaria endemic regions were likely to be the single, most important source, contributing 1-15 times more imported cases than infected visitors. Malaria prevalence in mainland Tanzania is 9.2% (in children under the age of 5) and ranges from 3.6% -10.2% in the coastal regions closest to Zanzibar [251]. There are a number of ferry and airline companies travelling to and from Zanzibar, with hundreds of individuals arriving and leaving Zanzibar on a daily basis, not to count the numerous private fishing boats that may not leave via the main ports. This makes border controls almost impossible to establish. Other options could be screening of high risk travel groups, providing free prophylactics to Zanzibar residents travelling to the endemic mainland, and using insecticides in aeroplanes and ferries to stop the importation of mosquitoes. Estimating malaria importation remains technically and operationally challenging [247], but more precise estimates of Zanzibar's importation risks are urgently needed [227].

Due to the perceived threat of malaria importation, the technical feasibility assessment also suggested that intervention coverage needs to remain high (LLIN coverage of 75% or above), even after malaria elimination is achieved [227]. Vectorial capacity is reduced whilst vector control interventions are maintained, but once vector control is relaxed malaria transmission may return to baseline, making resurgence highly probable following reintroduction of malaria [4]. Resistance to pyrethroids also pose a serious threat to the sustained efficacy of vector control in Zanzibar. Furthermore, the near extinction of *An. gambiae s.s.* (usually

attributed to the success of IRS and LLINs against this endophilic and anthropophilic species) has resulted in *An. arabiensis* now being the main species of mosquitoes in Zanzibar [223]. *An. arabiensis* is an efficient malaria vector that feeds indoors and outdoors, on human and non-human hosts, and is not optimally controlled by indoor interventions. Vector control in Zanzibar is likely to need other tools in addition to LLINs and IRS [223].

The 50 countries that successfully eliminated malaria during the Global Malaria Eradication Programme launched in 1955 had higher GDP than countries that did not succeed in elimination [4]. In the 34 WHO listed malaria eliminating countries the gross domestic product has increased by 3.5% per year between 2000 and 2010 [199]. It is clear that economic wealth and urbanisation leads to declining malaria. It is also probable that a decline in malaria leads to economic growth, which in return would help a country maintain elimination once it is achieved [4]. Importantly housing structure improvements associated with accumulating wealth, such as closing of eaves, ceiling installation, window screens and use of tin roofs can prevent or discourage mosquitoes from feeding on humans [4]. I believe economic growth will precede malaria elimination in Zanzibar, and that molecular methods will play an important role in elimination efforts.

8.2 HOW WILL MOLECULAR METHODS ASSIST MALARIA ELIMINATION?

An ideal surveillance system would involve highly sensitive diagnostic tests, frequent malaria incidence counts, defining of geographical and demographic risk groups, real-time data regarding drug resistant parasites and insecticide resistant mosquitoes, accurate monitoring of transmission intensity, and recording of climatic data regarding rainfall. Data collected by mobile phones and SMS messaging, or directly though shared databases, could allow rapid deployment of interventions limiting further transmission, and could also allow data sharing for optimally coordinating activities between areas [196].

In Study II we found Chelex-100 extraction to be the most sensitive method for DNA extraction from RDTs and filter paper, after which this method was used in the following studies. Chelex-100 extraction provides a higher yield of DNA, and is therefore better suited for extraction of samples containing low-density infections, but may not provide DNA of equal quality as column based extraction methods. The DNA-containing supernatant, obtained with Chelex-100 extraction from filter papers, is often yellowish in colour especially if more than one 3mm filter paper punch is used. This suggests contamination of the sample with remaining haemoglobin, which may be inhibitory in some PCRs. The rigorous boiling used in Chelex-100 extraction can enhance DNA degradation during sample processing [231], and Chelex-100 extracted DNA is thought to be more susceptible to degradation during sample freeze-thawing [252]. In Study III we developed a highly sensitive PCR method specifically designed for screening low-density samples collected on filter paper and extracted by Chelex-100. The PCR amplifies a small fragment of DNA, in order to reduce the impact of DNA degradation, and targets the high copy number *cytb* gene, in order to improve

the sensitivity of the method. This method was used in Study IV for screening for malaria in filter paper samples collected in cross-sectional surveys.

It is important to note that PCR detection limits vary with extraction method [49, 253, 254], with PCR protocol [49, 255], and also between laboratories [205, 256, 257]. Parasite densities that are on the cusp of the detection limit can alternate between being PCR positive and negative, and reproducibility of results may be low [207, 216]. Increasing blood sample volume can increase PCR sensitivity [76], but may be difficult to implement with large scale collection of samples in the field. Cross-sectional surveys also only provide a snap shot in time and may underestimate true parasite prevalence [258]. Furthermore, all PCRs presented in these studies were conducted in a research laboratory at Karolinska Institutet. As discussed in Study V, PCR is not yet available in resource limited settings due to the need of expensive, specialised equipment, reagents and know-how [77]. The methods presented here may be useful for research purposes, but most likely not for point-of-care or mass screening in field settings. Some attempts have been made to make PCR more accessible in the field, such as the use of an "in house" mobile laboratory for DNA extraction and real-time PCR screening in Cambodia [259]. The mobile laboratory enabled screening of 5000 individuals in less than 4 weeks (on average 240 samples per day), providing treatment of parasite carriers within 24-48 hours after sampling. Lab-on-chip PCR diagnostics could also overcome some of the challenges of malaria diagnosis and surveillance in the field. This involves a disposable plastic chip with a desiccated hydrogel containing reagents required for *Plasmodium* specific PCR and a low-cost (~2000 US$), portable, real-time PCR machine for DNA amplification. No sample processing is needed with the lab-on-chip, and the study reported a detection limit of 2 parasites/µL blood. Chips could be manufactured to test multiple targets simultaneously such as different pathogens, different species and genetic markers [260].

In Study V we report results from the hitherto largest implementation of LAMP in a field setting, for centralised, mass-screening of asymptomatic malaria. LAMP has several advantages over PCR for field use, as it doesn't require expensive equipment, has faster time-to-result, and provides results that can be read by eye. We employed a field friendly kit, which we found to be highly sensitive and simple to use. LAMP could potentially be adopted by malaria elimination programs for more accurate estimation of parasite prevalence in cross-sectional surveys, and for mass screening and treatment purposes. DNA contamination is however, a problem with nucleic acid amplification based methods that needs to be addressed. We suggest that a higher throughput (e.g. 96 well plate), affordable closed system may help reduce, but still will not eliminate, the risk of contamination. Logistical issues in Zanzibar such as the unreliable supply of power from the mainland also poses unique challenges for wide scale implementation [251]. Adopting the lab-on-chip format for LAMP, that permits diagnosis using a single-use, electricity-free device (e.g. battery driven or heated by an exothermic reaction), could offer a possible alternative [51].

In Study IV we characterise temporal trends in PCR-determined asymptomatic *Plasmodium* infections. We report that there is a declining, albeit persistent, reservoir of parasites present

at low densities. The results highlight the need for sensitive molecular methods for identifying residual parasite reservoirs more accurately. More accurate characterisation of the residual parasite reservoir can also provide important information for malaria control programmes, allowing for reorientation and targeting of control activities. PCR-based testing has revealed a higher proportion of *P. malariae* than previously observed in Zanzibar. We report *P. falciparum* as the predominant species, followed by *P. malaria* which was present in up to 43% of infections in 2009. Other studies in Zanzibar have shown that 40% of PCR-detectable malaria infections contained non-falciparum species [216], including reports of *P. ovale* and *P. vivax* [216, 261]. Zanzibar introduced the use of combo RDTs, that detect all species of malaria, but non-falciparum infections tend to be of lower density and may require more sensitive tools for detection [199]. *P. vivax* and *P. ovale* are very rare in Zanzibar, but an increase in prevalence could pose a problem for elimination efforts [216]. As burdens of *P. falciparum* decrease, malaria eliminating countries may need new strategies to diagnose, treat and interrupt transmission of non-falciparum malaria [199]. *P. vivax* and *P. ovale* hypnozoites are hard to detect when dormant in the liver, and can result in relapses of malaria if not treated correctly. Areas where *P. vivax* and *P. ovale* infections are prevalent may for example, require MDA with primaquine to eliminate infections [219].

Parasite densities are likely to be correlated with transmission intensity, immunity, rates of re-infections and multiplicity of clonal subtypes [206]. In Study IV we found that parasite densities declined after 2005, after which the majority of the PCR detected infections had parasite densities lower than 10 parasites/µL. We also reported a shift in age; in 2005 children aged 5-15 were most likely to have malaria whilst in 2011 and 2013 the burden was highest in young adults aged 15-25. Increasing age has been significantly associated with lower parasite densities, and Okell *et al*. (2012) found significantly higher proportion of subpatent infections in older children and adults when compared to young children. This is most likely explained by greater immunity in older individuals, likely due to cumulative exposure and more developed immune systems [205]. This argument does not however, hold in high transmission settings where parasite densities are greater and the effect of age is overcome by time since last infection [205]. In Study IV we also use microsatellites to estimate the MOI, and found that the MOI declined after 2005 but the genetic diversity remained high. Microsatellites and genetic barcoding can also be used for tracking of parasites to identify the geographic origin of *P. falciparum* strains. This may be especially interesting in tracking the spread of artemisinin resistance [262] and to distinguish local and imported malaria infections [199].

In summary, logistical constraints such as high costs, need for know-how, and the risk of contamination may limit the implementation of molecular methods at a point-of-care level. However, on a programmatic level molecular tools can provide important insights into the characteristics of the remaining parasite population, and will in my opinion be very useful for evaluating malaria elimination efforts and for providing important data that can be used for reorientation of elimination strategies. Treating all malaria infections is a prerequisite for malaria elimination. We have shown that standard malaria diagnostic tools including

microscopy and RDT are not sensitive enough to detect low-density asymptomatic infections. The cost effectiveness of for example deploying LAMP for MSAT needs to be evaluated against MDA, which overcomes the need for sensitive diagnostic tools but may increase the risk of drug resistance. A combination of reactive case detection, based on index cases, and FSAT or targeted MDA could be viable options in areas of low transmission where risk factors of malaria are not well defined [201]. FSAT still requires the use of sensitive diagnostic tools and targeted MDA (employing different classes of antimalarials then those used for individual case management [190] combined with a transmission limiting treatment such as primaquine [199]) requires close surveillance of molecular markers of drug resistance.

8.3 WHAT CAN WE CONCLUDE FROM THE DECLINING PREVALENCE OF DRUG RESISTANCE POLYMORPHISMS IN ZANZIBAR?

We observed a decrease in molecular markers previously associated with amodiaquine tolerance/resistance, both in symptomatic (Study I) and asymptomatic individuals (Study IV) in Zanzibar, despite wide scale use of ASAQ over the last decade. The methodology evaluated in Study II was also implemented in Study IV for drug resistance surveillance using malaria positive RDTs passively collected at health facilities in North A and Micheweni districts in 2013. Allele frequencies in these symptomatic samples were compared with allele frequencies in asymptomatic samples collected during a cross-sectional survey conducted the same year.

The decrease in molecular markers associated with amodiaquine tolerance/resistance, observed in Study I and IV, and the lack of the putative artemisinin resistance mutations in the *pfkelch13* gene reported in the additional results, may support the sustained efficacy of ASAQ as first-line treatment in Zanzibar. However, this conclusion would have to be confirmed by *in vivo* efficacy trials as markers of drug resistance may not predict clinical outcomes [10]. Several hypotheses to explain these results were put forward, including the importation of wild type parasites from the mainland Tanzania, a selection by artesunate *per se*, and the impact of parasite fitness associated with these alleles. Understanding the potential contribution of these hypotheses would require further investigations employing for example, tracking of parasite strains using microsatellite data to understand whether or not the parasites are imported, *ex vivo* assessment of the parasite susceptibility to artemisinins and *in vitro* assessment of parasite fitness. *In vivo* efficacy trials are also useful for assessing increases in parasite tolerance, but this is difficult to conduct in Zanzibar due to the low malaria prevalence, which limits the recruitment of patients and also results in low reinfection rates during the follow-up period. ASAQ has shown to select *pfcrt* 76T and *pfmdr1* 86Y, Y184 and 1246Y after treatment, but little has been reported regarding trends in transporter polymorphisms after prolonged use of ASAQ, and it would be interesting to see if other areas with ASAQ as first-line treatment observe similar trends as in Zanzibar.

ASAQ and AL have shown to select in opposite directions after treatment [116], but the selection pressure seems to be stronger with AL than ASAQ. In contrast to the lack of reported selection of SNPs after long term use of ASAQ, increases of *pfcrt* K76 and *pfmdr1* N86, 184F and D1246 have been reported in areas where AL has been used as first-line treatment [128, 263-265]. AL could be more prone to the selection of resistance, as resistance to the partner drug lumefantrine is associated with wild type alleles, and the spread of resistance is therefore not inhibited by fitness costs of mutations as is for amodiaquine. Furthermore, *pfcrt* K76, *pfmdr1* N86 and D1246 have been associated with decreased susceptibility to artemisinins *in vitro* [146-149], and it could be that artemether and lumefantrine select in the same direction, whilst artesunate and amodiaquine select in opposite directions. This just goes to show the importance of monitoring trends in molecular markers of drug resistance, and also the importance of regular clinical trials to monitor the *in vivo* response, in order to preserve the efficacy of ACTs.

Finally we found that the decline in molecular markers was more pronounced in symptomatic compared to asymptomatic infections (additional results), and that asymptomatic infections had a higher prevalence of SNPs (Study IV and additional results). However, small sample sizes, and the lack of data regarding fever and treatment histories and age of these individuals make it difficult to draw any conclusions from these results. Age related immunity, treatment history and district could be important confounding factors. Similar findings have however, been reported in Uganda and Benin, where the prevalence of wild-type genotypes were higher in symptomatic compared to asymptomatic infections. It has been suggested that asymptomatic infections represent an important reservoir for resistance genes that confer fitness disadvantage relative to wild-type alleles, and may therefore contribute to the epidemiology of drug resistant malaria [250]. We conclude that differences in the ability to carry and clear drug resistant parasites are likely to depend on a complex interplay between parasite fitness, host immunity, transmission intensity and drug pressure.

Parasite isolates can differ in their ability to invade erythrocytes, and consequently in their multiplication rates and number of merozoites they produce. It is conceivable that less fit *P. falciparum* isolates, that harbour mutations associated with significant fitness costs, produce low-density infections that are less likely to be detected by microscopy and more likely to be asymptomatic [65]. At the same time, higher gametocyte densities have been associated with mutations in *pfcrt*, *pfmdr1*, *dhfr* and *dhps* increasing the transmission potential of drug resistant parasites [266]. Host immunity also seems to play an important role in selection of drug resistance. Antimalarial drug resistance generally arises in areas of low transmission where there is little immunity to malaria. In these areas most infections are symptomatic and therefore treated, providing a greater drug pressure than in areas of high transmission; the lack of immunity may also reduce clearance of drug resistant parasites enhancing transmission [95]. In Zanzibar the drug pressure remains low, since the majority of infections are asymptomatic due to residual immunity after a recent decline in transmission. The low drug pressure may contribute to the lack of selection of resistance markers over time. On the other hand, a study in Kenya showed that submicroscopic infections were more common in

individuals reporting the use of non-ACT-based antimalarials in the preceding two weeks compared with individuals not reporting use of antimalarials [267], and data regarding previous use of antimalarials in the asymptomatic and symptomatic individuals would, of course, have been very valuable information. Finally, differences in human genetics in asymptomatic and symptomatic infections could also be a variable explaining the differences in the prevalence of SNPs. Amodiaquine is metabolised to DEAQ mainly by cytochrome P450 2C8 (CYP2C8). CYP2C8 low metaboliser alleles are present at 3.6% in Zanzibar [268]. Interestingly, a study conducted in Zanzibar showed that carriers of the low activity CYP2C8*3 had increased frequency of infections harbouring *pfmdr1* 86Y and 1246Y after treatment with ASAQ, perhaps due to the longer half-life for AQ in these individuals [269].

In conclusion, we report a decrease in molecular markers previously associated with amodiaquine tolerance/resistance, both in symptomatic and asymptomatic individuals in Zanzibar. These results may indicate sustained efficacy of ASAQ as first-line treatment in Zanzibar. However, this would have to be confirmed by *in vivo* efficacy trials, as markers of drug resistance may not predict clinical outcomes. Finally, the decline in molecular markers was more pronounced in symptomatic compared to asymptomatic infections, and asymptomatic infections had a higher prevalence of alleles associated with resistance. Understanding these intriguing observations require further investigation both *in vivo*, with clinical trials, and *in vitro* with further molecular methods.

8.4 FUTURE PERSPECTIVES

We are planning to assess a high throughput version of LAMP developed by FIND in the upcoming cross-sectional survey that will be conducted in May-June 2015. ZAMEP are also in the process of registering single low-dose (0.25 mg/kg) primaquine to be given on the first day of ACT treatment, as per WHO recommendations. A two arm *in vivo* efficacy trial (ASAQ and ASAQ + primaquine) is planned to be conducted in Zanzibar in the near future. This will be logistically challenging, but important to carry out since the last efficacy trial was conducted in 2005.

Other things to consider in Zanzibar may include:

- Evaluation of MSAT with LAMP versus targeted MDA programmes.
- Case-control study design, for identifying risk factors of malaria.
- Routine monitoring of antimalarial drug resistance markers and insecticide resistance in mosquitoes.
- Accurate monitoring of malaria transmission by serology.
- Gaining a better understanding of the transmissibility of low-density infections. What proportion of submicroscopic infections harbour or produce gametocytes, and at what densities? What is the transmissibility of these infections assessed by membrane and skin feeding assays?
- Assessing the risk of malaria importation. Cross-sectional surveys conducted on ferries arriving and leaving Zanzibar could be an interesting study to conduct. This could allow for collecting more accurate information than what can be obtained with mobile phone usage data on malaria parasite importation and travel history.

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