Understanding the contribution of malaria parasites to continued residual malaria in a pre-elimination setting in South Africa: implications for elimination strategies

by

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Summary

Remarkable progress has been made in averting malaria cases and reducing malaria mortality globally. However, an incomplete understanding of the factors driving the continued transmission in pre-elimination settings challenges further successes in malaria control. For the evidence-based deployment of effective strategies targeting the parasite reservoir in humans, a better understanding of the contribution of asymptomatic parasite carriage to continued transmission is necessary. Primaquine (PQ) is a drug that targets and clears mature gametocytes, the transmissible forms of the malaria parasite. However, the implementation of PQ treatment strategies to reduce *Plasmodium falciparum* transmission is rather slow. The perceived safety issue surrounding PQ use in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals and local drug regulations are partly responsible for this slow uptake. When exposed to high doses of PQ, G6PD-deficient individuals risk haemolysis that may be life-threatening. In addition, the human cytochrome P-450 enzymes modulate PQ efficacy. This thesis evaluated locally relevant G6PD-deficiency genotypes and CYP2D6 variants in a pre-elimination setting in South Africa. The low prevalence of these variants supports PQ deployment as a transmission-blocking strategy towards malaria elimination in the country.

To better understand which populations should be targeted, the thesis subsequently investigated the contribution of asymptomatic parasite carriage to continued transmission in South Africa. We focussed on genetically characterising the currently circulating parasites in the Vhembe District in Limpopo Province, an area that continues to experience malaria transmission. While the parasites are homogenous based on genotypes, clear distinctions are shown for “local” parasites in contrast to those “imported” from neighbouring countries. When we analysed drug resistance markers, we observed high levels of resistance to sulphadoxine-pyrimethamine and no evidence for artemisinin resistance in the parasite population. This supports the need for continued surveillance to maintain and prolong the efficacy of the existing drugs. Importantly, this thesis uncovers the presence of apparently chronic low-density *P. falciparum* parasite infections, only detectable by sensitive telomere-associated repetitive element 2 qPCR. Elimination strategies need to consider these low-density infections that may play a role in ongoing transmission.

The transmission potential of (low density) infection depends on the production of mature male and female gametocytes. Mosquitoes need to ingest at least one gametocyte of both sexes to become infected with malaria. The thesis describes the development of a novel multiplex qPCR for detecting *P. falciparum* male and female gametocytes. The *P. falciparum* male gametocyte enriched transcript (*pfmget*, PF3D7_1469900) and female specific (*ccp4*, PF3D7_0903800) mRNA targets were used in qPCR assays on mosquito blood meal

samples. This approach fuelled a pilot study to explore the possibility to use mosquito blood meals as a source of parasite material for xenodiagnoses of malaria, either by qPCR or by rapid diagnostic tests.

Taken together, this thesis addresses several relevant gaps in our understanding of the role of asymptomatic parasite carriers to ongoing transmission in South African settings. The novel data presented in this thesis may contribute to the rational implementation of malaria interventions in South Africa with the ultimate aim of achieving countrywide malaria elimination and ultimately global malaria eradication.

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List of Abbreviations

ACTs	Artemisinin combination therapies
AP	Aminopyridines
CNV	Copy number variants
COI	Complexity of infections
CRT	Chloroquine resistance transporter
CQ	Chloroquine
CYP2D6	Human cytochrome P450 2D6 enzyme
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DDT	Dichlorodiphenyltrichloroethane
E8	Elimination 8
EM	Extensive metaboliser
FACS	Fluorescence-activated cell sorting
FOI	Force of infection
FSAT	Focal screening and testing
FPBS	Filter paper blood spots
G6PD	Glucose-6-phosphate dehydrogenase
GDP	Gross Domestic Product
GFP	Green fluorescence protein
GLURP	Glutamate-rich protein
GMEP	Global malaria elimination programme
GTS	Global technical strategy
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HRP	Histidine rich protein
IFA	Immune-fluorescence assays
IM	Intermediate metaboliser
IMPs	Imidazopyridazines
IRS	Indoor residual spraying
ITNs	Insecticide-treated bed nets
LDH	Lactate dehydrogenase
LysoPC	Lysophosphatidylcholine
MB	Methylene Blue
MDA	Mass drug administration
MAO	Monoamine oxidase
MOI	Multiplicity of infections
MSAT	Mass screening and testing
MSP-1	Merozoite surface protein 1
NAA	Nucleic acid amplification
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHC	Primary healthcare clinic
*Pf*K13	*Plasmodium falciparum* kelch 13
PfMGET	*Plasmodium falciparum* magnetic enriched protein
PM	Poor metabolizer
PI3K	Phosphatidylinositol-3 kinase
PI3P	Phosphatidylinositol-3-phosphate
PQ	Primaquine
QT-NASBA	NASBA Quantitative nucleic acid sequence-based amplification
RCD	Reactive case detection
RDT	Rapid diagnostic tests
RFLP	Restriction fragment length polymorphisms
RT-PCR	Real time polymerase chain reaction
SDS	Sodium dodecyl sulphate

SMC	Seasonal malaria chemoprevention
SNP	Single nucleotide polymorphisms
SP	Sulphadoxine pyrimethamine
sWGA	Single whole genome amplification
TARE-2	Telomere associated repetitive element -2
UM	Ultra-rapid metaboliser
WHO	World Health Organization

Chapter 1

Literature review

1.1 Malaria burden

Malaria continues to impose a tremendous global health burden with 3.2 billion people at risk of malaria and approximately 435 100 malaria deaths reported in 2017 [1]. The majority of these deaths (93%) occurred in Africa. There were an estimated 219 million cases globally, with the World Health Organization (WHO) Africa region accounting for 92% of these cases [1]. The burden of malaria correlates closely with the rate of economic development, with malaria forming an impediment to economic growth in malaria-endemic countries [2]. Nations affected by malaria are among the poorest in the world and it is a vicious cycle, with poverty causing disease, and disease causing poverty. The cost of malaria prevention, treatment and the accompanying loss of productivity due to morbidity and mortality, impacts negatively on the income of the poorest households in many endemic settings [3]. In addition, malaria impedes nations' prosperity including effecting fertility and population growth, decreasing private and public sector investment's and increasing medical costs [4]. Countries in which a majority of the population live in regions with intensive malaria infections, experienced an average per capita gross domestic product (GDP) of 0.4% per year in contrast to other countries with no malaria where average growth was 2.3% per year [4].

Despite the enormous burden of malaria, considerable progress has been made in the last 15 years. In this period, malaria parasite prevalence reduced by approximately 50% globally [5]. However, the reduction in malaria transmission intensity has not occurred equally between countries or within countries, with greater declines in many Southern African countries in contrast to larger parts of West and Central Africa [6]. Following this remarkable progress in the fight against malaria, recent trends suggest a picture of stagnation of progress and plateauing of donor funds [7]. This worrying trend suggests recent gains may be under threat: malaria cases have consistently increased globally between 2014 and 2016, with 5 million more cases reported in 2016 (Fig 1.1) [7]. In addition, 11 countries out of 34 that were accelerating towards elimination by 2020 have reported an increase in local cases in 2016 relative to 2015 [6]. South Africa, with a country incidence rate of 0.18/1000 per annum [8], witnessed a rebound in cases with an increase of 3 768 cases recorded between 2015-2016 [1, 9].

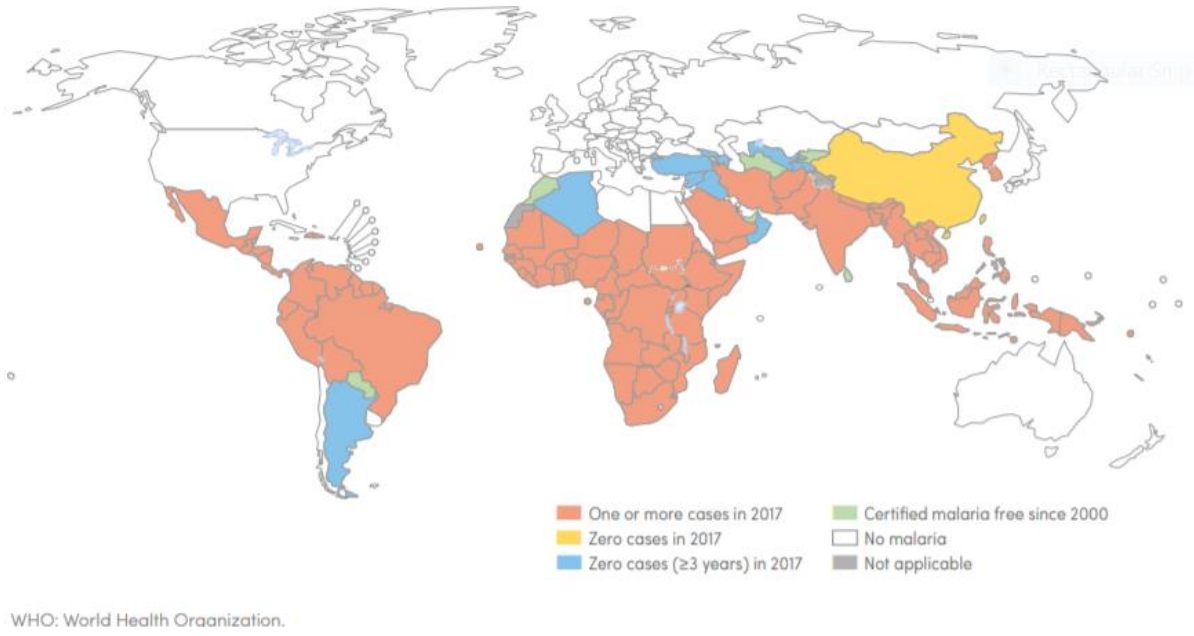

Figure 1.1: The global distribution of malaria. The map shows the countries and regions with indigenous malaria cases from the year 2000 and their status by 2017. The different shades of colours as highlighted show the current malaria endemicity levels. Map sourced from [1].

1.2 Malaria biology and pathology

Six species of *Plasmodium* parasites are currently associated with naturally acquired malaria infections in humans: *P. falciparum, P. vivax, P. malariae, P. ovale, P. knowlesi* and *P. cynomolgi* [10, 11]. *P. falciparum* is responsible for most severe morbidity and mortality of all the *Plasmodium* species and is widely distributed in sub-Saharan Africa [12]. *P. malariae* causes malaria in humans throughout the tropics and subtropics, and is more widely reported at greater prevalence than *P. ovale* [13]. *P. malariae* distinguishing characteristic is its ability to persist in a single host for decades [14, 15]. Until fairly recently, *P. knowlesi* and *P. cynomolgi* infections were thought to be limited to macaques, their natural host. However, studies from Southeast Asia have consistently revealed a high incidence of human *P. knowlesi* malaria [10] and sparse reports of *P. cynomolgi* [11] leading to their recognition as additional human malaria parasites. While the cases of *P. cynomolgi* are not yet a public health concern, *P. knowlesi* parasites are a considerable cause of (severe) morbidity in parts of Southeast Asia [16]. Outside Africa, *P. vivax* is the most widespread *Plasmodium* parasite and is responsible for half of the malaria deaths [17]. *P. vivax* is rare in most of the indigenous African populations and this was previously thought to be due to lack of expression of erythroid Duffy antigen that prevents the parasite from exploiting the entry mechanism on the erythrocyte surface [17, 18]. However, a growing body of evidence indicates sparse *P. vivax* infections in

Duffy-negative individuals in Africa [19]. In contrast to other malaria parasite species, *P. vivax* and *P. ovale* frequently develop dormant hypnozoites in the liver that are responsible for relapses [13, 20] and complicate malaria treatment.

P. falciparum malaria, responsible for a large proportion of global malaria mortality, causes a spectrum of clinical presentations ranging from asymptomatic parasite infections to severe disease and fatalities [21]. Uncomplicated malaria manifests itself in a variety of clinical presentations including fever paroxysms (>37.5°C), headaches, rigours of cold shivers and joint pains [22]. Other symptoms of uncomplicated malaria include diarrhoea, nausea, vomiting, and loss of appetite, dizziness, sore throat, muscle weakness and lethargy [21]. If not detected and treated in time, severe illness may develop, in particular in individuals with limited previous exposure to malaria. The WHO defines severe *P. falciparum* malaria as acute malaria with signs of severe illness and/or evidence of organ dysfunction occurring in the absence of an alternative cause and in the presence of *P. falciparum* asexual parasitaemia [23]**.** Signs of severe malaria may occur alone or in combination and include impaired consciousness, prostration, multiple convulsions, acute respiratory distress syndrome, acidosis, hypoglycaemia, severe malaria anaemia (haemoglobin concentration of <5 g/100 mL; haematocrit <15%), renal impairment, jaundice, pulmonary oedema, significant bleeding, shock, hyperparasitaemia and haemoglobinuria [24].

1.2.1 The life cycle of *Plasmodium*

As *P. falciparum* is the major species responsible for mortality and morbidity in Africa, this thesis will mainly focus on this species. The *P. falciparum* life cycle consists of two distinct stages, the asexual stages in the human host and the sexual stages in the female *Anopheles* mosquito vector (Figure 1.2). Infection in humans begins after the inoculation of sporozoites into the bloodstream by a blood-feeding mosquito [25]. The sporozoites rapidly invade liver cells within 30-60 minutes, where they replicate and divide to produce between 10 000 to >30 000 daughter merozoites [21]. The infected liver cell ruptures, releasing merozoites into the bloodstream, where they invade erythrocytes and begin the asexual reproductive stage. Symptoms typically develop 4-8 days after the initial erythrocyte invasion [26], with the replication cycle of the merozoites within the erythrocytes lasting up to 48 hours [27].

A minor proportion (<10% but frequently <1%) of asexual parasites stochastically develop into sexual gametocyte forms [25], the mature forms of which can be transmitted from the infected human back to a feeding mosquito. This sexual commitment is regulated by a signalling pathway during schizont development, which controls the expression of the transcription factor

ap2-g as the master regulator of sexual differentiation [28, 29]. Recently, host-derived lipid lysophosphatidylcholine (LysoPC) was demonstrated to control *P. falciparum* cell fate by repressing parasite sexual differentiation [30]. Once sexually committed, each schizont produces daughter merozoites of a single sex; the sex ratio of gametocytes in the human peripheral blood is typically female-biased (3-5 females for 1 male) [31, 32]. Gametocytogenesis is a prolonged process in *P. falciparum* parasites and involves maturation through five morphologically distinct stages (stage I-V). For a large part of this development, that takes 10-15 days to complete, developing gametocytes are not in the circulation but primarily sequestered in the bone marrow [33].

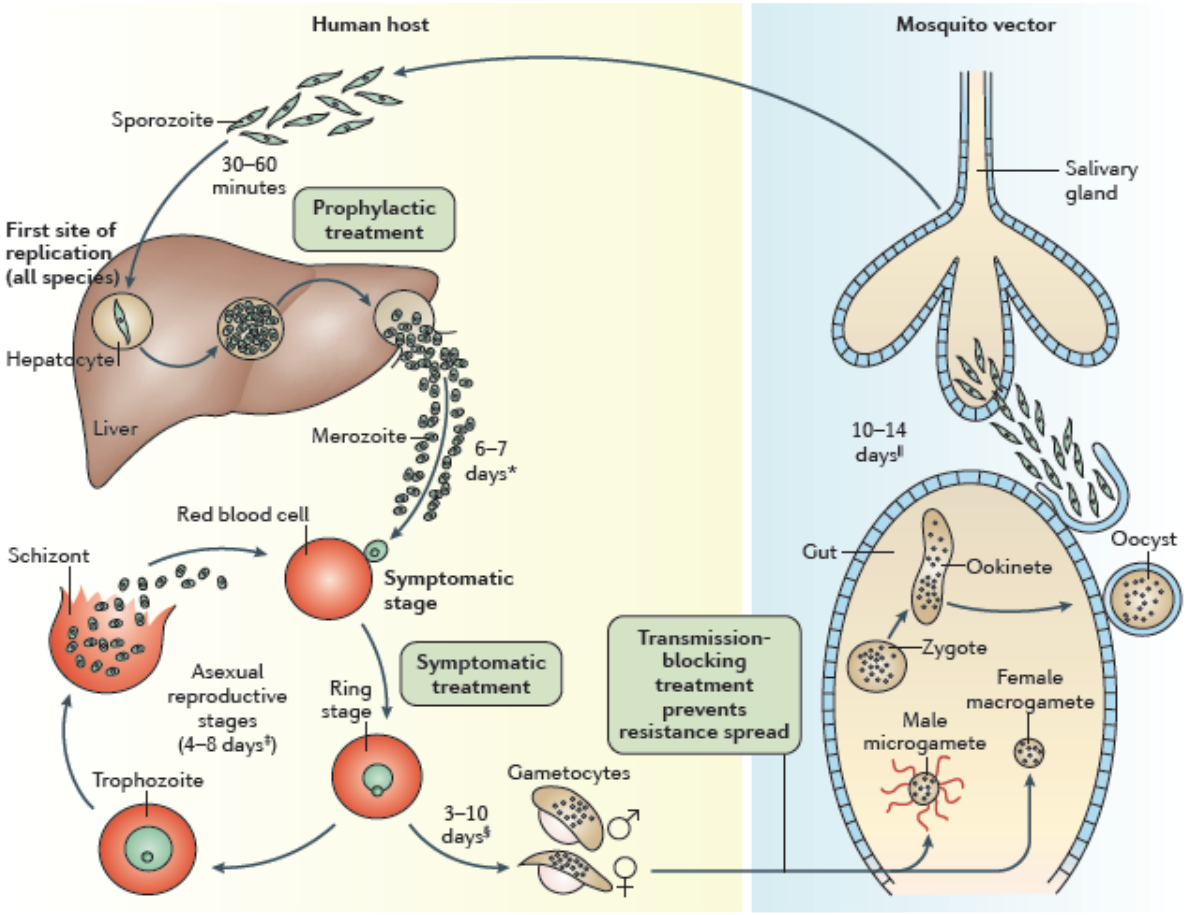

Figure 1.2: The life cycle of *P. falciparum* and treatment options. The cycle begins with the injection of sporozoites into the human bloodstream by female Anopheles species mosquitoes during a blood meal, they undergo liver stage and erythrocytic development before gametocytes are taken up again during feeding by mosquitoes to re-initiate the cycle. Figure reproduced with permission from [21].

Immature stage gametocytes (I-IV) are not found in human peripheral blood, but sequester in the extravascular space of the human bone marrow where they undergo development before re-entering the circulation once mature [33-35]. Microscopically, the initial gametocyte stages (I and early stage II) are similar to asexual stages parasites; morphological differences appear

only 72-96 h post-invasion (Figure 1.3). Morphological differences between male and female gametocytes are clearly visible from stage III gametocytes onwards [36]. Female gametocytes have a smaller nucleus, more concentrated pigment and differently coloured cytoplasm compared to male gametocytes where the nucleus is larger and the pigment is more diffuse [25]. After maturation, stage V gametocytes might actively move or passively diffuse to the skin vasculature where they are ingested by female mosquitoes during a blood meal [37]. Within the mosquito, these mature gametocytes activate to form male and female gametes. Gametocytes activation is triggered by the different mosquito environment that includes a drop in temperature by 5°C, elevated pH from 7.4 to 8.2 and the increased xanthurenic acid concentration [25, 38, 39]. Following activation, male and female gametes fuse to form zygotes that undergo meiosis and develop further into motile ookinetes that can penetrate the mosquito midgut to form oocysts. Within the oocyst, the parasite undergoes many rounds of mitosis to create thousands of haploid sporozoites. Upon oocyst rupture, the sporozoites migrate to the mosquito salivary glands, rendering the mosquito infectious upon its next bite.

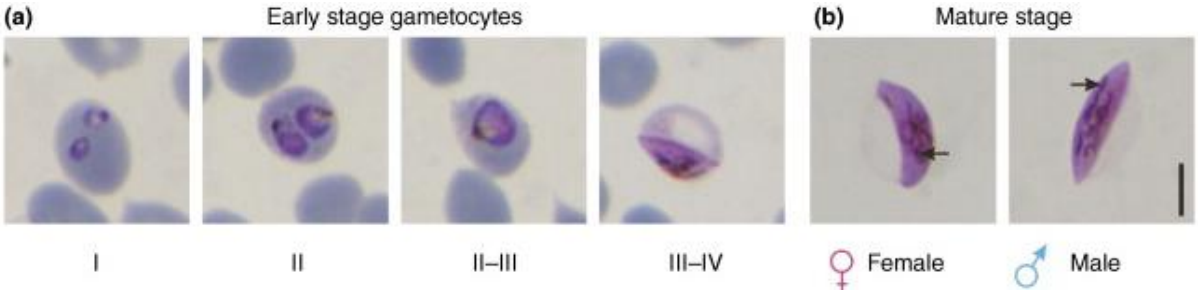

Figure 1.3: The five developmental stages of *P. falciparum* gametocytes. The figure shows giemsa stained slides of the sequential development of *P. falciparum* gametocytes from (a) the early stages I through IV and (b) the mature stage V showing both female and male gametocytes. The concentrated pigment of the female gametocyte in contrast to the dispersed pigment of the male gametocyte is shown by the black arrows. This figure is reprinted from[40] with permission of the publishers, Elsevier.

Factors associated with transmission efficiency

1.2.2 Mosquito factors

The abundance of vectors, competent species and the frequency in which they take infected blood meals from individuals is the most important factor determining transmission success. This is dependent on the effective contact rates between humans and mosquitoes. The extent to which different populations are sampled by mosquitoes differs and can play an important role in determining sources of onward transmission [41]. Not all parasite clones are equally infectious to all mosquitoes. Ookinetes must escape the mosquito immune system, with the sexual stage parasite protein, *Pfs47* playing a key role in this process [42]. Mosquito innate

immunity, feeding behaviour, longevity and duration of sporogonic development also influence the chances of successful infection [27].

1.2.3 Human factors

Human activities and behaviour, such as spending plenty of time outdoors [43], differences in attractiveness to mosquitoes for example due to differences in body sizes [41] and odour profiles [44] influence contact with mosquitoes and hence transmission opportunities. This relationship between humans and mosquitoes varies between endemic settings and among individuals within the same settings. Naturally acquired immunity of the human host are also highly important in determining parasite kinetics and transmission. Naturally acquired immunity affects asexual parasite densities and thus indirectly the development of gametocytes [25]. Naturally acquired immunity to *Plasmodium* sexual stage antigens (gametocytes and gametes) has also been described and may reduce transmissibility of gametocytes once ingested by mosquitoes [45]. The waves of gametocytaemia that typically follow clinical episodes of uncomplicated malaria suggest a possible role of nonspecific immune effectors including tumour necrosis factor in parasite killing [46]. In addition, the delayed appearance of *P. falciparum* gametocytes may be a strategy to escape the toxic levels of human cytokines during the ensuing febrile paroxysms [47]. Whilst naturally acquired antibody responses can influence transmission efficiency, the importance of this type of immunity for transmission dynamics remains to be demonstrated.

1.2.4 Parasite factors

Many parasite factors influence transmission potential. Total parasite density is positively associated with gametocyte density, albeit loosely for *P. falciparum* [48, 49]. However, relatively high concentrations of gametocytes may be observed in low-density infections [25], making it impossible to predict transmission potential from total parasite burden. Gametocyte sex ratio is a key determinant of transmission success. As the sex ratio is typically female biased, the production of a higher number of male gametocytes compared to female gametocytes increases the likelihood of transmission success particularly at lower gametocyte densities [31]. The gametocyte sex ratio is also influenced by the presence of competing parasites strains as well as anaemia that favours males during the course of an infection [31, 50]. Differences in sex ratios may also be associated with parasites' response to host immunity and environmental stress factors affecting gametocytogenesis [31, 50]. While transmission success increases as gametocyte density increases, transmission is nevertheless still observed at low gametocyte densities [51]. Possible explanations for this include the clustering of gametocytes in the human vasculature during mosquito blood meals [52]. Low gametocyte

densities typically result in lower rates of mosquito infections and even lower numbers of oocysts in infected mosquitoes. It has been demonstrated that the majority of oocyst–positive mosquitoes, including those with low oocyst densities, are infectious and capable of continuing the transmission cycle to the next human host [53]. This implies that even mosquitoes with low infection burden (oocyst density) are infectious and relevant for control efforts. It further implies that individuals who give rise to low infection prevalence and density in mosquitoes, e.g. low-density asymptomatic parasite carriers, may form an important source of onward transmission to mosquitoes and should be considered in elimination efforts [54].

1.3 Malaria control and elimination

1.3.1 Malaria elimination and eradication strategies: past and future

The current aim for global malaria eradication is not new: the global community has previously embarked on malaria eradication (defined as the permanent reduction to zero of the worldwide incidence of infection caused by human malaria parasites) [23]. This initiative, spearheaded by the Global Malaria Eradication Programme (GMEP) [55], depended heavily on the application of dichlorodiphenyltrichloroethane (DDT) for mosquito control and chloroquine for treatment of infections in humans [55]. During the GMEP, malaria was permanently eliminated from many regions and many countries have maintained their malaria-free status since then [56]. However, substantial gains in malaria control and elimination strategies were lost in other countries and followed by resurgences, sometimes epidemics [57]. The earlier eradication initiative was eventually abandoned in 1969 due to social and operational challenges, including the realisation that eradication was not feasible in the short term in certain countries [55]. While the WHO maintained its commitment to eradication, it did not specify an end date for the attainment of this goal. More recently and encouraged by the global reduction in malaria cases in the last 15 years, there is a renewed push for malaria elimination and eradication. Elimination entails the complete interruption of transmission within a defined geographical area [58], eradication equals sustained worldwide elimination. The WHO guidelines on elimination recognize that malaria transmission represents a continuum [59]. Program actions are identified across the spectrum of transmission intensity from high to low settings. The four defined phases of the malaria elimination continuum are; malaria control (incidence >five malaria cases per 1,000 population at risk), pre-elimination (incidence between zero and five malaria cases per 1,000 population at risk), elimination (incidence <one malaria case per 1,000 population at risk) and prevention of reintroduction (zero malaria cases per 1,000 population at risk) per malaria season [59]. South Africa is in the pre-elimination phase of the continuum [60].

The WHO Global Technical Strategy for Malaria (GTS) set the ambitious goals of reducing malaria case incidence and mortality rates by at least 90%, eliminating malaria in at least 35 countries and preventing the resurgence of malaria in all malaria-free countries by 2030 [61, 62]. The strategic framework consists of three major pillars: 1) ensuring universal access to malaria prevention, diagnosis, and treatment; 2) accelerated efforts towards elimination and attainment of malaria-free status and 3) transformation of malaria surveillance into a core intervention [61]. The key pillars are supported by elements of innovation and research in addition to a strong enabling environment to maximize the impact of the existing life-saving tools.

1.3.2 Malaria elimination: current status and determinants of success

According to WHO, 21 countries have the potential to reach malaria elimination by 2020 with Sri Lanka and Paraguay most recently certified as malaria-free. The successful reduction in malaria cases has mainly been achieved by targeting the vector in the elimination strategies through the use of either long-lasting insecticidal treated bed nets (ITNs) and/or indoor residual spraying (IRS) [5]. These interventions are estimated to have halved the prevalence of *P. falciparum* and reduced the incidence of clinical disease by 40% between 2000-2015 as well as averting 68% of cases [5]. Vector control that reduces the host-vector contact has substantially contributed to a reduction in transmission [63]. However, getting to zero cases for a majority of countries will require additional tools, including novel approaches with chemotherapeuticals [64] and, potentially, vaccines [65]. Unfortunately, the majority of countries earmarked for elimination by WHO have suffered setbacks in their bid to achieve this goal [66]. To protect the gains achieved thus far, it is important to understand the reasons for the apparent lack of progress in these countries that had high expectations of achieving elimination.

1.3.3 Challenges facing malaria elimination

The challenges facing elimination can be broadly classified under parasite, vector, human and environmental factors (Table 1.1). These include areas of high transmission due to favourable ecosystems, highly competent vectors, ongoing malaria transmission, drug and vector resistance and large segments of the population not served by the health systems [64]. In low transmission settings, the realisation that transmission varies between and within countries and is influenced by different mosquitoes and parasite species as well as different health systems means that intervention programmes need to evolve as transmission changes from high to low transmission settings [64]. As malaria transmission is heterogeneous, not one single strategy will solve all the challenges. Rather, countries need to adapt specific tools for

their local epidemiology and health systems context [64]. Thus, WHO frames national elimination as a continuum with reorientation and reorganisation of programmes rather than the achievement of milestones for specific phases [59].

Table 1.1: Factors and challenges facing malaria elimination. Table compiled from [67, 68]

Factors	Challenges
Vector	→ Changes in mosquito feeding and biting behaviour; → Increasing insecticide resistance; → Genetic differences amongst vectors; → Vector movement.
Parasite	→ Submicroscopic parasitaemia; → Asymptomatic infections; → Incomplete understanding of gametocyte stages; → Parasite genetic diversity; → Hypnozoites (for *P. vivax* and *P. ovale*); → Emergence and spread of drug resistance; → Lack of effective vaccines
Human and programmatic challenges	→ Lack of adherence to control strategies, → Reduced risk perception in low transmission settings; → Behaviour change; → Human movements; → Plateauing in funding (donor fatigue); → Lack of political commitment i.e. lack of country ownership of elimination efforts; → Weak health systems; → Inadequate surveillance; → Lack of affordable drugs leading to counterfeits; → Weaknesses in supply chain management.
Environment	→ Unpredictable climate effects; → Heterogeneity in transmission; → Continued favourable conditions for mosquito productivity and transmission.

1.4 The toolbox for malaria elimination

1.4.1 Targeting the parasite reservoir for elimination

The WHO recommends artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria caused by *P. falciparum* parasites. The ACTs that combine 2 active ingredients with different mechanisms of action, are still the most effective antimalarials available to date. As the global malaria community shifts focus from malaria control to elimination and potential eradication, there is a need for a different approach to the use of antimalarial chemotherapies, aiming to break the cycle of disease transmission rather than only curing individual patients [69]. This potentially requires the treatment of asymptomatic parasite carriers, ideally with a therapeutical that can be administered as a single dose and is safe and well tolerated by the entire population, including pregnant

women and infants [70]. While the ideal drug meeting all these stringent requirements is currently unavailable, also with the currently available drugs it is possible to refocus efforts to include asymptomatic individuals who are plausible contributors to transmission [69]. Antimalarial treatment administered irrespective of symptoms can considerably reduce the disease burden in treated populations. During seasonal malaria chemoprevention (SMC), full courses of blood schizonticides are provided to young children during the transmission season to prevent clinical malaria [71]. SMC is highly efficacious in reducing malaria burden when given regularly to large segments of the populations in malaria-endemic regions and has particularly been useful in the Sahelian region [72]. Ideally, drugs that are used for malaria elimination should also prevent malaria transmission by clearing or sterilizing gametocytes and, in the case of *P. vivax*, clear hypnozoites [69, 73]. In the context of elimination, transmission-blocking drugs would reduce the gametocyte reservoir in asymptomatic individuals and in this way contribute to transmission reduction (Figure 1.4). Ideally, drugs for malaria elimination can improve the transmission blocking properties of standard ACTs currently used as mainline treatment recommended by the WHO [74, 75].

Methylene blue (MB) [76], primaquine (PQ) [77], and several other compounds including recently described potent imidazopyridazines (IMPs) and 2-aminopyridines (2-APs) [78] have activity against late-stage gametocytes. For *P. falciparum*, PQ is the only currently registered drug that is useful in reducing gametocyte levels. However, uptake of PQ in many malaria-endemic settings has been rather slow [79]. The perceived safety issue surrounding PQ use in glucose-6-phosphate dehydrogenase (G6PD)-deficient individuals and local drug regulations are in part responsible for this slow uptake [80]. The WHO recommends a single low-dose of 0.25 mg/kg of PQ alongside ACTs for treatment of *P. falciparum* malaria in elimination settings are areas threatened by artemisinin resistance [79]. In trials with the single low dose 0.25 mg/kg PQ, the percentage of people who were infective to mosquitoes 3 to 4 days post-treatment was reduced from 14% to 2%, with a smaller effect observed at day 8 (reduction from 4% to 1%), with no evidence of harm [81]. In a trial in Mali, the addition of single low-dose PQ to sulfadoxine-pyrimethamine (SP) and amodiaquine or MB to dihydroartemisinin-piperaquine led to complete blockage of human-to-mosquito transmission by day 2, and a larger reduction in transmission than that offered by SP and amodiaquine or dihydroartemisinin-piperaquine alone [82].

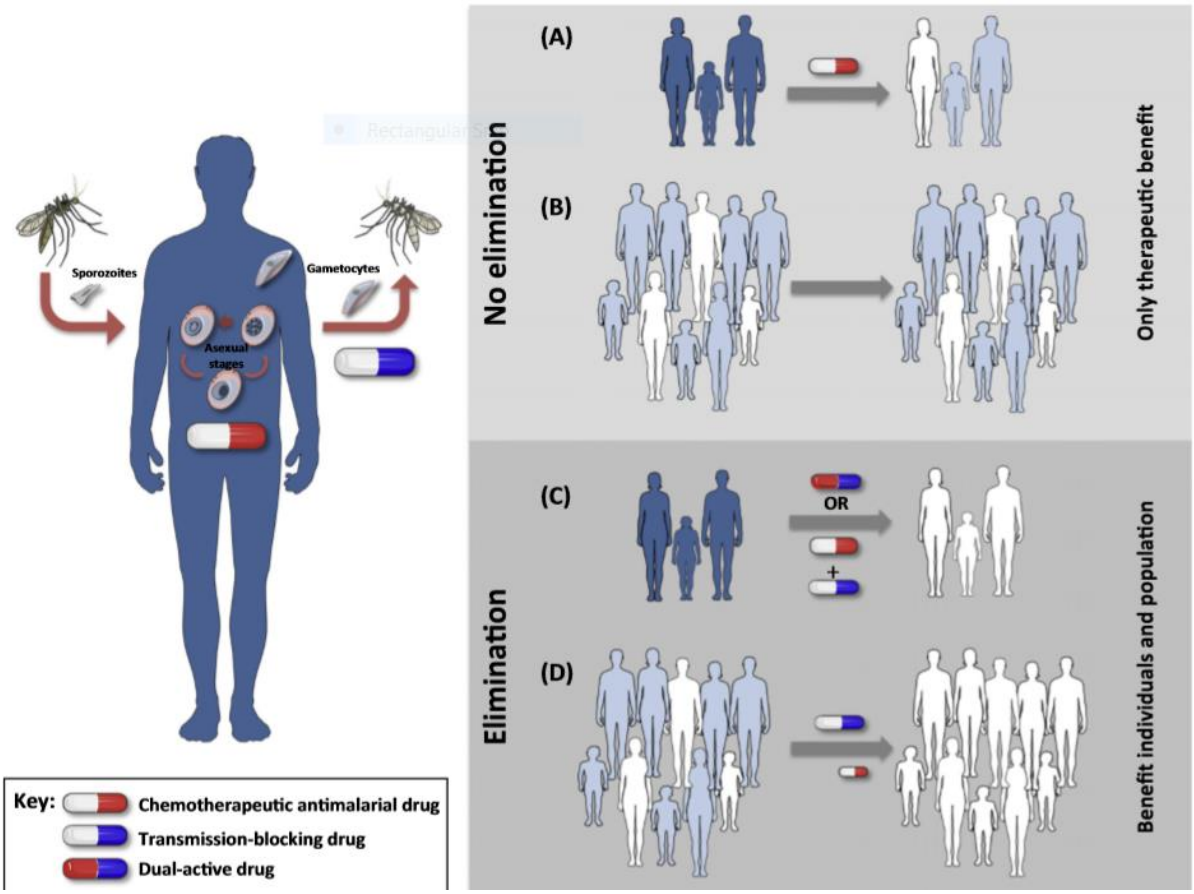

Figure 1.4: Malaria control and elimination through parasite targeting chemical interventions. The development of new transmission-blocking drugs may result in a drug targeting only gametocytes or dual activity against gametocytes and asexual stages. To treat symptomatic patients (blue), chemotherapeutics are used to kill pathogenic asexual forms of the parasite, but transmissible sexual gametocytes may not be eliminated (A). Asymptomatic, gametocytes carriers (pale blue) remain undetected, untreated, and sustain parasite transmission to the mosquito vector (B). These scenarios will not eliminate malaria, as gametocyte reservoir has to be cleared with either a dual-acting drug or a combination of a drug curing malaria and a transmission-blocking drug (C). At the population level, administration of a transmission-blocking drug to asymptomatic gametocyte carriers will eliminate gametocytes and prevent transmission by disrupting the lifecycle (D). Low-density infections in asymptomatic individuals should concurrently be treated with a chemotherapeutic drug. Figure from [73] with permission from the publishers, Elsevier.

In addition, the efficacy of PQ may be influenced by polymorphisms in the human cytochrome P450 2D6 (CYP2D6) that are linked to the reduced capacity to metabolize the drug [83]. Many allelic variants of the *cyp2d6* genes affecting substrate metabolism have been described. The CYP2D6 alleles are classified as variants leading to abolished, decreased, normal or increased enzyme activity [84]. The efficacy of the reduced low dose PQ in lowering transmission in individuals with CYP2D6 variants is unclear. The parent PQ molecule is a pro-drug that is metabolised into active and effective metabolites through interactions with several CYP enzymes particularly CYP2D6 and monoamine oxidase (MAO) [85]. However, the

essential pathways for PQ activation have not been elucidated. The MAO pathway is thought to be responsible for initial catalysis, that is, deamination to the aldehyde to yield carboxy-primaquine the major plasma metabolite of PQ in humans [86]. Carboxy-primaquine lacks toxicity and is ineffective against gametocytes. The hydroxylation of PQ mediated by CYP2D6 creates unstable metabolites like 5-hydroxyprimaquine that can redox cycle to the corresponding quinone-imine species [84]. The redox cycling of these metabolites and the resulting oxidative stress from PQ metabolism are thought to be responsible for PQ efficacy and toxicity [84].

Irrespective of the drug used, the most important component of successful parasite-based interventions is the achieved coverage among all parasite carriers. Mass drug administration (MDA) involving the provision of malaria chemotherapy to whole populations except those with drug contraindications without testing [87], is one approach leading to a significant reduction in cases. This has been experienced in moderate-low transmission in Zambia [88] and may lead to complete elimination of malaria if frequently repeated in small isolated settings such as Aneityum [89]. In general, the long-term efficacy of MDA requires almost 100% intervention coverage and repeated rounds in combination with effective vector control [90]. An alternative for MDA, mass screening and treatment (MSAT) involves mass screening regardless of symptoms and treatment if test-positive [91]. MSAT again depends on high coverage and on the sensitivity of the diagnostic used. While mass blood surveys can generally detect asymptomatic infections in high transmission settings, in low transmission settings such infections are challenging to identify due to low parasite densities [90]. MSAT is currently not recommended by the WHO while MDA may be considered in some settings aiming for elimination [92].

Reactive case detection (RCD) is yet another approach for parasite detection that is often considered following or in conjunction with MDA or MSAT. It is based on the assumption that infections cluster at small geographical scale [91, 93]. In case of evidence of malaria parasites infections in an index case (e.g. a patient coming to a clinic with malaria) individuals within a defined radius around the index case, are screened to identify and treat symptomatic and asymptomatic cases [91, 94]. RCD has yet to demonstrate the best conditions under which it could drive regional elimination strategies in field settings. Areas that have piloted RCD have seen limited impact [95-97]. A mathematical modelling study in Zambia concluded that RCD could only be effective in areas where transmission has recently been introduced rather than in an all-encompassing low-transmission setting [98].

1.4.2 The importance of asymptomatic malaria infections for transmission

Asymptomatic parasitaemia is defined as the presence of parasites without any apparent malaria symptoms. Asymptomatic parasite carriers commonly do not seek treatment. Asymptomatic malaria is at least in part explained by naturally acquired immunity that develops following natural malaria exposure [99]. Children may experience many bouts of malaria attacks that enables the majority of them to develop protection against severe disease as juveniles while still experiencing clinical episodes. With age, clinical immunity develops that prevents malaria-exposed individuals from symptoms and leads to a state where parasites may be present without accompanying symptoms [100]. Some of these infections may occur at densities undetectable by microscopy or rapid diagnostic tests (RDT), so-called submicroscopic infections or sub-patent infections [101] (Figure 1.5). Up to 75% of infections detected during community surveys in high transmission settings are asymptomatic [27, 102]. While parasite prevalence generally is lower in low transmission settings, the proportion of infections present at submicroscopic parasite densities is largest in these settings [103].

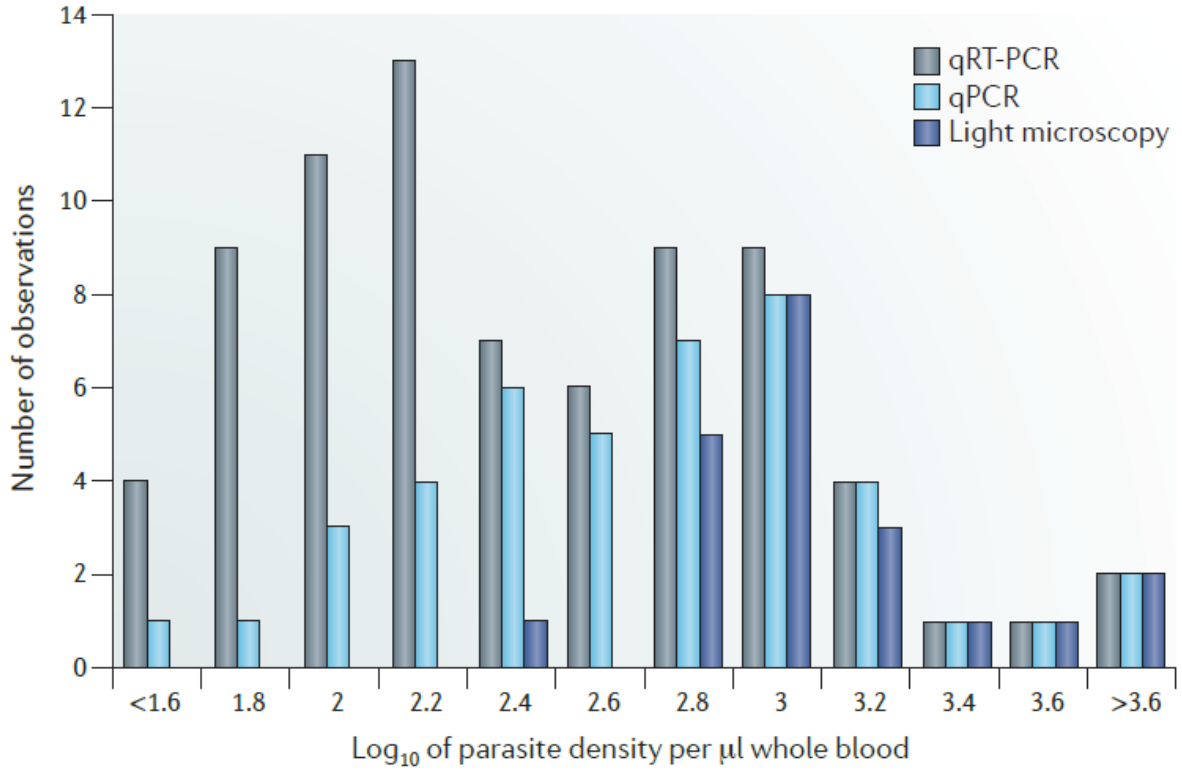

Figure 1.5: Sensitivity of malaria parasite detection tools of microscopy and nucleic acid amplification detection methods for diagnosis of *P. falciparum* infections. Parasite detection using microscopy, quantitative PCR (qPCR) and quantitative real-time PCR (qRT-PCR) were applied to the same blood samples from asymptomatic individuals. 18S ribosomal RNA transcripts by qRT-PCR is the most sensitive, followed by 18S rDNA detection qPCR and microscopy is the least sensitive. Figure reproduced from [27] with permission from publishers.

In *P. falciparum* natural infections, gametocytes oftentimes comprise <5% of the total parasite biomass [25]. The use of sensitive molecular tools has shown that low-density (submicroscopic) gametocyte carriage is prevalent in all endemic settings [104, 105]. Numerous field studies have shown that mature gametocytes are detectable in patients presenting at clinics with symptomatic malaria infections in African settings [106], similarly, mature gametocytes are also detectable in asymptomatic individuals [107]. Transmission studies where infectivity is determined by either feeding venous blood of different populations to mosquitoes or allowing mosquitoes to feed directly on the skin of infected humans allow a quantification of the contribution of different populations to onward transmission. Such studies in high endemic settings indicate that low-density infections and submicroscopic gametocytes are capable of infecting mosquitoes [104, 108]. However, it is currently unclear if a similar pattern occurs in low transmission settings.

1.4.3 Understanding gametocyte production and transmission in populations

Factors that drive the production of gametocytes are currently unknown, especially in low transmission settings [67]. Similarly, it is unknown whether mosquito infectivity differs at different times of day [109] or whether gametocytes cluster in the skin where it is more likely that they are taken up by mosquitoes. Gametocytes might also influence the attractiveness of human hosts to mosquitoes [44]. An increasing body of evidence indicates that vector mosquito species can differentiate between malaria-infected and uninfected individuals based on odour profiles and skin volatiles [110, 111]. Whilst the importance of these factors (circadian rhythms, clustering, gametocyte-induced infectiousness) for transmission is currently unclear and may be very small, it is evident that mosquito exposure is a major determinant in the contribution of different populations to transmission. In a multiregional assessment study from both high and low transmission settings in Africa, infectivity of mosquitoes was 4-fold higher in children <15 years of age compared to older individuals [41]. However when estimates are adjusted [112] for the number of mosquito bites received, body size and demographic composition of the population, then all individuals contributed equally to transmission [108].

Gametocyte sex ratio is a critical factor affecting transmission with an optimal ratio of three or four female gametocytes to one male gametocyte observed in natural settings [25]. This ratio is dynamic and varies during the course of an infection, between clones, between endemic regions and between years in the same regions [25, 137]. The development of sensitive molecular tools to quantify male and female gametocytes could inform on the effect of drugs on sex ratios and in an epidemiological context, the relevance of clonal diversity and sex ratios in malaria transmission. The earliest marker of sexual stage development (found in all gametocyte stages and sexual stage-committed asexual parasites up to 24 h after merozoite

invasion) is expression of *pfs16* [138]. Additionally, *pfs25* has been widely used to quantify and distinguish gametocytes from other blood stages using either reverse transcription PCR (RT-PCR) or quantitative nucleic acid-based amplification (QT-NASBA) [27]. *Pfs25* expression is upregulated in female gametocytes [139] thus making it an important target for sensitive detection and quantification of female gametocytes [140]. Male-specific *pfs230p* and *pfmget* (male gametocyte–enriched transcript) markers have been tested for the detection of mature gametocytes but their sensitivities were found to be relatively low compared to *pfs25* [141]. A recently described novel gametocyte biomarker, *Pfg17* was suggested as an alternative and excellent biomarker for detecting asymptomatic reservoirs compared to *pfs25* [142]. The detection of gametocytes with high sensitivity is informative in identifying individuals in populations that contribute to transmission. However, gametocytes need to be present at sufficiently high densities (~5 gametocytes/μL or more) and in a permissive sex-ratio to plausibly result in mosquito infections [113].

1.5 Tracking infections: genotyping of *P. falciparum* to inform elimination

Assessing the infectivity of different populations by mosquito feeding assays is one way of examining the transmissibility of malaria in populations. The complexities of the duration of infections, longitudinal gametocyte production and mosquito exposure (and thus opportunities to transmit), however, make it complicated to translate these findings to transmission potential or the number of secondary infections arising from a single infection. This is one area where genotyping can play an important role. Different parasite strains can vary in their ability to invade erythrocytes [114], the number of merozoites they produce [115] and transmission potential [116]. Genotyping of *P. falciparum* parasites is useful in understanding parasite biology and epidemiology [117], assessing transmission reduction, identifying sources of reservoirs of incident infections as well as tracking and surveillance of parasites for enhanced resolutions (detailed assessment) [118].

Genotyping assays enable the detection and quantification of different *P. falciparum* clones to assess clone-specific multiplication rates, the duration of infection and the role of distinct clones in transmission. Genotyping may be performed using highly polymorphic antigen markers including merozoite surface protein (*msp)1, msp2* and glutamate-rich protein (*glurp)* [119], microsatellite markers (repetitive DNA sequences made up of repeats of about 2-8 nucleotides in length), single nucleotide polymorphisms (SNP, markers that looks at single changes in the sequence), copy number variants (CNVs) as well as amplicon and whole genome sequencing [120]. Polymorphic antigen markers like *msp1, msp2*, and *glurp* have been useful to distinguish reinfection from recrudescence as well as monogenomic from polygenomic infections [121]. Due to their high genetic diversity, microsatellite markers are

more useful in tracking parasite relatedness as well as studying specific regions of the genome, for example, drug resistance loci. Organelle markers like mitochondrion and/or apicoplast-specific genes are useful for tracing population history due to lack of recombinations [120, 122].

1.5.1 Multiplicity of infections

Multiplicity of infection (MOI) is a relevant indicator of the force of infection in populations [123] and can be readily assessed by polymorphic markers such as *msp1* and *msp2* genes. The *msp1* and *msp2* genes exhibit great length and sequence polymorphism, remain stable throughout the erythrocytic lifecycle, making them suitable molecular markers for genotyping *P. falciparum* populations [119, 124]. The *msp1* gene can be divided into 17 blocks according to the level of sequence divergence [125]. The block 2 region of the N-terminal of the *msp1* gene is the most diverse and three allelic families (K1, Mad20, and Ro33) have been identified [126]. On the other hand, the *msp2* gene can be divided into five domains with most diversity occurring in its dimorphic central repeats (block 3), which are flanked by variable non-repetitive blocks 2 and 4 and conserved blocks 1 and 5 [127]. Two allelic families: 3D7 and FC27 are found in natural parasite populations [128]. Length and sequence polymorphism inherent in these genes make them readily detectable by gel electrophoresis of PCR amplified products [124]. However, interpreting PCR gels of single samples can be subjective and difficult, more so when multiple genotypes are present making it challenging to distinguish between allelic families [129]. An alternative sensitive approach is the use of capillary electrophoresis that deploys fluorescently labelled primers specific for either 3D7 allelic family or the FC27 allelic family of *msp2*. Genotypes are subsequently distinguished by their fluorescent dye and by their size which is determined by an automated sequencer and software [129, 130].

Depending on transmission intensity, a fraction of the population may have multiclonal infections (>1 parasite genotypes present) [131]. This MOI, the number of different parasite clones co-infecting a single host [132], is also referred to as complexity of infections (COI) [133]. MOI may arise from either superinfection, whereby a single individual is infected by different parasites from multiple mosquitoes, or co-transmission where a single mosquito transmit multiple parasite clones at once [122]. The rate at which a susceptible individual contracts malaria is termed force of infection (FOI), or when traced with current molecular tools frequently referred to as the molecular FOI (mFOI). Measurement of mFOI is performed by following individuals longitudinally and assessing the number of clones acquired over a given time.

MOI and mFOI can give insights in malaria epidemiology and changes in malaria epidemiology. A lower mean *P. falciparum* MOI has been reported in areas of low and

moderate malaria transmission intensity as compared to areas of high transmission intensities [134]. MOI is also more susceptible to changes in malaria transmission than overall parasite prevalence as it allows conclusions to be drawn about certain parasite genotypes that persist after interventions [135]. As an epidemiological metric, MOI can thus aid in understanding infection dynamics and changes in transmission intensity [136]. One important caveat of measuring MOI is that not all clones may be easily detectable, especially when single observations are used. The MOI in single infections depends on the diversity of the marker used for analysis and the relative abundance of rare clones in PCR results [137]. Clone density fluctuations over time and sequestration of parasites in deep capillaries may mask their detection. Consequently, it is more informative to perform longitudinal sampling [138].

1.6 Local transmission vs imported cases

One of the current challenges to malaria control and elimination in South Africa is the importation of malaria cases from neighbouring countries with higher malaria transmission intensity. Since local malaria transmission requires different control measures than imported malaria, the ability to distinguish locally transmitted cases from imported malaria parasites is becoming increasingly important during malaria elimination strategies. For instance, in South Africa, the increasing cases of imported malaria from neighbouring high malaria-endemic countries continue to hamper South Africa's elimination agenda and may seed local transmission. A review of malaria cases in South Africa from 2010 to 2014 reported 41% of all malaria cases in the country as imported, with neighbouring Mozambique and Zimbabwe accounting for 87% and 6% of these cases, respectively [139]. These endemic countries influence malaria cases in South Africa through returning travellers, population movements and through both documented and undocumented economic immigrants. Imported malaria cases are thus defined as infections whose origin can be traced back to known malaria-endemic regions outside South Africa to which the individual travelled within a period of 1 month [140]. Local malaria cases, in contrast, are defined as infections acquired within a malaria-receptive area of South Africa where there is no history of travel to another malaria-endemic country and where local transmission cannot be ruled out. Unclassified cases occur when local transmission is unlikely but the malaria patient cannot be traced to account for their recent travel patterns [140]. However, this classification is not entirely accurate due to recall bias in self-reported travel history and thus genotyping can provide a more accurate classification [141].

1.7 The local context: malaria elimination in southern Africa

Eight southern African countries have formed the Elimination 8 (E8) initiative, a multi-country coordinated effort to achieve malaria elimination in the region. The four frontline countries of South Africa, Botswana, Namibia and Eswatini (previously Swaziland) aim for elimination by 2020 [142], thus setting the stage for subsequent elimination in the other E8 member states of Angola, Mozambique, Zambia, and Zimbabwe by 2030 [143]. Countries in the E8 region experienced reductions in malaria cases between 2002 and 2012, a stagnation in the number of cases from 2012-2015 with a resurgence experienced from 2015-2018 [144]. This resurgence is attributed to poor vector control coverage, weaknesses in surveillance, changes in vector profile and insecticide resistance as well as increased vulnerability due to connected cross-border zones [139, 145]. The challenges facing E8 countries in their push towards elimination and possible solutions are summarised in Table 1.2. After reviewing the challenges, the E8 has outlined 3 key pillars to help the region accelerate towards elimination. These include integrated vector management strategy, universal integrated patient and parasite management, enhanced surveillance with supporting elements of robust programme and health systems. The crosscutting issues affecting this region is mainly the population mobility and lack of a synchronised malaria containment strategy.

Table 1.1 Challenges and possible solutions for malaria elimination in the E8 region. Table adapted from [144, 145].

Component	Challenges	Solutions
Integrated vector management	→ Lack of entomology data to support evidence-based vector control strategies; → Poor IRS coverage; → Procurement challenges of IRS commodities; → Inadequate supervision of spray operators; → Limited entomological surveillance.	Robust monitoring of → Vector species composition; → Bionomics; → Insecticide resistance.
Integrated patient management	→ High malaria case fatality rates; → Imported malaria due to population movements and migration within and across international boundaries.	→ Achieve universal coverage; → Availing quality-assured prevention and treatment to the general population and vulnerable groups.

Integrated Parasite management and surveillance	→ Inadequate epidemic detection and rapid response systems; → Lack of coverage and access to malaria testing and treatment in some localities; → Inadequate epidemic detection and rapid response systems.	→ Improve regional data and information sharing for evidence-based decision-making and resource allocation; → Provide incidence management support for prompt response at the regional, national and subnational levels. → Vector incrimination studies
Programme management and health systems	→ Lack of technical capacity for elimination activities at subnational levels; → Funding gaps interrupting of elimination service delivery; → Long turnaround times in procurement systems to enable prompt outbreak responses.	→ Enhanced malaria workforce; → skilled for technical and programmatic coordination, supervision and implementation.

1.8 South Africa's elimination efforts: past and present

South Africa is in the southern most fringes of malaria distribution on the African continent and has historically had remarkable success in malaria control over the years. It was earmarked for malaria elimination by 2020 according to the E8 plans [144] and is classified by the WHO as a pre-elimination setting [146]. However, a recent review of the progress and challenges faced by the country in its bid to achieve elimination noted several concerns [139]. Historical records show that epidemics were common during 1830-1900s [147]. However, intense larvicidal measures, together with case management using quinine, contained the situation and led to a drop in malaria cases despite periodic epidemics. The introduction of DDT for IRS led to the eradication of *An. funestus* in South Africa with no reported malaria epidemics until the mid-1980s [147]. Occasional epidemics in the period following the 1980s have been associated with the spread of chloroquine (CQ) resistant parasites. A drug policy change from CQ to SP contained this situation leading to low levels of malaria in the ensuing years [60]. However, from the mid-1990s, a trend of increasing malaria cases was recorded peaking in 1999-2000 transmission season. This was attributed to various issues including the policy change earlier in 1995 to move away from DDT for IRS and the subsequent reappearance of pyrethroid resistant *An. funestus* and the emergence of sulphadoxine-pyrimethamine (SP)-resistant parasites [147]. A relaxed border control, allowing the influx of Mozambican immigrants from the high-transmission setting, and favourable climatic conditions may also have contributed to the rise in malaria from the mid-1990s onwards. The subsequent reintroduction of DDT for IRS in addition to the replacement of SP with ACTs drastically

reduced malaria transmission (Figure 1.6) and this time the reduction seemed sustainable for many years to come.

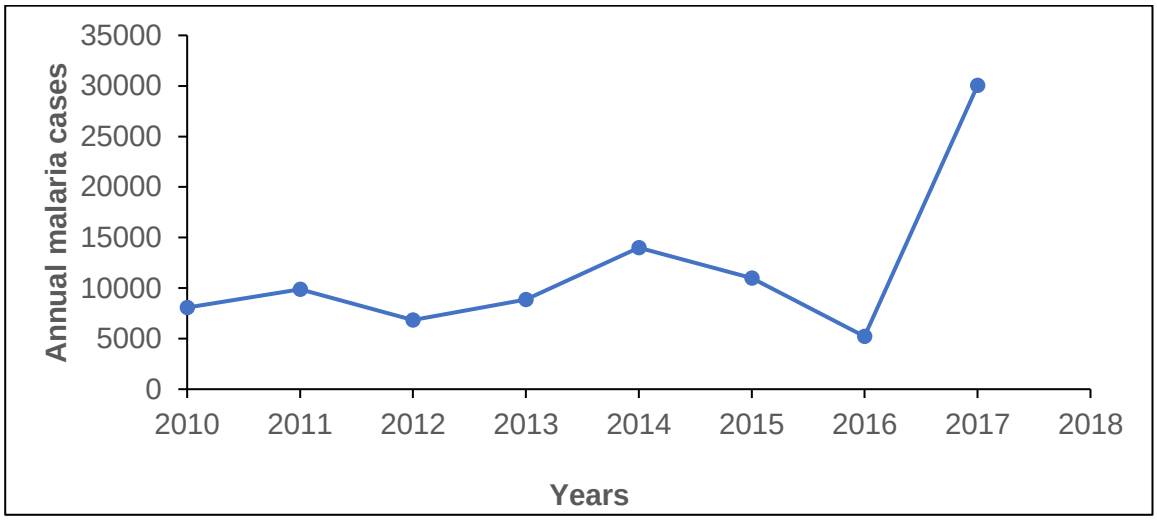

Figure 1.6: Annual number of malaria cases (confirmed and presumed) in South Africa between 2010 and 2017. Figure compiled with data from World Health Organization [1, 148].

A suite of control interventions have been deployed in South Africa to help keep malaria at the low levels with large parts of the country being classified as malaria-free [60]. However, the disease is still endemic in the north-eastern areas of KwaZulu-Natal and the low altitude regions of Mpumalanga and Limpopo Provinces (Figure 1.7). These endemic provinces are bordered by Eswatini, Botswana, Mozambique, and Zimbabwe with different malaria-endemicity patterns. The 3 malaria-endemic provinces have different levels of success with malaria control with transmission heterogeneously distributed in each of the endemic provinces. Using the WHO elimination continuum, the provinces are in different phases of the control or pre-elimination. Within KwaZulu Natal province, the only 3 malaria-endemic municipal districts (uMkhanyakude, uThungulu, and Zululand) are in the elimination phase. In Mpumalanga, out of 3 districts, only a single municipal district (Ehlanzeni) is in the elimination phase. In Limpopo Province, out of the 5 malaria-endemic districts (namely Capricorn, Greater Sekhukhune, Mopani, Vhembe, and Waterberg), Vhembe municipal district stands out. That district is responsible for over 60% of the national malaria burden and is thus still in the control phase [139].

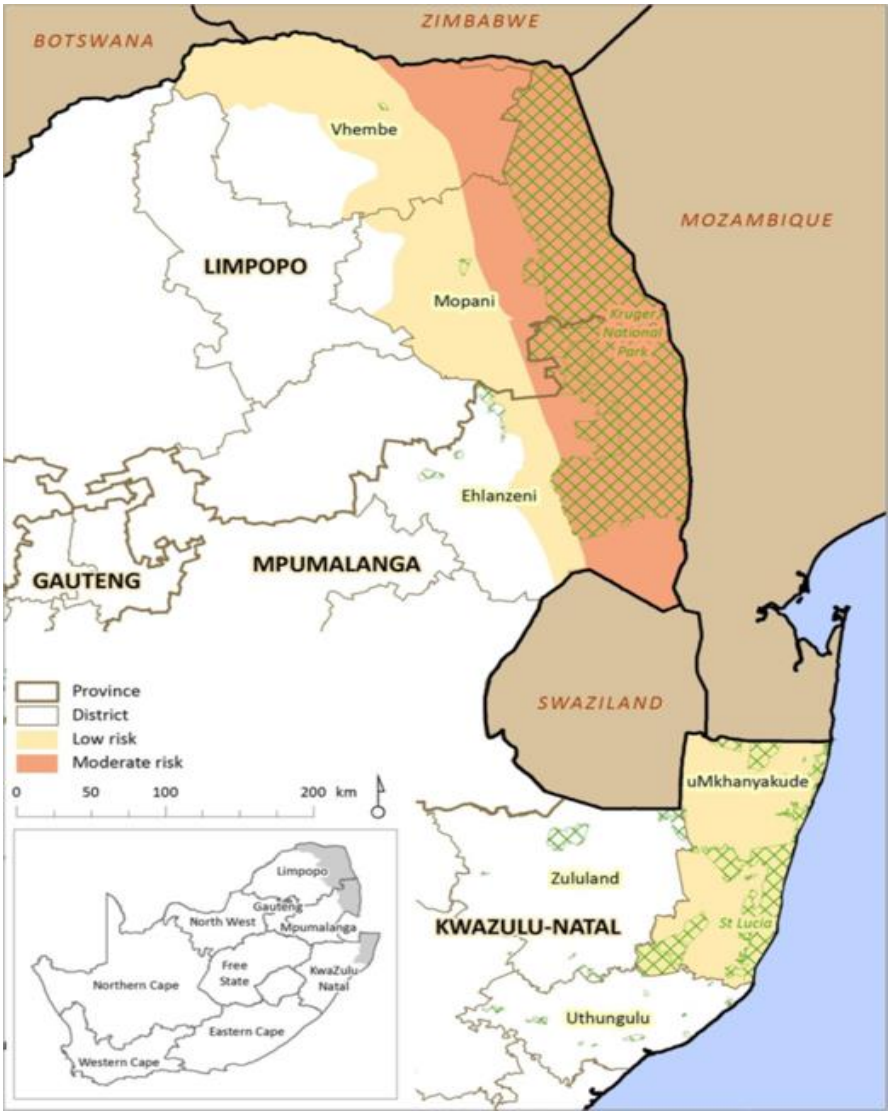

Figure 1.7: Malaria risk map for South Africa showing the malaria-endemic provinces and surrounding Elimination 8 member states [139]. This figure is freely available for use under Creative commons attribution license 4.0 (CC BY 4.0).

1.8.1 South Africa's current malaria and vector situation

P. falciparum is currently and has always been responsible for the majority of malaria cases in South Africa (>95%), with *P. malariae*, and *P. ovale* occasionally reported either as mixed infections or singly [149]. Malaria transmission is seasonal, beginning in the rainfall months of September to May per transmission seasons with peaks usually in December, January, and April. These transmissions peaks usually coincide with increased movements witnessed during the Christmas and Easter holidays [139]. The main vectors in South Africa belong to the *An. gambiae* and *An. funestus* complexes [147]. *Anopheles funestus* has been implicated in malaria epidemics and outbreaks in the country [150]. In addition, *An. arabiensis* and *An. merus* have also been implicated in local malaria transmission [151]. *An. arabiensis* is widely distributed in all the malaria-endemic districts of South Africa. Due to its outdoor resting

(exophilic) and biting behaviour (exophagic), *An. arabiensis* is less amenable to control by IRS and may thus play an important role in explaining persistent low-level transmission in South Africa [147]. In contrast, *An. funestus,* an anthropophilic and endophilic species, is decreasing in importance for malaria transmission in South Africa, perhaps due to the sustained use of IRS [147]. The recent detection of first outdoors-caught female *An. vaneedini* with *P. falciparum* sporozoites in KwaZulu-Natal province [152], may have implications for the currently used vector control approaches in the country and calls for increased surveillance for vector bionomics. However, further proof of infection is necessary as this report was based on very limited samples.

1.9 Challenges of malaria elimination in South Africa

Residual transmission is defined as the persistence of malaria transmission following the implementation in time and space of a widely effective malaria programme [23]. The sources of and risks for residual transmission may vary by location, time and the existing components of the current effective malaria control programme [153]. Several challenges can be defined in the current context of malaria elimination in South Africa, including changing vector populations, increasingly heterogeneous transmission dynamics and substantial imported malaria. As such, data is needed to address our incomplete understanding of the drivers of continued residual transmission in malaria transmission hotspots. In this context, it is critical to ascertain whether residual transmission in South Africa arises due to vector behavioural changes/plasticity or if there is a parasite component involved. For the latter, the role of parasite genotypes in driving continued transmission needs to be defined as well as to determine the extent to which a hidden parasite reservoir contributes to this. However, sensitive detection and characterisation of both sexual and asexual parasites [27] is required to understand the contribution of the parasite component to continued transmission. Additionally, evaluation of the potential use of evidence-based targeting of the parasite reservoir with transmission-blocking drugs like PQ is required, particularly for the South African setting. However, whether PQ can be safely used in such interventions depends on our understanding of human genetic factors that are involved in mediating PQ metabolism and toxicity. If this information indicates that PQ could be safely deployed in the South African population, this leads to the next question as to who should be treated. Therefore, an updated evaluation of the parasite reservoir is required with highly sensitive and specific molecular tools. These sensitive molecular tools for parasite detection may further be deployed for vector surveillance as complementary tools to existing surveillance approaches.

1.10 Aim

To characterise the contribution of the parasite reservoir and its role in continued residual malaria transmission in the Vhembe District of South Africa in order to inform the deployment of anti-parasite tools for malaria elimination.

Objectives

Chapter 2 investigates the existing prevalence of G6PD deficiency and CYP2D6 variants in Vhembe District and how they may influence PQ efficacy, safety and thereby PQ deployment.
Chapter 3 aims to characterise the parasite reservoir in the population in Vhembe District, through *Plasmodium* parasite detection and speciation, drug resistance profiling and multiplicity of infection.
Chapter 4 explores the utility of new surveillance tools to aid in elimination. Novel male and female specific gametocyte markers are used to quantify parasites from mosquito blood meals as an alternative source of human blood for surveillance. Additionally, the suitability of ultrasensitive and regular RDTs for xenosurveillance is investigated.

The following outputs resulted from this study:

Published manuscript:

Awandu SS, Raman J, Makhanthisa TI, Kruger P, Frean J, Bousema T, Niemand J, Birkholtz LM (2018): Understanding human genetic factors influencing primaquine safety and efficacy to guide primaquine roll-out in a pre-elimination setting in southern Africa. Malaria journal. 7(1):120.

Awandu SS, Raman J, Bousema T and Birkholtz LM (2019). Ultra-low-density *Plasmodium falciparum* infections in African settings. Clinical Infectious Diseases Feb 15. pii: ciz147. doi: 10.1093/cid/ciz147. [Epub ahead of print]

Manuscript in preparation

Xenosurveillance of *Plasmodium falciparum* through mosquito blood meal analysis. SS Awandu, A Siebler, GJ van Gemert, M van de Vegte-Bolmer, L Meerstein-Kessel, Kjerstine Lanke, Wouter Graumans, L Birkholtz, T Bousema, B Knols.

International conference proceedings:

SS Awandu, J Raman, K Hafmans, P Kruger, H Gupta, J Niemand and L Birkholtz. High levels of submicroscopic and asymptomatic *Plasmodium* infections in a low transmission southern African setting; implications for elimination. 7th Multilateral Initiatives on Malaria Pan African Conference, Dakar Senegal (April 2018). Oral presentation.
SS Awandu, J Frean, P Kruger, J Niemand, J Raman and L Birkholtz. Towards malaria elimination; host genetic factors determining potential primaquine use in Vhembe District,

Limpopo Province, South Africa. Malaria: From Innovation to Eradication, Kampala Uganda (February 2017). Poster presentation.

SS Awandu, J Raman, J Niemand, P Kruger, T Kruger, B Louw, L Birkholtz. Glucose 6 phosphate dehydrogenase (G6PD) deficiency phenotype and genotype distribution in point-of-care settings in Vhembe district, Limpopo Province, South Africa. American Society of Tropical Medicine and Hygiene (ASTMH 65th Annual Meeting) Atlanta, Georgia, United States of America (November 2016). Poster presentation.

SS Awandu, J Raman, J Frean, P Kruger, B Louw, J Frean and L Birkholtz. Prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficient phenotypes and genotypes at select point of care health settings in Vhembe District, Limpopo Province, South Africa. 2nd South African Malaria Research Conference, Pretoria South Africa (July 2016) Oral presentation.

SS Awandu, J Raman, J Niemand, P Kruger, T Kruger, B Louw, L Birkholtz. Molecular epidemiology of malaria transmission in Vhembe District, Limpopo Province, South Africa. São Paulo Schools of Advanced Science - Science of Eradication: Malaria course. São Paulo Brazil (September 2015). Poster presentation.

SS Awandu, J Raman, J Niemand, P Kruger, T Kruger, B Louw, L Birkholtz. Molecular epidemiology of malaria transmission in Vhembe District, Limpopo Province, South Africa.1st Southern African Malaria Research Conference, Durban South Africa (August 2015). Oral presentation.

Chapter 2

Human genetic factors influencing primaquine safety and efficacy to guide primaquine roll-out in a pre-elimination setting in southern Africa

This work has been published as follows: Awandu S.S., J Raman, T. I. Makhanthisa, P Kruger, J Frean, T Bousema, J Niemand and L Birkholtz. 2018. "*Understanding human genetic factors influencing primaquine safety and efficacy to guide primaquine roll-out in a pre-elimination setting in southern Africa.*" *Malaria Journal* 17 (1):120. doi: 10.1186/s12936-018-2271-z.

2.1 Introduction

South Africa is one of eight southern African countries that are signatories of the E8 initiative, a multi-country coordinated effort to achieve malaria elimination. The four frontline countries (South Africa, Botswana, Namibia, and Eswatini) aim for elimination, in various timeframes by 2020 [94], thus setting the stage for subsequent elimination in the other E8 member states [154]. In an effort to accelerate the region towards malaria elimination, novel parasite and integrated vector tools are being explored.

For treatment of patients with *P. falciparum* malaria, the WHO advocates the use of a single low-dose (0.25 mg/kg) of PQ together with artemisinin-based combination therapy in malaria-eliminating countries [79]. PQ is effective against the transmissible gametocyte stage of *P. falciparum* parasites and may reduce onward transmission to mosquitoes if deployed at the community level [155, 156]. It is also the only commercially-available drug active against hypnozoites in *P. vivax* and *P. ovale* infections [157]. However, country-specific efforts towards low-dose PQ deployment for *P. falciparum* transmission reduction are being impeded by the perceived safety issue, surrounding PQ use in G6PD-deficient individuals. When exposed to high doses of 8-aminoquinoline anti-malarial agents like PQ and tafenoquine, G6PD-deficient individuals risk suffering haemolysis [157, 158]. However, at the recommended low dose, the risk of haemolysis is thought to be substantially reduced and PQ is considered safe [79].

G6PD deficiency is an X-linked genetic disorder that displays great polymorphism with about 186 described mutations [159], resulting in the most common enzymopathy in the world [158]. G6PD deficiency is clinically observed mainly in hemizygous males or homozygous females, with heterozygous females presenting with a wide spectrum of aberrant enzyme activities due to mosaicism. Circumstantial evidence suggests that G6PD deficiency imparts some resistance against malaria as evidenced by the overlap in the geographical distribution of the deficiency with present and past malaria-endemicity [160], although the exact nature of such

potential protection is unknown. Whilst earlier studies reported G6PD deficiency protection against severe forms of malaria [161, 162], recent data from a large multi-centre, case-control study and a meta-analysis suggests protection from cerebral malaria and high parasitaemia and not severe malarial anaemia, mainly in heterozygous individuals in African settings [163, 164]. Although the complexity of this disorder presents a diagnostic challenge, certain G6PD variants, including the African and Mediterranean variants, have been used to probe the general G6PD deficiency status in populations [165]. G6PD variants are classified according to their residual enzyme activity into three types [166]. These range from the most severe presentations but rare type 1, that present with congenital nonspherocytic anaemia, while type 2 is usually asymptomatic in the steady state until exposed to exogenous triggers. These type 2 variants are the equivalent of class II and III of the WHO classification system [159] and predispose individuals to substantial risk of haemolytic anaemia. Type 2 variants include the Mediterranean, A-, Mahidol, Canton, Vanua Lava and Seattle variants amongst others [167]. Type 3 variants manifest phenotypically as normal G6PD activity with no clinical issues.

In sub-Saharan Africa, three variants are most commonly described, namely the B wild-type variant associated typically with normal G6PD activity and the G6PD African variants, A+ and A- [167]. The African variants (“A”) are amongst the most common in the world with a wide enzyme activity range [158]. The A+ variant carries an A376G mutation in exon V that results in mild enzyme deficiencies. By contrast, the more common A- variant which carries an additional mutation in exon IV (G202A) is associated with mild clinical presentations such as jaundice and dark urine when exposed to 8-aminoquinolines [167]. Several other A- mutations (A542T, G680T, and T968C) have been detected in West African individuals [168, 169].

PQ efficacy is further modulated by the CYP2D6 enzyme [85], which metabolizes ~25% of all clinically prescribed drugs [170]. CYP2D6 is highly polymorphic with varied enzymatic activity as a result of allelic sequence variations and protein structural rearrangements. These genotypic and phenotypic variations affect drug metabolism, probably increasing the risk of treatment failure or potential toxicity [84]. To date, more than 113 allelic variants (and sub-variants) of the *cyp2d6* gene have been reported which can be classified into four distinct phenotypic groups. Poor metabolizers (PMs) have two copies of defective alleles resulting in reduced expression of a particular CYP2D6 enzyme. Intermediate metabolizers (IMs) are heterozygous for a defective allele and a functional allele resulting in slightly reduced enzyme activity. Extensive metabolizers (EMs) carry two functional alleles and have normal enzyme activity, while ultra-rapid metabolizers (UMs) have more than two functional gene copies and elevated enzyme activity [171]. The PMs with the CYP2D6*4 polymorphisms are associated with PQ treatment failure in *P. vivax* [172], whereas IMs harbouring CYP2D6*17 mutations have been linked with diminished enzyme activity in black Africans [173]. Whilst the impact of

CYP2D6 metabolizer status on the efficacy of single low-dose PQ for gametocyte clearance is currently unknown, it is conceivable that the gametocytocidal effect of PQ would be reduced if lower plasma concentrations of the active PQ metabolite are achieved [174].

Prevalence of G6PD deficiency and CYP2D6 polymorphisms in a population are thus important considerations for public health interventions involving the deployment of 8-aminoquinoline drugs like PQ [80]. In sub-Saharan Africa, the prevalence of G6PD deficiency is estimated to range between 5% to 37.5% [175], and CYP2D6 allele frequencies range from 1% to 33% [176]. South Africa has previously (1940s-1980s) reported fewer cases of G6PD deficiency (5.3%) [177, 178] and CYP2D6 (2.6%) [179] mutations compared to its neighbouring countries, most likely a consequence of genetic differences among the distinct ethnic groups [180, 181]. However, recent population movements and immigration [182] may have led to shifts in the frequency of the enzyme deficiency and CYP2D6 variants across the country. Due to a paucity of current data, regionally relevant evidence of G6PD deficiency and CYP2D6 variant metrics are needed. These will inform implementation of single low-dose PQ policy in pre-elimination settings within South Africa.

This study aimed to determine the current prevalence of both G6PD deficiency and CYP2D6 variants in representative populations in a malaria-endemic Vhembe region of South Africa. G6PD deficiency measurements were also compared to phenotypic screening with a potential point-of-care tool in mass screen and test scenarios. This is the first study employing genotyping to evaluate locally relevant G6PD deficiency and CYP2D6 variants in a pre-elimination setting of South Africa.

2.2 Materials and methods

2.2.1 Ethics statement

Ethical approval for the study was obtained from the ethical review committees of the University of Pretoria (Ethics reference No. 406/2014) and the Limpopo Department of Health (Ref 4/2/2). Signed informed consent in the local language (Tshivenda) was obtained from all participants prior to sample collection. Parents or guardians of children less than 7 years of age gave written, informed consent, while children between the ages of 7 and 17 years gave assent before sampling. An adult witness was required to co-sign the informed consent document.

2.2.2 Study site and population

This study was conducted in the Vhembe District, a 25 597 km^2 area in Limpopo Province, a malaria-endemic region in the northernmost part of South Africa. Malaria transmission primarily occurs during the months of September to May, with limited transmission generally occurring over the winter months of June and July [183]. Vhembe District is responsible for more than 60% of all reported malaria cases in South Africa [139], with 1 543 cases and 18 deaths noted in the region in the 2015/16 transmission season, when sampling occurred [9, 184]. The district is bordered by Mozambique to the south-east, Zimbabwe to the north and Botswana to the north-west. The climate is typically subtropical with mild winters and wet, warm summers with average rainfall per annum of 820 mm [185]. The district experiences frequent droughts with areas that are predominantly semi-arid [186].

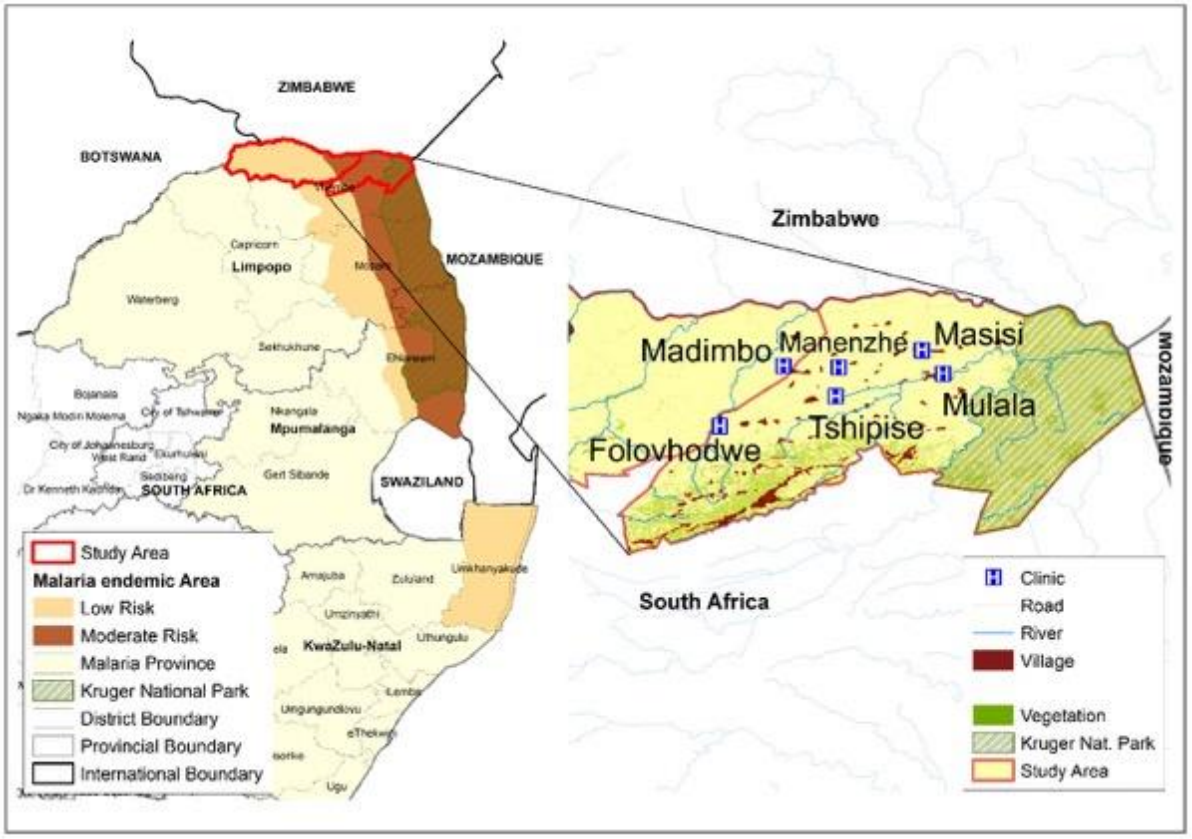

Figure 2.1: Map of the study site in Vhembe District, Limpopo Province, South Africa. The six primary health care clinics (H) and village catchment areas where participants were recruited are indicated. The study district in Limpopo Province is shown alongside other malaria-endemic provinces of KwaZulu-Natal and Mpumalanga, is in close proximity to Botswana, Zimbabwe, and Mozambique. Study participants were recruited from six primary health care clinics (PHCs): Folovhodwe, Madimbo, Manenzhe, Masisi, Mulala and Tshipise in Mutale and Musina local municipalities (Fig. 2.1) during October and November 2015. Once recruited into the study in one particular clinic, patients were ineligible for subsequent enrolment even if they presented at a different clinic. The district has an estimated population of 1 347 235 with a population density of 52.6 persons per km^2 [187]. The area is mainly inhabited by the Venda ethnic group, with sizable Zimbabwean immigrant populations (Shona and Ndebele ethnic groups) that work on the commercial farms and reside in the area.

2.2.3 Questionnaire administration and sample collection

A brief questionnaire on demographics (specifically age and gender), travel history, history of fever or any malaria symptoms, ethnicity and residence was administered to the consenting participants. Upon completion of the questionnaire, fingerprick filter paper (Munktell TFN card, Lasec, Cape Town, South Africa) blood spots were collected from the participants for *P. falciparum* parasite detection and human genotyping studies. Sample size in this pilot study was constrained by the availability of CareStart™ G6PDd rapid diagnostic test kits.

2.2.4 Malaria parasite detection

Peripheral fingerprick blood samples were tested for *P. falciparum* parasite antigens using RDT kits detecting the histidine-rich protein 2 (First Response® Malaria Antigen *P. falciparum* card test HRP2, Premier Medical Corporation, India) in accordance with the South African National Malaria Diagnosis Quality Assurance guidelines [188, 189]. The test was performed on individuals presenting with fever at the PHCs. The presence of *P. falciparum* parasites was confirmed by multiplex polymerase chain reaction (PCR) as previously described [190].

2.2.5 G6PD phenotypic screening

The CareStart™ G6PD RDT (Access Bio Inc., New Jersey, USA) was administered concurrently with the malaria RDT on 248 participants at the PHCs as per the manufacturers' guidelines. Briefly, a fingerprick blood sample (2 µl) was collected through the sample pipette, applied into the sample well and 2 drops (~100 µl) of assay buffer added. The test was developed at room temperature (25°C) for 10 min, after which the results were read by two blinded primary evaluators, with a third blind evaluator acting as a tiebreaker to make the final decision in case of discrepancies. Normal G6PD individuals were classified according to the development of a distinct purple coloured test band, while in G6PD-deficient patients, no purple coloured test band developed. A test was declared invalid when either no or incomplete blood migration took place.

2.2.6 G6PD genotyping

DNA was extracted from blood-saturated filter paper discs of 3 mm diameter using the Chelex method [191]. Specific regions of the *g6pd* gene were amplified and subjected to restriction fragment length polymorphisms (RFLP, all restriction enzymes sourced from New England Biolabs, Inc., USA) to detect mutations as previously described [165]. A 308 base pair (bp) amplicon was amplified from genomic DNA and subjected to *Fok*I digestion for 60 min at 37°C. Uncut fragments were classified as G6PD variant B, while samples where two fragments of 184 bp and 124 bp were produced, were classified as G6PD A+ variants. Only samples

positive for G6PD A+ mutations underwent further PCR amplification and RFLP analysis to detect additional mutations. Subsequent digestion was performed with *N1a*III (G202A; 211 bp, 81 bp, and 130 bp), *Bsp*EI (G542T; 130 bp, 80 bp and 50 bp), *Bst*NI (G680T; 115 bp, 98 bp and 29 bp) and *Nci*I (T968C; 282 bp, 162 bp and 120 bp) for 60 min at 37°C (except for *Bst*NI where digestion occurred at 60°C). All G6PD deficiency restriction products were resolved on 3% agarose/TAE gel with ethidium bromide staining. Images were captured on UVDoc HD2 transilluminator (UVITEC, Cambridge, UK).

2.2.7 CYP2D6 genotyping

Genotyping of CYP2D6*4 was performed by PCR amplification of a 309 bp segment of the *CYP2D6* gene followed by overnight digestion with *Bst*NI at 60°C as previously described [192], resulting in a 309 bp fragment for *wt/wt* CYP2D6*4; 309 bp, 201 bp and 108 bp fragments for *wt/mt* CYP2D6*4 or 201 bp and 108 bp fragments for *mt/mt* CYP2D6*4. CYP2D6*17 genotyping was performed by PCR amplification of a segment of the *cyp2d6* gene of interest and subsequent overnight digestion with *Bts*CI as previously described [192], resulting in 407 bp, 328 bp, 79 bp, 67 bp and 55 bp fragments for *wt/wt*. For CYP2D6*17 *wt/mt* the resulting fragment sizes were 407 bp, 328 bp, 79 bp, 67 bp and 55 bp; *mt/mt* CYP2D6*17 resulted in 328 bp 79 bp, 67 bp, and 55 bp fragments. For all the CYP2D6 variants, the PCR and restriction products were resolved on 1.5% agarose/TAE with ethidium bromide staining gel. Images were captured on UVDoc HD2 transilluminator.

2.2.8 Sanger sequencing

All G6PD A- positive PCR products were confirmed with Sanger nucleotide sequencing. After purification with the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany), samples resuspended in HiDi™ formamide were sequenced using an ABI PRISM BigDye® terminator V3.1 cycle sequencing kit and processed on ABI PRISM 3130xl and ABI PRISM 3500xl genetic analyzers (Applied Biosystems™/Life Technologies, Carlsbad, USA). BLAST queries were conducted for preliminary identifications of the sequences and thereafter analysed with MAFFT v 7.182 and CLC sequence viewer software.

2.2.9 Data analysis

Data were analysed with SPSS version 20 (IBM Corp. NY, USA). Descriptive statistics were used to report the demographics of the participants, with phenotype and genotype being presented per PHC. Differences in proportions of individuals carrying the G6PD deficiency, CYP2D6*4 and CYP2D6*17 wildtype and mutant alleles were compared by chi-squared (χ^2) tests. Odds ratios (ORs) were used to assess any association of G6PD deficiency, CYP2D6*4

and CYP2D6*17 occurrence based on gender. To test for conformity with the Hardy-Weinberg equilibrium (HWE), allele frequencies were analysed both by PHC and overall. The expected female allele frequencies were calculated based on male genotype prevalence (A+, A- and B) then compared with observed values using chi-squared tests as described [165]. The negative predictive value (NPV) of the CareStart™ G6PD deficiency kit was determined using the A-variant PCR-RFLP genotyping results as the 'gold standard' [193]. Agreement between the two test results was evaluated using Cohen's Kappa coefficient (κ), with the following scale used to score strength of agreement; 0.01-0.20 slight; 0.21-0.40 fair; 0.41-0.60 moderate; 0.61-0.80 substantial and 0.81-1.00 almost perfect [194]. A *P*-value of <0.05 was considered statistically significant.

2.3 Results

2.3.1 Study participant characteristics and malaria parasite detection

From the six PHCs, a total of 248 participants of all age groups were sampled when they visited the centers for care and consented for recruitment into the study. More female participants (n=193, 78%) compared to male participants (n=55, 22%) were enrolled, with study participants' median ages of 31 years for females (range 1-94) and 21 years for males (range 1-81). The majority (97%, 241/248) of participants self-identified as South African Venda ethnicity. Other ethnic groups screened included Pedi (1.2%, 3/248) and Tsonga (0.4%, 1/248), with 1.2% (3/248) Shona from Zimbabwe. None of the participants harboured detectable *P. falciparum* parasites by either *P. falciparum* RDT or single-step, multiplex PCR.

2.3.2 G6PD genotyping

The prevalence of the A+ and A- African G6PD variants among the study participants was determined by genotyping. Of the 248 participants screened, just over half (52%, 129/248) carried the G6PD normal B allele (Figure 2.2). The A376G mutation in exon V was detected in 32% (79/248) of the participants, identifying these individuals as carrying the A+ variant. A total of 13% (32/248) of the participants were G6PD A- heterozygous individuals. Only a minority of participants (3.2%, 8/248), were hemizygous or homozygous for the G6PD A- (A376G/G202A) variant. Among males, G6PD A- hemizygous individuals constituted 11% (6/55), while among females, 1% were G6PD A- homozygous (2/193). All G6PD A- variants identified contained A376G/G202A mutations, which was independently confirmed by DNA sequencing. The A542T, G680T or T968C mutations were not detected in the study population.

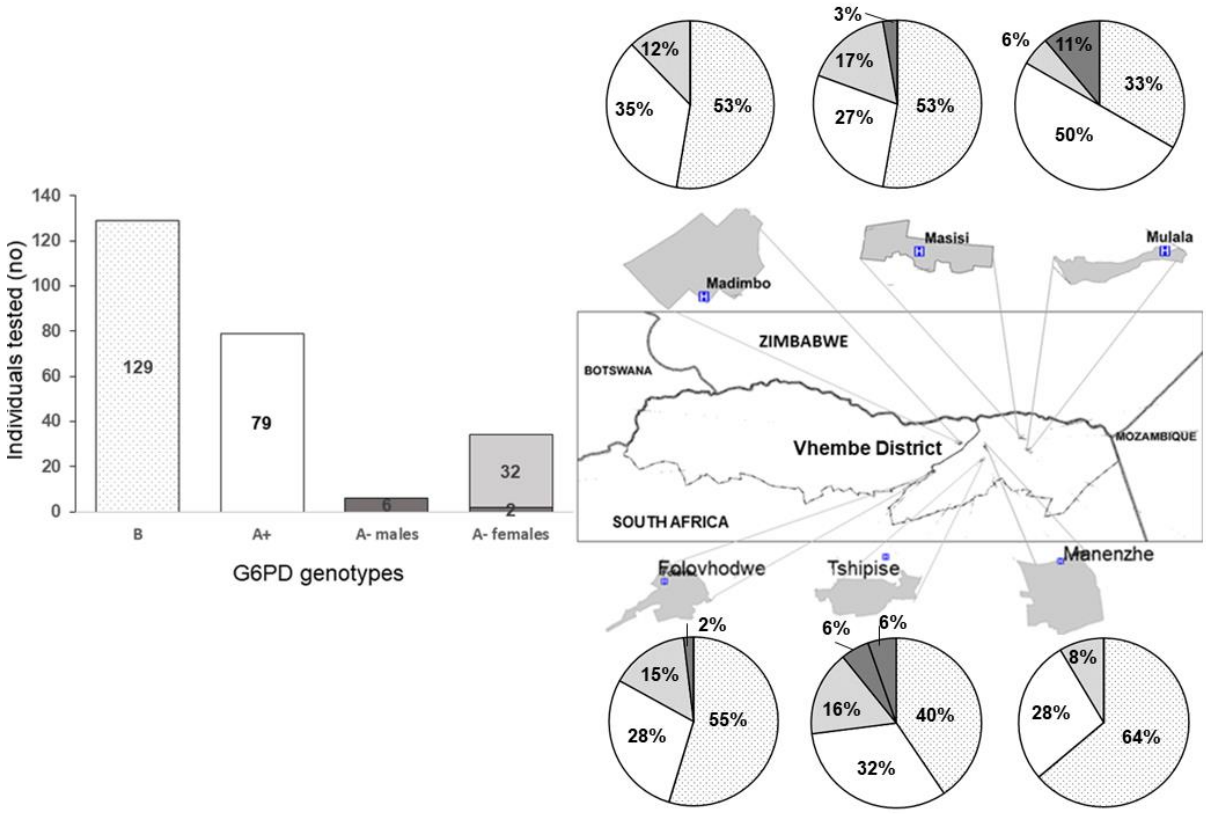

Figure 2.2: G6PD genotype prevalence. A) G6PD genotypes (B patterned bar; A+ (A375G) white bar; A- (A375G/G202A) heterozygous light grey bar, hemizygous and homozygous dark grey bar) identified with PCR-RFLP and confirmed by sequencing for all the study participants. B) G6PD deficiency stratified for each of the 6 primary health care clinics in Vhembe District, Limpopo Province, South Africa. (B patterned wedge; A+ (A375G) white wedge; A- (A375G/G202A) heterozygous light grey wedge, hemizygous and homozygous dark grey wedge).

When comparing the proportions of participants with G6PD A+ and A- variants in the population, considerable variations were observed among localities (P = 0.01 for A+ and P = 0.05 for A- genotypes, respectively). In Manenzhe PHC, the highest proportion (64%) of G6PD B variants but lowest proportion (8%) of G6PD A- variants were detected. All the A- participants from Madimbo and Manenzhe PHC were A- heterozygous females (Fig. 2B). Mulala PHC recorded the highest proportions of A- hemizygous participants (11%) while homozygous participants (6%) were only identified from Tshipise PHC. All of the A- deficient participants G6PDd were of Venda ethnicity.

The genotypic frequencies were tested to confirm adherence to the HWE. According to the principle, the genetic variation in a population should remain constant from generation to generation in the absence of disturbing factors. The HWE measurement indicated that in general, female gene frequencies did not significantly differ from predicted male gene frequencies (χ^2 >0.05). However, departures from HWE were seen for the G6PD deficiency variants for the general population (χ^2 = 0.007) and in Tshipise PHC (χ^2 = 0.009) (Table 2.1) where all of the study homozygous participants were reported. When comparing G6PDd A+

and G6PDd A- genotypes, no distinctive association with gender was observed *(P* = 0.107 and *P* = 0.441, respectively).

Table 2.1: **Frequency of G6PD genotypes in 6 primary health care clinics in Vhembe District, Limpopo Province, South Africa.**

	Genotypes											
	Males				Females							
	No.	A^-	A^+	B	No.	A^-A^-	A^+A^-	BA^-	A^+A^+	BB	BA^+	HW $P(\chi^2)$
Folovhodwe	10	1	4	5	43	0	5	3	0	24	11	0.880
Madimbo	6	0	0	6	51	0	1	6	2	24	18	NC
Mananzhe	9	0	3	6	38	0	1	3	2	24	8	0.548
Masisi	11	1	2	8	25	0	1	5	1	11	7	0.197
Mulala	7	2	2	3	11	0	1	0	3	3	4	0.317
Tshipise	12	2	1	9	25	2	1	5	1	6	10	0.009*
Totals	55	6	12	37	193	2	10	22	9	92	58	0.007*

G6PD genotypes: Male (X*Y) hemizygous deficient A-, male (XY) normal A+ or B; female (X*X*) homozygous deficient = A-/A-; female (X*X) heterozygous deficient = A+/A- or B/A-; and female (XX) normal =A+/A+, B/B or B/A+.
**P*-values <0.05 indicate significant differences from predicted G6PD allele frequencies in females based on male allele frequencies for A-, A+ and B alleles using the Hardy-Weinberg equation ($\chi 2$).
NC = not calculated as all males tested in Madimbo clinic carried the G6PD B variant.

2.3.3 Phenotypic G6PD screening

The CareStart™ G6PD deficiency RDT test kit was used for phenotypic evaluation of G6PD status in the test population, to explore its utility as a point-of-care tool. The CareStart™ G6PD deficiency RDT identified 13% (33/248) of participants as G6PD- deficient (Table 2.2), with males showing a higher prevalence of G6PD deficiency (18%; 10/55) compared to females at 12% (23/193) (odds ratio 1.642; 95% confidence interval 0.7293 – 3.6992, *P* = 0.229). All the identified G6PD-deficient individuals bar one (Tsonga) were of Venda ethnic origin.

The CareStart™ G6PD deficiency RDT was able to detect all the genetically determined G6PD-deficient hemizygous males and homozygous females (Table 2.2) with the highest risk of haemolysis. Comparison of the RDT phenotyping results with that of the G6PD A- (A376G/G202A) PCR-RFLP genotyping indicated the specificity of 91.3% (95% CI 86.7-94.8) with a fair Cohen's Kappa agreement (κ = 0.310). A more substantial agreement was observed among the males (κ = 0.711) than among the females (κ = 0.202). The negative predictive value for identifying hemizygous, homozygous and heterozygous participants by RDT was 0.88 (95% CI 0.85-0.91) compared to genotyping.

Table 2.2: **G6PD deficiency status amongst male and female participants per primary health care clinic according to the CareStart™ G6PD RDT.**

		Males (n, %)		Females (n, %)	
CareStart™ G6PDd RDT classification		Deficient (10, 18%)	Normal (45, 82%)	Deficient (23, 12%)[b]	Normal (170, 88%)[c]
Primary Health Care Clinics[a]	Folovhodwe	2 (4%)	8 (14%)	7 (4%)	36 (19%)
	Madimbo	1 (2%)	5 (9%)	1 (0.5%)	50 (26%)
	Manenzhe	1 (2%)	8 (14%)	5 (3%)	33 (17%)
	Masisi	2 (4%)	9(16%)	3 (1%)	22 (11%)
	Mulala	2 (4%)	5 (9%)	5 (3%)	6 (3%)
	Tshipise	2 (4%)	10 (18%)	2 (1%)	23 (12%)

[a] No of participants and (%) indicated.
[b] From the deficient females by CareStart™ G6PD RDT test, 2 were homozygous, 7 heterozygous and 14 normal.
[c] From the 170 normal females, 25 were heterozygous and 145 normal.

2.3.4 Cytochrome P450 genotype and phenotype distribution

Mutations in the *cyp2d6* gene that could interfere with PQ activation were evaluated on the 248 collected samples (Table 2.3). Allele frequencies of 0.03 and 0.19 were detected for the CYP2D6*4 and CYP2D6*17 alleles, respectively. Only 2 of the study participants (0.8%, 2/248) were classified as *mt/mt* PM with activity scores (AS) of 0 with a minor proportion (6%, 15/248) classified as *mt/mt* IM (AS of 0.5 - 1.0), using the AS metric as described [195]. The majority of the population (231/248, 93%) was classified as extensive or normal metabolizers. No association between gender and either the CYP2D*4 (χ^2, P = 0.731) or CYP2D6*17 (χ^2, P = 0.906) genotype was noted.

Table 2.3: **Prevalence of CYP2D6*4 and CYP2D6*17 alleles and inferred phenotypes amongst study participants.** The genotypes were determined by PCR-RFLP. Genotypes: wildtype/mutant (wt/mt) and mutant/mutant (mt/mt). All genotyping experiments were performed in duplicate in two independent experiments. Inferred phenotypes: Intermediate metaboliser (IM) and poor metaboliser (PM).

CYP2D6 alleles	Enzyme activity	Heterozyg. (*wt/mt*)	Homozyg. (*mt/mt*)	Allele frequency	Activity score	Inferred phenotype
CYP2D6*4	Non-functional	12	2	0.0323	0	PM
CYP2D6*17	Reduced	67	15	0.1956	0.5 - 1	IM

2.4 Discussion

This study reports on the prevalence of G6PD deficiency as well as CYP2D6*4 and CYP2D7*17 mutations in a southern African malaria pre-elimination setting, with the aim of informing a low-dose PQ policy. The G6PD A- prevalence of 11% among hemizygous males was comparable to that previously reported in South African Venda ethnic group [196]. Higher G6PD deficiency prevalence among males was previously reported in ethnic groups residing in close proximity to neighbouring Mozambique and Zimbabwe [178, 196], consistent with the observations in this study. These countries have higher malaria transmission intensity compared to South Africa, which could explain their higher G6PD deficiency prevalence of up to 19% in males [197, 198]. Modelling the median population estimates for G6PD allele frequency predict low frequencies of 3.3% for South Africa [166]. However, maps aggregate average frequency data to national levels and may mask sub-national variation and local heterogeneity in G6PD deficiency. By contrast, in Eswatini, a study on PQ pharmacovigilance in two health facilities reported the absence of G6PD deficiency amongst 102 participants [199].

The presence of A- (A376G/G202A) variant was confirmed in G6PD-deficient individuals, corroborating its previously described widespread distribution [177, 196]. Other G6PD variants such as the A542T, G680T or T968C mutations previously reported in the Gambia and Senegal [168, 169], were not identified in the current southern African study population. This is consistent with findings from two large randomized controlled multicentre studies in Africa [165, 200]. While the study aimed to inclusively genotype different G6PD deficiency variants identified as important in African settings, the presence of additional G6PD deficiency variants in the locality cannot be ruled out. Future studies using more novel and robust genotyping methods are envisaged, as they could identify novel mutations that may impact PQ roll-out [180].

PQ is an important addition to the standard malaria treatment regimen to aid in transmission reduction, especially for countries on the cusp of elimination [157]. PQ efficacy may be influenced by individual and ethnic genetic differences amongst populations [84, 201] and is reliant on metabolic activation. The PM and IM CYP2D6 phenotypes observed (3.2% and 19.5%, respectively) may result in PQ treatment failure at the recommended 0.25 mg/kg dose [83]. These findings on CYP2D6*4 allele frequency and predicted PM phenotype is consistent with earlier reported 1.3% [179] and 4% [202] prevalence among the Venda ethnic group. Indigenous African blacks have consistently been reported with a high frequency of the CYP2D6*17 allele, in agreement with reported data in this study [179, 181, 203].

As South Africa moves towards malaria elimination, novel interventions such as the WHO-recommended low-dose PQ strategy need further exploration. However, individuals with the CYP2D6*4 variants that may be unresponsive to PQ treatment and CYP2D*17 variants, who may require higher PQ dose to ensure therapeutic efficacy, is cause for concern in the country [83]. While there is little conclusive evidence on the effect of PQ on community-wide transmission if deployed in mass drug administration campaigns [204], efficacy studies point to a marked reduction in the duration of gametocyte carriage [155, 205]. The PQ-associated gametocyte clearance and its outcomes on mosquito infectiousness depend on malaria transmission settings and the choice of artemisinin combination therapy. It has been demonstrated that PQ has modest benefits on asymptomatic individuals with lower parasite densities prior to initiation of combination PQ treatment with either artemether-lumefantrine [174] or dihydroartemisinin-piperaquine [205]. That PQ should not be administered to infants aged <6 months, breastfeeding women of infants aged <6 months as well as pregnant women (South Africa has a 12% teenage pregnancy rate and higher fertility rates among non-urban women) [206], identifies the treatment gaps for PQ, with impact dependent on the proportion of the population treated [207].

Accumulating evidence from Africa [208-210] and Asia [211], suggests that single-dose PQ at 0.25 mg/kg as a gametocytocidal drug to block transmission would be safe in areas where G6PD deficiency A- and other type 2 variants predominate, comparable to the settings in this study site. Encouraging evidence from neighbouring Eswatini, a country with a similar G6PD deficiency prevalence profile to South Africa, suggests the roll-out of a low-dose PQ policy with no prior genetic profiling requirement, at least in none G6PD-deficient participants by RDT, is associated with good safety profiles [199]. However, stringent pharmacovigilance and patient monitoring systems should be put in place to mitigate any adverse events. Further, the low levels of CYP2D6 PM and IM is encouraging for the use of single low-dose PQ as an

adjunct to current malaria control tools and may accelerate efforts to eliminate malaria in South Africa.

This study was limited by the small sample size and constrained by the availability of the G6PD RDT tests. Moreover, a potential selection bias towards females (78%) was present in the sample set, and findings may therefore not be directly extrapolated to estimate the overall population structure and deficiency prevalence in the study area, which can be rectified through more general population sampling, rather than sampling only at PHC facilities. Since the study only deployed a G6PD deficiency RDT, enzymatic activity validation is ultimately required to evaluated functional loss of G6PD activity to disentangle female heterozygotes from deficient and the non-deficient females. Female gender segregation would control for the profound effects of comparing male hemizygous and female heterozygous on the diagnostic performance of G6PD tests [212].

2.5 Conclusion

Phenotypic and genotypic analysis both detected a low prevalence of individuals carrying the G6PD A-, CYP2D6*4 PM variants and modest prevalence of the CYP2D6*17 IM variants in the study area. Taken together with the MOI, drug resistance profiles and the submicroscopic infections in the study area, these findings suggest that the deployment of single low-dose PQ to clear gametocytes and stop onwards transmission. Single low-dose PQ would pose a minimal risk to the area populations and could be useful in elimination strategies, in localized areas of South Africa. While PQ will target the mature gametocytes, its use with ACTs for elimination requires an understanding of the parasites driving the continued residual transmission of malaria. The subsequent chapter explores and describes the parasite pool that may play a role in sustaining this residual transmission.

Chapter 3

Detection and characterization of currently circulating malaria parasites in South Africa

3.1 Introduction

South Africa has revised its target of halting malaria transmission within its borders from the year 2018 to 2023. This change in elimination timelines from the earlier national plans reflects the challenges for successful elimination facing the country [146, 213]. Across the region, countries like Namibia, Botswana and Zimbabwe are also experiencing a plateauing or even decline of recent successes [7, 9]. In Vhembe and Mopani districts in Limpopo Province, malaria transmission is ongoing despite the deployment of vector control strategies [9]. A recent increase in malaria cases has been observed in these districts despite vector control interventions that are effective in neighbouring districts [60, 214].

Parasite-targeted interventions may support approaches to accelerate towards elimination. Quality-assured light microscopy is considered as the gold standard for malaria diagnosis even though it is not the most sensitive of the available diagnostic tools [215]. The alternative RDTs are prone to false positive results due to *Plasmodium falciparum* histidine-rich protein-2 (*pfhrp2)* antigen persistence in blood, sometimes for weeks after successful treatment [216] and potentially by *pfhrp2* gene deletions [217]. The South African malaria control program includes a reactive case detection and surveillance system that relies on microscopy that potentially misses low-density infections [139]. However, these low-density infections may contribute to transmission [54, 218, 219]. In addition, with the declining transmission, a higher proportion of infected individuals remain asymptomatic [220] and are unlikely to present for treatment. To accelerate towards elimination, sensitive molecular tools may thus be of use to identify the infectious reservoir, including nucleic acid amplification (NAA) methods [221]. The use of molecular methods typically leads to a higher number of detected infections; the detectable fraction varying between endemic settings and the population at risk [27, 54, 222]. Malaria case management for uncomplicated malaria in South Africa relies on ACTs [188] that retain high efficacy in the region. However, the recent isolated report of indigenous artemisinin-resistant *P. falciparum* clones in West Africa [223] and other *Plasmodium falciparum* kelch-13 (*PfK13*)-independent treatment failures in United Kingdom patients who travelled to Africa [224], highlights the need for continued and enhanced surveillance for any local emergence of artemisinin resistance. NAA tools that allow detection of drug resistance markers is critical

to determine whether the currently used antimalarial regimens, including non-artemisinin partner drugs, retain efficacy.

Importation of parasites due to population movements from neighbouring high-transmission endemic countries is a further threat to elimination [148]. Identifying and classifying cases as imported or local may be based on travel history but such assessments can be supported by genotyping of the parasite population. In high-transmission endemic settings, a higher MOI is observed [132] while MOI typically reduces in low transmission settings and following successful control [225]. As such, low MOI may imply the success of control interventions leading to a low force of infection and ultimately a small number of circulating parasite clones [117]. Further genetic characterisation of imported infections may concentrate on drug sensitivity patterns.

This chapter thus explores the sensitive detection and subsequent genetic characteristics of malaria parasites in Vhembe District in terms of species, MOI and drug resistance profiles to provide a picture of the circulating parasites. To put the findings into the perspective of global and regional elimination agenda, parasite data from "imported" cases from other malaria-endemic countries are compared to "local" South African cases.

3.2 Materials and Methods

3.2.1 Ethics Approval

Ethical approval for the study consent has been described in Chapter 2, section 2.2.1. (Appendices 1 and 2).

3.2.2 Study populations and sample collections

Samples for this study were sourced in Vhembe District ('Vhembe District survey samples') during the 2014/2015 malaria transmission season (survey 1) and 2015/2016 malaria transmission season (survey 2), both in clinics and asymptomatic populations. Samples from asymptomatic populations were collected during surveys in communities, surrounding clinics and migrant farms. Community members and farm workers were mobilised by the Limpopo Department of Health malaria control personnel to present at a central location for malaria testing and collection of samples. The officers in charge of the primary health care clinics (PHCs) assisted in scheduling sample collection from their facilities. RDT-positive samples were also available from select PHCs in the Vhembe district (termed 'Vhembe RDT-positive samples'). Additionally, samples were sourced from individuals visiting the malaria centre in Tzaneen (termed 'Tzaneen samples'), with diverse travel history including travelling to high-

transmission areas in neighbouring Mozambique. Finally, the study accessed samples from Johannesburg (termed the 'Johannesburg samples'), which mainly consisted of imported cases of malaria infections from patients at the Chris Hani Bharagwanath hospital. Samples from Johannesburg did not have accompanying identifying information or information about donor characteristics such as age or gender (Figure 3.1).

3.2.2.1 'Vhembe samples'

In Vhembe district, a total of 1475 filter paper blood spot (FPBS) samples were collected from participants spanning two malaria transmission seasons (termed Vhembe District survey samples). These participants comprised three distinct groups: 1) migrant farm workers, 2) locals attending PHCs for any treatment indication and 3) community members living in the catchment areas of the PHCs (Figure 2.2). The three groups were purposely selected to sample patients as well as asymptomatic infections during the study period. The first 520 samples were collected during survey 1; an additional 955 samples were collected during survey 2 in the peak malaria transmission season in South Africa. In addition to these survey samples, a total of 205 *P. falciparum* positive RDTs were collected from participants who were symptomatic from several clinics (Makuya, Mulala and Masisi clinics) in Vhembe District Limpopo Province, South Africa. These were termed 'Vhembe District RDT positive samples'.

3.2.2.2 'Tzaneen samples'

Blood samples, in the form of archived RDTs (n = 101), were collected from patients presenting at the malaria testing facility of the malaria institute in the town of Tzaneen, Mopani District, Limpopo Province, South Africa. Tzaneen is situated in close proximity to malaria-endemic Mozambique and the Kruger National Park, popular holiday destinations for the people of Tzaneen and the rest of South Africa. Moreover, the town is surrounded by numerous agricultural enterprises that offer seasonal work to migrant workers, typically originating from Mozambique and Zimbabwe. Individuals who suspect they have malaria are tested for malaria, free of charge when they visit the Tzaneen testing facility. Personal details of the patients including demographics like age, gender, the area of residence and travel history are recorded on arrival. Thereafter, a malaria RDT and microscopy was performed on the participants' samples to determine malaria infection status. Samples were collected over a two-month period; from 9th March until the 8th of May 2015 and stored individually in zip-lock packets containing silica gels at room temperature before processing.

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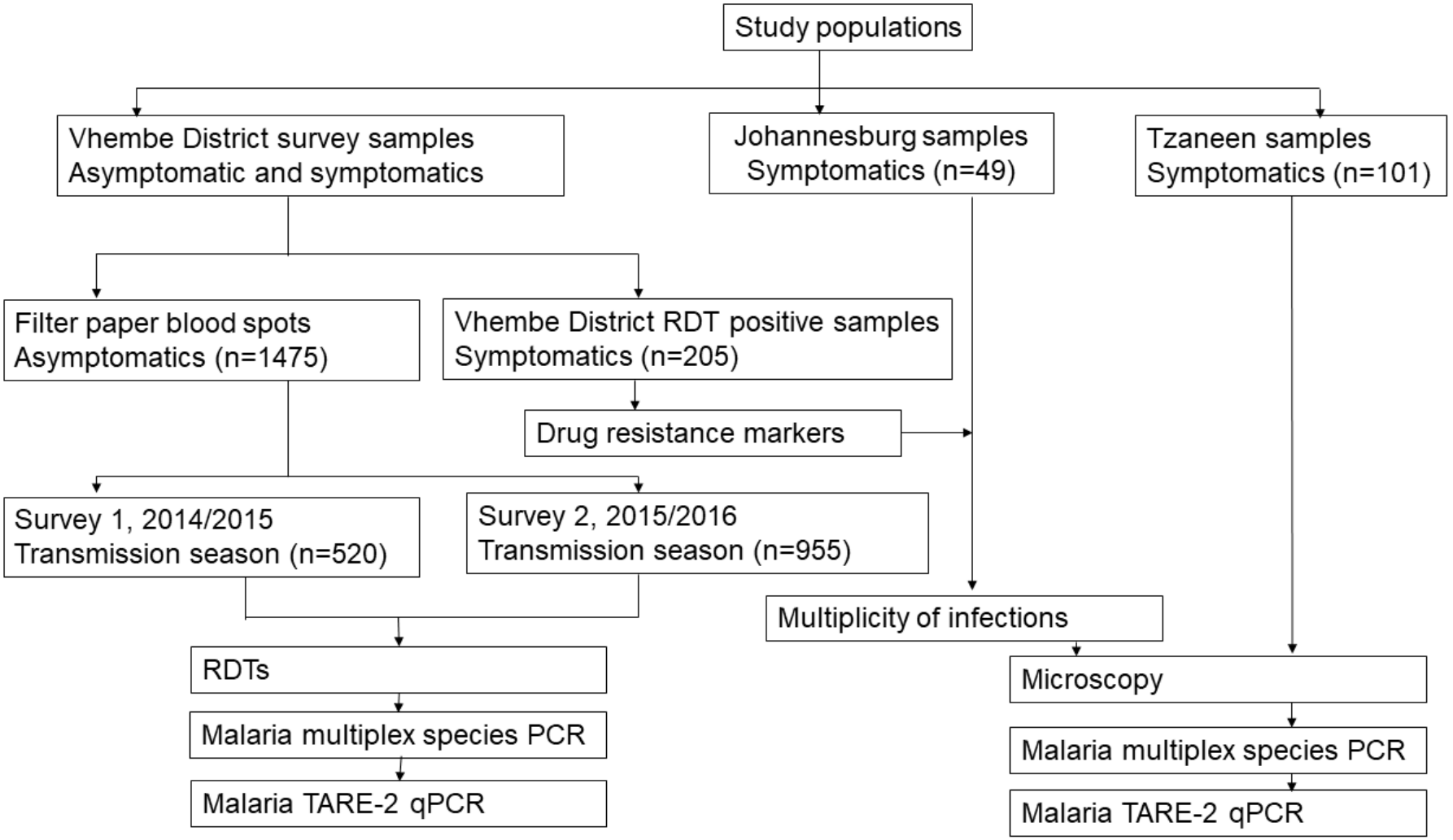
Figure 3.1: A schematic of the study participants, sample profiles and tests performed



3.2.2.1 'Johannesburg samples'

A total of 49 genomic DNA samples from malaria positive patients presenting at the Chris Hani Bharagwanath Hospital located in the Soweto township area of Johannesburg (Gauteng Province, South Africa) were used for MOI genotyping.

3.2.3 Microscopy

Thick film microscopy was performed for the identification of the different *Plasmodium* species; *P. falciparum, P. vivax, P. ovale,* and *P. malariae*. Thick blood smears were prepared by qualified health workers from finger prick peripheral blood, stained and examined at the Malaria Institute in Tzaneen or at the Bharagwanath Hospital in Johannesburg. When no parasites were detected in 100 fields of the blood film it was declared negative. If parasites were identified, an additional 100 fields were examined for speciation with the WHO plus system shown below used to give an indication of the parasite load [226].

+ = 1-10 parasites per 100 thick film fields.
++ = 11 - 100 parasites per 100 thick film fields.
+++ = 1 - 10 parasites per single thick film field
++++ = more than 10 parasites per single thick film field

3.2.4 RDT interpretation and storage

Finger prick blood samples were tested by histidine-rich protein 2 based RDT (First Response® Malaria Antigen *P. falciparum* card test HRP2*,* Premier Medical Corporation, India) in accordance with the South African National Malaria Diagnosis Quality Assurance guidelines [227]. All RDT samples were subsequently stored in a cardboard box with or without preservatives at room temperature.

3.2.5 DNA extraction from RDTs

DNA was isolated from the blood samples captured in the RDT matrix (nitrocellulose strip). The RDT cassettes were opened using sterile scissors and forceps and the nitrocellulose strips containing the parasite DNA removed. The strips were cut in half and the portion containing the blood sample was used for extraction. This portion was cut into small pieces (approximately 2x2 mm) to enhance DNA recovery. Sterility was maintained between samples, by cleaning the scissors and forceps with 70% ethanol and wiping dry with clean paper towels. The pieces of nitrocellulose membrane were incubated in 500 µl of 0.5% saponin (w/v in 1× phosphate buffered saline, PBS) at room temperature overnight and DNA extraction performed using the Chelex-100 method as described [228]. After overnight incubation, centrifugation was performed (3 min at 10 000*g*) and the supernatant discarded. Nitrocellulose sample pieces were washed with 1 ml 1× PBS and centrifuged (1 min, 10 000*g*),

followed by the removal of supernatant. Thereafter, 75 µl sterile nuclease-free water and 25 µl of 20% (w/v) Chelex suspension were added to the sample and incubated in a digital heat block at 99°C for 10 min. Chelex was separated out by centrifugation (1 min, 10 000*g*), after which the supernatant containing the DNA was transferred into a fresh tube. A final centrifugation step (1 min, 10 000*g*) was performed, ensuring that all the chelex were settled at the bottom and to enhance the purity of the DNA. DNA supernatant was collected into a fresh tube and stored at -20°C until used. DNA concentration and purity (A_{260}/A_{230} $A_{260}/_{280}$) were determined spectrophotometrically using a GeneQuant™ Pro (Amersham Biosciences, Amersham Place Little Chalfont Buckinghamshire, UK).

3.2.6 DNA extraction from filter paper blood spots

Parasite DNA was extracted from FPBS following the Chelex method as described elsewhere [191]. Briefly, a 0.25 mm round piece of the FPBS was cut and placed in a labelled 1.5 ml Eppendorf tube. To each Eppendorf tube, 1 ml of 5% (w/v) sodium dodecyl sulphate (SDS) in 1× PBS was added and then incubated overnight at room temperature. The following day, the Eppendorf tubes with the FPBS were inverted twice and subsequently washed twice (1 min, 10 000*g*) with 1× PBS. Distilled water (150 µl) and 50 µl of 20% w/v chelex were then added to the Eppendorf tube, which was boiled (100°C) for 8 minutes. Thereafter, the sample was left at room temperature for 5 minutes, briefly centrifuged and the supernatant collected and transferred to a new labelled tube. Approximately 100 µl of DNA was obtained from every sample, aliquoted into two equal proportions and stored at -20°C until required for further downstream analysis. The purity and concentration of the extracted DNA from these samples were measured spectrophotometrically as described in section 3.3.3.

3.2.7 DNA extraction by commercial extraction kits

DNA from the Johannesburg samples were extracted by qualified staff at the Chris Hani Bharagwanath Hospital using a commercially available DNA extraction kit. The samples were subsequently transferred to the University of Pretoria, Malaria Parasite Molecular laboratory (M2PL) and stored at -20°C until required for experiments. DNA concentration and purity were again determined spectrophotometrically as described in section 3.3.3.

3.2.8 Multiplex PCR for *Plasmodium* speciation

Since parasites were not detected by microscopy or RDT in any of the samples from surveys in Vhembe District, a single-step multiplex PCR targeting *Plasmodium* species-specific sequences in the 18S ribosomal-RNA gene was performed [190]. Briefly, a 25 µl reaction

contained 1× Kapa 2G Fast Multiplex Mix (Kapa Biosystems¸ Wilmington, USA), 10 µM of forward and 15 µM of multi-reverse primer and 5 µl DNA template. Primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. The thermal cycling conditions are as shown (Table 3.1) and PCRs were performed in Applied Biosystems® 2720 Thermal Cycler (Applied Biosystems®, Waltham, Massachusetts, USA).

Table 3.1. Primer sequences for the *Plasmodium* species identification multiplex PCR

***Plasmodium* species**	**Primer name and sequence (5'-3')**	**Secondary product sizes**	**PCR conditions**
P. falciparum	5'-AAC AGA CGG GTA GTC ATG ATT GAG-3'	275 bp	Denaturation 94°C for 5 min, 30 cycles of 94°C for 15 s, 60°C for 2 min, 68°C for 2 min 4°C till needed.
P. vivax	5'-CGG CTT GGA AGT CCT TGT- 3'	300 bp	
P. malariae	5'-CGT TAA GAA TAA ACG CCA AGC G-3'	412 bp	
P. ovale	5'-CTG TTC TTT GCA TTC CTT ATG C-3'	375 bp	
	Multi-reverse primer		
	5'-GTA TCT GAT CGT CTT CAC TCC C-3'		

3.2.9 Ultra-sensitive TARE-2 qPCR

To test for the presence of very low densities of *P. falciparum*, a qPCR targeting the telomere-associated repetitive element (TARE-2) region was conducted on pools of 5 samples each (1 µl per sample) as described [229] using the QuantStudio™ 12K flex Real-Time PCR System platform (Applied Biosystems, Massachusetts, USA). Briefly, 200 nM each of forward (5'-CTA TGT TGC ACT TAC ATG CAY AAT-3') and (5'-TGA CCT AAG AAG TAV AAT AAT GAT GA-3') reverse primers were used in a 25 µl total reaction volume using SYBR Green-based real-time quantification of amplicons (PowerUP SYBR® Green mix at 1×, Applied Biosystems). Quantification of samples was performed using a standard curve of a 10-fold dilution of ring stage *P. falciparum* (3D7 strain) parasites ($6.8x10^{3}$ to $6.8x10^{-2}$ parasites/µl) with a cut-off for positive signal set at a C_T value of below 37. Cycling conditions consisted of 2 min pre-incubation at 50°C, initial denaturation of 10 min at 95°C, then 45 cycles of 15 s denaturation and 1 min of annealing and elongation at 95°C. Melt curves for each specific experiment were inspected to identify false positive results. True positives were differentiated from the primer dimer and unspecific PCR products based on the amplicons' melting temperature as visually inspected on the melt curve plots (Appendix 3). Sample pools were run in duplicates, with the assay repeated in triplicate for any discrepancies. Samples were subsequently defined as positive if at least two of the three replicates were positive. Negative control samples were prepared by mixing 5 pools of malaria-negative blood samples from volunteer donors.

3.2.10 Multiplicity of infections genotyping PCR

To test for MOI, a primary PCR targeting conserved regions of block 3 of the *msp2* gene and the secondary pcr targeting *msp2* specific allelic families of 3D7 and FC27 clones was performed [230]. Briefly, for the initial reaction (reaction N1) molecular grade water (SABAX), 1× KAPA Taq (KAPA Biosystems, South Africa) and 0.5 µM each of the S2 forward and S3 reverse primers per sample were added (Table 3.2). Samples were centrifuged for 90 s at 10 000*g* to reduce the chances of picking any carryover chelex. After vortexing and quick-spinning, 8 µl of the mix was added to 2 µl of the extracted DNA. A blank negative control and positive controls were run per experiment. Samples were run on the Applied Biosystems 2720 Thermal Cycler. For the secondary PCR (reaction N2), 0.5 µM each of the S1 forward and S4 reverse primers were used with 1 µl diluted primary PCR product from reaction N1 as a template. The cycle conditions for the secondary PCR were as shown in Table 3.2. After optimizations, the samples were subsequently diluted 1:10 for Johannesburg samples and 1:20 for the Vhembe positive RDT samples to improve the visualisation of the bands into distinct genotypes.

PCR products were analysed by gel electrophoresis for different band sizes of the different *Plasmodium* species: *P. falciparum* = 276 bp; *P. vivax* = 300 bp; *P. ovale* = 375 bp and *P. malariae* = 412 bp. Multiple bands were interpreted as a mixed species infection. Distinctly visible bands from the secondary PCR products were interpreted as different *P. falciparum* clones. Clones between 200-700 base pairs were interpreted as part of the FC27 family, while 400-750 base pairs were interpreted as part of the 3D7 family [230]. When there were clones between both ranges visible, it was assumed that there was a mixed infection from both families.

Table 3. 1: Primer sequences and thermocycling conditions for MSP2 genotyping PCR

PCR reaction	Primer	Sequence	Thermocycling conditions
N1	S2 forward	5'-GAG GGA TGT TGC TCC ACA G- 3'	95°C for 5 min, followed by 30 cycles of (94°C for 25 s, 50°C for 30 s, 65°C for 2 min), 72°C for 5 min.
	S3 reverse	5'-GAA GGT AAT TAA AAC ATT GTC- 3'	
N2	S1 forward	5'-GAG TAT AAG GAG AAG TATG- 3'	95°C for 5 min, followed by 30 cycles of (94°C for 25 s, 50°C for 60 s, 72°C for 2 min), 72°C for 5 min.
	S4 reverse	5'-CTA GAA CCA TGC ATA TGT CC - 3'	

3.2.11 Drug resistance genotyping

The presence of molecular markers associated with drug treatment failures to commonly used antimalarials on all the *P. falciparum* positive samples was assessed. Mutation-specific nested PCR and RFLP were used to analyse select codons in chloroquine resistance transporter (*crt*), multidrug resistance drug-protein 1 (*mdr1)*, dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes to identify the presence of mutant alleles [231, 232]. All primer sequences and PCR conditions are presented in Appendix 4. The primary PCR reactions contained 1× Kapa Taq ReadyMix (Kapa Biosystems, Lasec Company, Pretoria, South Africa), 0.4 µM of forward (F1) and reverse (R1) primers with 1 µl of DNA template in a 20 µl total volume. For the secondary PCR, final concentration of 0.4 µM of forward (F2) and reverse (R2) primers with a 2 µl aliquot of the primary amplification product in a 20 µl total volume were used. Samples were run on the Applied Biosystems 2720 Thermal Cycler.

To distinguish between wildtype and mutant parasite clones, RFLP was used to cleave DNA at the codons for drug-resistance markers: *crt76*, *mdr1-86*, *dhfr51*, *dhfr*59, *dhfr*108, *dhfr*164 and *dhps437* and *dhps*540 codons. For each sample, a 5 µl secondary PCR product was incubated with 2 units of the corresponding mutation-specific restriction enzyme (New England Biolabs, Beverly, MA) and buffer to a total of 15 µl and incubated overnight at the designated incubation temperatures (Appendix 5). Codon analysis was used to categorize and code the results of the molecular assays of *dhfr* and *dhps* as described and summarized in Figure 3.2 [231]. In brief, each codon of *dhps* and *dhfr* alleles was categorized as wildtype, mixed (both wildtype and mutant genotype detected in the same infection) or mutant (pure mutant genotype present). The *dhfr* and *dhps* genotypes for each sample were then categorized as “wildtype,” when no mutations were detected in a sample; as “single,” when the parasites of a sample contained a single mutation, as “double mixed,” when some parasites of a sample contained both the mixed and the single genotype; and as “double pure,” when mutation in both of the codon were detected in a single sample, as “triple mixed,” when the samples contained parasites with mixed mutations in all 3 codons; and “triple pure,” was categorized when in a single sample, 3 pure mutations were detected at all the 3 codons [231]. To classify the genotype of *dhfr*/*dhps*, the same method of classification was used. Genotypes for *dhfr*/*dhps* were then categorized wildtype, single, double, triple, quadruple, quintuple mixed or quintuple pure.

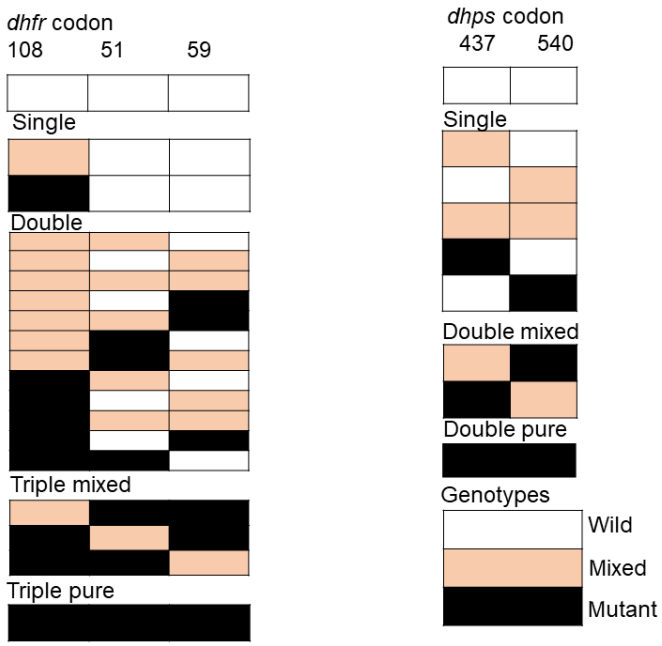


Figure 3.2: Codon analysis scheme for results of genotyping for *Plasmodium falciparum* dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) mutations. For each infection, genotypes were coded (for example, "wildtype", "single", or "double") based on the minimum number of mutations whose presence could be inferred on the basis of polymerase chain reaction results. Wildtype genotypes are shown by clear bars, mixed genotypes by orange bars with mutant genotypes shown by black bars. Figure adapted with permission from the publishers [233].

3.2.12 Gel electrophoresis

PCR products were resolved on 2% agarose gel by electrophoresis in 0.5 x Tris-acetate-EDTA buffer (40mM Tris-acetate and 1 mM EDTA, pH 8.0) and visualised with 0.5 µg/ml ethidium bromide staining alongside a 100 bp ladder (Promega). Images were captured on UVDoc HD2 transilluminator (UVItec Ltd., Cambridge, UK).

3.2.13 Statistical analysis

The study participants and laboratory data were analysed with either SPSS version 20 (IBM Corp. NY, USA) or GraphPad Prism version 8.0 for Windows (GraphPad Software, La Jolla California USA). Descriptive statistics such as mean and standard deviations were used to report the demographics of all the study participants, when available. For MOI, descriptive statistics were used to describe the number of *P. falciparum* clones in Johannesburg, and the Vhembe District RDT positive samples. To compare MOI between Johannesburg and Vhembe District RDT positive samples, Mann-Whitney U test for two independent samples was performed. For drug resistance markers, a calculation of the prevalence for *crt*, *mdr1*, *dhfr* and *dhps* mutations and their associated genotypes was executed.

3.3 Results

3.3.1 Demographics of the samples collected from the different sites

Cross-sectional survey samples from Vhembe district (Vhembe samples, n=1475 plus additional 205 clinical RDT positive samples); clinical samples from Tzaneen (Tzaneen samples, n=101) and clinical samples from Baragwanath Hospital in Johannesburg (Johannesburg samples, n=49) were evaluated for demographics (Table 3.4). In general, mostly adults were sampled from all the study sites with more males than female observed from the Vhembe District survey samples while both gender were almost similar from the Tzaneen population.

Table 3.3: Demographic characteristics of the study populations

	Vhembe District survey samples (Asymptomatic cases, N=1475)	***Vhembe District RDT+ samples (Clinical cases N=205)**	**Tzaneen samples (Suspected clinical cases N=101)**	***Johannesburg Imported clinical cases (N=49)**
Males % (n/N)	34 (507/1475)	_	55 (51/92)	_
Females % (n/N)	67 (988/1475)	_	45 (41/92)	_
Mean age in years (SD)	34 (36.4)	_	35	_
Median age in years (IQR)	30 (21-43)	_	37	_
Self-reported fever % (n/N)	14.6 (215/1475)	100 (205/205)	100 (101/101)	100 (49/49)

*For the Vhembe District RDT positive samples and the Johannesburg samples, age and gender information were not available.
n = number of individuals of interest
N = Total number of individuals in the population
SD= Standard deviation
IQR= Interquartile range

3.3.2 Detection and speciation of malaria parasites with NAA techniques: Multiplex end-point PCR evaluations

All of the samples were subsequently evaluated for the presence of *Plasmodium* parasites with sensitive NAA amplification techniques. In cases where RDT and microscopy results were already available (e.g. the Vhembe RDT, Tzaneen and Johannesburg samples), the NAA data was directly compared with the RDT/microscopy data. Where not available (cross-sectional sampling for the Vhembe samples), RDT was performed in a subset of samples and the NAA data subsequently compared with this. NAA technique used was a multiplex-PCR speciation based on the 18S rRNA gene.

3.3.2.1 Vhembe samples

For the samples collected in the Vhembe District, RDTs was performed on a subset of 352 participants who all tested negative. All the samples (n=1475 from both surveys), were subsequently tested for *Plasmodium* parasites with the multiplex PCRs that allows for the simultaneous detection *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* parasites (Figure 3.2). No *P. falciparum* infections could be detected in any of the samples, however, one participant was identified with *P. ovale* parasites. This individual was a 25-year-old male immigrant from neighbouring Zimbabwe who was negative by RDT (*P. falciparum* HRP-2 based) (Figure 3.3).

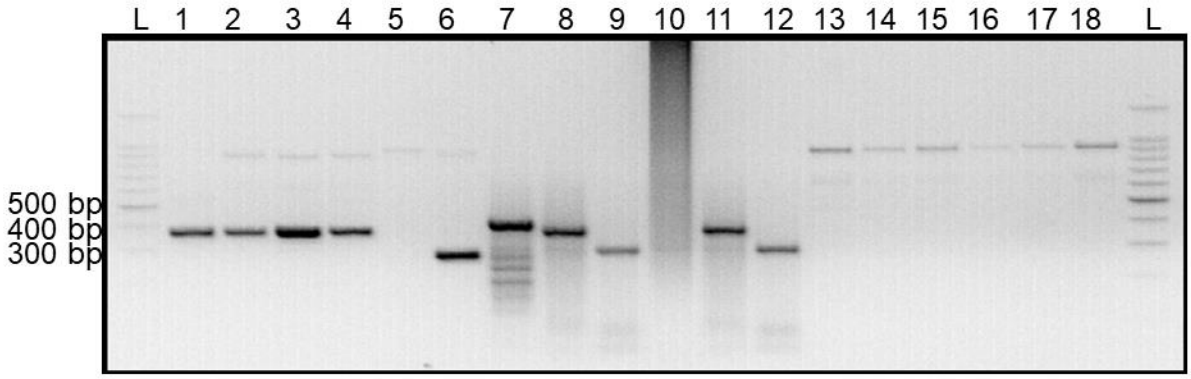


Figure 3.3: Multiplex PCR for identification and speciation of *Plasmodium* in selected samples the Vhembe survey. Selected samples (7) from the Vhembe District survey sample set were amplified with a multiplex PCR. Samples were analysed on a 2% agarose gel and bands visualised with EtBr staining. L: 100 bp DNA ladder. Lanes 1-4: A single *P. ovale* positive sample, re-extracted and diluted 1/10 resulting in a 375 bp band. Lane 5: Blank control well. Lane 6: *P. falciparum* control resulting in a 276 bp band. Lane 7: *P. malariae* control resulting in 412 bp band. Lane 8: *P. ovale* control resulting in a 375 bp band. Lane 9: *P. vivax* control resulting in a 300 bp band. Lane 11: *P. ovale* control resulting in 375 bp band. Lane 12: *P. vivax* control resulting in a 300 bp band; Lanes 13-18, 6 selected individual participant samples.

In addition, selected Vhembe District RDT positive samples were tested for malaria parasites by the multiplex PCR. Among these samples, 193 (94%) were confirmed positive for *P. falciparum* parasites while 12 that were RDT positive could not be confirmed by PCR (6% were negative), indicating the possibility of false positives detected by RDT.

3.3.2.2 Tzaneen Malaria Institute samples

For these samples, there was a complete agreement between samples identified as *Plasmodium* positive by microscopy and RDTs (6%, 6/101), with a higher positive proportion assigned to males (5/51, 9.8%) compared to females (1/41, 2.4%; *P*=0.22). Samples were subsequently evaluated using multiplex PCR to confirm parasite detection and allow speciation. A total of 13.8% (14/101) samples were confirmed to be *P. falciparum* positive including the 6 samples previously identified as *P. falciparum* positive via RDT and microscopy

(Figure 3.4). Moreover, 1 sample among the positive samples harboured a mixed *P. malariae* - *P. falciparum* infection that had been missed by microscopy.

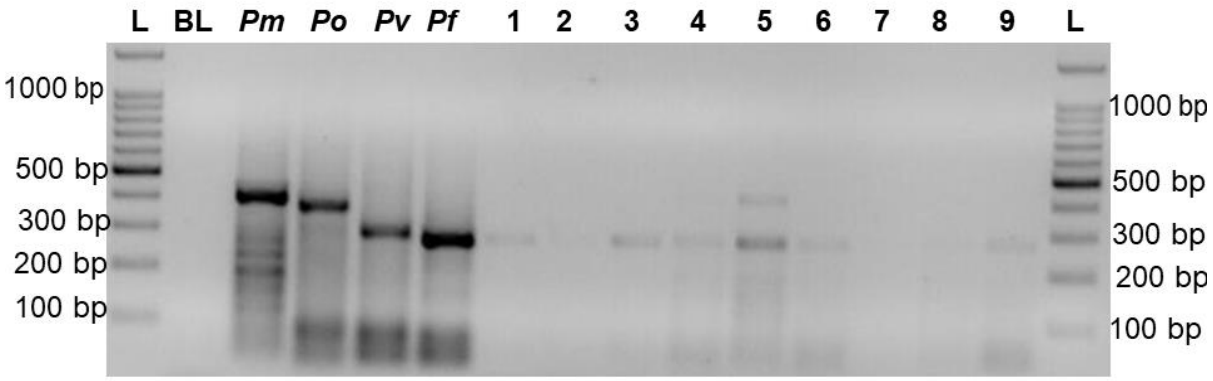


Figure 3.4: Multiplex PCR identification and speciation of *Plasmodium* in a subset of Tzaneen malaria institute samples. Selected samples (9) from the Tzaneen sample set were amplified with a multiplex PCR that allows for the simultaneous detection *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* parasites. Samples were analysed on a 2% agarose gel electrophoresis and bands visualised with EtBr staining. L: 100 bp DNA ladder. BL: blank negative control. *Pf*: Control *P. falciparum* sample resulting in a 276 bp band. *Pm*: Control *P. malariae* sample resulting in a 412 bp band. *Po*: Control *P. ovale* resulting in a 375 bp band. *Pv*: control *P. vivax* resulting in a 300 bp band and *Pf*: Control *P. falciparum* sample resulting in a 276 bp band. Lane 1-19, selected samples of Tzaneen malaria institute samples. Sample number 5 has mixed *P. malariae* and *P. falciparum* infections.

3.3.2.3 Johannesburg samples

All the Johannesburg samples, which were previously defined as *P. falciparum* positive by microscopy, were evaluated using the multiplex PCR to confirm the species. The presence of *P. falciparum* parasites was confirmed in all of the samples with no mixed infections detected (Figure 3.5). For this sample set, there was complete agreement therefore between the samples identified as positive by microscopy and PCR.

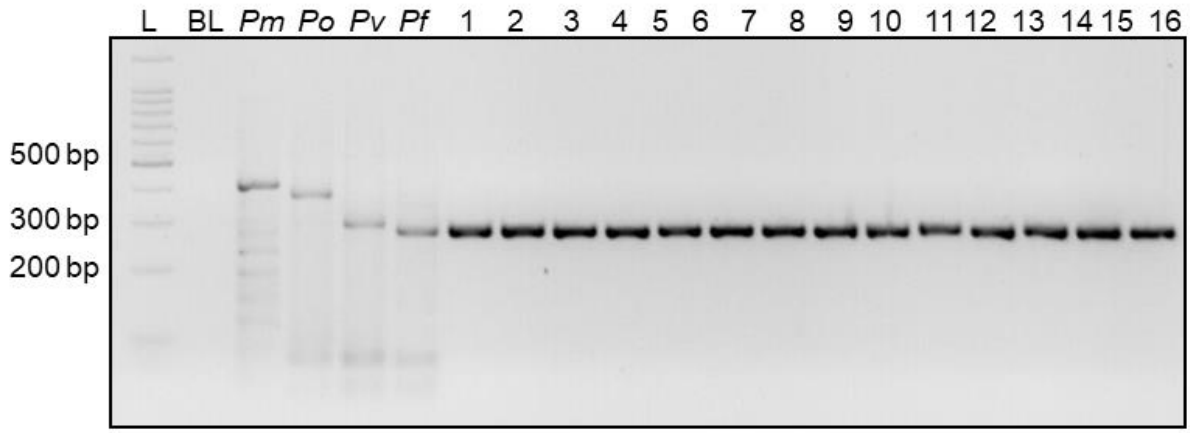


Figure 3.5: Multiplex PCR for *Plasmodium* species identification for the Johannesburg samples. Selected samples (16) from the Johannesburg sample set were amplified with a multiplex PCR that allows for the simultaneous detection *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax* parasites. Samples were analysed on a 2% agarose gel electrophoresis, and bands visualised with EtBr staining. L: 100 bp DNA ladder; BL: Blank negative control well. *Pm*: *P. malariae* control resulting in 412 bp band; *Po*: *P. ovale* control resulting in a 375 bp band; *Pv*: *P. vivax* control resulting in a 300 bp band. *Pf*: *P. falciparum* control resulting in 276 bp band. Participant samples from Johannesburg are numbered 1 to 16.

3.3.3 Detection and speciation of malaria parasites with NAA techniques: ultra-sensitive TARE-2 qPCR

Previous reports indicated that ultra-sensitive NAA techniques are required to detect very low parasite densities [229]. To ensure that such low parasite densities were not missed by the techniques used above, an ultra-sensitive qPCR based on amplification of the TARE-2 genes was used to detect parasites in the samples from Vhembe and Tzaneen. TARE-2 qPCR efficiencies, as determined on a *P. falciparum* 3D7 culture as control, were comparable to those previously published within the desired efficiency range of 90%–105% [229] (Table 3.4). In our hands, the TARE-2 assay could detect up to 1 parasite/µl based on the standard curve obtained (Figure 3.6 A).

Table 3.4: qPCR details and efficiencies of the TARE-2 assays between published data [229] and data generated in this study.

TARE-2 Assay	Slope	Efficiency	Intercept[a]	R^2	Platform	Amplicon length
Hoffmann *et al*, 2015 [229]	-3.75	84.7%	32.08	0.97	SYBR green	93 bp
This study	-3.39	97.3%	37.27	0.97	SYBR green	93 bp

[a] Intercept equals the C_T value of the DNA equivalent of five parasites added to the qPCR reaction.

Upon validation of the TARE-qPCR to detect up to 1 parasite/µl of blood according to the standard curve (Figure 3.6 A), pools of 5 individual samples, each containing 1 µl of extracted parasite DNA, were tested for the total 1475 samples. The TARE-2 qPCR detected *P. falciparum* infections in a total of 31 pools and, following individual qPCRs, in 57 participants (3.8%, 57/1475). All these samples had hitherto been missed by both the multiplex PCR and RDT (Figure 3.6 B).

Vhembe RDT positive samples were also tested by TARE-2 qPCR (Figure 3.6 C). Among these samples, 96% (19/205) were confirmed positive for *P. falciparum* parasites, whilst 4% (8/205) could not be identified with qPCR as positive, again confirming suspected false positives detected with the RDT, similar to the multplex PCR outcomes.

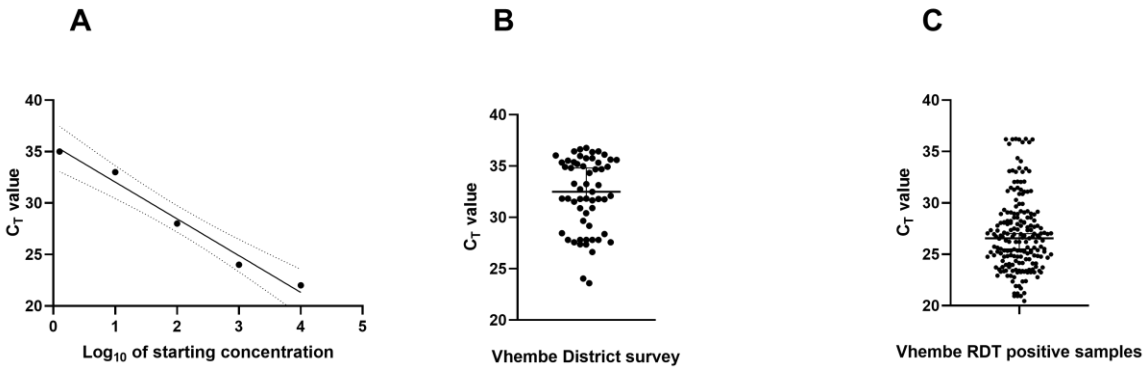


Figure 3.6. TARE-2 qPCR outcomes. A, shows validation of the TARE-2 qPCR showing the dilution series of duplicate measurements of the Log_{10} parasites/mL and the corresponding C_T value of standard curve based on cultured 3D7 parasite lines with 95% confidence bands of the best-fit line. **B,** Vhembe District positive samples with each dot representing individual samples and the corresponding C_T values. C, individual samples from Vhembe District RDT positive samples. For each category of samples B and C, summary lines are displayed: thick middle black lines indicate the median and thin black lines indicate the IQR.

The Tzaneen samples were also tested with the ultra-sensitive TARE-2 qPCR. A total of 31% of the participants were indicated to be *P. falciparum* positive (Figure 3.7) including 17 samples that were not previously detected with either RDTs, microscopy or multiplex PCR. Agreement between the assays was good with all the samples positive in microscopy, RDT and 18S rRNA PCR confirmed as positive by TARE-2 qPCR.

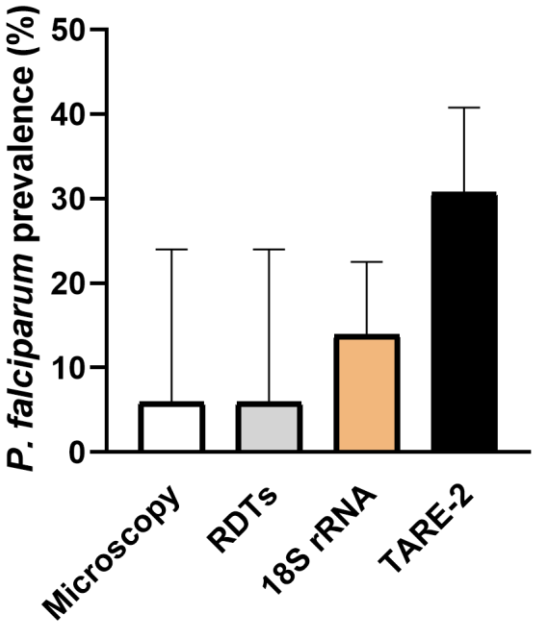


Figure 3.7: Overall *P. falciparum* prevalence in Tzaneen malaria institute samples by different diagnostic methods. *P. falciparum* parasites were detected using RDTs, microscopy, 18S rRNA nested PCR and TARE-2 qPCR. Errors bars shown 95% confidence intervals of the proportions.

3.3.4 Comparison of MOI between Johannesburg and Vhembe District samples

MOI was determined for *P. falciparum* positive samples from Vhembe district samples. The data from these samples were compared to samples from Johannesburg, originating from migrant workers outside of South Africa, with a presumably higher transmission compared to South Africa. The samples were tested for MOI using *msp-2* genotyping. Samples from Johannesburg had 1 to 5 different *P. falciparum* clones, a mean of 2.84 and a minority of single clone infections (Figure 3.8 A). Samples from Vhembe had far fewer clones, with a range of 1-3 (mean of 1.82) (Figure 3.8 B). This data indicated that less genetic complexity exists in the samples from a low transmission setting such as Vhembe.

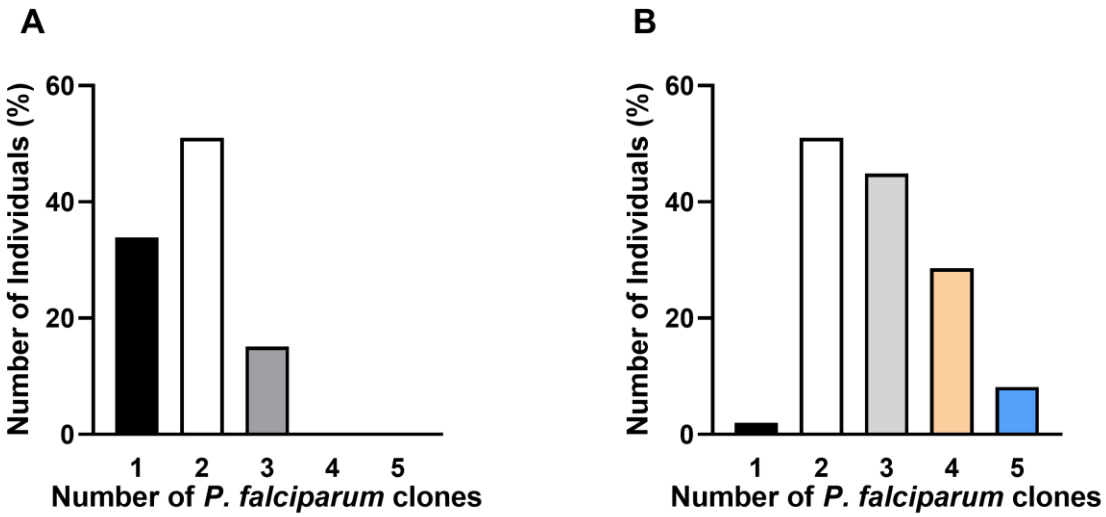


Figure 3.8: Comparison of multiplicity of infections in local (A, Vhembe District survey samples) versus imported (B, Johannesburg) *P. falciparum* clones. The number of *P. falciparum* clones are shown with different colours with monoclonal infections in the black shaded bars, 2 clones in white bars, 3 clones in grey bars, 4 clones in yellow bars and 5 clones in blue bars.

Within the Vhembe district samples, samples from Masisi PHC had the highest number of *P. falciparum* clones (1 to 3 clones, mean of 2.17), followed by Makuya PHC and Mulala PHC, with the mean number of clones at 1.36 and 1.51, respectively (Figure 3.9). Masisi PHC, the northern most clinic, had a significantly higher number of *P. falciparum* clones in contrast to both Makuya and Mulala (Mann Whitney U test, $P = 0.0001$).

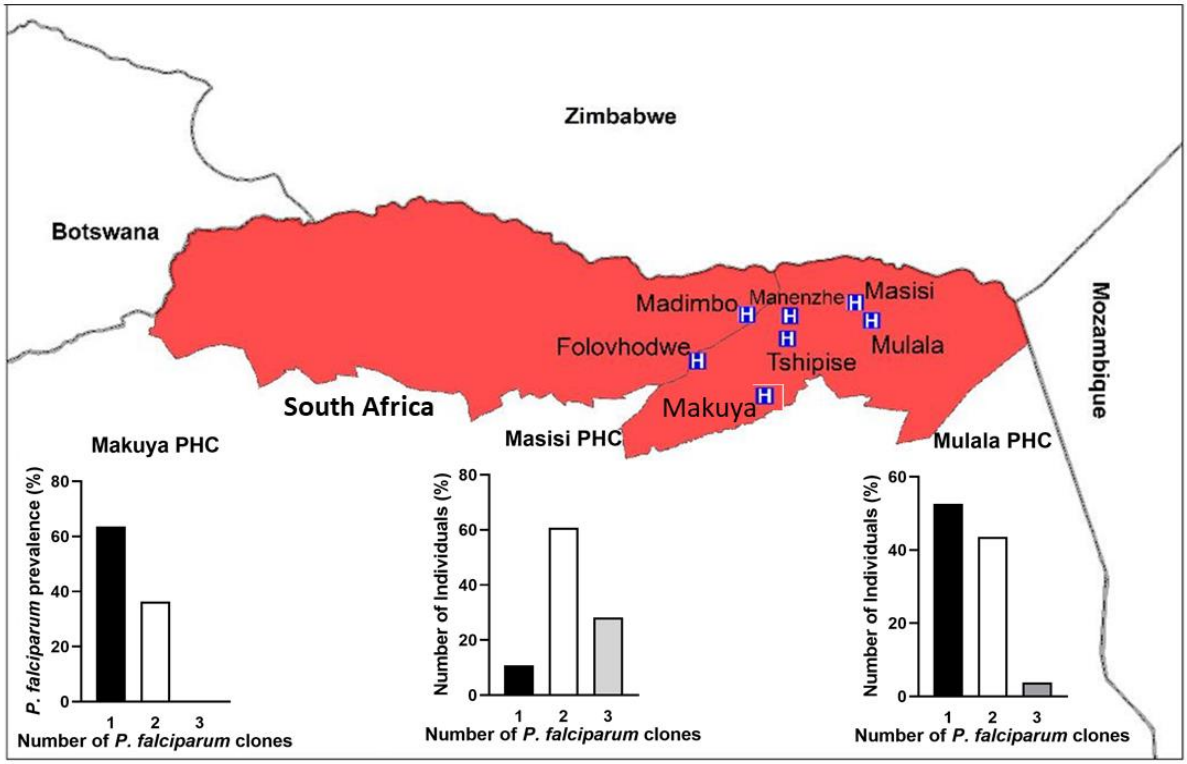


Figure 3.9: Multiplicity of *P. falciparum* infections in samples from Masisi, Mulala and Makuya PHCs. The Vhembe District (highlighted in red) with primary clinics (H) is shown alongside other E8 countries neighbouring South Africa. The multiplicity of infections of each of the 3 clinics are shown at the bottom of the figure. *P. falciparum* clones are shown with different colours; monoclonal infections in the black shaded bars, 2 clones in white bars and 3 clones in grey bars.

3.3.5 Drug resistance markers

Molecular genotyping was performed to determine variants in drug resistance markers on samples from Johannesburg and Vhembe District for *crt*76, *mdr1*-86, antifolate resistance monitored on *dhfr*50, *dhfr*51, *dhfr*59, *dhfr*108, *dhfr*164 and *dhps*436, *dhps*437, *dhps*540 and *dhps*581.

From the Johannesburg samples, the majority (83%) of the samples retained the Lys-76 *crt* wild-type allele (K76), indicative of chloroquine sensitivity, whilst only 17% (5/30) carried the mutant Thr-76 *crt* (T76) allele. Additionally, a minor proportion of the samples carried mutations at the *mdr186Y* allele (13%, 4/30) (Figure 3.10 A). The overall prevalence of SP resistance mutations at *dhfr*-51L, 59R, 108N, and 164L were 70%, 66%, 76% and 7%, respectively. For *dhps* 437G and *dhps* 540E, the prevalence was at 80% and 57% respectively, indicating high SP resistance. Combining 51I/59R/108N (*dhfr* triple mutant) gave a prevalence of 67% (20/30), *dhps* double mutant (*dhps* 437G/540E) was 47% (14/30) and combined *dhfr/dhps* quintuple mutant (*dhfr* 51I/59R/108N + *dhps* 437G/540E) was 40% indicative of substantial SP resistance.

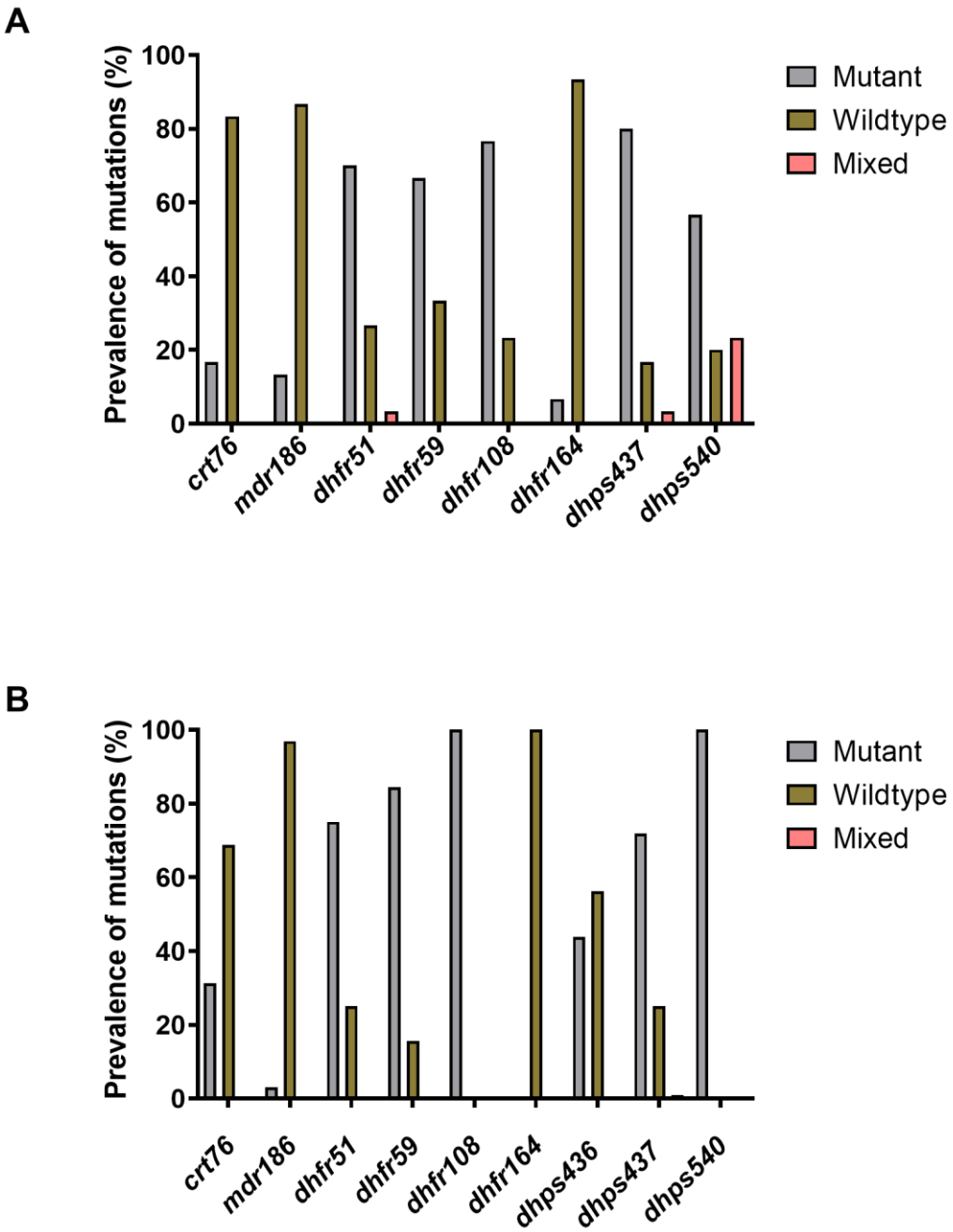


Figure 3.10: Prevalence of drug resistance marker point mutations. (A) Johannesburg samples and (B) Vhembe survey samples were analysed by PCR for markers for the *P. falciparum* chloroquine resistance transporter-76 (*crt76*), multidrug resistance protein-1-86 (*mdr186*), dihydrofolate reductase (*dhfr* 51-164) and dihydropteroate synthase (*dhps* 436-540) codons. Results of infections were coded wildtype, mixed or mutant.

Comparatively, for the samples evaluated from Vhembe, CQ resistance mutations were also at a low prevalence (22%, 7/32 of samples harboured mutations at *crt* T76 allele). Mutations in the *mdr186Y* allele were equally low, detected at 3% (1/32) prevalence (Figure 3.10 B). The overall prevalence of key SP resistance mutations at *dhfr*-51L, 59R, and *dhps*-437G were

generally high at 75%, 84%, and 72%, respectively. While the *dhfr* 108N and *dhps* 540E were fixed at 100%, in contrast, the *dhfr* 164L was not detected in the sample set (Figure 3.10 B). Combining 51I/59R/108N (*dhfr* triple mutant) was 75% (24/32), *dhps* double mutant (*dhps* 437G/540E) was 72% (23/32) and combined *dhfr/dhps* quintuple mutant (*dhfr* 51I/59R/108N + *dhps* 437G/540E) was 59%.

Taken together, there was no dramatic difference between the Johannesburg and Vhembe samples for CQ resistance or mutations in the multidrug resistance allele. The proportion of combined *dhfr/dhps* quintuple mutants in Vhembe sample set (59%), was significantly higher than in Johannesburg samples (40%), $P = 0.007$, indicating higher SP resistance.

3.4 Discussion

Here, we determined the prevalence of subpatent infections (beyond the detection limits of RDTs) in different populations in South Africa, assessed species and clonal complexity and molecular markers for drug resistance. Findings of 3.8% parasite prevalence by sensitive qPCR in Vhembe and 31% in Tzaneen compared to 5.9% by microscopy agree with other settings [234] and corroborate findings from neighbouring Namibia with 3.4% subpatent malaria infections by molecular methods [235]. These results are in line with a growing body of evidence demonstrating the sensitivity and superiority of NAA technologies for detection of low-density infections when compared to the conventional microscopy and RDTs [221].

The majority of the participants in this study from farms, communities and PHCs presented with no symptoms at the time of sampling. Asymptomatic infections were detected in all age groups sampled [234] and although some infections may have progressed to clinical malaria [236], or self-cleared shortly after detection [70, 237], it is plausible that a large fraction of the detected infections were chronic asymptomatic infections. In low transmission settings like South Africa with relatively low malaria exposure, immunity is assumed to be incomplete and infected individuals may be expected to develop symptoms. Consistent with this line of thought is the increased case fatalities amongst individuals with locally contracted malaria even though late presentations to the clinics may also play a role in these deaths [139]. Especially low-complexity infections may, on the other hand, also be well controlled in populations with relatively limited malaria exposure, low chances of super-infection and partial immunity [234, 238, 239].

The data herein are reported from a single cross-sectional survey that may not provide comprehensive information on parasite transmission dynamics in individual infections. Individuals may have been sampled during an ongoing infection and it is possible that parasite densities below the detection threshold at sampling time may rise to detectable levels with

clinical presentations at a later time point or alternatively become chronic asymptomatic carriers of infections [240].

Parasite persistence was not explicitly measured in this study, however, results from diverse transmission settings have reported some infections that may last 6 months or longer [236]. For example, in the pre-elimination settings of South East Asia, substantial proportions of infected individuals carried parasites for 2-3 months in Cambodia [241] and for 6-9 months in Myanmar [242]. Similarly, it is also possible that high-density infections would eventually develop from ultra-low-density infections seen in this study if no treatment is offered. It is currently unknown to what extent they may sustain transmission. Recently in low transmission setting of Ethiopia, it was demonstrated that asymptomatic infections are important in onward transmission to mosquitoes because of the relative abundance of such infections in the population and because low-density infections may rise in density over time to transmissible densities [219].

Despite the high sensitivity of the tools we used in this study, some infections may have been missed. Since blood samples used in this study were from filter paper blood spots that contain relatively limited blood volume, examining venous blood samples may have resulted in higher infection detection [243]. The sample volume will also have affected our estimates of MOI. The detectability of parasite clones strongly depends on their absolute density and relative density compared to other circulating clones [244] A significantly higher number of *P. falciparum* clones was found in the imported samples from Johannesburg (probably arising from high malaria transmission areas), compared to the samples from locals in Vhembe district. When comparing the clinics in Vhembe district, a significantly higher number of *P. falciparum* clones was found in the samples from the Masisi PHC (closest to the border) compared to the Mulala and Makuya PHCs that are further in the hinterland of South Africa. This may be attributed to the geographical location of the Masisi PHC close to malaria-endemic Zimbabwe, with mosquitoes plausibly acquiring infections from neighbouring higher endemic areas [245, 246]. To further understand malaria transmission patterns, the tools deployed in the current study may be expanded to include genotyping tools such as molecular barcoding that may provide insights in transmission patterns and sources and sinks of parasites [247]. However, the main challenge in the application of genotyping studies in low transmission areas are greater chances of inbreeding, leading to the production of clustered parasite offspring genotypes identical to the parents. Whilst relatively small SNP barcodes and panels of microsatellite markers [248, 249], can be used to monitor the changes in the diversity and structure of the parasite population, the signal may not be adequate to discern geographic origin and have limited resolutions in certain transmission settings [250, 251]. A solution may lie in using multiallelic loci that is more robust than biallelic loci [252]. In addition,

the granularity of the genetic data in regards to source of parasites can be augmented by leveraging on mobile phone technology to track and trace individual movement patterns [141].

Lastly, we examined drug resistance markers in the Limpopo Province and among imported infections from Johannesburg. Results demonstrate a high prevalence of sensitive *crt* parasites in Johannesburg and Vhembe. This is consistent with recent data that has shown a return of CQ sensitive parasites in African settings [253, 254]. The return of CQ sensitive parasites may also be influenced by the use of artemether/lumefantrine as a first line treatment therapy that has been demonstrated to select for wildtype 76K allele [255]. *Pfmdr1* as marker predictive of reduced efficacy of the non-artemisinin partner drug lumefantrine was low in Vhembe District (3%), consistent with previous findings [60], but appeared higher in Johannesburg (11%), similar to that reported from Mozambique [256]. This suggests that drug resistance markers may be influenced by parasite migration through population movement. *P. falciparum* isolates from Vhembe contained mutant alleles of K540E at 100%, possibly be explained by the use of co-trimoxazole as prophylaxis in HIV-infected persons [257] since SP is not used for antimalarial treatment in the region. However, the findings should be interpreted with caution as only a small portion of positive samples were tested for drug resistance markers from the Vhembe District sample set. With various countries in the southern African region implementing different drug treatment regimens, there is a need for continued surveillance for the emergence and spread of drug-resistant parasites in South Africa. An ideal situation would be a regional drug use policy, but this may not be possible as the individual states have different regulations and malaria-endemicity levels, a situation that regional initiatives can address

3.5 Conclusion

We demonstrated the sustained presence of low-density and asymptomatic *P. falciparum* infections across two transmission seasons in Vhembe District. Molecular tools differed considerably in their ability to detect low-density infections. Considering their prevalence, the South African malaria control programme may consider the deployment of sensitive diagnostic tools to identify this hidden reservoir of infections that are missed by the regular tools. Further characterisation of these infections demonstrated variation in parasite multiplicity of infection, indicative of variation in the force of infection, and variation in drug resistance genotypes that hint towards a role of population movement in the spread of drug-resistant parasites in the region. The next chapter explores the development of novel gametocyte markers and use of mosquito blood meals in xenosurveillance. These approaches can help in improved surveillance and monitoring systems targeting both the human and vector components of the residual transmission.

Chapter 4:

The detection of P. *falciparum* gametocytes and asexual stage parasites in mosquito blood meals – novel assay development

4.1 Introduction

Malaria mortality and morbidity estimates are largely based on routinely collected health data. Given that health information systems may be imprecise, particularly in resource-poor environments, estimates may utilize models that convert parasite prevalence to mortality rates [7]. Information that became available following the increased distribution of RDTs across Africa and improved reporting to the national surveillance systems, suggests that malaria caseload may have been considerably underestimated in many settings [5]. More challenges lie ahead: RDTs have several limitations that may reduce their utility in low-transmission settings, including the inability to detect low density parasitaemia [258]. Alternative or additional strategies for disease surveillance may thus be beneficial. In most endemic settings, the vast majority of individuals with detectable malaria parasites have no acute symptoms suggestive of disease or prompting health-seeking behaviour [70]. Whilst malaria interventions increasingly aim to include these 'asymptomatic infections', for example in screening campaigns by community health workers [259], low-density infections are frequently missed [27] despite their potential importance for onward transmission to mosquitoes [260].

The transmission of malaria to mosquitoes depends on the presence of mature male and female gametocytes in the peripheral circulation. Mosquitoes can become infected even when feeding on humans with submicroscopic gametocyte densities [48]. Since gametocyte sex ratio is female-biased but both genders need to be present for infection to be achieved, the density of male gametocytes is critical in determining infectiousness [261]. Molecular gametocyte detection tools such as RT-qPCR and QT-NASBA, with higher sensitivities than microscopy, have been deployed in field settings [262, 263] and may be used to predict the transmission potential of infected hosts [113]. However, molecular gametocyte detection is laborious, requiring a cold chain and is highly resource intensive [27]. Since gametocytes are commonly produced at some point during malaria infections to continue the life cycle, it is doubtful that the precise quantification of gametocytes is of relevance for public health surveillance [27]. From an operational perspective, it would be highly attractive to identify approaches that can detect and potentially quantify malaria transmission potential without relying on blood drawing or a cold chain [264, 265]. Alternative sampling approaches for

surveillance may be particularly beneficial in pre-elimination settings that need to find the last remaining pools of parasites [64].

Blood-fed mosquitoes caught resting indoors and outdoors contain blood samples from their human and other hosts. This may present an attractive source for blood samples for xenosurveillance [266], which would entail testing blood ingested by mosquitoes for the presence of human pathogens. This indirect sampling method, utilizing mosquitoes as a form of a biological syringe, has previously been considered for human pathogens [267] and allows simplification of surveillance, minimization of costs and negates the need for invasive sampling [268]. A limited number of studies indicate that this method is feasible for human pathogens [268-270]. Identification of pathogens of different taxa, including Dengue virus, West Nile virus, HIV-I, *Trypanosoma brucei gambiense*, and *Bacillus anthracis*, is possible for several hours after ingestion, depending on the respective stability of pathogen nucleic acid in the mosquito midgut [268-270]. Against this promising background of xenosurveillance for other pathogens, the validation of this approach for *Plasmodium* spp. could be of great interest, especially if samples may be pooled to detect sparse infections with high efficiency.

This study aimed at developing sensitive and robust assays to detect parasite antigen, DNA and RNA transcripts for surveillance of (transmissible) forms of the parasite in field settings. For this, mosquito blood meals were used to detect gametocytes, determine gametocyte sex ratios and identify asexual *P. falciparum* parasites using molecular methods and both regular and ultrasensitive RDTs.

4.2 Materials and methods

4.2.1 Ethics statement

The experiments detailed in this section were performed according to Good Laboratory Practices as approved by the Radboud Institute of Molecular Life Sciences ethics and animal welfare committee.

4.2.2 *In vitro* gametocyte culture

A semi-automated suspension culture as described [271] was used for the gametocyte culture. Briefly, for gametocyte production asynchronous *P. falciparum* (NF54 clone) were started on day 0 (0.5% parasites, 5% red blood cells) at 37°C under 4% CO_2, 3% O_2 and 93% N_2 continuous gas flow. Parasites were cultured in RPMI 1640 with L-glutamine (Life Technologies), supplemented with 5.94 g/L HEPES (BDH Biochemical) and 50 mg/L hypoxanthine (Sigma Aldrich). From days 8 till 12, N-acetyl glucosamine (NAG) was added (50 mM (Sigma Aldrich)) to remove residual asexual parasites. Culture medium was

automatically changed twice daily and gametocytes monitored by microscopy with Giemsa-stained blood smears. Cultures were harvested at day 14-16, with predominantly stage V gametocytes used in assays.

The generation of pure male and female sorted gametocyte populations to be used as controls was performed using the PFDynGFP/P47mCherry reporter lines and tested by immune–fluorescence assays (IFA) and microscopy as described [272]. Briefly, the PfDynGFP/P47mCherry line contains a GFP integrated under the control of the male-specific *dynein heavy chain* promoter and mcherry expression is regulated by the female-specific *P47* promoter in episomal plasmids. This expression of sex-specific fluorescent markers allowed the separation of male and female gametocytes by fluorescence-activated cell sorting [273]. Sorting was performed based on GFP signal only. On day 5 following NAG addition, $4x10^7$ stage III gametocytes were purified by running through magnetic-activated cell sorting (MACS) column (Miltenyi Biotec) for enrichment based on absorption of paramagnetic iron (III), sorted based on GFP expression and fractions of male (GFP+) and female (GFP-) gametocyte were subsequently cultured separately. On day 12, sorted populations that had progressed to stage V of gametocyte maturation were assessed by IFA using the gametocyte (rabbit anti-*Pfg*377) [274] and female gametocyte-specific antibodies (mouse anti-Pfg377, generated using immunizations with recombinant *Pfg*377) [275] and by microscopic examination of Giemsa stained smears. The IFA analysis showed that the GFP+ fraction was 100% *Pfg*27+/*Pfg*377– confirming them as males. This was affirmed by microscopy where male gametocytes showed the diagnostic cytoplasmic pink staining. By contrast, the GFP- fraction was 83% *Pfg*27+/*Pfg*377+ and thus confirmed as females. This was confirmed by microscopy where the females exhibited the diagnostic cytoplasmic blue staining on Giemsa smears [272]. These experiments thus confirmed the sex-specificity of the transgenic PfDynGFP/P47mCherry line (Appendix 6).

4.2.3 Mosquito husbandry and membrane feeding assays

Experiments were carried out using laboratory strains of *An. stephensi* (Sind Kasur Nijmegen strain) mosquitoes [276]. The mosquitoes were reared at 30°C and 70-80% humidity while exposed to a 12 h day and night cycle and maintained on 8% (w/v) sucrose solution. Venous blood of malaria-naïve volunteers was drawn into EDTA-containing tubes and kept warm in a 37°C heat-block. Cultured *P. falciparum* stage V mixture of NF54 clones of male and female gametocytes were added to the whole blood at 10-fold dilutions between $1.0x10^7$ and $1.0x10^3$ gametocytes/ml. A 300 µl aliquot of the mixture of gametocytes and EDTA-whole blood was transferred to an artificial small membrane feeder (14 mm diameter feed area) and offered to 3 to 4-day old *An. stephensi* mosquitoes for 20 min in a medium sized cage (W30 x D30 x H30

cm) at room temperature in the dark. Negative control mosquitoes were fed EDTA-whole blood with no gametocytes. After 15 min of feeding, blood-fed mosquitoes were removed from the cages, killed by exposure to CO_2 gas and their midguts punctured to empty the contents into tubes with 50 µl of RNAprotect Cell Reagent (Qiagen, Hilden, Germany). Mosquitoes were either frozen immediately at -20°C or maintained on glucose at the insectary until they were required for experiments at 6, 12 and 48 h.

4.2.4 Mosquito homogenizations

For mosquitoes whose midguts were not punctured, homogenization was performed by bead beating as described previously [277]. Briefly, silica beads were added to mosquitoes in RNAprotect Cell Reagent up to a total volume of 500 µl in tubes. Mosquitoes were subsequently homogenized by vigorous mechanical plate shaking for 10 s (Biospec, Mini Beadbeater 96). The tubes were centrifuged for 1 min at 10 000*g* to spin down the homogenate and beads before extractions.

4.2.5 Total nucleic acid extractions

Mosquito homogenates or intact mosquitoes with punctured midguts were used for total nucleic acid extraction using the bead-based MagNaPure LC Total Nucleic acid Isolation kit (Roche, Basel, Switzerland). The automated nucleic acid extraction process relies on magnetic bead technology to purify nucleic acids after lysis with special buffer containing chaotropic salts and proteinase K with several washing steps. The HS500 standard extraction protocol was followed to elute total nucleic acids into 50 µl final volume from a starting volume of 500 µl.

4.2.6 Multiplex qPCR-based detection of *P. falciparum* parasites

The quantities of *P. falciparum* parasites and blood volume in the mosquito midgut were quantified by qPCR targeting the 18S rRNA small subunit gene and human β-tubulin (white blood cell marker), using primer and probe sequences as described [278]: for the 18S rRNA amplification forward primer 5'-GTA ATT GGA ATG ATA GGA ATT TAC AAG GT-3', reverse primer 5'-TCA ACT ACG AAC GTT TTA ACT GCA AC-3', TaqMan probe 5'FAM TGC CAG CAG CCG CGG TAA TTC with BHQ1-3' quencher. The probe was labelled with a 6-carboxy-fluorescein (FAM) reporter and 6-carboxy-tetramethylrhodamine (TAMRA) quencher [278]. For the human β-tubulin, forward primer sequences were 5-'AAG GAG GTC GAT GAG CAG AT-3' and reverse primer 5'-GCT GTC TTG ACA TTG TTG GG-3' with Texas red probe 5'-TTA ACG TGC AGA ACA AGA ACA GCA GCT with BHQ2-3' quencher. Probes were sourced from Life technologies (Applied Biosystems) and primers were from SIGMA ALDRICH. For

the *P. falciparum* qPCR, 5 µl of extracted total nucleic acids were used as template in a total reaction volume of 20 µl of a TaqMan® Multiplex master mix (Applied Biosystems) in the presence of final concentrations of 100 µM probe and 50 µM primer. Cycling conditions were initial denaturation for 3 min for 95°C with 40 fast cycles of 10 s at 95°C and 30 s at 60°C. All reactions were run using hard-shell 96-well PCR plates (Bio-Rad) and quantification based on a standard curve of trendlines on the CFX96™ real-time PCR detection system (Bio-Rad).

4.2.7 Development of multiplex qPCR-based detection for male and female gametocytes

Using previously published data [279] and genes selected from in-house experiments (van Biljon, PhD thesis, University of Pretoria), promising gametocyte-specific transcripts were identified. These genes were selected based on specific and increased upregulation in stage V of gametocyte development and downregulation in asexual stages after microarray experiments. They were ranked based on an integrated gametocyte-specificity score, on the ability of different transcriptomic and proteomics datasets to discriminate known gametocyte genes from asexual genes following probabilistic integration [279]. Ultimately, the male-specific marker, *P. falciparum* gametocyte enriched target (*pfmget*, Pf3D7_1469900) [272] and the female marker (*ccp4*, Pf3D7_0903800) [279] were selected for multiplex qPCR development as they demonstrated high levels of expression in gametocyte stages and good discriminatory characteristics for detecting male and female gametocytes, respectively [272, 273, 280].

For gametocyte male and female marker detection, a one-step reverse transcriptase (RT)-qPCR was thus set up targeting the female-specific *ccp4* gene [279] and male-specific *pfmget* gene (Table 4.1). In brief, 5 µl of extracted total nucleic acids were used as template in a total reaction volume of 20 µl of a Luna® Universal Probe One-Step RT-qPCR Kit (New England Biolabs Inc., Massachusetts, USA) with 100 µM probe and 50 µM primer. Cycling conditions were initial reverse transcription process at 55°C for 15 min, followed by denaturation for 1 min at 95°C, then 45 cycles of 10 sec at 95°C and 1 min at 60°C. Probes were sourced from Life technologies (Applied Biosystems) and primers were from SIGMA ALDRICH. All reactions were run using hard-shell 96 well PCR plate (Bio-Rad) and quantification based on a standard curve of trendlines on the CFX96™ real-time PCR detection system (Bio-Rad).

Table 4.1: Gene IDs, primer sequences and product length of male and female gametocyte markers.

Gene_ID	Primers 5-3'	Length	Tm	Product Length
PF3D7_0903800 (*ccp4*)	Forward CAC ATG AAT ATG AGA ATA AAA TTG	24	55	120 bp
	Reverse TAG GCG AAC ATG TGG AAA G	19		
Probe sequence and fluorophore	AGC AAC AAC GGT ATG TGC CTT AAA ACG Texas Red			
PF3D7_1469900 (*pfmget*)	Forward CGG TCC AAA TAT AAA ATC CTG	21	60	120 bp
	Reverse GTG TTT TTA ATG CTG GAG CTG	21		
Probe sequence and fluorophore	CAG CTC CAG CAT TAA AAA CAC FAM			

The detectability of male and female gametocytes using these newly described markers, was investigated by a single-step multiplex qPCR on 5 µl of human blood (Figure 4.1).

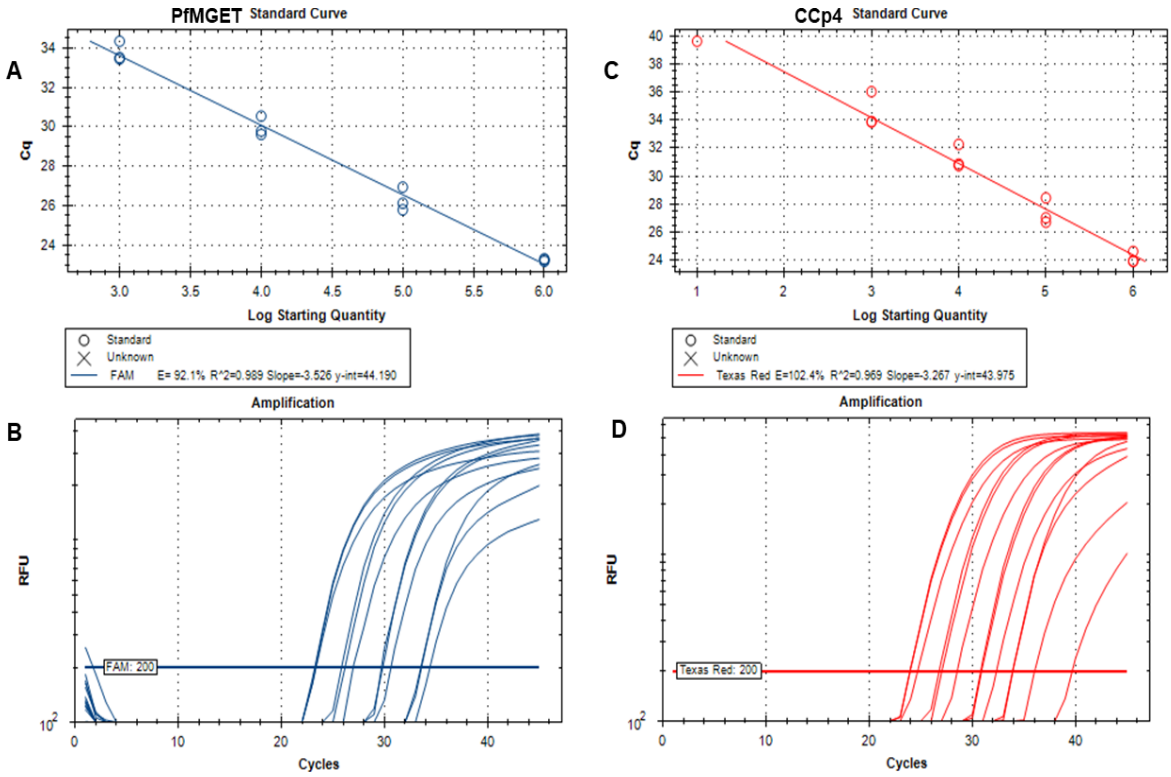


Figure 4.1: Standard curves and amplification images of male (*pfmget*) and female (*ccp4*) gametocyte markers. Standards curves was performed for **(A)** male marker fluorescently detected with FAM dye and **(B)**, female marker with Texas Red dye on experiment's performed in triplicates on control material. The amplifications plots are shown for male **(C)** and female **(D)** markers respectively.

Based on the outcomes of using human blood, the utility of the same multiplex qPCR to distinguish between male and female gametocytes from mosquito blood meals was investigated.

4.2.8 Feasibility of using RDTs to detect asexual *P. falciparum* in mosquito blood meals

To test for the feasibility of using asexual parasite load in mosquitoes for xenosurveillance, both regular and ultrasensitive RDTs were used to detect parasites at $1.0x10^{7}$-$1.0x10^{4}$ parasites/ml. Fully fed mosquitoes were used in the RDT experiments immediately after feeding, 15 min, 1 h, 6 h and 24 h post-feeding. At each time point, the midguts of fully blood-fed mosquitoes were punctured into the sample well of either the regular (CareStart™) or the highly sensitive (Alere™) RDTs that both target the *PfHRP-2* antigens. The results were read after 20 minutes as per the manufacturer's instructions (Figure 4.2).

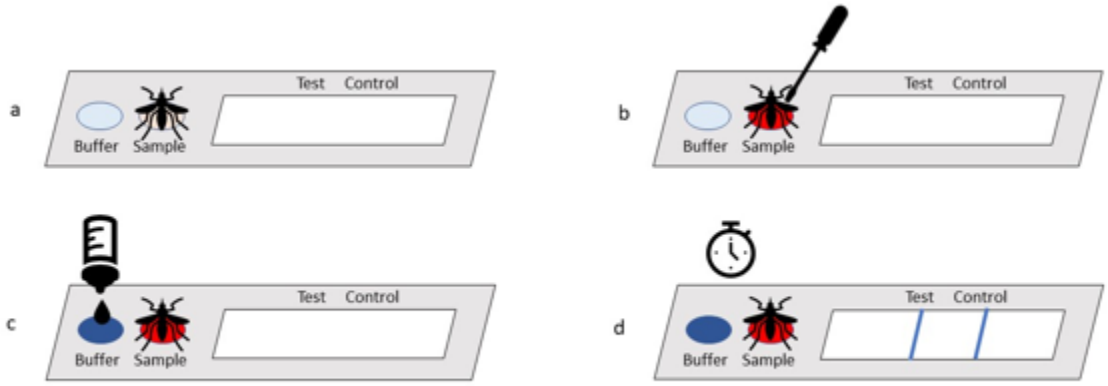


Figure 4.2: Schematic on performing rapid diagnostic tests with mosquito blood meals. (a) Blood-fed mosquito is placed on a newly opened RDT; (b) mosquito midgut is punctured using a needle and blood meal evenly spread on the sample well; (c) drops of the manufacturer buffer is applied to buffer well; (d) result can be interpreted after 20 min as recommended by the manufacturer.

To determine the feasibility of using pooled mosquitoes in field settings, infected mosquitoes were pooled with uninfected mosquitoes. Pooling of mosquitoes involved puncturing one mosquito infected with either $1.0x10^{6}$ or $1.0x10^{5}$ ring stage parasites/ml and mixing it with either 4 (1:5) or 9 (1:10) uninfected mosquito blood meals. For these pooling experiments, mosquitoes were homogenized into a single tube with the provided RDT buffer as diluent. A total of 5 µl of samples were loaded into the RDTs using a pipette (Figure 4.3).

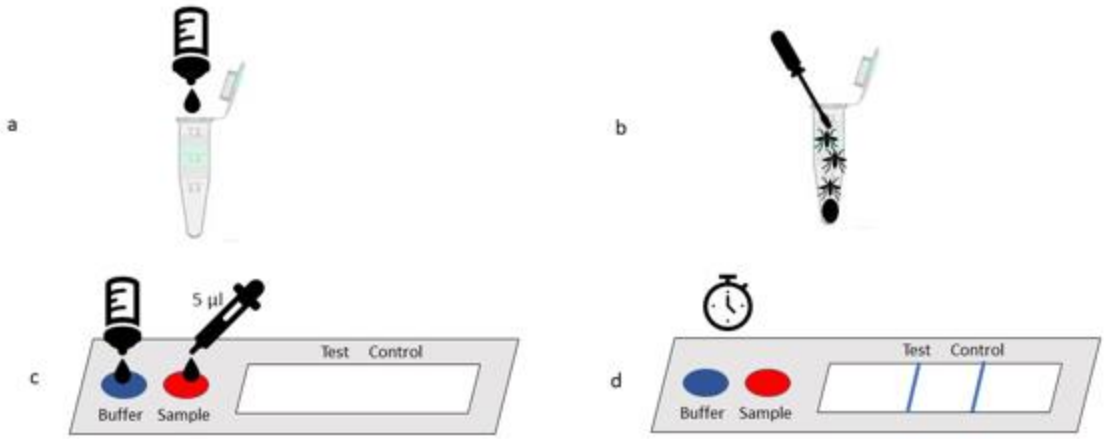


Figure 4.3: Schematic on performing the rapid diagnostic test with pooled mosquitoes. (a) One drop (approximately 30 µl) buffer was added into an Eppendorf tube; (b) The required number of mosquitoes (2, 4, 5, 6 and 10) were added to the tube and homogenized carefully; (c) A total of 5 µl homogenate was pipetted and transferred to the sample well, subsequently two drops of buffer was added to the buffer well; (d) results we reported after 20 min as per manufacturer's recommendations.

4.2.9 Statistical analyses

All analyses were performed with SPSS version 20 (IBM Corp. NY, USA) and GraphPad Prism 8.0 (Graph Pad Software Inc., La Jolla, CA). Students' t-tests were performed to determine differences between bead-beating *vs*. no bead beating before nucleic acids extraction. A two way ANOVA was used to determine the effects of timing on the DNA detection of the qPCR assay.

4.3 Results

4.3.1 Detection of male and female gametocytes in mosquito blood meals

To determine whether mosquito blood meals can be used as blood sample source to determine gametocyte sex ratio in multiplex qPCR assays, male (*pfmget*) and female (*ccp4*) gametocyte marker signals were tested in single blood-fed mosquitoes. Both the male and female markers were detectable at $1.0x10^6$, $1.0x10^5$ and $1.0x10^4$ parasites/ml within single mosquitoes, with female signal performing slightly better than the male signal with lower C_T values (Figure 4.4). No male or female gametocyte signal was detectable at $1.0x10^3$ parasites/ml dilution, and consequently, it was decided not to use this concentration of parasites in subsequent experiments

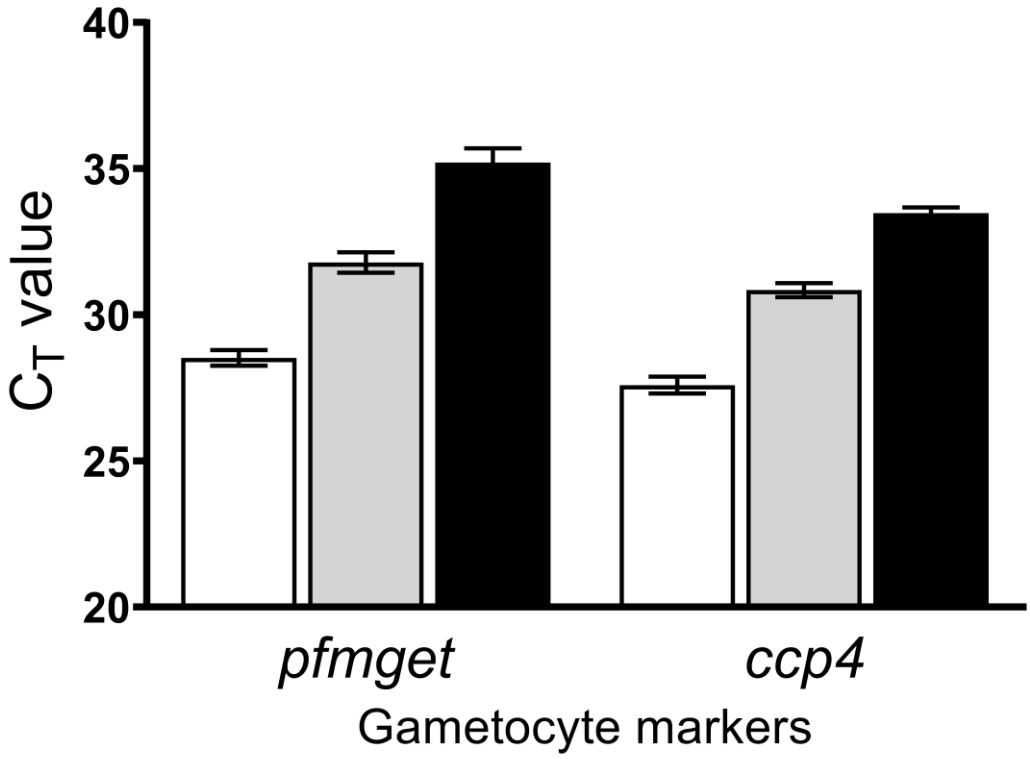


Figure 4.4: Male (*pfmget*) and female (*ccp4*) gametocyte marker transcripts detection in multiplex qPCR experiments in mosquitoes. All experiments were performed with blood meals from single mosquitoes. White bars represent $1x10^6$, grey bars represent $1.0x10^5$ and black bars represent $1.0x10^4$ parasite concentrations/ml respectively. Error bars represent SEM of 5 replicates.

4.3.2 Asexual stage parasite detection in mosquito blood meals.

To determine the quantity of *P. falciparum* parasites in mosquito blood meals, 18S rRNA signal was tested by qPCR. To measure the amount of blood meal ingested by individual mosquitoes, the human white blood cell marker, β-tubulin, that is expressed in all leukocytes and thus a useful surrogate marker for bloodmeal size, was used. Using 5 µl of culture material as input, the qPCR assay was consistently able to detect $1.0x10^4$ parasites/ml, the lowest tested concentration, as reported previously [26] (Figure 4.5A). Human β-tubulin showed no variation between the different parasite concentrations when 5 µl of culture material with variable *P. falciparum* parasite densities was used for DNA extraction and qPCR (C_T value range 25.56 – 26.03, 3 replicates). Using these assays, the stability of parasite DNA signal post-feeding in the blood meals of mosquitoes that fed on parasite culture material and harvested at 1, 6, 12 and 24 h post feeding was investigated. At 24 h post feed, the signal was reduced (i.e. higher C_T values) for both 18S rRNA and human β-tubulin targets across both tested parasite concentrations ($1.0x10^7$ and $1.0x10^6$ parasites/ml) (Figure 4.5B), but only significant for the human β-tubulin target (*P*=0.002).

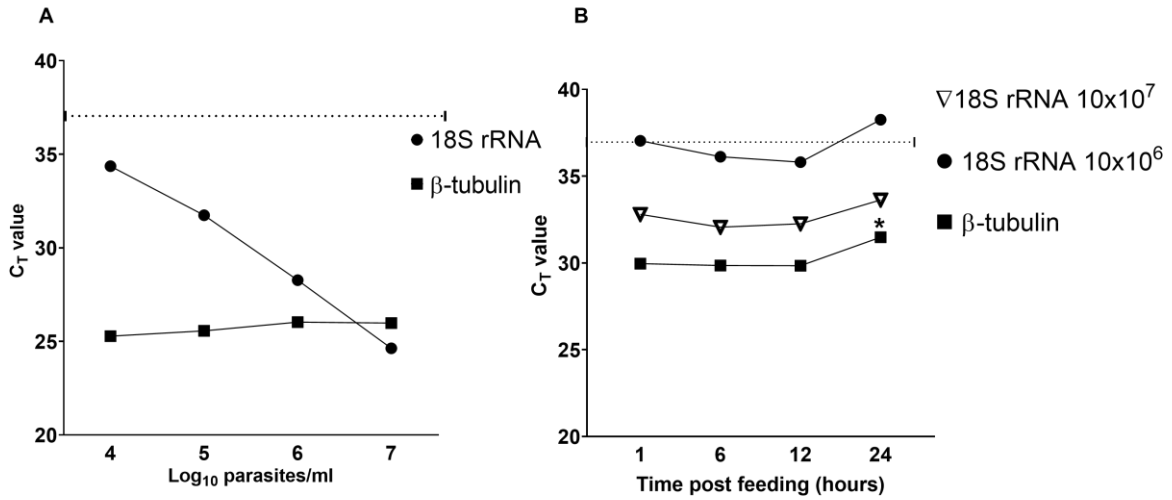


Figure 4.5: Detectability of asexual parasites by 18S rRNA and leukocytes by β-tubulin markers in multiplex qPCR experiments. A, log starting parasite concentration for 18S rRNA and β-tubulin markers with C_T value 5 µl volume of control culture material. B, detectability of 18S rRNA and β-tubulin concentrations at various timepoints post feeding on single mosquito blood meals. The threshold of positivity set at C_T of <37 is shown with a dotted line.

4.3.3 Effect of homogenization on malaria parasite detection signal

To determine whether the method of mosquito processing affects qPCR outcomes, comparisons were made between mosquitoes homogenized through bead beating or solely by midgut puncturing. The female gametocyte marker signal was weaker in bead beaten mosquitoes compared to non-bead beaten mosquitoes at $1.0x10^5$ (P=0.005) and $1.0x10^4$ parasite concentrations/ml (P=0.0017) (Figure 4.6 A). In addition, there was a marked loss in qPCR signal on the male gametocyte marker, with a greater loss in signal as demonstrated with increased C_T value in bead beaten mosquitoes for all 3 parasite concentrations (P<0.001) (Figure 4.6 B).

For asexual parasites, bead beating significantly reduced the ability to detect 18S rRNA targets in the blood meal samples in experiments performed in 5 replicates ($1.0x10^5$, $1.0x10^6$ and $1.0x10^7$ parasites/ml, P=0.0103, P<0.001 and P=0.0101, respectively) (Figure 4.6C). This reduction was evident in both 18S rRNA with raised C_T values (~1.18) compared to not bead beaten mosquitoes. For the human β-tubulin marker (Figure 4.6 D) there were no significant differences between bead beaten and not bead beaten mosquitoes at $1.0x10^7$ parasites/ml (P=0.1205). However, there were significant differences at $1.0x10^6$ parasites/ml (P=0.014) and $1.0x10^5$ (P=0.0017) parasites/ml.

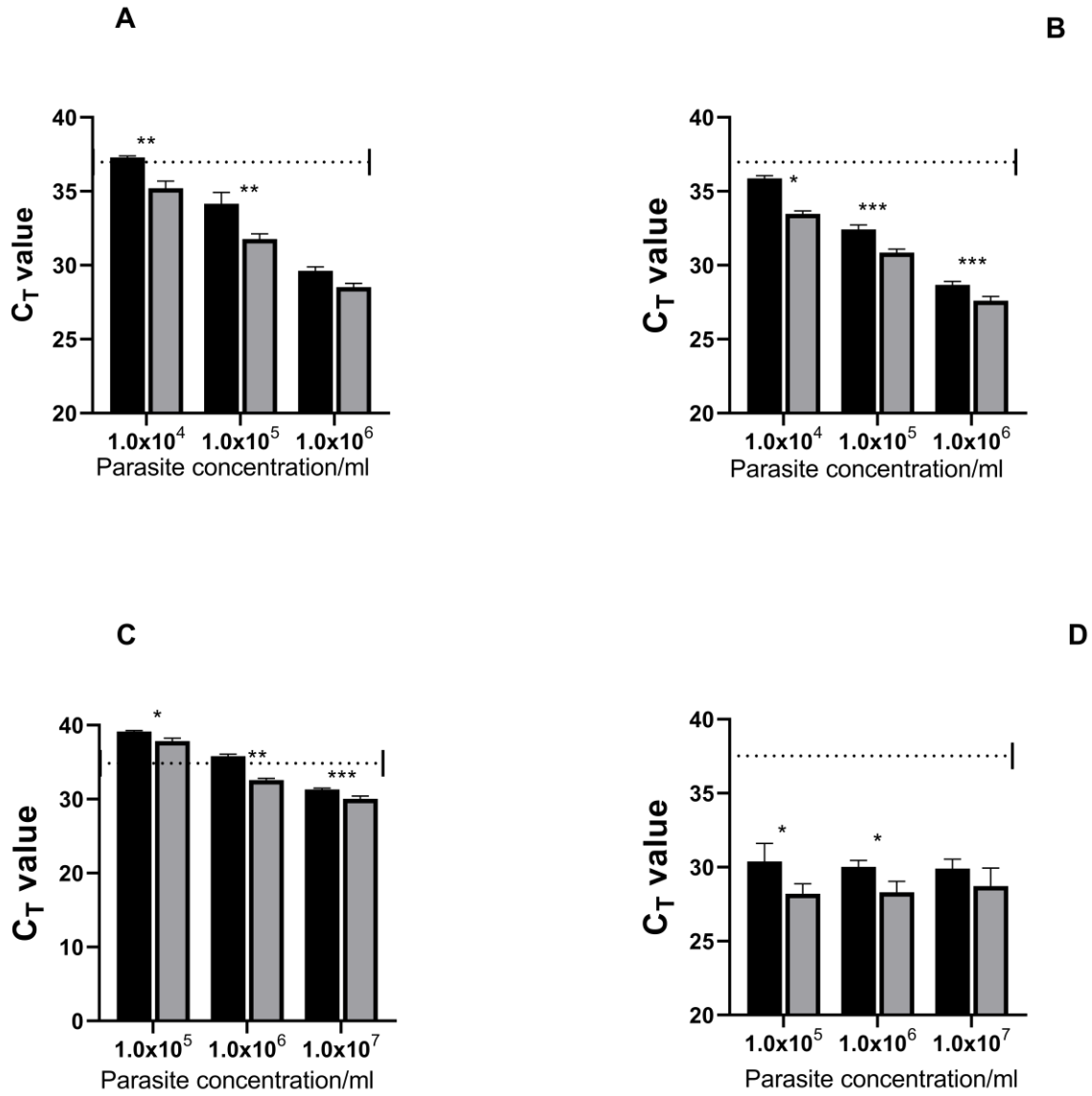


Figure 4.6: Effects of homogenization method on qPCR detection of signal in individual mosquitoes. (A) Female gametocyte (*ccp4*), (B) male gametocyte (*pfmget*), (C) 18S rRNA and (D) human β-tubulin markers in mosquito blood meals are compared at various concentrations. Black bars represent bead beaten mosquitoes with grey bars representing non-bead beaten mosquitoes (homogenised by midgut puncture only). The threshold of positivity of C_T value of <37 is shown with a dotted line. All experiments were performed in 5 replicates of individual mosquito blood meals. Error bars represent SEM of 5 replicates. Statistical significance was performed using 2-way ANOVA * P<0.01, *** P<0.001, ** P<0.005.

4.3.4 Detectability of malaria parasites in pooled mosquitoes

Pools of 2 and 4 positive mosquitoes were compared to individual mosquitoes to assess whether qPCR signals would improve (Figure 4.7). Pooling of mosquitoes led to statistically significant improvement in both signal detection of male and female gametocyte markers. This improvement was statistically significant at $1.0x10^6$ and $1.0x10^5$ concentrations of parasites/ml tested (P<0.01) but not at $1.0x10^4$ (P=0.2441). The signal was not different between pools of

4 mosquitoes compared to 2 mosquitoes (*P*=0.2202), except for the female gametocyte marker at 1.0x10^5 gametocytes (*P*=0.0017).

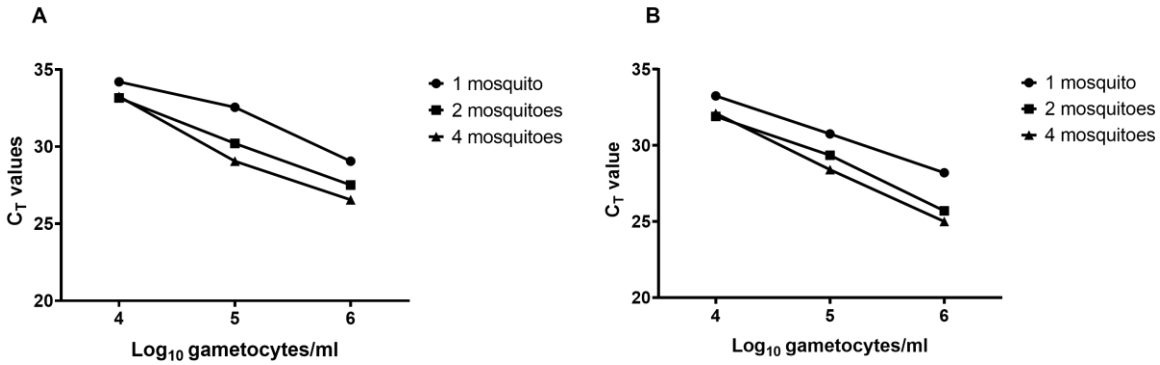


Figure 4.7: Effect of pooling mosquitoes on detection of sex-specific gametocyte markers. The male (A, *pfmget*) and female (B, *ccp4*) gametocyte markers were detected in single, 2 and 4 mosquitoes respectively at different gametocyte concentrations. The expected linear increase in C_T values with decreasing concentration of parasites was observed.

On asexual parasites, the pooling approach did not result in a lower C_T value or any better signal detection (Table 4.2) and the detectability of parasites was rather poor in general. Detection of the 18S rRNA signal was not achieved at 24 h post-feed for any of the tested concentrations or pool sizes.

Table 4.2: Results of qPCR pooling experiments, on 18S rRNA target. DNA extraction performed on pools of 2, 4 and 6 mosquitoes at differing concentrations and time points.

Parasites/ml blood	15 min	1 h	6 h	24 h
2 mosquitoes				
1.0x10^6	37.05	38.07	38.12	Undetermined
1.0x10^5	Undetermined	Undetermined	Undetermined	Undetermined
1.0x10^4	Undetermined	Undetermined	Undetermined	Undetermined
4 mosquitoes				
1.0x10^6	36.51	37.91	36.40	Undetermined
1.0x10^5	Undetermined	Undetermined	Undetermined	Undetermined
1.0x10^4	Undetermined	Undetermined	Undetermined	Undetermined
6 mosquitoes				
1.0x10^6	38.12	37.17	37.29	Undetermined
1.0x10^4	39.54	Undetermined	Undetermined	Undetermined

4.3.5 Feasibility of using RDTs to detect *P. falciparum* in mosquito blood meals

Having tested the utility of mosquito blood meals in detecting gametocyte and asexual stage parasites using molecular methods, the feasibility of using RDTs for xenosurveillance was explored in pilot experiments that were limited in scope due to the limited availability of RDTs. Tests were carried out using both the *PfHRP2* antigen-based regular and ultrasensitive RDTs using mosquitoes fed on different concentrations of asexual stage parasite spiked blood meals. Both RDTs and uRDTs showed clear test lines for high parasite concentrations ($1.0x10^6$) 15 minutes directly after the feed (Figure 4.8), thus demonstrating the general feasibility of using both tests on blood meal ingested by the mosquito for the detection of *P. falciparum*.

Figure 4.8: A snapshot of RDT and uRDT outcomes performed with mosquito blood meal, 6 h post-feeding with different concentrations of parasite/ml of blood and uninfected blood. Mosquito carcass can be seen in the sample well, test and control lines for the tests are clearly visible. The top panel indicates the ultra-sensitive RDT from ALERE™ and below the regular RDT from CareStart™. On each RDT, the amount of parasites /ml blood is indicated and 5 µl of sample per well was added.

Both regular RDT and uRDT were able to detect a concentration of $1.0x10^4$ parasites/ml blood in the fresh blood meal, whereas a control RDT with the same concentration without ingestion by the mosquito rendered a negative result (Table 4.3). All tests performed on non-infected blood showed valid negative results (i.e. no false positives), indicating no detrimental interference of unknown substances in the mosquito gut with the test devices.

Table 4.3: Outcomes of RDT/uRDT experiment at different time points, parasite concentrations and pools of mosquitoes. Rapid diagnostic tests can detect *Plasmodium falciparum* using the mosquito blood meal as a sample source.

	Time after feed			
Parasite per ml	Up to 15 min		6 h	
	RDT	uRDT	RDT	uRDT
Feeding material				
$1.0x10^7$	(1/1)	-	-	-
$1.0x10^6$	(1/1)	(1/1)	-	-
$1.0x10^5$	(1/1)	(1/1)	-	-
$1.0x10^4$	(0/1)	(1/1)	-	-
Uninfected blood	(0/1)	(0/1)	-	-
Individual mosquitoes (Number of test positive/total number performed)				
$1.0x10^7$	(1/1)	-	(3/3)	
$1.0x10^6$	(1/1)	(1/1)	(3/3)	(3/3)
$1.0x10^5$	(1/1)	(1/1)	(2/3)	(2/3)
$1.0x10^4$	(1/1)	(1/1)	(0/3)	(0/3)
Uninfected blood	(0/1)	(0/1)	(0/1)	(0/1)
Pooled mosquitoes (Number of mosquitoes per pool, infected: uninfected)				
$1.0x10^7$	-	-	(3/3) for (1:4) (2/2) for (1:9)	-
$1.0x10^6$	-	-	(2/3) for (1:4) (1/1) for (1:9)	(3/3) for (1:4) (1/2) for (1:9)
$1.0x10^5$	-	-	-	(0/3) for (1:4) (0/1) for (1:9)

At 6 h post-feeding, both RDT and uRDT were able to detect the presence of *P. falciparum* at a concentration of $1.0x10^5$ parasites/ml blood in two out of three tests in individual mosquitoes (Table 4.3). While both RDT and uRDT could detect parasites in pools with 1 infected parasite and 4 or 9 uninfected mosquitoes (1:5 and 1:10 pooling) with $1.0x10^7$ and $1.0x10^6$ parasites/ml in the positive mosquito, no test was positive at $1.0x10^5$ parasites/ml. Follow up tests up to 48 h post-feed were unsuccessful, possibly due to complete digestion of the blood meal, as none or very little of the blood meal could be transferred onto the test well.

4.4 Discussion

In this study, the feasibility of detecting *P. falciparum* parasites in mosquito blood meals is demonstrated by both multiplex qPCR and RDTs at clinically relevant parasite loads. We demonstrated for the first time that gametocytes can be quantified in mosquito blood meals. Our qPCR parasite detection thresholds are consistent with previous studies assessing molecular detection of nucleic acids of *Plasmodium* and other human pathogens [269, 272, 279]. Whilst the detectability of infections in single blood meals is promising, sensitive detection of sparse malaria infections after pooling of mosquitoes is essential for the approach to be attractive for xenosurveillance. In our pooling experiments, parasite infections below $1.0x10^7$ parasites/ml that are typically associated with asymptomatic malaria [237, 243] were frequently missed.

Detecting malaria parasites in mosquito blood meals can serve multiple purposes. It might support investigations into the long-standing hypothesis that parasites and specifically gametocytes may sequester in the skin of malaria-infected patients [281-283]. This might explain why mosquito infection rates are typically higher in mosquitoes that fed directly on the skin of gametocyte carriers compared to mosquitoes that feed on venous blood in an artificial membrane feeder system [284]. In this context, our findings that both male and female gametocytes can be detected in mosquito blood meals is an exciting step towards a direct assessment of transmission potential from different blood compartments. Using our approach, we were able to directly demonstrate that mosquitoes have ingested a blood meal that was likely to be infectious. The amount of the blood meal ingested by mosquitoes using a β-tubulin marker indicated very limited variation in blood meal sizes among our homogenous mosquito colony. Our novel multiplex assay targeting male and female gametocyte transcripts with intron-spanning primers has some important advantages. As qRT-PCR assays also amplifies residual DNA extracted together with RNA, DNase I treatment is necessary for accurate results. Concerns that DNAse I treatments would lead to loss of signal [285], highlights the potential value of intron-spanning primers. *Pfs25* (PF3D7_103100) has routinely been used to quantify gametocyte burden [106, 286, 287] but these previous estimates are potentially imprecise as *pfs25* is considerably enriched in females or is potentially female specific [288]. The choice of male-specific gene transcript (*pfmget*) and female specific (*ccp4*) allows the estimation of gametocyte sex ratio, which can be an important determinant of gametocyte infectivity and informative in trials of gametocytocidal drugs with differential effects on male and female gametocytes [272, 289]. Since mosquitoes must ingest at least 1 male and female gametocyte to become infectious, and the targets tested in this study are capable of detecting gametocytes at concentrations below the minimum estimates needed for infectivity [51], we

consider the novel multiplex male-female gametocyte qRT-PCR highly promising. We also regard the currently proposed methodology suitable for research on the potential sequestration of gametocytes in skin tissue.

The observation that homogenization through bead beating resulted in a considerable loss of signal in asexual stages and gametocytes was unexpected. This method has previously been used in field studies [290] and clinical trials [291] and was considered to be a high throughput method without loss in signal. Previous anecdotal evidence of a lower luminescence signal after bead beating mosquitoes infected with transgenic parasite lines has been reported [272], but we anticipated the DNA target to remain unaffected by bead beating. We introduced a new method of rupturing of mosquito midguts by needles to release contents into tubes with RNAprotect reagent and bypassing the bead beating step. Our approach significantly improved signal detection. The detrimental effects of bead beating may be due to the release of endogenous RNAses/DNAses or other inhibiting or degrading substances including the carcass otherwise retained in the mosquito with the alternative puncturing method. Arguably, RNA stabilizing agents should protect the integrity of the RNA [292], but this was not the case in our laboratory outcomes. Our findings may thus need to be incorporated in future studies using readouts requiring mosquito homogenization.

The results obtained by qPCR on mosquito blood meals were somewhat disappointing. The lower detection limits for the qPCR assay was $1.0x10^6$ parasites/ml in contrast to RDTs that could detect up to $1.0x10^4$ parasites/ml. This may be attributed to a technical limitation of malaria molecular diagnostics, the intrinsic chance effect of capturing of scarce parasite material that depends on the volume of blood used [222]; DNA degradation is unlikely since the human white blood cell marker was detectable without evident loss in signal. The limited qPCR sensitivity may be mitigated by pooling of mosquitoes to increase volumes and targeting of high copy number DNA sequences [229]. On the other hand, pooling of mosquitoes will typically result in the dilution of the signal since only a minority of blood meals is expected to have parasites. The ultra-sensitive RDT was not superior to regular RDTs in detecting *Plasmodium* infections when using the mosquito blood meal as a sample source. This is in contrast to the previous observations that uRDTs have a 10-fold increased detection than other commercially-available *HRP2* based RDTS [293]. However, the uRDT outperformed the regular RDTs during control experiments on mosquito feeding materials. This may possibly be due to the limited quantity of blood transferred to the test device. The average blood meal of *Anopheles* mosquito varies between 2 µl to 8 µl while the manufacturers recommend using 5 µl of the blood. Since the mosquito initiates digestion of the blood meal immediately after feeding, the stability of *HRP2* in the midgut may also affect readouts [266]. Earlier studies

established the relative stability of viral RNA inside the midgut for up to 24 h post-feeding [268, 270]. Recent findings indicate *HRP2* half-life to be up to 3 days in human blood [294].

Based on the limited number of observations in this study, further investigations are necessary to validate this approach for xenosurveillance. These include the effect of longer time periods between the mosquito feeding and influence on RDTs readouts as well as performance of aldolase or pLDH based RDTs kits. The growing body of evidence suggesting the presence of *HRP2* deleted parasites in Africa [217] supports testing of other antigen-based RDTs. Finally, recent studies have used different species of mosquitoes in xenosurveillance trials [266, 295] and exploring other *Anophelines* for detection of other human pathogens or vector-borne diseases can be useful [268].

4.5 Conclusion

This study presents an experimental approach to detect *P. falciparum* and its life stages in mosquito blood meals. Male and female gametocyte transcripts were successfully quantified across a spectrum of densities that possibly contribute to onwards transmission to mosquitoes. Our approach may thus identify mosquitoes that will plausibly become infected following the blood meal. Our approach with mosquito homogenization and pooling is currently insufficiently sensitive for xenosurveillance, regardless of whether parasites are detected based on qPCR or RDT. More sensitive approaches are needed to identify the sparse mosquitoes that ingested a parasite positive blood meal, especially when considering the parasite densities that are typically present in asymptomatic parasites carriers.

Chapter 5

Concluding discussion

The history of malaria elimination efforts shows remarkable successes in some countries but failures in others [64]. The failure of the GMEP in many African settings offers key lessons that the reinvigorated global push for malaria elimination can learn from to avoid future disappointments [55]. The price of failure for countries that have tried to eliminate malaria and failed has been considerable [296] as exemplified by the recent surge in cases in Venezuela. For South Africa, that has historically managed malaria transmission to low levels in contrast to its neighbours, the final hurdle of getting to zero local malaria cases has been challenging, coming very close but not attaining elimination. The 2018 World Malaria Report indicated 22 100 confirmed indigenous malaria cases in South Africa, the highest number since 2011 [1]. This calls for reflection and perhaps new strategies to stem the tide of increasing malaria cases: optimism in the South African malaria community of achieving malaria elimination by 2018 (deemed realistic in the context of very few malaria cases in 2015) has been replaced by the realisation that a change in strategy may be necessary. In this thesis, we aimed to understand ongoing malaria transmission in South Africa with a specific focus on the contribution of the parasite component to continued transmission in the Vhembe District of South Africa. The aim was to provide evidence that may inform a better deployment of parasite-centred tools to support malaria elimination efforts. In the concluding discussion section, the lessons from the research that is reported on in this thesis are summarized and placed into the context of the South African elimination agenda.

In this thesis, we provide evidence for the safe deployment of PQ in South Africa, based on the local low prevalence of G6PD deficiency that should limit PQ recipients risk of (severe) haemolysis [77]. Ultimately, PQ would need to be administered as widely as logically and ethically feasible. Considering that interventions with PQ already exclude pregnant women and children below 5 years of age, not covering school going children in interventions may further reduce community-coverage to a level where a community-wide impact of PQ becomes unlikely. As such, the implementation of PQ for elimination in South Africa requires tailored approaches to near complete coverage of the entire eligible population. Coverage levels at or above 80% are deemed essential for mass drug administration campaigns [207, 297]; if PQ would be deployed in mass screening and treatment approaches a similarly high coverage will be essential for impact. It is thus highly important to consider hard-to-reach populations particularly farm workers who are at the margins of health systems coverage. While the single

low dose PQ as an adjunct to ACTs is recommended by the WHO without prior testing in populations and our findings support this by highlighting the low prevalence of G6PD deficiency in South African communities, there are other complications with PQ use. Since important research in *P. vivax* infections on PQ efficacy [172] was reported, concerns over the necessity of PQ bioactivation by certain CYP enzymes (CYP2D6) exist. We showed a very low prevalence of poor and intermediate metabolizers in our study population. Whilst our genotyping approach may not have been inclusive of all CYP2D6 variants, and CYP2D6 phenotyping would have been ideal yet logistically nearly impossible [195], our findings are the first of its kind in the target population for malaria elimination and informative. The sparse CYP2D6 variants that we detected may reduce the efficacy of PQ by reducing plasma levels achieved upon PQ dosing [174], potentially to levels below the minimum gametocytocidal concentration. Efficacy may further be compromised by the weight-based dosage that may not have the desired outcomes in all individuals. Taken together, we provide an additional layer of information in which the national malaria programme can use in its review and discussions on PQ as an adjunct to its current elimination strategy.

Our study for the first time describes the presence of low-density *P. falciparum* parasites circulating in different age groups in both local populations and immigrants in South Africa across two transmission seasons. While the presence of asymptomatic infections has been shown in low transmission settings neighbouring countries of Botswana [235] and Eswatini [298], this is the first large-scale report for South Africa. With this data, the hypothesis of submicroscopic (subpatent) parasites contribution to sustained, continued malaria transmission in the pre-elimination settings in South Africa is accepted. The outstanding question is what these low-density infections mean for the country in terms of sustaining ongoing transmission. Low-density infections generally correlate with low infectivity to mosquitoes. In a study in Kenya, the proportion of children with microscopically confirmed gametocytes that were capable of infecting mosquitoes was identical to that of children with submicroscopic gametocytaemia however the latter group infected fewer mosquitoes [48]. Because of their high abundance in populations, low-density infections can be responsible for a large proportion of the (sparse) transmission events [41, 290]. In our study, we failed to detect infections by microscopy in some populations, the only confirmed infections being present at submicroscopic levels. These low-density infections, if left undetected and untreated have the potential of increasing in density to levels able to infect mosquitoes [290]. In the seasonal and low transmission settings of Sudan, asymptomatic carriage of *P. falciparum* sustained long-lasting infectious reservoir that seeded transmission for malaria outbreaks after the onset of rains [299]. It is possible that a similar situation may play out in South Africa, where transmission is limited to the summer months.

While data presented in this thesis were acquired during the summer months of ongoing malaria transmission seasons, data from the dry season were not captured. The availability of the low transmission parasite transmission data would further broaden understanding of the residual transmission and how it is maintained particularly during the winter season. The factors contributing to the persistence of asymptomatic infections are incompletely understood. This may be determined by inter-individual differences in health status, host immune status and parasite factors [27]. Important for the context of parasite-driven interventions: the detectability of asymptomatic infections is strongly influenced by the timing of the sampling period and the sensitivity of the diagnostic method. The daily dynamics of *P. falciparum* clones in Tanzanian children were found to be unstable over time and fluctuated from day to day [138]. More recently in Vietnam, parasite densities of persistent infections were reported to oscillate from ultralow-density infections and frequently developed to high-density infections [236]. If sampling is performed at a single time point, surveys may identify asymptomatic infections that later progresses to becoming symptomatic, prompt treatment seeking behaviour and results in clearance by antimalarials [237]. A large fraction of the low-density infections may also be cleared without antimalarial drugs [241, 290]. The parasite density threshold that is needed to result in fever, is important in determining whether the host develops symptoms or maintains long-term asymptomatic carriage [300]. This threshold differs between immune and non-immune individuals, age, transmission intensity and, in some settings, ethnicity [237]. To move beyond the studies presented in Chapter 3 and truly understand both the dynamics of infections and the importance of low-density infections for transmission, longitudinal studies are warranted. Such studies should aim to unravel how low parasite densities are controlled and maintained so efficiently in the population and assess gametocyte production or, ideally, mosquito feeding assays to examine their transmission potential at different time points over the course of infections [27].

Individuals harbouring low-density asymptomatic parasitaemia are unlikely to seek treatment [237] further complicating efforts to treat them. While it is evident that sensitive diagnostics and surveillance tools are required, it is unclear where and how programmes deploy these sensitive techniques. Regular RDTs in screening and treatment campaigns are unlikely to be sufficiently sensitive to identify and treat the last cases to achieve elimination [95, 301]. For example in South Africa, the malaria surveillance and outbreak investigations teams have had limited success with the identification of additional cases in surrounding geographical localities of the index case with the existing tools [302]. It is sometimes argued that sensitive diagnostics are not essential; Sri Lanka and Paraguay recently eliminated malaria using the existing tools without focussing on detection of subpatent and asymptomatic infections. However, it is important to acknowledge that these countries had different challenges to the ones faced by

South Africa, including less efficient mosquito vectors. The use of sensitive molecular tools, as presented in Chapter 3, provides a new dimension to the detection of subpatent infections that are potentially missed by the regular detection tools. In some circumstances where infections are widespread and the use of molecular diagnostics is complicated, elimination programmes may opt for mass drug administration (MDA) without prior screening, like recently reported in Zambia [88]. In other settings or scenarios where infections are too sparse to justify community treatment, molecular diagnostics provide an option that is more acceptable to public health practitioners as it reduced the number of treatments administered to non-infected populations. MDA may also be targeted following the detection of (subpatent) infections in communities [303] or be followed by mass screening and treatment with sensitive diagnostics.

With increased drugs and RDTs targeting *P. falciparum* in Africa, the epidemiology of the malaria species distribution is changing [304]. Increases in the proportion of malaria infections caused by other malaria species like *P. vivax, P. ovale and P. malariae* have been reported [305]. While this thesis only records one single *P. ovale* infection, it is important to note that current diagnostics deployed across large sections of Africa only target *P. falciparum* and it is possible that the cases of the other malaria parasites may be rising or are systematically underestimated [306]. Historical records show a high prevalence of *P. vivax* and *P. ovale* in South Africa in the early 1900s, however with the introduction of IRS in the 1950s the cases have been rare [147]. The increasing reports of *P. vivax* in Africans lacking Duffy antigen receptor expression suggests a previously unrecognized burden of this parasite species in sub-Saharan Africa, including southern Africa [307]. For the vast majority of African countries where health systems are not optimal and quality assured microscopy is not guaranteed except in reference laboratories, it could also mean that the other malaria parasites are misidentified or misclassified [308]**.** As the region pushes towards elimination with focus on *P. falciparum*, the sparse evidence of these other malaria species potentially contributing to transmission may justify the use of more inclusive detection tools to aid in the detection of non-*falciparum* malaria and comprehensive malaria surveillance.

In the absence of firm evidence for the presence of mutations associated with artemisinin resistance in Africa [309-311] we focused on the non-artemisinin partner drugs in ACTs. It is reassuring that no evidence of any resistance to the most important ACTs partner drug in our set-up was observed. However, we need to acknowledge that our choice of partner drug resistance as measured by *mdr*1-86 genotyping is not comprehensive. It should be taken into consideration that not all antimalarials have established resistance markers and new markers are being identified. A best-case scenario would be to test the available markers as soon as they become validated and perform regular monitoring at several sentinel sites to identify any rise in resistance levels very early on, before clinical treatment failures are observed at

population-wide scale. In pre-elimination settings including Vhembe where it is logistically challenging to identify and follow up participants for *in vivo* drug efficacy trials, we showed that the use of genetic markers is a more feasible approach for studying parasite resistance. Such genetic approaches can include the use of more robust techniques including a SNP-based array containing several markers, an approach that can provide greater information compared to the few markers that were included in our study [312].

The thesis highlighted differences between local *P. falciparum* parasite clones in South Africa and imported parasite clones. Reports from seasonal transmission settings in Sudan showed that multiple genotypes persisted for longer periods of time compared to single clones [299]. Similarly, the proportion of mixed-genotype clones and the number of genotypes per individual was higher among asymptomatic than symptomatic infections [313]. As transmission declines, it is expected that also in asymptomatic infections fewer clones are circulating. Our detection of low clonal complexity in local populations, suggests that interventions in South Africa have reduced the force of infection successfully. This is supported by previous studies demonstrating that a high number of clones predicted an increased risk of disease [314] while fewer clones predicted a reduced number of cases [315]. Arguably, the detection of clones in this study was undertaken with gel electrophoresis that is not sensitive to detect minority clones as well as quantify clone density [316]. Perhaps more sensitive techniques, like capillary electrophoresis, single amplicon sequencing with many SNPs and genome-wide SNP panels would have identified subtle differences between related clones [316]. Another potential approach to increase the resolution of the available parasite genetic information is the selective whole genome amplification (sWGA), that uses computationally selected short oligonucleotide probes as primers to preferentially bind to target genomes [317]. These alternative approaches including amplicon sequencing are more efficient for genotyping multiallelic short-range haplotypes and microsatellites with more consistent coverage of regions of interests and at lower costs than whole-genome sequencing [141]. While the study was not designed to study clone dynamics, this is an important facet of understanding the continued residual transmission. Since the genotyping approaches are particularly suitable and informative in moderate-low endemic settings (being able to support the distinction between local and imported malaria), future investigations with such tools in South Africa are warranted.

Whilst we identified populations with asymptomatic infections and argue that they may be a source of onward transmission to mosquitoes, we have as yet no direct evidence for this. The relationships between parasite density, gametocyte density, gametocyte sex ratio and the probability of mosquito infection is multifaceted [113]. Gametocyte sex ratio is a relevant factor influencing transmission potential [113, 318]. However, it has been very difficult to determine

the sex ratio in natural infections due to low gametocyte densities and the absence of specific and sensitive markers to identify and quantify male and female gametocytes. Even though several markers including *pfg17*, *pfs25*, *pfGK*, *pf230p* and *pf13* have been used [319, 320] they all have limitations. One very practical limitation is the resource intensiveness and the reliance on DNAse treatment prior to RNA quantification. However, our development and validation of male and female gametocytes markers, detectable from low blood volumes in mosquito blood meals by multiplex qPCR, provide the conclusive new markers that are able to address the knowledge gap. While the signal for male and female gametocyte markers was below the detection limit in the lower parasite concentrations as obtained from the field samples, pooling strategies may help in improving the signal. The utility of these novel markers may be useful in linking mosquito infectivity and the number of male and female gametocytes ingested in the blood meal. This is relevant since mosquitoes sample different blood compartments compared to those typically examined in malaria surveys. The subdermal capillaries are currently *terra incognita* in terms of parasite densities and possibly parasite sequestration. In membrane feeding assays, very obvious and intriguing differences in successful mosquito infections in skin feeding assays compared to direct membrane feeding assays have been observed [284]. While this could be attributable to several factors, the quantification of male and female gametocytes in the mosquito midgut post-feeding may help in understanding whether there is clustering of gametocytes in the subdermal capillaries [321, 322]. In addition, the approach may produce better estimates of gametocyte densities in the blood compartments relevant for transmission and may thus better predict infectivity. Regardless of the blood sample used, the quantification of male and female gametocytes in a multiplex assay would greatly reduce the workload and uncertainties in quantifying total gametocyte density and sex ratio, as compared to two independent assays. Taken together, our results open up possibilities of incorporating entomological surveillance data to perform xenosurveillance and inform transmission metrics to support malaria elimination efforts.

For maximum utility, our approach to detect parasites in mosquito blood meals would ideally be sufficiently sensitive to detect sparse (low-density) infections in pools of mosquitoes. If this is realistic, our xenosurveillance approach may classify as a non-invasive diagnostic that does not require a blood sample [323] yet can identify parasite carriage at the population level. Several other non-invasive sampling approaches have been piloted including the use of saliva and urine as well as skin volatiles [21] and malaria diagnosis by trained dogs ([324]. Our results in chapter 4 describe modest experiments to examine the utility of xenosurveillance for malaria parasites. While our results are novel, there are limitations such as the small number of observations on mosquitoes. Even with these limitations, it is obvious that our approach has challenges in terms of sensitivity. To be useful to replace community surveys to detect

malaria parasites (such as in Chapter 3), the xenosurveillance approach in chapter 4 should allow a large number of mosquitoes to be easily surveyed in pools to provide a snapshot of the prevailing parasite numbers. In that context, our findings were sobering. Infections with parasite densities observed in chapter 3 are unlikely to be detected in mosquito blood meals, either in single mosquitoes or pools of mosquitoes. Even higher density infections rapidly become undetectable if pooled with uninfected blood-fed mosquitoes. In this sense, our methodology requires optimization. Some reason for optimism in this respect comes from a recent study that used a similar pooling strategy to identify *P. vivax* infected blood meals in anthropophilic *Culex* mosquitoes. In that study, infections detected by xenosurveillance were spatially correlated with *P. vivax* infections in humans [295]. The novel xenosurveillance described here can similarly be adaptable to the existing malaria information system (MIS) platform for automated collections of timely surveillance data [325, 326].

The work in this thesis, therefore supports the South Africa elimination initiative in several ways. We uncovered a potentially important asymptomatic reservoir of infections in local and migrant populations. The molecular signatures of drug sensitivity suggest that current antimalarials can be used to clear asexual parasites in these infections, potentially supported by PQ that is likely to be safe and efficacious based on the WHO recommended dose and the genetic characteristics of the target population. During elimination strategies, alternative strategies to identify (pockets of) parasite carriers may become increasingly important. The ability to detect parasites and transmissible parasites in mosquito blood meals may form a starting point for non-invasive xenodiagnostic strategies. Improved knowledge of residual malaria transmission, as presented in this thesis, is urgently needed in South Africa where, despite implementation of the current existing strategies, elimination efforts have not yet brought success.

Malaria elimination is a laudable objective with recent success stories from Sri Lanka and Paraguay providing hope and optimism for South Africa. The observation that once malaria elimination is successfully achieved, it remains stable with very little resurgence is a call to action for a robust effective elimination strategy [56]. The projected benefits of achieving elimination in South Africa and associated positive impact on many sectors negatively affected by the existing residual transmission calls for rational approaches to target residual transmission.

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Appendices

5.1 Appendix 1. Approval Certificates of the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria

LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH

Enquiries: Latif Shamila Ref:4/2/2

Shengu Shagari Awandu
University of Pretoria
Private Bag X323
Arcadia
0007

Greetings,

RE: Molecular epidemiology of malaria transmission in Vhembe District, Limpopo Province, South Africa.

The above matter refers.

1. Permission to conduct the above mentioned study is hereby granted.
2. Kindly be informed that:-
   - Research must be loaded on the NHRD site (http://nhrd.hst.org.za) by the researcher.
   - Further arrangement should be made with the targeted institutions.
   - In the course of your study there should be no action that disrupts the services.
   - After completion of the study, a copy should be submitted to the Department to serve as a resource.
   - The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
   - The above approval is valid for a 3 year period.
   - If the proposal has been amended, a new approval should be sought from the Department of Health.

Your cooperation will be highly appreciated.

Head of Department 30/04/2015 Date

18 College Street, Polokwane, 0700, Private Bag x9302, POLOLKWANE, 0700
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Figure 1. Approval Certificate for monitoring malaria transmission in field isolates in South-Africa. This letter from the Faculty of Health Sciences Research Ethics Committee (University of Pretoria) gives a 5 year approval for monitoring malaria transmission in field isolates in South-Africa.

5.2 Appendix 2 University of Pretoria Ethics approval document

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

30/10/2014

Approval Certificate
New Application

Ethics Reference No: 406/2014

Title: Molecular epidemiology of malaria transmission in Vhembe District, Limpopo Province, South Africa.

Dear Mr Shehu Shagari Awandu

The **New Application** as supported by documents specified in your cover letter for your research received on the 26/09/2014, was Provisional approved by the Faculty of Health Sciences Research Ethics Committee on the 29/10/2014.

Please note the following about your ethics approval:
- Ethics Approval is valid for 4 years.
- Please remember to use your protocol number (**406/2014**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Additional Conditions:
- Provisionally approved, pending permissions of Musina and Mutale Hospitals.

We wish you the best with your research.

Yours sincerely

*** Kindly collect your original signed approval certificate from our offices, Faculty of Health Sciences, Research Ethics Committee, H W Snyman South Building, Room 2.33 / 2.34.*

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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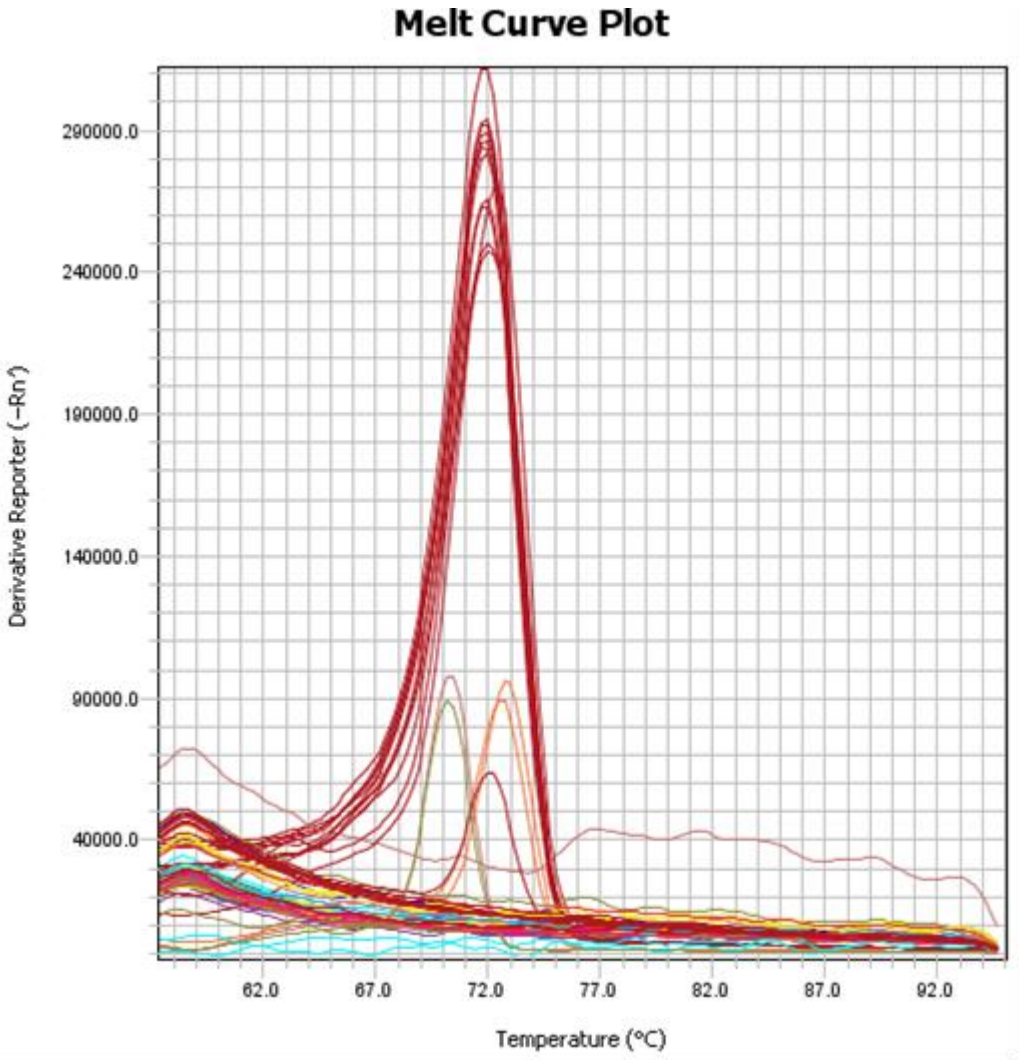
5.3 Appendix 3. Melt curve plot for tare-2 qPCR experiments.



5.4 Appendix 4 Primers and sequences for drug resistance genotyping markers

Table 7.2. Sequences of primers and PCR conditions used for the detection of the *crt* K76T, *mdr*1 N86S, *dhfr* N51I, C59R, S108N/T, I164L SNPs and *dhps* A437G and K540E.

Primer		Sequence (5'-3')	Product size (base pairs)	PCR condition
1st Amplification PCR				
mdr86	mdr86 F1: mdr86 R1:	GCGCGCGTTGAACAAAAAGAGTACCGCTG GGGCCCTCGTACCAATTCCTGAACTCA	450	95 °C, 5' followed by 44 cycles (92 °C, 30"; 56 °C, 30"; 65 °C for 45"); 72 °C, 15' and 4 °C for 5' or ∞
crt76	crt76 F1: crt76 R1:	GCGCGCGCATGGCTCACGTTTAGGTGGAG GGGCCCGGCGGATGTTACAAAACTATAGT TACC	206	95 °C, 5' followed by 44 cycles (92 °C, 30"; 56 °C, 30"; 65 °C for 45"); 65 °C, 10' and 4 °C for 5' or ∞
		2nd Amplification PCR		
mdr86	mdr86 F2: mdr86 R2:	TTTACCGTTTAAATGTTTACCTGC CCATCTTGATAAAAAACACTTCTT	291	95 °C, 5' followed by 25 cycles (92 °C, 30"; 51 °C, 30"; 65 °C for 45"); 72 °C, 15' and 4 °C for 5' or ∞
crt76	crt76 F2: crt76 R2:	TGTGCTCATGTGTTTAAACTT CAAAACTATAGTTACCAATTTTG	145	95 °C, 5' followed by 30 cycles (94 °C, 30"; 51 °C, 45"; 65 °C for 45"); 65 °C, 10' and 4 °C for 5' or ∞
		1st Amplification PCR		
dhfr51 and *dhfr59*	dhfr51/59 F1: dhfr51/59 R1:	GCGCGCTAATAACTACACATTTA CCCGGGCTCTTATATTTCAATTT	147	95 °C, 5' followed by 45 cycles (92 °C, 30"; 45 °C, 30"; 65 °C for 45"); 72 °C, 10' and 4 °C for 5' or ∞
dhfr108 and *dhfr164*	dhfr108/164 F1: dhfr108/164 R1:	GGGGGGCAGTTACAACATATGTGA GGGGGCACATTCATATGTACTATTT	414	95 °C, 5' followed by 45 cycles (92 °C, 30"; 45 °C, 30"; 65 °C for 45"); 72 °C, 10' and 4 °C for 5' or ∞
dhps540 and *fdhps581*	dhps540/581 F1: dhps540/581 R1:	GGGCCCAAACAAATTCTATAGTG GGCCGGTGGATACTCATCATATA	256	95 °C, 5' followed by 45 cycles (92 °C, 30"; 58 °C, 30"; 65 °C for 30"); 72 °C, 10' and 4 °C for 5' or ∞
dhps436 and *dhps437*	dhps436/437 F1: dhps436/437 R1:	GGGGTATTAAATGTTAATTATGATTCT GGGGTCACATTTAACAATTTTATT	249	95 °C, 5' followed by 45 cycles (92 °C, 30"; 48 °C, 30"; 65 °C for 30"); 72 °C, 10' and 4 °C for 5' or ∞
2nd Amplification PCR				

dhfr51 and *dhfr59*	dhfr51/59 F2:	CTAGGAAATAAAGGAGTATTACCATGGAAA TGGA	113	95 °C, 5’ followed by 25 cycles (92 °C, 30”; 50 °C, 30”; 65 °C for 30”); 70 °C, 10’ and 4 °C for 5’ or ∞
	dhfr51/59 R2:	ATTTTTCATATTTTGATTCATTCACATATGTT GTAACTGTAC		
dhfr108 and *dhfr164*	dhfr108/164 F2:	CTAATTCTAAAAAATTACAAAATGT	256	95 °C, 5’ followed by 25 cycles (94 °C, 30”; 50 °C, 45”; 65 °C for 30”); 70 °C, 10’ and 4 °C for 5’ or ∞
	dhfr108/164 R2:	TTTCTTTTCTAAAAATTCTTGATAAACAACG GAACCTCTTA		
dhps540 and *dhps581*	dhps540/581 F2:	GCGCGCGTTCTAATGCATAAAAGAGG	201	95 °C, 5’ followed by 25 cycles (92 °C, 30”; 60 °C, 30”; 65 °C for 30”); 72 °C, 10’ and 4 °C for 5’ or ∞
	dhps540/581 R2:	CCCGGGTAAGAGTTTAATAGATTGATCAGC TTTCTTC		
dhps436 and *dhps437*	dhps436/437 F2:	TGTTCAAAGAATGTTTGAAATGA	148	95 °C, 5’ followed by 25 cycles (92 °C, 30”; 60 °C, 30”; 65 °C for 30”); 72 °C, 10’ and 4 °C for 5’ or ∞
	dhps436/437R2:	CCATTCTTTTTGAAATAATTGTAAT		

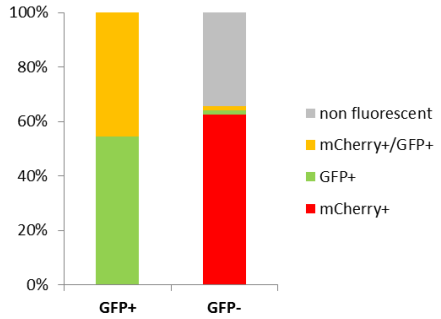
5.5 Appendix 5 Restriction fragment length polymorphisms profiles

Table 7.3. Digestion protocol of amplified products used for the detection of the *crt* K76T, *mdr1* N86S, *dhfr* N51I, C59R, S108N/T, I164L SNPs and *dhps* A437G and K540E

Codons	Buffer (10x)	Restriction enzyme	Product size (bp)	Incubation temperature
Pfmdr1 86	NEbuffer 3.1	*AflIII* cleaved with **mutant**	126 and 165	37 °C
Pfcrt76	NEbuffer 3.1	*ApoI* cleaved with **wild**	46 and 99	50 °C
Pfdhfr51	Buffer EcoRI	*EcoRI* cleaved with **wild**	35 and 78	37 °C
Pfdhfr59	Buffer Tango	*BsrGI* cleaved with **wild**	43 and 65	37 °C
Pfdhfr108	NEbuffer 3.1	*AluI* cleaved with **wild**	46 and 210	37 °C
Pfdhfr164	NEbuffer 3.1	*PsiI* cleaved with **wild**	42 and 214	37 °C
Pfdhps540	NEbuffer 3.1	*FokI* cleaved with **mutant**	56 and 145	37 °C
Pfdhps437	NEbuffer 3.1	*AvaI* cleaved with **mutant**	69 and 79	37 °C

5.6 Appendix 6: Counts of live fluorescent gametocytes in the GFP+ and GFP- populations and summary of IFA results

A. Counts of live fluorescent gametocytes in GFP+ and GFP- populations



B. Double IFA with α-Pfg377 and α-Pfg27 antibodies on acetone fixed gametocyte smears of the GFP+ and GFP- populations.

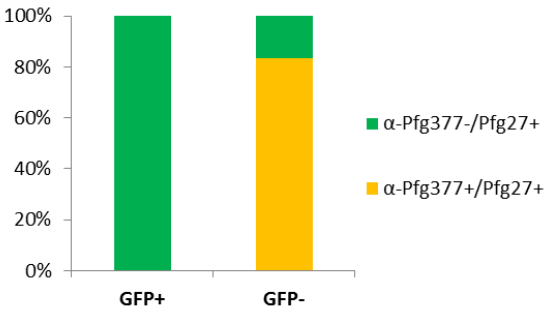


Figure adapted from Stone *et al*., 2016 with permission from the publishers.