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IMMUNITY AND IMMUNOLOGICAL SURVEILLANCE FOR MALARIA ELIMINATION IN TROPICAL ISLANDS

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Cover illustration: Geometric map of fantastical Lake Victoria in Kenya with its distinct islands by Mohd. Hanif Akmal Basir, all rights reserved ©

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Immunity and immunological surveillance for malaria elimination in tropical islands

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mother, Hjh. Siti Sani Sairan,

In memory of my father, Hj. Md Idris Md Yusoff

ABSTRACT

Malaria remains one of the most significant global public health challenges. Nearly half of the world's population remains at risk, largely in African Region. In the past decade, considerable progress has been made in the global fight to control and eliminate malaria. In some endemic countries, aggressive malaria control has reduced the malaria burden to a point where malaria elimination is becoming feasible. Nevertheless, sustained malaria control is crucial to prolong this downward trend for endemic countries. Understanding the contribution of local transmission, parasites movement, asymptomatic and sub-microscopic reservoirs can shape how active surveillances are used to pursue malaria elimination. Furthermore, a better understanding of the epidemiological effects of naturally acquired immunity against malaria is warranted to guide efforts to control or potentially eliminate the disease.

In five cross-sectional surveys in Kenya conducted between 2012 and 2014 (N = 10,430), malaria prevalence (i.e. microscopy and PCR) and clinical assessments were evaluated to investigate the distribution and extent of malaria infections on islands (Mfangano, Takawiri, Kibuogi, and Ngodhe) and a mainland area (Ungoye) in Lake Victoria. Malaria prevalence varied significantly among setting; highest in the mainland, moderate in the large island, and lowest in small islands. More than 90% of infected populations were asymptomatic, and 50% of them were sub-microscopic with age-dependency for both proportions. These observations provide support for the inclusion of MDA in the area. Using the two surveys in 2012 (N = 4,112), antibody responses to *P. falciparum* *Pf*AMA-1, *Pf*MSP-1_{19} and *Pf*CSP were measured in order to describe transmission patterns and heterogeneity in Lake Victoria. The overall seroprevalence in Lake Victoria was 64% for *Pf*AMA-1, 40% for *Pf*MSP-1_{19} and 13% for *Pf*CSP. A clear relation between serological outcomes of *Pf*AMA-1 and *Pf*MSP-1_{19} was observed with parasite prevalence and serology-derived EIR in heterogeneity in transmission. These observations collectively suggest that malaria serological measure could be an effective adjunct tool for assessing differences in transmission as well as for monitoring control and elimination in the high endemic area.

Using *msp1* and *csp* data from samples collected from 1996 to 2002, patterns of gene flow and population genetic structure of *P. falciparum* (N = 316) and *P. vivax* (N = 314) from seven sites on five islands (Gaua, Santo, Pentecost, Malakula, and Tanna) were analysed in order to understand the transmission and movement of *Plasmodium* parasites in Vanuatu. In general, genetic diversity was higher in *P. vivax* than *P. falciparum* from the same site. In *P. vivax*, high genetic diversity was likely maintained by a greater extent of gene flow among sites and islands. The results suggest that the current malaria control strategy in Vanuatu might need to be bolstered in order to control *P. vivax* movements across islands. To understand the impact of vector control interventions (i.e. ITNs) in Vanuatu, samples collected in 2003 (N = 231) and 2007 (N = 282) on Ambae Island were assessed for parasite infection (i.e. microscopy) and measured for antibody responses against three *P. falciparum*, three *P. vivax* and *Anopheles*-specific salivary gSG6 antigens. Decreases in seroprevalence were observed to all *P. falciparum* antigens but two of three *P. vivax* antigens, consistent with the pronounced decrease in parasite prevalence from 19% in 2003 to 3% in 2007. Seroprevalence to gSG6 also reduced significantly, suggesting that reduced exposure to vector bites was important to decrease in parasite prevalence. Together, decrease in both parasitological and seroepidemiological malaria metrics from 2003, and 2007 implied that reinforced vector control played a major role in the reduction of malaria transmission on Ambae Island.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Malaria är fortfarande en av de mest betydande globala utmaningarna för folkhälsan. Nästan hälften av världens befolkning lever fortfarande i malariaendemiska områden, till stor del i Subsahariska Afrika. Under det senaste årtiondet, har betydande framsteg gjorts i den globala kampen för att kontrollera och eliminera malaria. I vissa endemiska länder, har aggressiv malariakontroll minskat bördan till en punkt där malariaeliminering blir genomförbart. Trots detta, är upprätthållet av malariakontroll avgörande för att fortsätta i den nedåtgående trend för endemiska länder som har präglat 2000-talet. Genom att öka förståelsen för den lokala transmissionen, överföring av parasitpopulationer mellan öar samt asymtomatiska och submikroskopiska reservoarer kan man bidra till att forma hur malariaövervakningen ska utformas för att uppnå eliminering av malaria. Dessutom är en bättre förståelse av de epidemiologiska effekterna av naturligt förvärvad immunitet mot malaria befogad för att vägleda åtgärder för att kontrollera eller potentiellt eliminera sjukdomen.

I fem tvärsnittsstudier i Kenya, genomförda mellan 2012 och 2014 (*N* = 10,430), utvärderades malariaprevalensen (d.v.s. mikroskopi och PCR) och kliniska bedömningar för att undersöka fördelningen och omfattningen av malariainfektioner på öarna (Mfangano, Takawiri, Kibuogi och Ngodhe) och ett fastlandsområde (Ungoye) i Victoriasjön. Malariaprevalensen varierade avsevärt mellan de olika förhållandena; högst på fastlandet, måttlig på den största ön och lägst på de mindre öarna. Mer än 90 % av den infekterade populationen var asymtomatisk och 50% av dem var submikroskopiska med åldersberoendet i båda grupperna. Dessa observationer ger stöd för införandet av MDA i området. Med hjälp av insamlat provmaterial från de två undersökningarna år 2012 (*N* = 4,112), mättes antikroppssvaret mot *P. falciparum Pf*AMA-1, *P*fMSP-119 och *Pf*CSP för att beskriva spridningsmönster och heterogenitet i Victoriasjön. Den övergripande seroprevalensen i Victoriasjön var 64% för *Pf*AMA-1, 40% för *Pf*MSP-119 och 13% för PfCSP. En tydlig koppling mellan serologiska resultat från *Pf*AMA-1 och *Pf*MSP-1_{19} observerades med parasitprevalensen och serologiskt erhållna EIR för transmissionsheterogeniteten. Dessa observationer föreslår att malariasserologiska åtgärder kan vara ett effektivt verktyg för att bedöma skillnader i transmission såväl som för övervakningskontroll och eliminering i detta högendemiska område.

Med hjälp av msp1 och csp data från prover som samlats in från 1996 till 2002, analyserades mönster av genflöde och den populationsgenetiska strukturen hos *P. falciparum* (*N* = 316) och *P. vivax* (*N* = 314) från sju platser på fem öar (Gaua, Santo, Pentecost, Malakula och Tanna) för att förstå överföring och rörelse av Plasmodiumparasiter på Vanuatu. Generellt var den genetiska mångfalden högre i P. vivax än *P. falciparum* från samma plats. I *P. vivax* bibehölls troligen hög genetisk mångfald av en större grad genom genflöde mellan platser och öar. Resultaten tyder på att den nuvarande malariakontrollstrategin på Vanuatu kan behöva kompletteras för att kontrollera rörelser av *P. vivax*-populationer över öarna. För att förstå effekterna av vektorkontrollinterventioner (d.v.s. ITNs) på Vanuatu, utvärderades prover som samlats från 2003 (*N* = 231) och 2007 (*N* = 282) på Ambaeön för parasitinfektion (d.v.s. mikroskopi) och antikroppssvar mot tre *P. falciparum*-antigen, tre *P. vivax*-antigen och *Anopheles*-specifika salivära antigenen gSG6. Minskningen i seroprevalens observerades för alla *P. falciparum* antigener men enbart två av tre för *P. vivax* antigen, vilket stämmer överens med den uttalade minskning av parasitprevalens från 19% 2003 till 3% 2007. Seroprevalensen för gSG6 minskade också betydligt, vilket indikerar att minskad exponering för vektorbett har spelat en viktig roll i minskningen av parasitprevalensen.

ABSTRAK

Malaria merupakan salah satu penyakit berjangkit utama dunia. Hampir separuh populasi dunia berdepan dengan risiko jangkitan malaria terutamanya di benua Afrika. Sejak sedekad lalu, kemajuan besar telah dicapai oleh komuniti global dalam kawalan dan eliminisasi malaria. Kawalan yang berkesan oleh beberapa negara endemik telah berjaya mengurangkan penyakit malaria dan membolehkan program eliminisasi dilaksanakan. Walaubagaimanapun, kawalan yang mampan perlu untuk memanjangkan trend pengurangan ini. Pemahaman berkaitan dengan transmisi lokal penyakit, mobiliti parasit serta jenis penyakit yang bersifat asimptomatik dan submikroskopik mampu mendorong pengawasan yang lebih berkesan dalam mencapai status eliminisasi. Selain itu, pemahaman berkaitan kesan epidemiologi terhadap immuniti semulajadi terhadap malaria adalah penting dalam usaha kawalan mahupun eliminisasi penyakit malaria.

Dalam kaji selidik di Kenya pada tahun 2012 sehingga 2014 (10,430 orang), prevalen penyakit malaria dan penilaian klinikal telah dilaksanakan di lima pulau (Mfangano, Takawiri, kibuogi dan Ngodhe) dan sebuah penempatan di tanah besar (Ungoye) di kawasan Tasik Victoria. Prevalen malaria didapati berbeza iaitu berkeadaan tinggi di tanah besar, sederhana di pulau besar (Mfangano) dan rendah di pulau-pulau kecil. Lebih 90% pesakit malaria bersifat asimptomatik (tiada simptom) dan 50% dikalangan mereka dalam keadaan submikroskopik. Dapatan ini mengesahkan lagi bahawa MDA perlu dijalankan di kawasan Tasik Victoria. Dengan menggunakan dua kaji selidik pada tahun 2012 (4,112 orang), kesan antibodi terhadap antigen *P. falciparum* iaitu *Pf*AMA-1, *Pf*MSP-1_{19} dan *Pf*CSP telah dinilai untuk melihat kelainan bentuk transmisi malaria di kawasan Tasik Victoria. Pada keseluruhannya, seroprevalen di kawasan Tasik Victoria ialah 64% untuk *Pf*AMA-1, 40% untuk *Pf*MSP-1_{19} dan 13% untuk *Pf*CSP. Hubungan diantara hasil penilaian serologi ke atas *Pf*AMA-1 dan *Pf*MSP-1_{19} dapat dilihat dengan ketara dengan prevalen penyakit dan juga EIR. Dengan mengambil kira semua dapatan ini, penggunaan kajian serologi didapati mampu membolehkan perbezaan yang ketara transmisi penyakit dinilai terutamanya di kawasan-kawasan dengan endemik malaria yang tinggi.

Dengan menggunakan data *msp1* dan *csp* dari sampel yang dikumpulkan pada tahun 1996 sehingga 2002, bentuk aliran gen dan struktur genetik populasi *P. falciparum* (316 sampel) dan *P. vivax* (314 sampel) dinilai melibatkan tujuh kawasan dalam lima pulau (Gaua, Santo, Pentecost, Malakula dan Tanna) untuk memahami bentuk transmisi dan mobiliti parasit *Plasmodium* di Vanuatu. Pada keseluruhannya, kepelbagaian genetik dalam kawasan yang sama adalah lebih tinggi dalam *P. vivax* berbanding *P. falciparum*. Kepelbagaian genetik yang tinggi di dalam *P. vivax* mungkin disebabkan oleh darjah aliran gen yang besar di dalam pulau-pulau itu sendiri. Oleh itu, strategi kawalan malaria di Vanuatu perlu dipertingkatkan terutamanya di dalam kawalan penyebaran *P. vivax* diantara pulau-pulau terlibat. Untuk memahami impak kawalan vektor (ITN) di Vanuatu, sampel yang dikumpulan di Pulau Ambae pada tahun 2003 (231 orang) dan 2007 (282 orang) di nilai untuk prevalen infeksi dan kesan antibodi terhadap tiga antigen bagi *P. falciparum* dan *P. vivax* beserta satu antigen untuk nyamuk *Anopheles* iaitu gSG6. Penurunan sekata prevalen infeksi dari 19% pada 2003 kepada 3% pada 2007 dapat juga dilihat pada semua antigen *P. falciparum* dan hanya dua dari tiga *antigen P. vivax*. Seroprevalen untuk gSG6 juga menurun dan ini menggambarkan bahawa pengurangan dedahan terhadap gigitan vektor adalah penting untuk pengurangan prevalen penyakit itu sendiri. Pada kesuluruhannya, penurunan aras parasit dan seroepidemiologi dari tahun 2003 sehingga 2007 memperlihatkan bahawa peningkatan kawalan vektor memainkan peranan penting dalam penurunan transmisi malaria di Pulau Ambae.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers:

I. Chan CW, Sakihama N, Tachibana S, **Md Idris Z**, Lum JK, Tanabe K, Kaneko A. *Plasmodium vivax* and *Plasmodium falciparum* at the crossroads of exchange among islands in Vanuatu: implication for malaria elimination strategies.
PLoS One. 2015; 10(3):e0119475

II. **Md Idris Z**, Chan CW, Kongere J, Gitaka J, Logedi J, Omar A, Obonyo C, Machini BK, Isozumi R, Teramoto I, Kimura M, Kaneko A. High and heterogeneous prevalence of asymptomatic and sub-microscopic malaria infection on islands in Lake Victoria, Kenya.
Scientific Reports. 2016; 6:36958

III. **Md Idris Z**, Chim CW, Kongere J, Hall T, Drakeley C, Kaneko A. Naturally acquired antibody response to *Plasmodium falciparum* describes heterogeneity on transmission on islands in Lake Victoria.
Manuscript

IV. **Md Idris Z**, Chan CW, Mohammed M, Kalkoa M, Taleo G, Junker K, Arcà B, Drakeley C, Kaneko A. Serological measures to assess the efficacy of malaria control programme on Ambae Island, Vanuatu.
Parasites & Vectors. 2017; 10:204

Publication obtained during the course of the PhD studies but not included in this thesis:

Gitaka JN, Takeda M, Kimura M, **Md Idris Z**, Chan CW, Kongere J, Yahata K, Muregi FW, Ichinose Y, Kaneko A, Kaneko O. Selections, frameshift mutations, and copy number variation detected on the *surf 4.1* gene in the western Kenyan *Plasmodium falciparum* population.
Malaria Journal. 2017; 16(1):98

CONTENTS

LIST OF ABBREVIATIONS

ACKR1	Atypical chemokine receptor 1
ACT	Artemisinin-based combination therapy
AES	Average enlarged spleen
AMA-1	Apical membrane antigen 1
AL	Artemether-lumefantrine
API	Annual parasite incidence
AQ/PG	Amodiaquine plus proguanil
AS-AQ	Artesunate-amodiaquine
AS-MQ	Artesunate-mefloquine
AS-SP	Artesunate-sulfadoxine plus pyrimethamine
BMU	Beach management unit
CI	Confidence intervals
COX3	Cytochrome c oxidase III
CSP	Circumsporozoite
DARC	Duffy antigen chemokine receptor
DHA-PPQ	Dihydroartemisinin-piperaquine
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
G6PD	Glucose-6-phosphate dehydrogenase
gSG6	*Anopheles gambiae* salivary gland protein 6
HRP-2	Histidine-rich protein 2
IFA	Immunofluorescent antibody test
Ig	Immunoglobulin
IPTi	Intermittent preventive treatment in infants
IPTp	Intermittent preventive treatment in pregnancy
IQR	Interquartile range
IRS	Indoor residual spraying
ITN	Insecticide-treated net

LAMP	Loop-mediated isothermal amplification
LDH	Lactate dehydrogenase
LLIN	Long lasting insecticide treated net
MDA	Mass drug administration
MSP-1	Merozoite surface protein 1
NANP	Asn-Ala-Asn-Pro
NVDP	Asn-Val-Asp-Pro
OD	Optical density
PCR	Polymerase chain reaction
PR	Parasite rate
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RDT	Rapid diagnostic test
S	Sickle haemoglobin
SCR	Seroconversion rate
SP	Sulfadoxine-pyrimethamine
SRR	Seroreversion rate
WBC	White blood cell
WHO	World Health Organization

1 INTRODUCTION

MALARIA

1.1 The disease burden

Malaria is a protozoan disease transmitted by *Anopheles* mosquito. It remains one of the most prevalent infectious diseases in the world with an estimated 3.2 billion people at risk of being infected. In 2015, approximately 214 million cases (range: 149 – 303 million) of malaria occurred worldwide with 438,000 malaria deaths (range: 236,000 – 635,000), most of which were children aged less than five years. The African region remains the highest disease burden and accounts for 88 and 90% of the global clinical cases and deaths, respectively (1). At the beginning of 2016, malaria was considered endemic in 91 countries and territories, reduce from 108 in 2000 (2).

Malaria imposes an enormous socio-economic burden with high costs, both for individuals and governments (3). The costs for individuals are associated with the household health expenditures and productivity which include the purchase of antimalarial drugs, preventive measures, doctor fees and absence from school or lost days of work. For example, in Malawi, more than 50% of adults reported that their malaria illness affected their daily work (4) and time lost per adults Ghana varies between 1 and 5 days (5). The most direct economic impact for the governments is to reduce malaria prevalence where direct costs of treating malaria fall on governments. These include providing and maintain staffing of health facilities, purchase and supply antimalarial drugs as well as public interventions against malaria. These macroeconomic impacts, particularly in low-income countries, can lead to catastrophic health expenditures and more financial impoverishment.

Substantial progress has been made in fighting malaria. A concerted campaign with current interventions against malaria by the international community for the last 15 year have considerably reduced malaria disease incidence across the African continent (**Fig. 1**). Despite this progress, significant challenge remains, and many countries are still far from reaching universal coverage with life-saving malaria interventions (2). Even more than half (41) of the world's 91 endemic countries are on track to achieve 40% reduction in malaria cases and deaths by 2020, progress in low-income countries with high malaria burden has been particularly slow (6). Therapeutic and insecticide resistances to some key components of tools to fight malaria such as the highly effective first-line treatment artemisinin-based combination therapies (ACTs) and vector control of long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS) also pose a threat in public health challenges for malaria control and elimination (7).

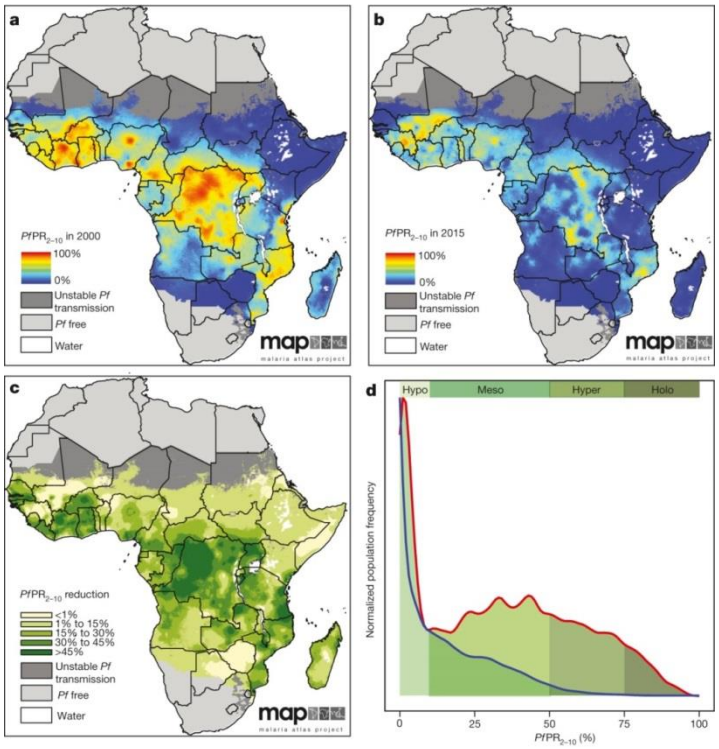

Figure 1 Change in infection prevalence 2000 – 2015. **a**, *Pf*PR_{2-10} for 2000. **b**, *Pf*PR_{2-10} for 2015. **c**, absolute reduction in *Pf*PR_{2-10} from 2000 to 2015. **d**, smoothed density plot showing the relative distribution of endemic populations by PfPR_{2-10} in years 2000 (red line) and 2015 (blue line). Reproduced from Bhatt et al. 2015 with permission from the Nature Publishing Group.

1.2 The parasite

Malaria is caused by protozoan parasites belonging to *Plasmodium* spp. (phylum *Apicomplexa*). *Plasmodium* spp. are indeed global pathogens and have complex life cycle alternating between vertebrate hosts and female *Anopheles* mosquitoes. Five plasmodial parasite species cause malaria in humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*. The two species namely *P. falciparum* and *P. vivax* are accountable for most malaria-attributed morbidity, but *P. falciparum* responsible for most-attributed mortality (2). The epidemiology of malaria varies geographically depending on seasonality and local transmission intensity.

P. falciparum is widespread in nearly all malaria endemic countries (tropical and subtropical), particularly predominant in sub-Saharan Africa and responsible for the majority of deaths due to malaria mainly in children under the age of 5 years (2). It is also prevalent in Asia and Latin America together with *P. vivax* in both mono and mixed infection (8, 9). More than 75% of *P. falciparum* infections that are detected during community surveys are without symptoms (i.e. asymptomatic) (10) and are associated with submicroscopic parasite densities (11). These asymptomatic infections can become symptomatic within days or weeks of initial detection (10, 12), or can remain asymptomatic for many months at variable parasite densities (11, 13).

P. vivax can be found mostly in Asia, Latin America and in small parts of Africa. Unlike *P. falciparum*, *P. vivax* infections include a dormant hypnozoites-liver stage that can lead to clinical relapse episodes (14, 15). In Asia, *P. vivax* and *P. falciparum* are the co-dominant species, albeit the distributions between the two species are different between countries (9, 16, 17). In South and Central America, *P. vivax* is the predominant species accounting for 71 – 81% of all malaria species (8). In eastern and southern Africa only 5% of total malaria infections are attributable to *P. vivax* (18). A major drive of the global *P. vivax* distribution is the influence inherited blood condition of Duffy negativity phenotype (19), which present at high frequencies in the majority of African populations (20). This genetic disorder will be described and discussed in more details in section 1.3.2.

P. malariae and *P. ovale* are much less prevalence compared to the two aforementioned species. In term of distribution, *P. malariae* is more or less sympatric with *P. falciparum* which mainly found in the region of sub-Saharan Africa and south-west Pacific (21, 22). Whereas, *P. ovale* spp. have a much more limited distribution to the area of tropical Africa and some islands in the West Pacific such as New Guinea, Indonesia and the Philippines (21, 23). Both species was observed as infrequent infections with prevalent detected by light microscopy rarely exceeding 1 – 2% for *P. malariae* and 3 – 5% for *P. ovale* (21). In West African population, *P. malariae* and *P. ovale* prevalence have been reported to peak at ages similar to those of *P. falciparum* (i.e. most common in children under 10 years old) and maximum parasitaemia rarely reached levels that were sufficient to introduce clinical attacks (24, 25). Furthermore, like *P. vivax*, *P. ovale* has long been thought to have a dormant stage (hypnozoites) that can cause relapses, but the evidence of the stage existence have never been demonstrated by biological experiments (26).

P. knowlesi, naturally occurs in long- and pig-tailed macaques, has recently been shown to cause primary human malaria in Sarawak, a state in Malaysia (27). It is now the most common cause of malaria in the country (28, 29) and has been increasingly observed elsewhere in Southeast Asia region (30-32). In this region, limited evidence suggests that asexual stages of *P. kowlesi* diagnosed by light microscopy are misidentified as *P. malariae* (27, 32-34), thus underestimate its true incidence. Unlike *P. malariae*, which multiplies every 72 h in blood and never results in severe infections, *P. knowlesi* multiplies within 24 h with high parasitaemia that can lead to death in humans (33, 35). Nevertheless, there is no evidence that sexual forms of *P. knowlesi* can develop in humans for human-to-human transmission (36).

1.2.1 *The parasite life cycle*

Plasmodium malaria is transmitted to the human host by female anopheline mosquitoes by inoculating microscopic motile sporozoites during a blood feed (**Fig. 2**). The sporozoites migrate rapidly through the dermis into the bloodstream which seek out and invade hepatocytes and the multiply. Nevertheless, of the about 100 sporozoites injected by a mosquito, only a few of those leaving the injection site to liver hepatocyte while the majority may enter lymphatics and drain to the regional lymph nodes where the adaptive immune

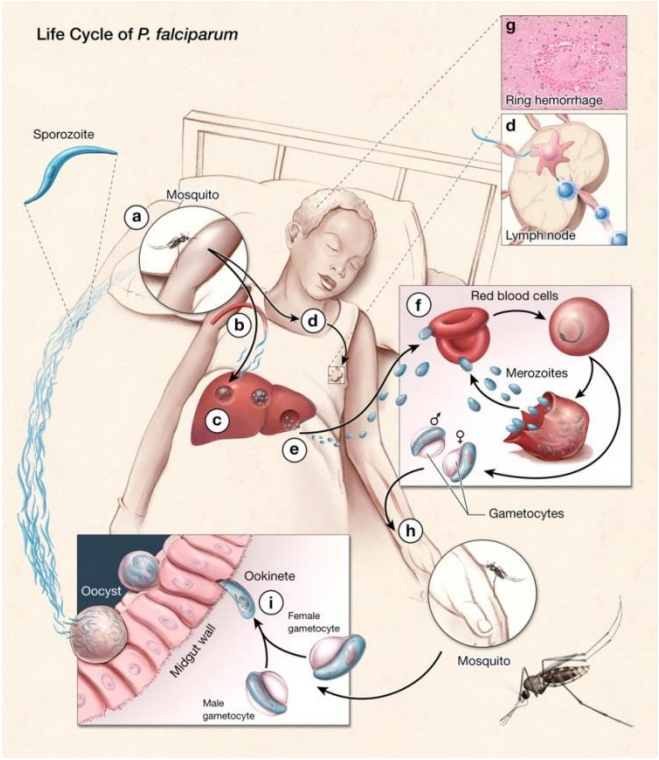

Figure 2 The life cycle of *P. falciparum* parasite. Reproduced from Pierce & Miller, 2015 with permission from the publisher. Copyright 2009.The American Association of Immunologists, Inc.

response is initiated (37, 38). Within a hepatocyte, a successful invasion of sporozoite can produce as many as 30,000 uninucleate-daughter merozoites in 5.5 to 8 days (39). When the exoerythrocytic schizonts rupture, the liberated merozoites release into the bloodstream where they quickly invade erythrocytes, commencing the erythrocytic stage (i.e. asexual cycle). An asexual cycle in the host's blood takes roughly 24 h for *P. knowlesi*, 48 h for *P. falciparum*, *P. vivax,* and *P. ovale* and 72 h only for *P. malariae*. The exponential expansion of parasite populations in the erythrocytic stage is responsible for the clinical symptoms of malaria.

The invading merozoite inside the erythrocyte (i.e. intraerythrocytic parasite) develops and mature from the ring stage to trophozoite and then to the final schizont stage. The infected erythrocyte eventually releases new merozoites (16 – 32 merozoites depending on species) (40) into the circulation that will, in turn, invade uninfected erythrocytes and repeat the cycle of blood schizogony. In a susceptible individual, the expansions of parasite populations have been shown to be between six times and 20 times per cycle (41).

After several erythrocytic generations, a small subset of merozoites undergoes sexual commitment and differentiates into male and female gametocytes (i.e. gametocytogenesis) that circulate independently in the peripheral blood. This differentiation is the next major stage of the parasite life cycle that involves in transmission by the mosquito vector. The exact timing of commitment and the triggers of parasite's sexual development involved are unclear (42, 43). Nonetheless, parasite exposure to different environmental stressors *in vitro* such as high host parasitaemia and drug treatment is correlated with an increase in the rate of

gametocytes (44). To complete the sexual cycle, these gametocytes need to be ingested when mosquito bites and infected host. Following ingestion by the mosquito, gametocytes form of *Plasmodium* experience a change in pH and a drop of temperature which together activate their maturation into gametes within the mosquito mid-gut (45). Sexually competent male gametes then fuse with female gametes to form a zygote which later develops into an ookinete. Ookinetes burrow from the mosquito midgut epithelial cell wall and form oocysts, which ultimately rupture releasing the sporozoites inside the mosquito. The sporozoites migrate within the mosquito body to the salivary glands where they stay until the mosquito takes a blood meal, at the same time delivers sporozoites to the next human host thus completing the life cycle (46).

1.3 **The host**

1.3.1 *Naturally acquired immunity*

Immunity against malaria parasite is complex, stage-specific and can be classified into natural (innate) and acquired (adaptive) immunities. Natural immunity to malaria is a rapid inhibitory response or an inherent refractoriness of the host against the introduction of the parasite and establishment of the infection. It is not dependent on any previous infections (47). Upon infection into human, the parasite induce a specific immune response, stimulating the cytokines and further activating host's various immune-dominant cells (i.e. monocytes, neutrophils, T-cells, natural killer cells) to react to the subsequent liver as well as blood stage parasite (48). Whereas, acquired immunity against malaria develops after infection. The protective efficacy of malaria acquire immunity varies depending on the characteristic of the host including the effect of exposure and age as well as transmission intensity (47).

Naturally acquired immunity against malaria is not sterile. Individuals living in malaria endemic areas acquired protective immunity to clinical symptoms only after years of repeated infections (49) (**Fig. 3**). After a few symptomatic infections, children particularly under 5 years of age, become immune to the most severe forms of malaria disease but remain susceptible to febrile illness (50). With cumulative parasite exposure over time, partial immunity to clinical disease is eventually acquired by the ability to control parasite density (47, 51). In adults, despite rarely suffering from clinical malaria episodes, sterilising immunity against infection is never fully achieved and they continue to be prone to re-infection and typically experience asymptomatic infections. In the case of naïve individuals, *Plasmodium* infection is almost symptomatic regardless of age, and clinical symptoms can easily be observed even at very low parasite density (47).

Long-standing evidences suggest that acquired immunity and protection from malaria exposure to *Plasmodium* parasites in endemic areas is largely mediated by Immunoglobulin G (IgG) (53, 54). This has been supported by many immune-epidemiological studies in endemic areas where antibody to parasite-specific antigens are significantly associated with protection in malaria clinical episodes (55-58). Several known mechanisms have been shown the ability of antibodies to limit the growth of blood-stages parasites as well as the

progression of clinical symptoms. These include opsonizing infected erythrocytes for phagocytic clearance (59) and blocking erythrocyte invasion (60).

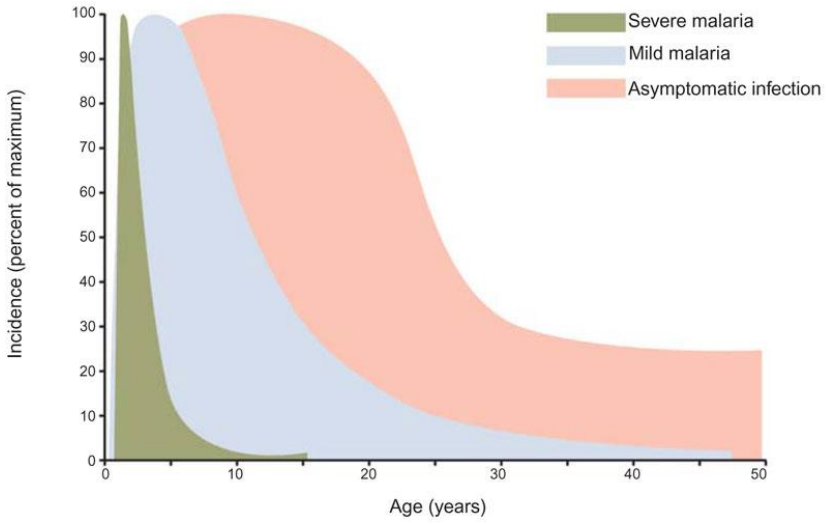

Figure 3 Changes over time of various indices of malaria in a population living in an endemic area. Adapted from Langhorne et al. 2008 (52) and reproduced with permission from the Nature Publishing Group.

Nonetheless, antibody responses to malaria infection as evidence seen in children and young adults are inefficiently generated, short-lived and waning rapidly in the absence of continued parasite exposure. In endemic areas, parasite-specific antibody levels appear to increase with age in stepwise manner and decay at a slower rate in young adults compared with young children in the same endemic area (61, 62). This phenomenon is ascribed to the defect in generating and maintaining long-lived memory compartment of B cells (63), probably due to the overwhelm of host's immune system to commit a sufficient number of antigen-specific B cells (64).

1.3.2 *Human genetics*

High mortality and widespread impact of *Plasmodium* parasite have played a crucial part in selective evolutionary force in current and past human demography and genetics (65, 66). In regions where malaria is prevalent especially in sub-Saharan Africa, naturally occurring genetics defence mechanisms have thought to evolve during the course of human evolution for resisting infection by *Plasmodium*. Human genetic resistance to malaria involved many genes and varied across populations (65). These genetic factors include enzymopathies (i.e. glucose-6-phosphate dehydrogenase (G6PD) deficiency), haemoglobin mutants (i.e. sickle haemoglobin), and red blood cell surface loci (i.e. Duffy antigen); to name a few.

G6PD is an important enzyme in glycolysis that catalyses the first reaction in the pentose phosphate pathway which plays and active role in the survival of erythrocytes. The *G6PD* gene is found on the X chromosome with more than 150 variants have been characterised causing different kinds of clinical deficiencies from mild to severe hemolysis (67). Given the

hemizygous states of males, in G6PD mutant-males all enzyme copies are deficient, as similar seen in homozygous females (66). Previous epidemiological studies have shown that the prevalence of malaria between endemic and non-endemic regions was significantly related to the distribution of G6PD deficiency (68, 69). This relationship reveals two important facts. While the G6PD deficiency provides excellent protections against malaria in particular for falciparum infection (69-71), it also can cause life-threatening hemolytic anaemia by using antimalarial drug (i.e. primaquine) and may even lead to death (72, 73).

Sickle haemoglobin (S) is a structural variant of normal adult haemoglobin. It is a result of as singles point mutation in the sixth codon of the beta globin gene (74). Sickle cell anaemia is an inherited disorder of homozygotes (SS) in which erythrocyte reveal an abnormal crescent shape (or sickle) containing abbarent haemoglobin. On the other hand, the sickle cell allele variant of AS heterozygotes, in which *A* indicates of the non-mutant form of beta globin gene, provide protection against malaria in sub-Saharan Africa and some other tropical areas (75-78). Cohort and case-control studies in many African countries have constantly found that 70 – 90% of AS heterozygotes protective against severe malaria (79-81). Parasite growth inhibition, impaired rosette formation and reduced cytoadherence of infected red blood cells are some of the hypothesised molecular mechanisms of protective sickle cell trait (AS) against malaria (82).

The Duffy antigen or Duffy antigen receptor for chemokines (DARC), also recently known as atypical chemokine receptor 1 (ACKR1), is a transmembrane receptor used by P. vivax to infect human red blood cells (83). The DARC gene has three major alleles types namely FY*A, FY*B, and FY*O (Duffy null) where FY*A and FY*B are the common allelic typed observed in non-African populations (84). FY*A is the most prevalence worldwide with the highest frequency in Asia than in Europe and relatively small frequency in southern Africa (20). The lack of expression of DARC in erythrocyte due to FY*O mutations has been shown to halt P. vivax infections (84, 85) and thus exhibit extreme geographic segregation with near fixation in equatorial Africa and nearly absence in both Asia and Europe (20).

1.4 The vector

Malaria is transmitted exclusively through the infective bites of female mosquitoes of genus the *Anopheles*. Among the 512 Anopheles species recognised worldwide, 70 species are able to transmit *Plasmodium* parasite to human hosts and 41 of which are the dominant malaria vector species (86, 87). Common characteristics of dominant vector species are their inclination to humans feeding, abundance, and longevity as well as elevate vectorial capacity (87).

The most efficient and effective dominant vector species of human malaria in Africa is the *Anopheles gambiae sinsu stricto* (88). It is a member of *An. gambiae* complex, which also contains *Anopheles arabiensis*, *Anopheles merus* and *Anopheles melas* (88-90). Also found in Africa are widespread of highly anthropophilic (i.e. preferring human beings to other animals) vector species namely *Anopheles funestus*, *Anopheles moucheti* and *Anopheles nili*

that have proved to be highly competent in malaria transmission and equally difficult to control (91).

The Asian-Pacific region has a greater number of dominant vector species than any other parts with at least nine out of 19 dominant species found are considered as species complex (89). For example, the Dirus and Minimus complexes both contain species considered particularly efficient in transmitting malaria in Southeast Asia region. Whereas in Asia-Pacific region, dominant vector species are dominated by three of the 12 members of the Punctulatus group; *Anopheles farauti* complex, *Anopheles koliensis* and *Anopheles punctulatus* complex (87). Among these, only the *An. farauti* complex expands eastward to the Solomon Islands and also found on the northern coast of Australia (87).

Environmental factors such as climate seasonality, temperature, rainfall patterns, humidity, the presence of vegetation and surface water play important roles in vector distribution and malaria biodiversity (86). Furthermore, human intervention and activities such as agriculture, urbanisation, deforestation and irrigation are also directly related to vector distribution and malaria transmission levels (92).

1.5 Clinical features of disease

The initial symptoms of malaria, typical to all different malaria species are non-specific and mimic a flu-like syndrome. Clinical findings in malaria are diverse and may range in severity from a headache to more serious complications. Based on severity, clinical features of malaria can be classified into uncomplicated malaria and severe malaria which differ in their treatment and prognosis.

1.5.1 *Uncomplicated malaria*

All signs and symptoms of uncomplicated malaria are non-specific and caused by the asexual or blood stage parasites. The hallmark of the malaria symptom is a fever. Following the infective bite of mosquito, infected individuals are generally asymptomatic for 10 to 30 days (i.e. incubation period; interval between infection and the onset of symptoms), but depending on parasite species can commence symptoms as early as 7 days, until parasite become detectable in blood (i.e. prepatent period) (93). In most *P. falciparum* and *P. vivax* cases, the incubation period is approximately two week and longest for *P. malariae*. Up to three days before the onset of fever, non-specific prodromal symptoms such as malaise, headache, myalgias, nausea, dizziness, sense of dizziness and vomiting may be experienced (94). Fever is often high, spiking up to 40^{o}C in children and naïve individuals, and can be associated with rigours in *P. vivax* infection (95). The classic malaria paroxysm consists of intermittent fever with chills and rigours occurring at the periodic interval of 24, 48 or 72 hours depending on the malaria species. It corresponds to the release of *Plasmodium* merozoites from schizont rupture during the blood-stage cycle. Thus, macrophages and monocytes are activated and further induces the release of proinflammatory cytokines (95). If uncomplicated malaria

treated with appropriate drugs, the symptoms remit over a few days, though often with considerable exhaustions.

1.5.2 *Severe malaria*

If the initial infection is not controlled either due to untreated or partially treated, the rapid progression to complicated or severe malaria can lead to death, particularly in falciparum malaria. The manifestations of severe malaria vary with both age and transmission level, which reflect the immune status of the populations (96). In Africa, three dominant syndromes namely cerebral malaria, severe anaemia, and respiratory distress are more associated with malaria deaths in children (97). Clinical features of severe malaria (i.e. in the absence of alternative cause), may include the presence of one or more of the features presented below, adapted from the WHO Guideline for the Treatment of Malaria (98).

a.	Impaired consciousness:	Coma Score < 11 in adults or < 3 in children.
b.	Prostration:	Generalised weakness; unable to sit, stand or walk
c.	Multiple convulsion:	More than two episodes within 24 hours
d.	Shock:	Compensated and decompensated shocks
e.	Pulmonary oedema:	Radiologically confirmed
f.	Significant bleeding:	Recurrent or prolonged bleeding from nose or gums.
g.	Severe malaria anaemia:	Hb ≤5 g/dL in children <12 years of age Hb ≤7 g/dL in adults (parasite >10,000/µL)
h.	Jaundice:	Plasma bilirubin >50 µmol/L (parasite >100,000/µL)
i.	Renal impairment:	Plasma bilirubin >265 µmol/L (blood urea >20 mmol/L)
j.	Acidosis:	Plasma bicarbonate <15 mmol/L or plasma lactate ≥5 mmol/L
k.	Hypoglycemia:	Blood or plasma glucose <2.2 mmol/L (<40 mg/L)
l.	Hyperglycemia:	*P. falciparum* parasitaemia >10%

1.6 **Endemicity and transmission**

Malaria endemicity is a proxy to indicate the malaria disease prevalence in a population. Malariologists have been long graded malaria endemicity according to the risk of infections as reflected in the proportion of the population having enlarged spleen (i.e. spleen rate; the percentage of sampled population with palpable enlargement of the spleen due to chronic exposure to malaria). 1951 WHO report on malaria conference in Equatorial Africa has classified endemicity as weighed by spleen rate surveys measured in the 2 – 10-year-old age group as follows: hypoendemic less than 10%, mesoendemic 11 – 50%, hyperendemic 51 – 75% (spleen rate in adults, high), and holoendemic more than 75% (spleen rate in adults, low). The similar report also classified endemicity as hypo-, meso-, hyper-, and holo-endemic based on parasite prevalence in children 2 – 10 years of age (99). Furthermore, a dynamic mathematical model using entomological determinant of malaria can further classified malaria endemicity into stable and unstable. This classification is taking into consideration that the stability of malaria is determined by the average number of feeds that a mosquito takes on human being during its life (100, 101). Nevertheless, the stable-unstable concept is rarely implemented. The reasons for this mostly due to the technical difficulties of obtaining

entomological-based metrics, issues related to measurement error and ethical concern of exposing human beings to malaria infection (102-104).

Malaria transmission can be defined as the process by which a malaria parasite completes its life cycle, involves parasites being uptake from a female anopheline mosquito through the skin, via the liver into human blood, and later from the infected blood back into the mosquito, leading to parasite development within a mosquito (105). The intensity of malaria transmission, a general concept describing the potential frequency of malaria transmission, varies enormously within endemic areas depending on the vectorial capacity of local mosquito populations, host immunity and malaria interventions (106). Measuring malaria transmission intensity is important in order to determine of the burden of malarial disease. Increases in the incidence of severe malaria disease and death have been shown to be associated with increasing malaria transmission intensity (107-109). Several points during the parasite life cycle can be used to measure the intensity of transmission using various metrics (110, 111). Each metric represents a quantity that is a major step in the transmission process.

1.6.1 *Entomological inoculation rate (EIR)*

The annual EIR (aEIR) is the number of infectious bites received per person per unit time, typically in year (ib/p/yr). It is the product of two components namely the human biting rate (i.e. *Ma*, the number of vectors biting and individual over a fixed period of time) and the sporozoite rate (i.e. SR, the fraction of mosquito with sporozoites in their salivary gland) (112). Catch and counting of mosquitoes by indoor or outdoor human landing catches, pyrethroid spray catches, and light traps can be used for measuring human biting rates. Whereas, dissection of mosquito salivary gland, serology and molecular method can be utilised to examining the caught mosquito for sporozoite. The gold standard method for estimating EIR is to measure SR and Ma directly (EIR = (total sporozoite positive tests/total mosquito tested) x (total mosquito collected/total catches)) (105). Another method of calculation has been proposed assuming that sporozoite data are available for all mosquito caught (EIR = total sporozoite positive mosquitoes/total catches) (102). Biases in different methods of catching mosquitoes (113) and interindividual differences in mosquitoes attractiveness (114), may contribute to the accuracy of EIR estimate, especially in the low transmission levels.

1.6.2 *Parasite rate*

Malaria parasite rate or prevalence (PR) is the proportion of the individuals in a given locale with detectable parasites in blood at given point in time. Since PR remains relatively constant in children aged 2 – 10 years (115), it has been widely used as a metric of transmission intensity particularly during the era of the Global Malaria Eradication Programme (i.e. PR exceeded 1 – 3%) (111). Examining blood sample for malaria parasite from a cross-sectional survey of a representative sample of the population such as school survey or the whole community can be rapidly measured PR. However, although PR can be estimated rapidly in populations, the accuracy and precision of PR are affected by many factors. The varying

distribution of parasite densities in a population (115), the recent used anti-malarial treatments, the method used for parasite detection (116, 117), and the seasonal variation of transmission (118, 119) were known to limit its utility for accurately measuring transmission intensity.

1.6.3 *Annual parasite incidence(API)*

The number of new parasitologically confirmed malaria cases per 1000 population per year is called the annual parasite incidence (API). Most countries have information on the API from routine surveillance and/or on the parasite prevalence from surveys. The WHO classifies geographical units according to local malaria transmission intensity based on API (2):

Very low transmission: areas have <100 cases per 1000 population; prevalence of *P. falciparum*/*P.vivax* malaria >0 but <1%.

Low Transmission: areas have 100 – 250 cases per 1000 population; prevalence of *P. falciparum*/*P. vivax* of 1 – 10%

Moderate transmission: areas have 250 – 450 cases per 1000 populations; prevalence of *P. falciparum*/*P. vivax* of 10 – 35%.

High transmission: areas have ≥450 cases per 1000 population; *P. falciparum* prevalence rate of ≥35%.

In moderate to high transmission settings, the relationship between API and transmission intensity is confounded by the relationship between exposure and acquired immunity. Whereas, in low transmission settings, the majority of the population are likely to have little clinical immunity against symptomatic disease (120, 121). Several factors affect the accuracy of API reflects transmission intensity. First, routine case data often do not discriminate between confirmed by diagnostic tests and those clinically diagnosed cases (121). Fortunately, since the launch of WHO's Test Treat Track (T3) campaign in 2012 (122), the proportion of cases that are correctly confirmed is increasing. Second, often information on whether identified cases are acquired locally or imported is not available, particularly in very low transmission areas where the proportion of imported cases may be substantial (123, 124).

1.6.4 *Serology*

Serological data offer an alternative means to estimate malaria transmission intensity under various malaria endemic settings (125-128). It is an ideal tool for rapid assessment of malaria transmission intensity and provided a theoretical advantage over EIR, and parasite prevalence in that single measurements reflect malaria exposure (i.e. infection) over an extended period (127). As exposed individuals can remain seropositive for antimalarial antibodies for a long period after infection (129, 130), integrating malaria exposure over time for assessing malaria endemicity can overcome the sampling biases associated with entomological and parasitological metrics such as seasonality and short-term fluctuations in transmission (131, 132). Also, the longevity of antibodies (i.e. reflect cumulative exposure to infection) means

that history of exposure can be constructed in situation of missing baseline data and predictions can be made even in the absence of active transmission (125). Age-specific serological data have also been used as evidence of reduction in malaria transmission and malaria elimination (131, 133, 134). Serological data (e.g. ELISA) are typically analysed using reversible catalytic models to estimate the antibody seroconversion rate (SCR; λ) - a function of antimalarial antibodies in the population or a rate at which seronegative individuals become seropositive (127, 135). The parameter of SCR (λ) is considered as a proxy to the 'force of infection' of malaria, as deflected through the immune responses of exposed malaria (136).

1.6.4.1 *Antigen selection for sero-surveillance*

Of more than 5,000 proteins expressed by the *Plasmodium* species, few have been examined in detail (137), and very few have been investigated as potential antigens for sero-surveillance. Properties of the different antigens could influence their selection for application in sero-surveillance assays, including immunogenicity, polymorphism, and antibody longevity (138). Antibodies to different malaria antigens are acquired at different rates relative to exposure (64, 139); thus, the selection also needs to consider according to the application and setting. Fast acquisition of malaria antibodies in early life for highly immunogenic and stable (i.e. long-lived) antigens will be essential for monitoring changes in transmission in low endemic settings, whereas those with shorter-lived responses will be more useful to reflect recent changes in exposure in moderate-to-high endemic settings. In addition, potential cross-reactivity of antigens from different malaria species (140, 141) and both sensitivity and specificity of surveillance assays are important in the context of elimination programs (142), to ensure high-risk subpopulations and geographical hotspots are correctly identified.

A panel of antigens, which are most studied as markers of exposure, observed immunogenicity and/or currently under development as vaccine candidate antigens were selected and included in the studies presented in this thesis. These selected antigens are described in brief below.

1.6.4.1.1 Antigens for *Plasmodium* sero-surveillance

AMA-1*:* Antibody responses to the merozoite antigens have been most studied as markers of exposure to *Plasmodium* (57, 138). One of them is apical membrane antigen 1 (AMA-1), a structurally conserved 83 kD type I integral membrane protein varying between 556 to 563 amino acids in most *Plasmodium* species (143). The protein made up of three domains and stabilised by eight disulphide bonds (144). It is expressed on the parasite's surface in the late schizont stage and long thought to be involved in red blood cell (145) and liver cell (146) invasion by *Plasmodium* merozoites. The surface location also makes the protein more exposed to human immune system and thus exhibit high antigenic diversity (147). There is extensive polymorphism among the sequence of genes coding for AMA-1. Most AMA-1 polymorphisms are dimorphic and either high or low in incidence (148). In a study in Papua

New Guinea showed that certain polymorphism frequencies differed between asymptomatic and symptomatic cases, suggested *Pf*AMA-1 might be one determinant of malaria morbidity (149). Polymorphisms also occurred in *Pv*AMA-1, although the regions under selective pressure might differ from those in *Pf*AMA-1 (150). Natural immune responses (both humoral and cellular) to AMA-1 are found in most people exposed to malaria, with antibody prevalence positively increased with age (151-154).

MSP-1: Merozoite surface protein 1 (MSP-1) is a high molecular mass protein (~180 kDa) that is proteolytically processed into fragments of 83, 30, 38, and C-terminal 42 kDa (MSP-1_{42}) (155, 156). During merozoite invasion, MSP-1_{42} is further processed into MSP-1_{19} and MSP-1_{33} which later remaining attached to the merozoite surface and present on ring forms in newly invaded red blood cells (157). MSP-1 is essentially dimorphic, albeit some parts of this large molecule are much more variable (158). Nonetheless, *MSP-1_{19}* gene is relatively conserved, and variability is restricted to 4 – 6 amino acid residues (159). Naturally acquired antibodies to MSP-1_{19} can impede erythrocyte invasion of merozoite by preventing the secondary processing that released this fragment from the rest of the MSP-1 complex (160).

Antibody responses both AMA-1 and MSP-1_{19} antibodies have been most studied as markers for exposure to *P. falciparum*. SCR for both *P. falciparum* merozoite antigens (i.e. *Pf*AMA-1 and *Pf*MSP-1_{19}) have been strongly correlated with other indication of transmission intensities such as EIR (based on the model of EIR upon altitude in Tanzania), parasite rate, and altitude (125-127, 161, 162). Immunological surveillance based on merozoite antigen SCRs has facilitated the identification of transmission host spots (161), changes in transmission intensity over time (126, 163-166), and seasonal variations in transmission (132, 167). Availability of *P. vivax* merozoite antigens (i.e. *Pv*AMA-1 and *Pv*MSP-1_{19}) have also been successfully demonstrated in regions where parasite prevalence was low (131, 168-171). Furthermore, the sensitivity of the serological assay can be tailored depending on transmission level. A highly immunogenic antigen such as AMA-1, or a combination of antigens such as AMA-1/MSP-1_{19} can be used in areas of low transmission (172). Whereas, less immunogenic antigens suitably used in high transmission areas, where seroprevalence to high immunogenic antigens approachers 100% very early in life (125).

CSP: Circumsporozoite (CSP) is the major surface protein of the sporozoite and forms a dense coat on the parasite's surface. The CSP protein from all species of *Plasmodium* are similar in overall size (400 amino acids) and is divided into three regions; NH2-terminal region, central repeat region and COOH-terminus (173). The repeat motifs in the central region of CSP protein comprise 37 tandem repeats of the tetrapeptide Asn-Ala-Asn-Pro (NANP) interspersed with four copies of Asn-Val-Asp-Pro (NVDP). Long thought that NANP repeat motifs of the CSP were identified as the target of protective antibodies (174, 175).

Unlike merozoite antigens, *Plasmodium* sporozoite antigen is exposed to the immune system for only short periods after mosquito inoculation, and anti-CSP antibodies would generally be detected in individuals with frequent or recent exposure (176). Some sporozoite rapidly

develops into liver stage, but others are taken up by macrophage, processed, and later be presented to the immune system (177). The small amount of antigenic materials and short time of contact with immune cells may underestimate the use of CSP antigen for malaria exposure in low transmission settings. It has been shown that CSP is not a reliable marker of malaria endemicity when the total EIR in the areas is <10 ib/p/yr (178). However, in hyperendemic areas, immunological surveillance based on *Pf*CSP has been reported to give reliable estimates of malaria endemicity and reflects the dynamic in seasonal transmission (177, 179). Also, *Pv*CSP has been associated with other measures of transmission intensity in Asia (180-182).

1.6.4.1.2 *Anti-salivary antibodies as biomarkers of exposure*

A measurement of human antibodies to *Anopheles* mosquito antigens is an alternative tool for describing exposure to malaria vectors. During a blood meal, mosquitoes inject saliva into the host's skin. This saliva contains a cocktail of active components that facilitate mosquito blood-feeding activity and counteract with host haemostasis and modulate immune responses (183, 184). Human produces IgG-, IgM-, and/or IgE-specific to injected mosquito salivary molecules (i.e. protein) following mosquito bites (infecting or non-infecting bites) (185-187). Such humoral responses towards salivary protein have proven to be a useful marker of human exposure to *Anopheles* vector bites (188-191) and could be performed in parallel with other serological measures of exposure.

gSG6: Recent transcriptomic studies on salivary glands of *An. gambiae*-females mosquitoes have identified over 70 putative secreted salivary proteins, and one of them is gambiae salivary gland protein 6 (gSG6) (192-194). The gSG6 protein is a small immunogenic protein (11 – 13 kDa) that is restricted to anopheline mosquitos and well conserved in the three major Afrotropical malaria vectors (i.e. *An. gambiae*, *An. arabiensis* and *An. funestus*) (195). Total IgG antibody responses to gSG6 peptides described *Anopheles* mosquito exposure in low vector density areas (196), in response to ITN-based vector control programs (197-199), and to reflect *Anopheles* heterogeneity in urban areas (185). Together with parasite antigens, gSG6 assays have shown to be sensitive to micro-epidemiological variations in mosquito exposure and provide a correlate of malaria risk as well as transmission (200-204). The short-lived nature of gSG6 appears to correlate with seasonal changes in *Anopheles* abundance with strong immunogenicity among rural populations in Burkina Faso (191, 204-206).

1.7 **Diagnosis**

Prompt and accurate diagnosis of malaria is crucial to the effective disease management and surveillance. Malaria diagnosis involves identifying malaria parasites or antigens/products in patient blood. For all patients suspected of malaria, WHO recommended prompt parasite-based diagnosis before any treatments are administrated (98).

1.7.1 *Microscopy*

Conventionally, malaria is diagnosed by light microscopy examination of stained blood smears, most commonly with Giemsa stain. Microscopic detection and identification of *Plasmodium* species in Giemsa remain the gold standard for laboratory diagnosis (207, 208), and remains relatively widespread as a point-of-care diagnostic in clinical and epidemiological settings (209). Malaria is diagnosed microscopically by preparing of thick and thin blood smears on a glass slide for the detection of parasites in the peripheral blood. Thick smears are useful for screening the presenting malaria parasite, parasite density and detecting of low-density malaria, whereas thin smears provide confirmation for malaria species. To prepare a thick blood smear, a blood spot is stirred in a circular motion with the corner of the slide, taking care not make the preparation too thick, and allowed to dry without fixative (208). Whereas, a thin blood smear is prepared by immediately placing the smooth edge of a spreader in a drop of blood, adjusting the angle between slide and spreader to 45^{o} and the smearing the blood with a swift and steady sweep along the surface (208). Parasites density (in parasites/µL) are estimated in thick blood smears by counting the number of asexual parasites against in 200 white blood cells (WBCs), where the average WBCs count of 8,000 cells/µL was assumed.

Microscopy technique is widely used in the management of malaria due to its simplicity, low cost, its ability to identify the presence of the parasites, the infecting species and assess parasite density. Despite proved to be a tremendously resilient and useful diagnostic tool, it is not without problems. There are still few limitations in the efficacy of microscopical diagnosis. The most obvious shortcoming is its relatively low sensitivity, particularly at detecting low parasite levels and resulted in underestimating malaria infection rates. Under optimal condition, an expert microscopist can detect up to 5 parasites/µL, whereas the average microscopist detects only 50-100 parasites/µL (210). Furthermore, the staining and interpretation process of microscopy slides are labour intensive, time-consuming, and require considerable expertise and trained health workers. Fatigue and the pressure to return results among the technicians (i.e. microscopists) may also lead to significant loss of efficiency and accuracy of the microscopy reading. Thus, constant monitoring of the workload of each technician may be required. Concerning the microscope, high-quality microscopes are expensive and often beyond the means of local health outposts, particularly in low-income countries. Access to portable and sturdy microscopes required for the field use are usually limited to only a few peripheral health facilities.

1.7.2 *Rapid diagnostic test (RDT)*

Unlike conventional microscopy, rapid diagnostic tests (RDTs) are all based on the same principle and detect malaria antigen, which uses antibody capture to detect soluble malaria antigens in blood flowing by immunochromatography i.e. migration of liquid across the surface of a nitrocellulose membrane. It is a simple lateral flow device, used a small amount of blood (5 – 15 µL), and does not require operation by laboratory equipment. RDTs commonly come in nitrocellulose strip and usually packaged in a plastic cassette or on a card.

Coloured test line result of RDTs have revolutionised malaria diagnosis by providing convenience and rapid turn-around time of only 15-20 min.

Most RDT products target a *P. falciparum*-specific antigen namely histidine-rich protein 2 (HRP-2), lactate dehydrogenase (LDH) or *Plasmodium* aldolase. Some tests detect *P. falciparum*-specific and pan-specific antigens (i.e. aldolase or lactate dehydrogenase (pLDH)) to distinguish non-*P. falciparum* infections from mixed malaria infections. HRP-2 is a water-soluble protein produced by asexual stages and young gametocyte of *P. falciparum* and expressed in abundance on the membrane surface of infected RBC (211, 212). On the other hand, both LDH and aldolase are enzymes found in the glycolytic pathway of the malaria parasite and produce by asexual and sexual stages of the parasite (213).

Currently, tests targeting HRP-2 contribute to more than 90% of malaria RDTs used worldwide, but the performance among different tests varied considerably. Several possible reasons including the specificities, sensitivities, numbers of false positives, numbers of false negative and temperature tolerances (214). The main problem associated with variability in both specificity and sensitivity of HRP-2 based RDTs is manufacturing process of the kits (215). Malaria transmission intensity, patient age, and lack of symptoms have also been demonstrated to influence specificity and sensitivity of RDTs, which can turn result in under- or overdiagnosis of the disease (216-218). Furthermore, the genetic variation in *Pf*HRP-2 amino acid sequence among parasite isolates from different geographical areas may lead to false-negative results from RDTs. Deletion (219) and a number of repeats and combinations (220) of *PfHRP-2* gene may contribute to the cause of diagnostic failure if the test. False-negative in results can also be explained by the absence of bands on an RDT either from excess antibodies or antigens (i.e. the prozone effect) (221). The HRP-2 antigen persists for weeks in the blood after an infection is cleared resulting in false positive results, thus limits the usefulness of *Pf*HRP-2 RDTs in high transmission settings (212, 214). Given that HRP-2 is indirect measures of parasite biomass (222) and the prolong presence after parasite clearance, results based on RDTs can also indicate a range of possible infection states, albeit less comparable than microscopy or molecular methods (i.e. parasite density as biological endpoint).

Even with those caveats, RDTs have proof to be a valuable tool for point-of-care diagnosis, particularly for use at the community level, in low-resource settings. Their use in field conditions allows prompt diagnosis of malaria in any febrile patients, reducing dependent on the presumptive treatment of confirmed cases as well as lessen the risk that patient will get sicker before a correct diagnosis is conducted.

1.7.3 *Molecular-based diagnosis*

Recent developments in molecular biological technologies have permitted extensive characterization of the malaria parasite and generating new strategies for malaria diagnosis. Molecular diagnostic platforms display high sensitivity, high specificity and their ability to detect extremely low-level infections. Nevertheless, the significant barrier of these methods

including required specially trained technician, relatively high operational cost, prone to contamination, complex methodologies, and the amount of infrastructure needed in the form of equipment, stable power and reagent storage (223).

1.7.3.1 *Polymerase chain reaction (PCR)*

PCR-based techniques have proven to be one of the most specific and sensitive diagnostic methods, particularly for malaria cases with low parasitaemia or mixed infections (224). PCR involves primer-directed amplification of a specific fragment of DNA by using thermostable Taq polymerase, under a specific set of alternating temperature cycling conditions. Most nucleic acid tests for malaria focus on the 18S ribosomal RNA (rRNA) (225-227), cytochrome b (*cytb*) (228), and recently cytochrome c oxidase III (*cox3*) (229, 230), which contains regions conserved across all malaria species and regions unique to each species. PCR is highly effective at identifying low-level infections that often missed by conventional techniques with a limit of detection of 0.5-5 parasites/μL (231-233). However, detection based on RNA which is much more abundant than DNA (up to 1000 copies of the 18 S gene per parasite) (234), can be even more sensitive (11), providing a more accurate detection of gametocytes (235, 236). To mention but a few, many of nested PCR (nPCR) (207, 231, 237, 238) and real-time PCR quantitative PCR (qPCR) (239, 240) have been previously developed for malaria detection, quantification and determination of malaria species. Moreover, PCR-based methods are now used routinely to help detect drug-resistant parasites (241-243) and evaluating other diagnostic tools (244-246).

1.7.3.2 *Loop-mediated isothermal amplification (LAMP)*

Unlike PCR, LAMP amplifies nucleic acids at a constant temperature (i.e. isothermal), without alternating temperature conditions. It is less time consuming than can be performed with a simple water bath or heating blocks, with results read by eye under UV light. The visual detection avoids the need for opening the reaction tube post-amplification, thus reduce the risk of contamination (247). In brief, a set of four to six specifically designed primers to recognise six distinct regions of the target DNA, as well as *Bst* DNA polymerase, are used for auto-cycling strand-displacement DNA synthesis. The unique design of primers results in DNA stem-and-loop formations in the amplification steps, which leaves a binding site constantly open for new primers to anneal (223). As little as one hour, the amplified products capable of achieving 10^8 fold (248), thus reduces the time-to-result (249). LAMP has been successfully developed to detect malaria in a field-stable format (123, 250) and the Loopamp™ MALARIA Pan/Pf Detection Kit has been evaluated in both laboratory (251) and field settings (252-254).

1.7.4 *Antibody assays*

Antimalarial antibodies can be detected using serological methods but cannot determine whether the antibodies result from current or past infection. The methods most commonly used to measure antibodies titres against malaria parasites are the indirect

immunofluorescence (IFA), and enzyme-linked immunosorbent assay (ELISA), which rely on detection of asexual blood stages antigens. Detection of antimalarial antibody is definitely not a substitute for the diagnosis of an acute attack of malaria (i.e. by microscopy and PCR) but rather used mainly in the screening of prospective blood donors to avoid transfusion-transmitted malaria (255, 256). Serological methods also can be used to diagnose of fever of unknown origin, especially in patients with tropical splenomegaly or inadequately treated for malaria, where the measured antibody titres reflect the intensity of contact with the parasite (257). The principle of serological assays is to rely on the delay antibodies development following infection of any *Plasmodium* species where specific antibodies are produced within two weeks of initial infection and remain three to six months after parasite clearance (208, 257). In endemic countries where reinfection is frequent, these antibodies may persist for months or year in semi-immune patients (127). Nevertheless, the utility of serological methods particularly for IFA is limited. IFA is time-consuming and difficult to automate. It requires fluorescence (UV) microscope and trained technician, making it operator-dependent and subjective particularly when dealing with serum samples with low antibody titres (257).

Serological methods can provide retrospective confirmation of malaria infection or history of infection and are currently used in many epidemiological surveys (128, 131, 163-165, 258-261). Despite being able to demonstrate exposure to infection in endemic populations, no serological methods reliably assess the extent of exposure in individuals. However, serological analyses can be used to a assess the risk of exposure among groups. Detection of anti-CSP antibodies in European travellers returning from malarious regions suggests that serology data can serve as indicators of the relative risk of infection in travellers to specific regions (262, 263).

1.8 **Treatment**

Malaria is an entirely curable disease if adequately treated. The main objective of malaria treatment is to establish a complete clearance of any forms of *Plasmodium* parasite in patients's blood thus eventually stop the progression of malaria complications or death. Malaria treatment should be governed by three main factors namely the infecting *Plasmodium* species, the clinical status of the patient and the drug susceptibility of the infecting parasites.

1.8.1 *Treatment of uncomplicated malaria*

The WHO recommended first-line treatment of artemisinin-based combination therapies (ACTs) for uncomplicated *P. falciparum* malaria in all endemic countries (98) due to its reliability, rapid action and few adverse effects (264). ACTs are composed of a fast acting drug derivatives (i.e. artemether, artesunate, or dihydroartemisinin) is given for 3 days with a slowly eliminating partner drugs (or longer eliminating half-lives), preferably in a fixed-doses combination(39). This combination results in the artemisinin component being protected from development of antimalarial drugs by the partner drug and vice versa (98). Currently, there are five ACTs for the first-line treatment; artemether-lumefantrine (AL), artesunate-

amodiaquine (AS-AQ), artesunate-mefloquine (AS-MQ), artesunate plus sulfadoxine-pyrimethamine (AS-SP), and dihydroartemisinin-piperaquine (DHA-PPQ) (98). In low transmission areas, WHO also recommend to include a single gametocytocidal dose of primaquine (0.25 mg/kg) for the treatment of uncomplicated malaria to prevent onward transmission (98). First-line treatment with ACTs has played and important role in reducing the malaria burden in Rwanda (265), São Tomé and Príncipe (266) as well as Zanzibar (267).

For most *P. vivax* and all *P. ovale* infections, the drugs of choice are a combination of chloroquine (total dose 25 mg/kg) with daily primaquine (0.25 mg/kg) for 14 days. For *P. malariae* and *P. knowlesi*, the drug of choice is chloroquine at standard dose and do not require radical cure with primaquine. Nonetheless, any ACTs can be given to non-falciparum infection together with primaquine accordingly, except for AS+SP where *P. vivax* is resistant (39). Other non-ACT treatments (i.e. second-line treatments) available include atovaquone plus proguanil (AQ/PG, Malarone), amodiaquine plus SP, and artesunate and/or quinine plus antibiotics such as tetracycline, doxycycline or clindamycin. Tetracycline and doxycycline are not suitable for pregnant women or children younger than 8 years (39).

1.8.2 *Treatment of severe malaria*

The main objective of antimalarial treatment for severe malaria is to save life. Severe malaria is a medical emergency and requires intensive care and careful management (39). Parenteral artesunate by intravenous or intramuscular is the treatment of choice for severe malaria worldwide (98), but artemether can be used if only artesunate is not available. In the case of both artemisinin derivatives are not available, quinine dihydrochloride can be given. Parenteral artesunate therapies have significantly reduced mortality compared to quinine both in Asia (22.4 to 14.7%) (268) and in Africa (10.9 to 8.5%) (269).

1.8.3 *Drug resistance*

The emergence of artemisinin-resistance *P. falciparum* in Western Cambodia and the Thailand-Myanmar border currently poses a great concern in malaria control and elimination (270-272). These artemisinin-resistance parasite populations are cleared slowly from the blood after ACT and treatment failure occur more often. Very recently, mutations in the Kelch propeller domain of *P. falciparum* (*Pfkelch13*) were discovered as a putative responsible for artemisinin resistance (273). Furthermore, resistance of *P. falciparum* to chloroquine and SP now occurs throughout most of the tropical world, but worsening across Africa. Nevertheless, chloroquine sensitivity is still retained in some part of Central America, North Africa, Middle East and Asia such as Peninsular Malaysia and part of the Philippines. Mefloquine resistance is still relatively unusual and has been shown to be associated with reduced susceptibility to quinine (274). Similar to artemisinin, high-level mefloquine resistance has also developed on the borders of Thailand and the adjacent countries (275-277).

1.9 Control and elimination

The WHO uses the following terminology to refer the hierarchy of potential public health efforts in dealing with malaria (278):

Malaria control: reduction of the disease incidence, prevalence, morbidity or mortality to a locally manageable level as a result of deliberate efforts; continued intervention efforts are required to sustain control.

Malaria elimination: interruption of local transmission (i.e. reducing the rate of malaria cases to zero) of a specified malaria parasite in a defined geographic area; continued measures are required to prevent re-establishment of transmission.

Malaria eradication: permanent reduction to zero of the worldwide incidence of infection caused by human malaria parasites as a result of deliberate efforts. Intervention measures are no longer needed once eradication has been achieved.

Unprecedented efforts of malaria community for the last decade to control malaria has witnessed a reduction in malaria cases globally, including renewed financial and political commitments thus reinviting the possibility of malaria elimination in some countries.

1.8.1 *Cornerstones in malaria control and elimination*

1.9.1.1 *Vector control*

Vector control is an essential component of malaria prevention. Personal protection measure namely insecticide-treated nets (ITNs), which comprise conventional (cITNs) and long-lasting insecticidal nets (LLINs) have become the most widely used form of vector control. In Africa, this method proved to significantly reduce morbidity and mortality via direct protection as well as in community-wide reduction in transmission (279-281). Only pyrethroid insecticides are approved for use on ITNs, and the nets require frequent retreatment, usually every 6 to 12 months. Whereas, insecticides such as deltamethrin or permethrin are used for LLINs which require no further treatment and have an expected lifespan of 3 to 4 years (282). The emergence and spread of insecticide resistance mosquito have threatened the effectiveness of ITNs. Currently, 27 countries in sub-Saharan Africa have reported pyrethroid resistance in Anopheles vectors (283).

Indoor residual spray (IRS) is a method against adult mosquitoes that involves applying a long-lasting insecticide to the interior walls and surfaces of the houses (e.g. ceilings) which serve as resting places for mosquitoes (i.e. endophilic). The most common insecticide used is pyrethroids. In sub-Saharan Africa, 7% of the population at risk were protected by IRS as vector control (2). Previously, IRS has been shown to disrupt malaria transmission significantly, reduce malaria incidence, and eliminate malaria vectors (284-286). Nevertheless, the use of IRS has declined in recent years due to lack of long-term effort from government, concern about insecticide resistance, and fear of its harmful effect on human health and environment (7).

Agents for biological control of malaria have been mainly developed against aquatic mosquitoes stages (i.e. larva and pupa) by introduction or manipulation of organisms to suppress vectors population. Larvivorous fish *Gambusia* (guppy fish) have been used for over a decade in mosquito control (287). The benefits of larvivorous fish are the fish relatively inexpensive; fish populations are self-sustaining even without the presence of mosquito larvae (288). Certain types of Gram-positive bacteria such as *Bacillus* spp. have also been found to be effective as bio-larvicides for control mosquito larvae (289).

1.9.1.2 *Improved diagnostics*

Improved diagnostics are crucial to monitor and measure changes in infections rates. The WHO in 2010 recommends parasitological confirmation by either microscopy or RDT to those who suspected malaria regardless of age and epidemiological settings (290). Current-generation RDTs are excellent for diagnosing individuals with symptomatic malaria who have relatively high parasite densities. In the African region, RDTs accounting for 40% if all cases tested in the region in 2011 (7). However, the current test cannot detect either the dormant liver stages (*P. vivax* and *P. ovale*) or the very low-levels blood-stage infections of any malaria species (291). With regard to primaquine (or unregistered tafenoquine drug), rapid testing of individuals deficient in the G6PD enzyme will enable the scale-up of effective *P. vivax* treatments.

1.9.1.3 *Effective treatment*

Timely access to effective treatment is fundamental to prevent rapid progression of malaria disease, particularly in children. Except in areas where chloroquine remains fully efficient, uncomplicated *P. falciparum* infections are recommended to be treated by ACTs as first-line treatment (7). The WHO recommends prompt treatment with recommended antimalarial medicines within 24 hours of the onset of fever, after confirmation of malaria through appropriate diagnostic tests (2). Most African countries are far below this target, with an only minority of fevers being treated promptly and efficiently (292-294). Nevertheless, tremendous increase globally have seen in the proportion of ACTs given to children with both a fever in the previous two weeks and a positive RDT at the time of survey from a median of 29% in 2010 – 2012 to 80% in 2013 – 2015 (2).

1.9.1.4 *Chemoprophylaxis*

Planned and intermittent administrations of antimalarial medicines with the consequent chemoprophylaxis effect is being used in high transmission areas to prevent infection and/or disease in several high-risk groups. Currently, sulfadoxine-pyrimethamine (SP) is the recommended chemoprophylaxis drug for both pregnant women and infants. Intermittent preventive treatment for pregnant women (IPTp) by SP has been endorsed as national policy in 35 sub-Saharan countries (7). In 2015, WHO reported that 31% of eligible pregnant women received three or more doses of IPTp among 20 countries (with sufficient data); a major increase from 6% in 2010 (2). In African nations with moderate-to-high malaria

transmission, WHO recommended that all infants at risk of *P. falciparum* infections should receive three doses of SP (IPTi) through immunisation services (295). The WHO also recommended the implementation of seasonal malaria chemoprevention (i.e. SP plus amodiaquine, AQ) for children under the age of five in areas of intense transmission season (i.e. sub-Sahel West African countries) (296).

1.9.1.5 *Vaccines*

The complexity of malaria parasite's life cycles have severely hampered vaccine development strategies since an immune response targeting one stage of malaria may not offer protection against a later stage (297). Significant progress has been made in the last few years, and this is yielding the most advanced malaria candidate vaccine RTS, S/AS01 which has completed Phase 3 trial. The vaccine consists of hepatitis B surface antigens virus-like particle incorporating a portion of *P. falciparum*-derived CSP protein and a liposome-based adjuvant (298). Interim results of the large phase 3 trials showed that vaccine efficacy estimates in preventing both infection and uncomplicated malaria fell by 55% among young children and 30% among infants, while efficacy against severe malaria was around 39% and 31.5%, respectively (299, 300). This falls short of the traditional goals of a vaccine but might give infants and small children a better chance of surviving when most vulnerable. Substantial progress has also been made with *P. falciparum*-derived vaccine namely *P. falciparum* sporozoite (PfSPZ) vaccine. It is composed of aseptic, purified, cryopreserved, attenuated (i.e. weakened) and metabolically active *P. falciparum* sporozoites (301), to generate an immune response to protect against malaria infection. Clinical trials have been promising (302-304), but short-term challenges include the feasibility with regard to large-scale of the manufacturing process, the potency of the vaccine and logistic delivering to those who need it most. Nevertheless, the PfSPZ vaccine is a very promising malaria vaccine candidate that is likely to be deployed first in the military and perhaps in travellers (305).

1.10 ***Malaria elimination***

The WHO classifies countries according to their malaria programme phase as follow (278):

Pre-elimination: test positivity rate <5% among suspected malaria cases throughout the year and API <5%.

Elimination: API <1; malaria is a notifiable disease with a manageable number of reported malaria cases nationwide.

Prevention of reintroduction: recently endemic countries with zero local transmission for at least three years; case management of imported malaria and maintaining the capacity to detect malaria infection and manage the clinical disease is required.

Malaria-free: certified malaria-free with last five years.

Most national malaria programmes considered malaria elimination is an attainable goal. Nevertheless, the decision to convert a malaria control programme that has successfully

achieved a high level of control, into an elimination is complex and should take into account technical and operational feasibilities as well as financial capacity (306, 307). Between 2000 and 2015, 17 countries eliminated malaria (i.e. attained zero indigenous cases for three years or more), where six of this countries have been certified as malaria-free by WHO (2). To date, there were 35 countries that met the malaria-eliminating criteria (i.e. countries that have a national or subnational evidence-based elimination goal and/or is actively pursuing elimination within its borders) (308) (**Fig. 4**) with most national or regional goals to achieve elimination by 2030 (278, 309). It is remarkable that among these countries, six belong to African region and its peripheries (Algeria, Botswana, Cape Verde, Comoros, South Africa, and Swaziland) with the potential to eliminate local transmission of malaria by 2020 (278).

The transition from sustaining control to elimination demands effective program management and additional activities. Although the aim of malaria control is to reduce morbidity and mortality in general population through access to prevention, diagnosis and treatment, elimination requires case-by-case focus, finding and treating symptomatic and asymptomatic infections, and taking action in specific foci to immediately prevent onward transmission (306). These aspects require enhanced laboratory support that cradle high sensitivity such PCR and serology. Furthermore, rigorous case investigation and reactive case detection activities are necessary for elimination settings to track secondary cases that arise in these specific foci. Unlike in control settings where universal coverage of vector control interventions is often the goal, elimination effort narrows the focus to high-risk groups based on malaria high-risk micro-foci based on geography and high-risk malaria groups based on demographic where cases and ongoing transmission are concentrated.

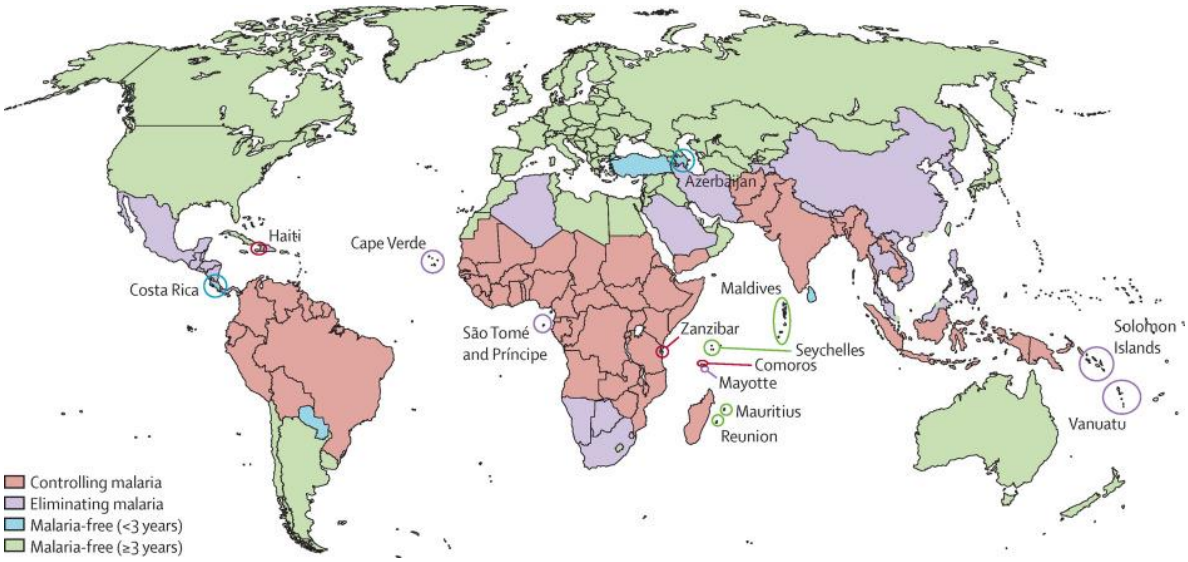

Figure 4 Categorisation of countries as malaria free, eliminating malaria, or controlling malaria, 2015. Reproduced from Newby et al. 2016 with permission from the Elsevier Ltd.

Nevertheless, major epidemiological shifts have occurred in malaria-eliminating countries. There are still important issues and challenges that need to be confronted in order to achieve malaria elimination. The following factors might contribute to a delayed success in malaria elimination program.

1.10.1 *Clustered reservoirs*

A noticeable epidemiological shift in areas approaching malaria elimination is where remaining parasites reservoirs are increasingly clustered in defined geographical areas (hotspots) (161, 310, 311). These hotspots may consist of several households or groups of households, maintain higher transmission of malaria with consistent of parasite reservoirs through the year (142). Factors determining hotspots are not entirely defined but include the proximity to mosquito breedings sites, household structural features, and human behaviour as well as genetic determinants (312). Meanwhile, malaria cases that are more clustered demographically into subpopulations with shared social, behavioural and geographical risk characteristics, referred as hotpops (161, 310, 311, 313). These hot populations are connected to the increasing importance of occupational and behavioural factors outside the home that put them in contact with infective bites (307). They may also act as parasite reservoirs, with many infections carried asymptomatically and with low parasites densities (142, 216, 314). Also, residual malaria transmission is some eliminating countries is concentrated in a few hard-to-reach populations. These populations include ethnic or political minority groups that typically mobile and impoverished, often driven to more remote areas by marginalisation, safety concern or economic opportunities (313, 315).

1.10.2 *Malaria importation*

Imported malaria is the main threat for malaria-eliminating settings to maintain elimination with the far greatest risk for countries neighbouring high-endemic areas (313, 315). The reintroduction of malaria to receptive malaria-free areas due to ever increasing movement of people around the world have been documented (316, 317), thus strengthen the notion that malaria is an international threat to health system worldwide. With regards to island countries, importation of malaria between islands such as in Solomon Islands and Vanuatu is a constant threat as these countries pursue island-by-island method of malaria elimination (318). Knowledge of the dynamics of population migration and cross-border malaria transmission is vital for developments of appropriate surveillance and response systems.

1.10.3 *Non-falciparum infections*

In many countries where *P. falciparum* has been successfully eliminated (i.e. countries in Europe and Central Asia), the relative burden to non-falciparum species increases and different challenges arise (123). In areas with both *P. falciparum* and *P. vivax*, the final challenge for elimination will be *P. vivax* (278, 307). P. vivax is less responsive to control interventions than P falciparum infections because of its unique characteristics such has the capability of become dormant in liver stage and often has parasite density lower than the level

of detection by diagnostic tests (319). Similar to *P. vivax*, detection of *P. ovale* infection is also a challenge because of it has a dormant liver stage. In Borneo Malaysia, *P. knowlesi* which has a macaque monkey reservoir can cause severe disease in human (33) and frequently misdiagnosed by microscopy by other species, often *P. malariae* (320). Targeting the reservoirs of infection in monkeys and possibilities of human-to-human transmission is still unclear. Therefore, malaria-eliminating countries may need new strategies to diagnose, treat and interrupt the transmission of these non-*falciparum* species.

1.10.4 *Asymptomatic and sub-microscopic infections*

Most infections for both *P. falciparum* and *P. vivax* in a population are likely to be asymptomatic (321-323), thus provide challenges for identification and treatment of infections in malaria elimination programmes. Typically, passive malaria surveillance will miss these asymptomatic individuals, but they remain infectious to mosquitoes (i.e. gametocyte carriers) (324). Without identification and targeting of this asymptomatic infectious pool, interruption of malaria transmission might not be attainable. A substantial proportion of malaria infections has parasite density lower that the threshold needed either microscopy or RDT detections i.e. sub-microscopic. Relative to all infections, the percentage of sub-microscopic is higher in areas with the recent decline in transmission (325). Although microscopic infections remain the cause of most malaria transmission, sub-microscopic infections have been estimated to result in 20 – 50% of all transmission episodes, particularly in low endemic settings (117, 325).

1.10.5 *Passive and active surveillances*

Passive detection systems are the cornerstone of detection in countries where malaria is controlled by enabling rapid case investigation and appropriate response (326). It relies on the health-seeking behaviour of symptomatic individuals to reporting health facilities and considered as the front line of detection of importation and local transmission. However, public health workers need continual training and guidance to maintain high clinical suspicion of malaria particularly for high-risk groups or patients who recently travelled to malaria-endemic areas (123).

Targeting asymptomatic parasite reservoirs by active case detection are crucial during the elimination phase (327), particularly in high-risk groups such as in hotspots and hotpops (142). It is recommended intervention in low malaria transmission settings (278) and is widely used, yet evidence for its cost-effectiveness is sparse. Methods with standard metrics to address the operational challenges of active case detection that includes high sensitivity of field diagnostic, parasite importation as well as achieving high coverage need to be developed (123, 142). Nevertheless, modified versions of active case detection have also been adopted in malaria-eliminating settings which include reactive and proactive case detection (306). Reactive case detection also called as focal screening and treatment, takes advantage of the fact that parasite carriages are more likely to be spatially or temporally clustered (328). It is triggered when a case is identified by passive case detection. Despite the widespread

operational implications (306), the most efficient radius for screening and standardised guidelines have not been established. For proactive case detection, geographical areas and high-risk groups are screened and treated at the same time without the trigger of the passively identified case for example mass screen and treat campaign (329). For this method to be successful, high coverage of the target population is crucial. To increase coverage of detection, whole communities should be screened, and those not at home during the visit should be recorded and revisited where possible (142).

1.10.6 *Mass drug administration*

Malaria mass drug administration (MDA) is a strategy whereby antimalarial drugs are given to the whole population in a defined geographical area without prior testing for parasitaemia and irrespective of the present of symptoms (330). MDA has been used as the main method for control and elimination of many neglected parasitic diseases (331) and with regard to malaria, it has overcome the issues of missed malaria infections due to insensitive malaria diagnostics (11). In one of MDA success story, high degree of community participation in Vanuatu is the key factor for the successful MDA campaign where weekly MDA of chloroquine, pyrimethamine/sulfadoxine, and primaquine for 9 weeks in 1991, before the onset of the rainy season, eliminated malaria from Aneityum Island (718 inhabitants) (332). Nevertheless, several fundamental challenges need to be addressed for MDA. The optimum combination of drugs, timing and total duration (i.e. rounds per year) of MDA need to be defined (123). Furthermore, the effectiveness of an MDA also depends on the therapeutic adequacy of the drug regimen, the coverage, and the chance of malaria reintroduction (333). The fear of accelerating drug resistance without a proper diagnosis of malaria in MDA is also a concern (98, 290). To reduce the risk of resistance emerging, one idea is to use different classes of antimalarial drugs with transmission-reducing effect (e.g. ACTs and primaquine). Political commitment should be in place in order to support the multiple rounds of MDA over several years with adequate resources of control measures (334). In general, a review by Newby et al. in 13 studies that interrupted transmission for over 6 months after the end of MDA conclude that implementing MDA in higher endemicity settings will reduce transmission, but in combination with other malaria interventions there is a much better chance of interrupting transmission when MDA is implemented in areas of low endemicity (335).

2 RATIONALE FOR THESIS

Islands can provide natural experiment models for complex biological process and a great potential for intervention studies. To our knowledge, Aneityum in Vanuatu is the only island in recent times where the cessation of malaria transmission has been successfully maintained for more than two decades. On Aneityum Island, malaria was eliminated with the combined package of mass drug administration, vector control and high community participation (332). Thus, observations from Aneityum can offer valuable insights into the concerns surrounding the feasibility of malaria elimination on island in high endemic area and the loss of anti-malarial immunity following elimination.

Our study sites are islands in Vanuatu, Oceania and Lake Victoria, Western Kenya. Both *P. falciparum* and *P. vivax* are prevalent in the formers islands, while mainly *P. falciparum* in the latter. Strong negative correlation between within-population genetic diversity of *P. falciparum* and geographic distance from sub-Saharan Africa over Africa, Asian and Oceania. The striking geographic patterns of isolation by distance overserved in *P. falciparum* mirror the ones previously documented in human and support the modern humans were infected prior to their exit out of Africa and carried the parasite along during their colonisation of the new world (336). In this scenario, the islands in Lake Victoria were the starting station, and those islands in Vanuatu were the last one.

In Vanuatu, a key to success of malaria control and elimination has been widespread community engagement and support through the involvement of various parties. Different approaches will be needed as systems and effort move from conditions for control and elimination to those surveillance and containment. New approaches to malaria monitoring systems such as genetic population and modelling transmission might help to monitor the incidence of the disease and improve management of resurgence in malaria elimination programs.

In Kenya, although malaria prevalence in on the downward trend in many areas, it remains high particularly around Lake Victoria basin despite similar tools for malaria control being deployed. There is an ongoing plan to test the feasibility of malaria elimination on the islands in Lake Victoria by applying the intervention strategy of Aneityum Island in Vanuatu. Large cross-sectional and seroepidemiological surveys conducted in this PhD programme will contribute to the establishment of baseline distribution of malaria to guide the implementation of elimination activities.

3 SCOPE OF THE THESIS

3.1 OVERALL AIM OF THE THESIS

To gain insight into malaria epidemiological characteristic and age-dependent immunity of endemic island populations in Kenya and Vanuatu.

3.2 SPECIFIC OBJECTIVES

I. To observe the patterns of gene flow and population genetic structures in malaria species on islands in Vanuatu.

II. To investigate the prevalence and geographical distribution of malaria infections on islands in Kenya.

III. To assessed the heterogeneity in malaria transmission on islands in Kenya by age-dependent antibody responses to *P. falciparum*-specific antigens.

IV. To evaluate the use of antibody response to *Plasmodium* infection and exposure to vector mosquito bites on the effect of vector control on island in Vanuatu.

4 MATERIALS AND METHODS

All material and methods used in this thesis are described in more detailed in Paper I-IV. Below are the general descriptions of the methodologies utilised in all four papers.

4.1 Study location and population

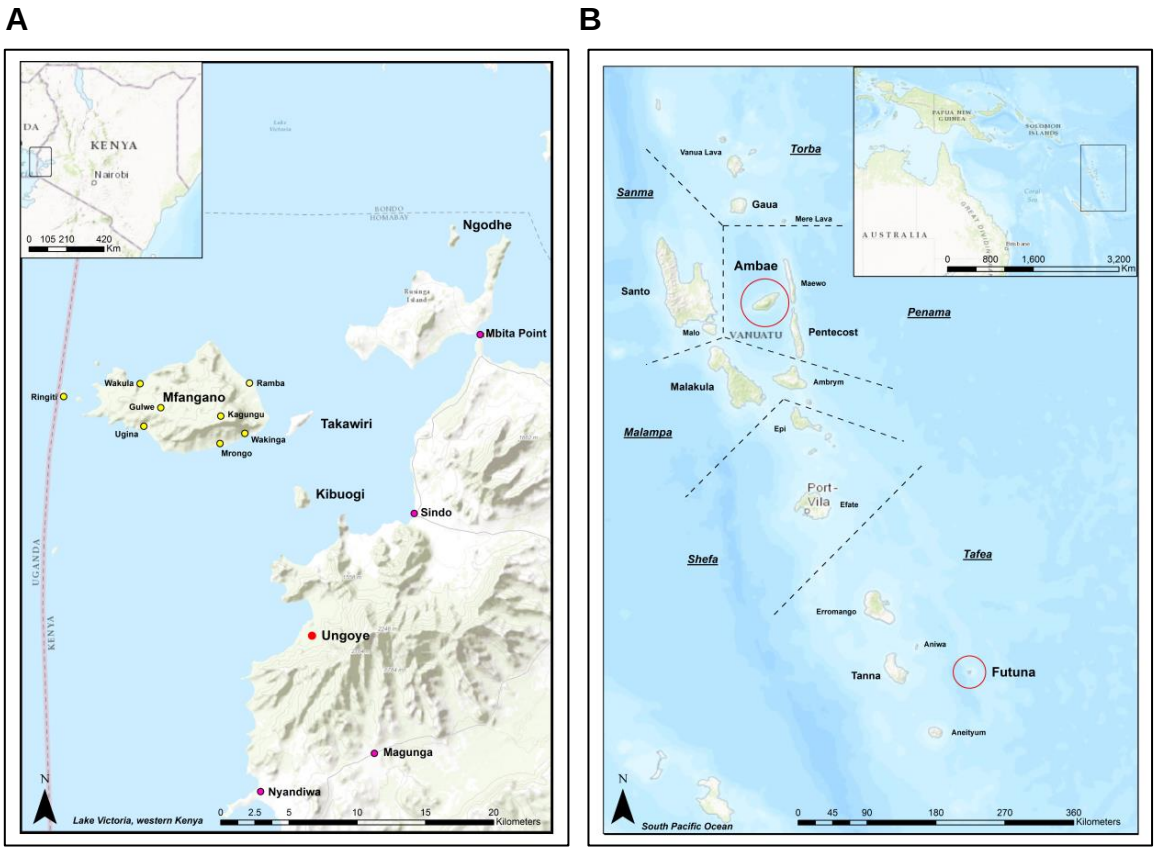

Figure 5 Maps of study settings in Kenya and Vanuatu. (**A**) Homa Bay County in western Kenya; three main areas involved namely mainland coast (Ungoye, red dot), large island (Mfangano Island) and small islands (Takawiri, Kibuogi, and Ngodhe). In Mfangano Island, eight catchment points were sampled (yellow dots) and grouped into three study sites i.e. east coast (Ramba, Wakinga, and Mrongo), highland (Kagungu and Gulwe) and west coast (Wakula, Ugina and Ringiti Island). Purple dots are the nearby town in the County. (**B**) Map of Vanuatu. The names of the six provinces in Vanuatu are capitalized and underlined, and approximate provincial boundaries are indicated by dash lines. Red circles are the two studied islands in Study IV. The maps were created with ArcGIS software version 10.4.

4.1.1 *Kenya*

All studies in Kenya (Study II and Study III) were conducted in Homa Bay County, located on the south shore of Lake Victoria, in western Kenya (**Fig. 5A**). The County covers an area of 3,183.3 km^2 with a population about 963,794 persons. The studies involved one large island (Mfangano) and three small islands (Takawiri, Kibuogi and Ngodhe) in Lake Victoria, and one coastal village (Ungoye) on the mainland. The dominant ethnic group in the study areas is Luo; Dholuo is primarily spoken, as well as the national language of Kiswahili. Detail information about the study settings in Kenya can be found in Paper I.

4.1.2 *Vanuatu*

Vanuatu is an archipelago of more than 80 islands in the South Pacific Ocean. Malaria is endemic on most of the nation's 68 inhabited islands (337). Nevertheless, a comprehensive elimination program initiated in 1991 on Aneityum Island (i.e. the southernmost island in Vanuatu) has achieved malaria elimination with a high degree of commitment from the local in 1999 (332). The country covers an area of 12,189 km^2 with a population about 243,304 persons and divided into six provinces. Five islands from five difference provinces were involved in Study I namely Gaua, Santo, Pentecost, Malakula and Tanna. Whereas, Study IV was involved two islands i.e. Ambae and Futuna (**Fig. 5B**). The inhabitants of Vanuatu (*Ni-Vanuatu*) are primarily of Melanesian descent and speak the official language of Bislama, although more than 100 indigenous languages are still actively spoken.

4.2 Sampling strategy

Island and village leaders were sensitised to study by trained field health workers and together provided information to community members at community meetings. Convenience sampling strategy was used in all surveys, both in Kenya and Vanuatu. In Kenya, five cross-sectional surveys were conducted between 2012 and 2014. Residents of the island or mainland village were asked to come to selected survey points such as beach management unit (BMU) community meeting hall, school or marketplace. In Vanuatu, cross-sectional surveys were conducted in different years; 1996 to 2002 (islands in Study I) and 2003 to 2011 (islands in Study IV). Islanders were asked to come to a central meeting point such as school or church, and families were invited from surrounding villages to be part of the survey.

4.3 Clinical assessments

Axillary body temperature was determined using a digital thermometer. Fever was defined as a temperature exceeding 37.5^{o}C. Haemoglobin level was measured with the HemoCue Hb 201 analyzer automated device. A measurement below 11 g/dL was classified as anaemic. Children aged 12 years and below were examined for enlarged spleen according to Hackett's method, regardless of fever or malaria status.

4.4 Blood collection

A blood sample was obtained by finger prick for thick and thin blood smears (5 ul each), microcuvette for haemoglobin measurement, and two spots of blood (70 µl each) were collected from 75 mm Micro-hematocrit capillary tube on Whatman ET31 Chr filter paper. Blood spots on filter paper were air-dried and stored in plastic bags.

4.5 Ethical considerations

All field studies were conducted in accordance with the Declaration of Helsinki (338). Study participants were informed by local interpreters of the purpose and procedures of the study. Informed consent was obtained from all the study participants, or from parent/guardians of children, prior to study enrolment. Ethical approvals were obtained from the Kenyatta

National Hospital/University of Nairobi-Ethics and Research Committee in Kenya, Ministry of Health in Vanuatu, and by the Committee on the Ethics of Human Research of Karolinska Institutet in Sweden.

4.6 Laboratory methods

4.6.1 *Blood slide microscopy*

Thin and thick blood smears were prepared on site, stored in slide boxes and transported daily to the main laboratory. Thin blood smears were stained with methanol, and all smears were stained with 3% Giemsa solution for 30 min and examined under oil emersion (1000x magnification) by local experienced microscopists. Blood smears were defined as negative if no parasites were found after examining with 100 high power microscopy fields. For all positives samples, malaria species were identified, and asexual parasites forms were counted against 100 (Vanuatu) or 200 (Kenya) leukocytes. *P. falciparum* gametocytes were separately recorded. Parasite density was estimated from parasite counts, assuming that there were 8,000 leukocytes per µL of blood. All slides were independently re-examined by two experienced microscopists blinded to first microscopy reading results. Discrepancies between the two readings were resolved by a third experienced microscopist.

4.6.2 *Polymerase chain reaction (PCR)*

The nested PCR protocol to detect *Plasmodium* DNA was used as described previously (229). Briefly, total DNA was extracted from three discs (6 mm) of blood spot using commercialised DNA extraction kit and was eluted in 150 uL of the provided buffer. A nested PCR using primers targeting the *Plasmodium* mitochondrial cytochrome c oxidase III (*cox3*) gene was used that include genus-specific (one set) and species-specific (four sets) primers. The primary PCR was carried out in 20 µL reaction, and the amplification product was analysed by 0.8% agarose gel electrophoresis, with an expected band of 940 bp. The secondary PCR was performed individually for each of the four *Plasmodium* species and was carried out in a 20 µL reaction containing 2 µL of the diluted primary PCR product. PCR products were analysed by 2% agarose gel electrophoresis, with expected band in the range of 87 and 233 bp.

4.6.3 *Genotyping*

Genotyping analysis for both *msp1* and *csp* genes have been described previously for *P. falciparum* (339, 340) and *P. vivax* (341, 342). Briefly, a subset of microscopy-positive samples from each site in Study 1 was randomly selected, extracted for genomic DNA and amplified by PCR. Direct sequencing of the PCR product was performed using commercialized sequencing kit where sequence primers were designed for coverage of target regions in both directions; 25 primers for *msp1* (chromosome 9) and 7 primers for *csp* (chromosome 3). DNA sequences were aligned using Clustal X software.

4.6.4 *Enzyme-linked immunosorbent assay (ELISA)*

Antibody response to malaria in serum samples against crude schizont extract (SE), recombinant proteins, and peptide were measured as described previously (343). A 3 mm disc was punched from each blood spot and serum was eluted in reconstitution buffer to the equivalent of 1/200 dilution (equal to 2.1 µL of blood). All sera from Kenya samples were tested for IgG antibodies to two recombinant blood-stages *P. falciparum* antigens namely *Pf*AMA-1, *Pf*MSP-1_{19}, and $NANP_5$ repeat peptide of CSP. Whereas, all sera from Vanuatu samples were tested for antibodies to three *P. falciparum* (*Pf*SE, *Pf*AMA-1, and *Pf*MSP-1_{19}) and three *P. vivax* (*Pv*SE, *Pv*AMA-1, and *Pv*MSP-1_{19}) antigens, as well as gSG6. Briefly, antigens were coated on ELISA plates and incubated overnight at 4^oC. Plates were washed three times and the blocked with 1% skimmed milk solution for 3 h. After washing, 50 µl of reconstituted blood spot solution were added in duplicate and incubated overnight at 4^oC. A positive control consisting of a pool of hyper-immune sera was included on each plate. The plates were washed five times and 50 µl of horse-radish peroxidase (HRP)-conjugated rabbit anti-human IgG antibody were added to all wells and incubated for 3 h. After further series of washes, 100 µl of substrate solution were added, and reactions were stopped after 15 min. The optical density (OD) was measured at 450 nm in an ELISA reader. The duplicate sample OD values were examined, and values that differed more than 20% (ODs >0.2 which differ by more than a factor of 1.5) were dropped, and possibly repeated.

4.7 **Statistical analyses**

Statistical analyses were performed using STATA/SE version 13.1 and GraphPad Prism version 5.03. Differences in proportions were tested two-sided using the Chi-squared test or the Fisher's exact test together with the corresponding exact binomial 95% confidence intervals (95% CIs). The average enlarged spleen (AES) index was calculated as the sum of the number of children in each spleen size class multiplied by the class number (0 – 4), divided by the total number of palpable spleen. For serological data, duplicate OD values were corrected by subtracting the appropriate blank value and averaged. Averaged OD values were transformed into titres against a standard control curve generated from hyperimmune sera to normalised between plates as previously described to adjust background reactivity (343). The titres of antibody responses were estimated using the equation dilution/[maximum OD/(OD test serum – minimum OD) – 1], where results were presented as median titre and together with interquartile range (IQR). Differences in antibody responses (i.e. continues data) in were assessed either by age-adjusted linear regression or the Mann-Whitney U test or the Kruskall-Wallis with Dunn's multiple comparisons *post-hoc* tests. The finite mixture model to determine seropositivity and the reverse catalytic model for age-seroprevalence data are described in detail in section 4.5. Univariate and multivariate logistic regressions were used to identify factors associated with *Plasmodium* infections or seropositivity. Statistical significance was determined as $P < 0.05$ in all studies.

4.8 **Modelling**

Seropositivity was determined by fitting a finite mixture model to normalised OD values assuming two Gaussian distributions, one for seronegative individuals and another for seropositive individuals as described previously (126). The mean OD values plus three standard deviations associated with the seronegative groups was used as the cut-off value for seropositivity. Finite mixture models were fitted to the dataset by STATA/SE's *fmm* command. For example:

Syntax: fmm depvar [*indepvars*] [if] [in] [weight], component (#) mixtureof (*density*)
Command: fmm 'normOD', component (2) mix (normal)

Seroprevalence data was stratified into yearly age groups and analysed using a reverse catalytic modelling approach under a binomial sampling assumption, as described elsewhere (125, 127). This method provides estimates of the mean annual rates of conversion to seropositive (i.e. seroconversion rate, SCR [λ]) and reversion to seronegative (seroreversion rate, SRR [ρ]) status, averaged over the population. The common SRR was estimated from the model using maximum likelihood as described previously (127). Reverse catalytic models were fitted to the dataset by STATA/SE *revcat* command. For example:

Syntax: revcat *depvar agevar* [*datevar*] [weight] [if] [in] [, minage [#) change (*numlist*) age smooth rr lambda (*varlist*) init (*matname*) level (#) *maximize_option*]
Command: revcat 'anypos' 'ageyears' if 'ageyears'>1 & 'island'= =1 & 'survey'= =1, plot
Fixed SRR
Command: revcat 'anypos' 'ageyears', lambda (island)
constraint 1 [log_rho]_b[_cons]= 'common rho'
revcat 'anypos' 'ageyears' if 'island'= =1 & 'survey'= =1, constraint (1) pred (anypos1)
revcat 'anypos' 'ageyears' if 'island'= =1 & 'survey'= =2, constraint (1) pred (anypos2)
twoway (line anypos1 anypos2 ageyears, sort) if 'ageyears'<=80

The serological derived EIR was estimated using *Pf*AMA-1 and *Pf*MSP-1_{19} seroconversion rates and a calibration curve derived from determined values as described previously (125). The log-log regression equations are as follows:

*Pf*AMA-1: 10e((log10(SCR) + 1.1058) / 0.3838)
*Pf*MSP-1_{19}: 10e((log10(SCR) + 1.507) / 0.4745)

5 RESULTS AND DISCUSSION

5.1 PAPER I

***Plasmodium vivax* and *Plasmodium falciparum* at the crossroads to exchange among islands in Vanuatu: Implications for malaria elimination strategies**

Chim W Chan, Naoko Sakihama, Shin-Ichiro Tachibana, Zulkarnain Md Idris, J Koji Lum, Kazuyuki Tanabe, Akira Kaneko

As mentioned in the introduction, island provides a unique opportunity to interrupt malaria transmission due to the relative geographical isolation and confined population. In Vanuatu, a nation with 68 inhabited islands, malaria transmission has decreased since the 1990s as a result of malaria control measures and general improvement in the health of the community (318). Identifying routes of parasite transmission and gene flow by population genetic study may provide information particularly in understanding the sustainability of some of the approaches taken in malaria control and elimination. The aim of this study was to compared the patterns of gene flows and population genetics structures in *P. falciparum* and *P. vivax* on islands in Vanuatu. *P. falciparum* and *P. vivax* isolates were collected during malariometric surveys conducted at seven sited on five islands (Gaua, Santo, Pentecost, Malakula, and Tanna) from five provinces in Vanuatu between 1996 and 2002. Molecular analyses involved genotyping and/or sequencing of *msp1* and *csp* genes of both parasite species. Specific calculations were used to examined the haplotype diversity and genetic variations within populations and studied islands.

We found that *P. vivax* was more genetically diverse than *P. falciparum* in Vanuatu. The observed difference in genetic diversity may be partially due to the structural difference in orthologous gene between these two species (344, 345). High frequencies of *P. vivax* multiple-genotype infections than *P. falciparum* were also observed in the current study. It has been shown that high frequencies of multiple-genotype infections facilitate meiotic recombination in the *Anopheles* mosquito vectors, leading to the generation of novel genotypes (346).

The extent of parasite genetic diversity in Vanuatu was further analysed to determined pattern of gene flow between *P. falciparum* and *P. vivax* in the population. Our finding revealed that gene flow among *P. falciparum* in seven study sites was restricted, in contrast to the higher degree of gene flow among *P. vivax* populations. Consistent with our observation in seven-site analyses, gene flow among *P. falciparum* populations was very minimal but more widespread among *P. vivax* populations on different islands. The higher degree of inter-island gene flow in *P. vivax* populations might be expedited by its ability to form dormant hypnozoites together with rapid development and emergence of gametocytes. Furthermore, unlike those with blood-stage parasites, P. *vivax*-hypnozoite carriers are typically asymptomatic and might, therefore, be less averse to long-distance travel (e.g. between

islands). The shorter period of extrinsic development of *P. vivax* compared to *P. falciparum* may also facilitate the *P. vivax* transmission in the area.

5.2 PAPER II

High and heterogeneous prevalence of asymptomatic and sub-microscopic malaria infections on islands in Lake Victoria, Kenya

Zulkarnain Md Idris, Chim W Chan, James Kongere, Jesse Gitaka, John Logedi, Ahmeddin Omar, Charles Obonyo, Beatrice Kemunto Machini, Rie Isozumi, Isao Teramoto, Masatsugu Kimura, Akira Kaneko

There is an ongoing plan to test the feasibility of malaria elimination on the island in Lake Victoria by applying the interventions strategy of Vanuatu. It is crucial to understand the extent of malaria infections in Lake Victoria before similar tools for malaria control and elimination being deployed. For this, five cross-sectional surveys were conducted between 2012 and 2014 in Lake Victoria basin covering a large population of one large island (Mfangano), three small islands (Ngodhe, Takawiri, Kibuogi) and one coastal village (Ungoye) on the mainland. Individuals provided a finger-pricked blood samples to assess malaria infection by microscopy, RDT and PCR. Physical assessments including body temperature, anaemia, and splenomegaly test were also performed in all surveys.

We found that malaria prevalence in Lake Victoria was high (mesoendemic) and varied among the studied areas. By PCR, parasite prevalence was highest in Ungoye (54.5 – 79.3%), moderate in Mfangano (29.7 – 55.5%), and lowest on small islands (8.2 – 26.5%). This varying observation may due to the relative abundance of vector species on the island and mainland (347) areas as well as human activities such as man-made holes and roadside ditches that contribute to the availability of vector larval habitats found in the late rainy season in Lake Victoria (348). We also found that intra-island variability in malaria prevalence in eight catchment areas in Mfangano Island indicating micro-geographical factor may also lead to the variation in malaria endemicity.

We then stratified malaria prevalence into categorical depending on the exact malaria species detected by both microscopy and PCR. As expected, *P. falciparum* was the predominant species, consistent with previous studies in other part of Lake Victoria (349-351). *P. malariae* and *P. ovale* were less common, and no *P. vivax* infections were observed. With regard to mixed-species infections, the majority of *P. malariae* infections were double co-infections with *P. falciparum* (*n* = 760 by PCR). Triple co-infections accounted for 22.3% (95%CI: 19.9 – 24.8) of all mixed-species infections.

When malaria prevalence and parasitaemia were stratified into age group, we found that both outcomes were strongly age-dependent. Parasitaemia was highest among children aged ≤5 years and decreased with age, but in contrast, malaria prevalence was highest in older children (6 – 10 and/or 11 – 15 groups) and decreasing in adolescents and adults. Similar to parasitaemia, the proportion of gametocytaemic malaria carriers was highest in children aged

≤5 years and decreasing significantly with age. We expect the proportion of gametocyte in children to be much higher than the reported prevalence due to the low sensitivity of microscopy for detecting all relevant densities of gametocytes (352). Age was also a contributing factor for *Plasmodium* spp. infections in the study areas. *P. falciparum*-specific prevalence was highest in the 11 – 15 group, whereas *P. malariae*- and *P. ovale*-specific prevalence highest in 6 – 10 group.

One of the highlights of this study is the uncovering of the breadth of asymptomatic malaria in the Lake Victoria basin. We found that the vast majority (89.7% by microscopy and 91.8% by PCR) of malaria infections in the study areas were asymptomatic i.e. malaria positive but not accompanied by febrile symptoms. Among these asymptomatic individuals, 10.2% (95% CI: 8.8 – 11.6) of them were also carrying gametocytes, and they may play a major role as a source for malaria transmission (11, 353, 354). Furthermore, most asymptomatic infections were observed in children 15 years and under and were not uncommon in the adult population (**Fig. 6A**). As potential gametocytes carriers, this asymptomatic adult population represents an important reservoir for malaria transmission.

By using sensitive molecular methods (i.e. PCR), we have the advantage to assess sub-microscopic infections (PCR positive but microscopy negative) in the population to further understand the true infection burden in the study areas. Our observation found that the proportion of sub-microscopic infections increase with age from 40.1% (95% CI: 36.9 – 43.4) in children ≤5 years to 75.5% (95% CI: 69.7 – 80.1) in adult ≥30 years. This observation was

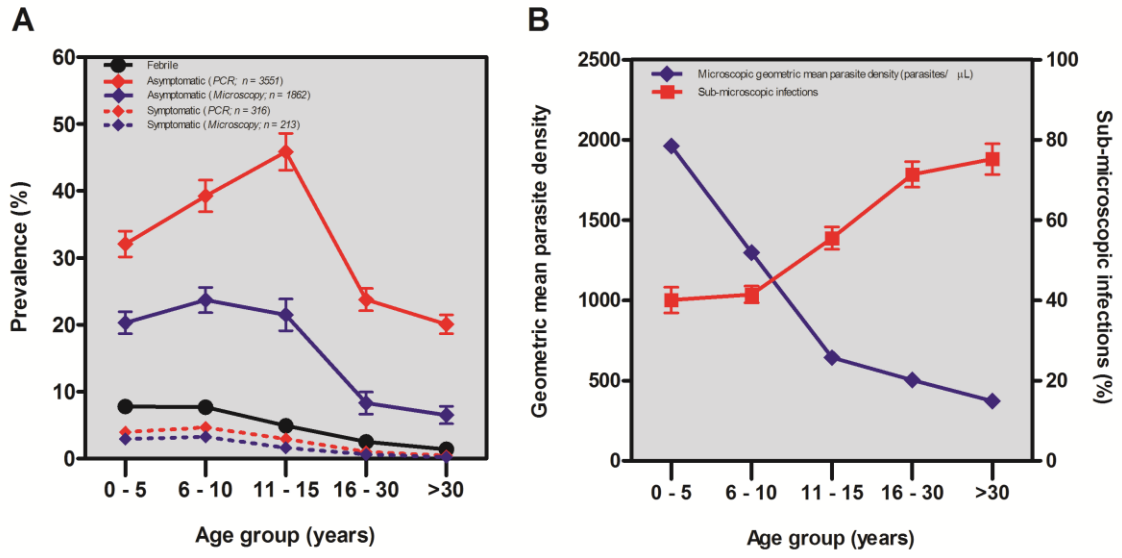

Figure 6 (A) Age trends of febrile illness, asymptomatic and symptomatic malaria infection by microscopy and PCR. **(B)** The relationship between the proportion of sub-microscopic and parasite density among infected individuals. Error bars represent 95% confidence intervals.

in contrast by the decreases in parasite prevalence as well as parasite densities in the studied population (**Fig. 6B**). Inverse correlation between the proportion of sub-microscopic infections and in both parasite prevalence and density has been well established in previous

meta-analysis study (325). Furthermore, the majority of sub-microscopic individuals were also asymptomatic (94%, 95% CI: 92.8 – 95.0). This pool of sub-microscopic asymptomatic individuals may yet be another factor of malaria transmission in Lake Victoria, although the minimum parasite density necessary for transmission is unknown.

5.3 PAPER III

Naturally acquired antibody response to *Plasmodium falciparum* describes heterogeneity in transmission on islands in Lake Victoria

Zulkarnain Md Idris, Chim W Chan, James Kongere, Tom Hall, John Logedi, Jesse Gitaka, Chris Drakeley, Akira Kaneko

Based on the observation made in Paper II, we decided to study with more attention on serological aspects of *P. falciparum* infections; the predominant species in Lake Victoria. The main aim of this study was to use age-dependent antibody responses to *P. falciparum* AMA-1, MSP-1_{19} and CSP to look for evidence of heterogeneity in transmission intensity in an area of high endemicity in Lake Victoria. In recent years, antibody response to one or more *P. falciparum*-specific antigens have been explored as alternative means to estimate malaria transmission intensity (125, 127). In this study, malaria prevalence data from previously conducted surveys in 2012 were compiled. Antibody responses to aforementioned antigens were measured in 4,112 samples; data were normalised and expressed as seroprevalence and SCR using a mathematical model.

Our finding demonstrates a clear relation of serological outcomes for *P. falciparum*-merozoite antigens (AMA-1 and MSP-1_{19}) with parasite prevalence and serology-derived aEIR in the heterogeneity of malaria transmission in Lake Victoria. For example, across the study sites, SCRs correlated significantly with parasite prevalence by PCR with R^2: 0.96 and R^2: 0.94 for AMA-1 and MSP-1_{19}, respectively. Furthermore, the aEIR equivalents estimated from merozoite antigens corresponded well with different levels of parasite prevalence by PCR: 7 – 12 ib/p/yr Ungoye and Mfangano (40.8 – 57.3%) and 2 – 4 ib/p/yr in small islands (11.3 – 16.2%). These results were in concordant with previous observations on the utility of seroprevalence to *P. falciparum*-merozoite antigens and the annual rate of SCR as a reliable estimate of the level of transmission (126, 128, 165, 168, 355, 356). Nevertheless, antibody responses to CSP did not follow the same pattern as in merozoite antigens. The reason for this contrary pattern may lie on the dynamic of CSP and merozoite antigens subjecting on the stage at which they are expressed. The availability of CSP to the immune system is of much shorter duration and relatively short life expectancy in the blood (357), than is that of merozoite antigens which are produced continuously in large number.

We further assessed the relationship between the intensity of malaria exposure and development of antibody response over time. We observed an early rate of antibody acquisition, with relative prominent responses among children, was seen towards particular antigens, such as AMA-1, and to lesser extend toward MSP-1_{19} (**Fig. 7**). Our data suggested that three patterns of antibody acquisition occurred with age in our study sites: rapid (AMA-

1; >75% seropositive by 20 years of age), moderate (MSP-1_{19}; >40%), and slow (CSP; <20%). Differential recognition of these antigens by the immune system or differing antibody-specific half-lives may be contributing to the variations in antibody response patterns. Nevertheless, we believed that antibody responses directed towards these early response-type antigens might appear to provide a more sensitive marker of recent previous exposure among young children.

To determine factors that may contribute to *P. falciparum* seropositivity in our study sites, we used multivariate logistic analysis adjusting for correlation between observations from the same explanatory variables. Except for age, we found that the associations between measured antibody response to *P. falciparum* antigens and the risk of malaria were inconsistent. For example, only antibody responses to AMA-1 were negatively associated with anaemia in Mfangano, Takawiri and Kibuogi. In contrast, when analysis was restricted to children ≤12 years, antibody responses to MSP-1_{19} were positively associated with anaemia (Ungoye and Mfangano) and splenomegaly (all study sites). Potential reasons for these risk inconsistencies in our study areas include differences in the intensity and stability of transmission, allelic variations of specific antigens and IgG subclass switching (51).

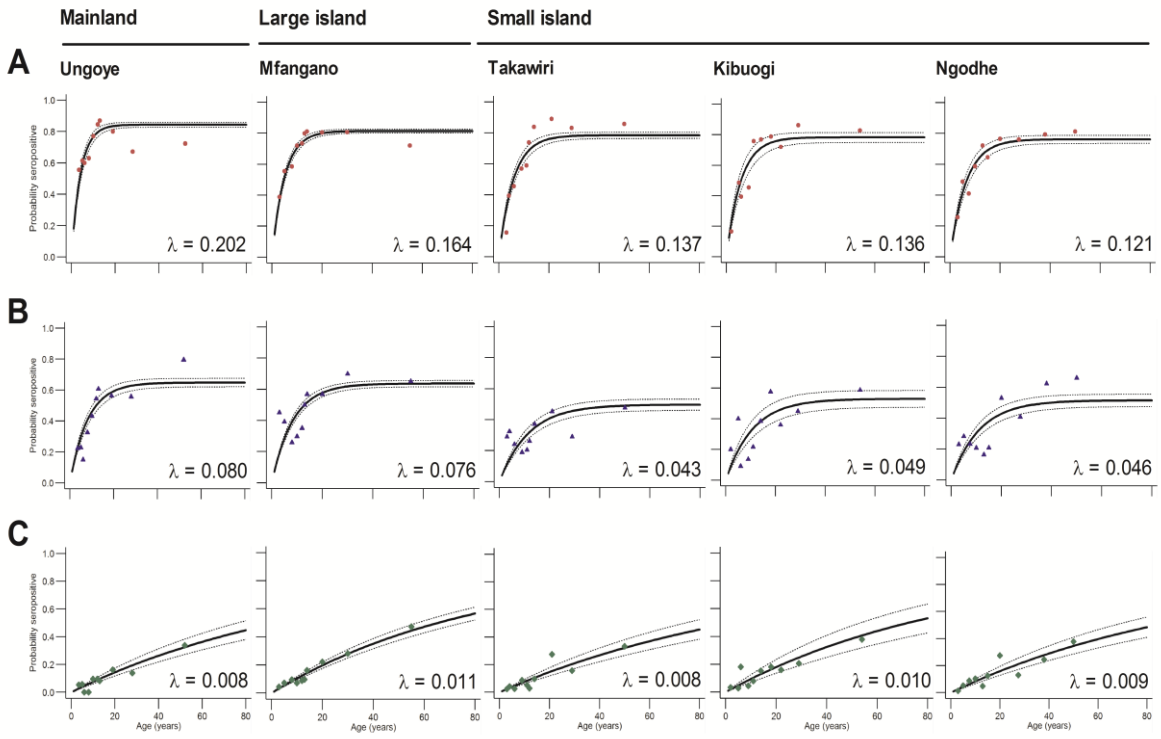

Figure 7 Annual probability of seroconversion rate for *P.falciparum*-specific antigen by age in each area. Maximum-likelihood fits from reversible catalytic equilibrium model from each setting are shown. λ, the area-specific annual rate of seroconversion. **(A)** AMA-1, **(B)** MSP-1_{19}, and **(C)** CSP. The model was constrained to fit a single value for the annual probability of common seroreversion rate (ρ). Point indicated observed seroprevalence and solid lines show model-predicted seroprevalence. Broken lines are 96% confidence intervals.

5.4 PAPER IV

Serological measures to assess the efficacy of malaria control programme on Ambae Island, Vanuatu

Zulkarnain Md Idris, Chim W Chan, Mubasher Mohammed, Morris Kalkoa, George Taleo, Klara Junker, Bruno Arcà, Chris Drakeley, Akira Kaneko

As mentioned above in Paper III, we found that malaria transmission intensity can distinctly be estimated by cumulative malaria exposure in a population. We hypothesised that modelling changes in age seroprevalence could be used to evaluate specific interventions in a particular area. The aim of this study was to assess the naturally acquired antibody responses to two malaria species namely *P. falciparum* and *P. vivax* as well as exposure to vector mosquito bites, on the effect of vector control intervention i.e. ITN. Filter-paper blood samples from 2003 and 2007 in Ambae Island were used in this study. At the time of the surveys, parasite prevalence was solely determined by microscopy. Antibody responses to *P. falciparum* (*Pf*SE, *Pf*MSP-1_{19}, and *Pf*AMA-1), *P. vivax* (*Pv*SE, *Pv*MSP-1_{19}, and *Pv*AMA-1), and *An. gambiae* (gSG6) antigens were measured in both surveys. Age-specific seroprevalence was analysed as previously mentioned in Paper III.

We observed a significant decrease in parasite prevalence together with significant decreases in seropositivity to most antigens tested between 2003 and 2007 (**Fig. 8**). This observation suggested that reinforced vector control intervention played a major role in the reduction of malaria transmission on the island. Based on the seroprevalence profiles, we found that serological responses generally increased with age and malaria transmission intensity decreased dramatically between the two years. SCRs estimated for *P. falciparum* antigens were higher than ones for *P. vivax* antigens, reflecting the more intense transmission and the predominance of the former species on the island, particularly in 2003. Changes in *P. falciparum* transmission were readily detected, and the estimated decreases in transmission intensity were consistent across the three antigens tested. Whereas, the decreases in *P. vivax* transmission over the two survey years were more variables across the antigens.

We demonstrated for the first time the use of gSG6 antigen to evaluate exposure to *Anopheles* vector bites in the Pacific setting. Antibody responses to gSG6 antigen have been previously described to be a reliable indicator of human Afrotropical malaria vectors (191, 200, 205, 358, 359). We found that the exposure to *Anopheles* mosquito bites was greatly reduced: 39% (95% CI: 32.6 – 45.6) in 2003 to 0.7% (95% CI: 0.1 – 2.5) in 2007 (**Fig. 8B**). This reduction in vector exposure suggested the positive impact of ITN distribution after 2003 on vector population density and/or changes in vector behaviour e.g. preference and aggressiveness towards humans. Furthermore, as the standard aEIR becomes harder to determine area accurately at low transmission settings (197), serological tools to measure exposure to vectors may be made more relevant.

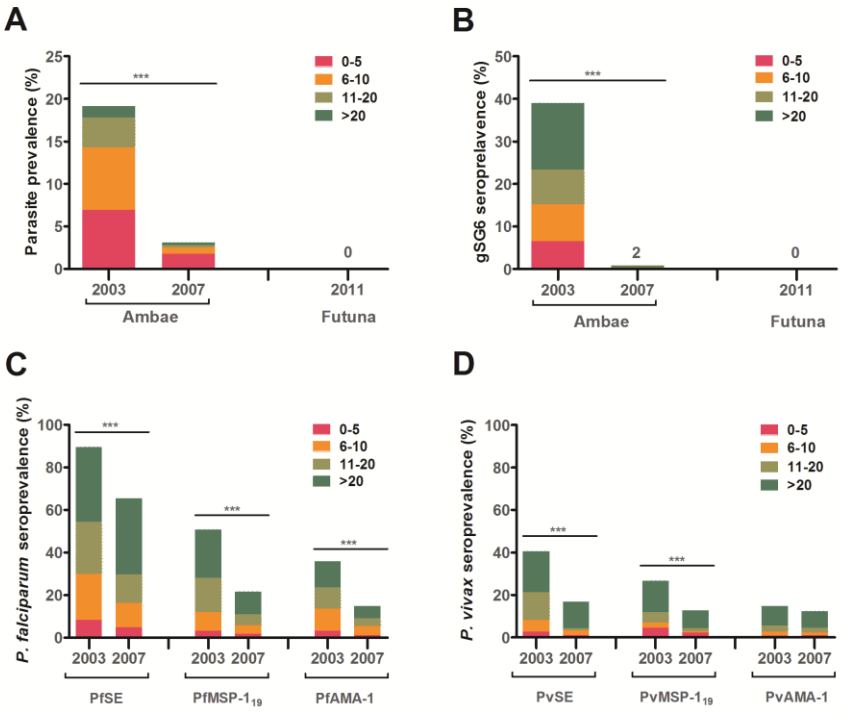

Figure 8 Age-specific parasite prevalence by microscopy and antibody response in Ambae Island and Futuna Island. Graphs are divided by year of sampling and in four age groups for **(A)** Microscopy, **(B)** *An. gambiae* salivary gland gSG6 antigen, **(C)** *P. falciparum* antigens and, **(D)** *P. vivax* antigens. Serological analyses for *P. falciparum* and *P. vivax* antigens were performed for samples from Ambae Island only. Numbers above bars show the numbers of positive individuals.

Some limitation of this study include:

- The relatively small numbers of individuals sampled for each year may lead to poor estimation precision of current SCR and limit the likelihood of identifying significant changes point in malaria transmission over time.
- The convenience sampling method used can result in an overestimation of malaria incidence in the area.
- While the recombinant *An. gambiae* gSG6 antigen could be used to measure exposure to *An. farauti* bites in Vanuatu, the limited conservation between SG6 protein from the two species likely result in lower detection sensitivity.
- Parasite prevalence was determined solely by microscopy which likely to underestimated the true prevalence in the low endemic area. Incorporating molecular-based detection method to assess sub-microscopic infections will be critical in monitoring malaria control measures.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

A few concluding aspects and future directions are discussed below based on the paper included in the thesis:

I. In Vanuatu, both *P. falciparum* and *P. vivax* showed different levels of genetic structure as well as different patterns of gene flow and population structures. *P. vivax* showed a high level of genetic diversity and a greater degree of inter-island gene flow than *P. falciparum*, suggesting the more centrally integrated measures to control *P. vivax* movement across islands in Vanuatu. In co-endemic areas, *P. vivax* tends to be more resilient than *P. falciparum* due to its low-density parasitaemia and unique biological features of hypnozoite, thus able to propagate further transmission. Hypnozoite can be destroyed by primaquine but the drug must be given as a 14 days treatment course, which might deter many patients from complying and without adequate G6PD test can cause severe hemolysis. While the elimination strategy in Vanuatu may adequate for *P. falciparum*, elimination programmes need increasingly new diagnostic and therapeutic tools that are adapted to particular properties of the *P. vivax* species. Reducing malaria transmission further to achieve malaria elimination in Vanuatu requires new strategies including reorientation of the current malaria control activities with greater need of financial supports.

II. Malariometric studies revealed that areas in Lake Victoria had high local heterogeneity in malaria prevalence; lowest in small islands, moderate in large island, and highest in the mainland. Importantly, high proportion of asymptomatic and sub-microscopic infections in the area justified the plan on future implementation of the MDA packages. Also, high asymptomatic individuals and low detection of gametocyte carriers by microscopy warrant the integration of molecular detection tools into all epidemiological studies to better understanding local malaria epidemiology. Effective and efficient malaria interventions require a good understanding of transmission dynamic in time and space. Refine spatial and temporal analyses to identify geospatial clustering patterns must be intensified for targeting interventions. In the relatively mobile community in Lake Victoria, the interplay between malaria importation and subsequent local transmission required strategic planning to understand the relationship between vulnerability, receptivity and local malaria dynamics for the future of malaria elimination efforts in Lake Victoria.

III. Naturally acquired immunity against *P. falciparum* malaria antigens can be a proxy measure of malaria transmission in a high endemic area in Lake Victoria. SCR of different immunogenicity of blood-stage falciparum antigens showed a consistent correlation with infection prevalence and able to marked distinct variation in transmission intensity. Recent changes in exposure and to smaller and more short-term trends in high transmission settings are possibly due to the quick acquisition of

antibody responses to the long half-life of many other blood-stage antigens. Longitudinal investigation to study more in depth the repertoire of immune responses to various antigens in populations and how it is related to protection against infection could be employed in particular if involved changes in transmission after malaria elimination.

IV. The implementation of vector control of ITNs was clearly effective in suppressing malaria transmission on Ambae Island. Amid this success, both *P. falciparum* and *P. vivax* are still lingered on the island, although in very low prevalence with a slight predominant of the former species. In the context of malaria control, sustaining high coverage of vector control interventions is critical in reaching malaria elimination in Vanuatu. Existing tools need to be optimised and should be adapted to human and vector behaviour in future intervention efforts. The only malaria vector in Vanuatu, *An. farauti s.s* mosquitoes are known to be exhibit early and outdoor biting behaviour, thus probably less liable to conventional interventions. Development and implementation of integrated vector control interventions targeting outdoor and early biting transmission before sleeping time is of high priority to protect vulnerable populations.

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