Investigating transmission of malaria parasites to *Anopheles farauti* mosquitoes in Papua New Guinea

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This thesis is presented for the degree of Doctor of Philosophy at the James Cook University, College of Public Health and Veterinary Sciences

DECLARATION

I, Lincoln Timinao, declare that the work presented in this thesis is of my own account for my PhD research and contains work, which has not been previously submitted for a degree at any tertiary education institution. Where assistance has been provided by others, I have declared this in the statement of candidature contribution.

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Signature

11.11.2022

Date

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Abstract

Malaria can be transmitted by both symptomatic and asymptomatic individuals to Anopheles mosquitoes. Human-to-mosquito transmission represents a bottleneck where the malaria parasite numbers drop from billions in infected individuals to anything as low as 2 gametocytes, to infect the mosquito. This bottleneck is a target of transmission blocking vaccines and antimalarials. The efficacy of these transmission blocking vaccines (TBV) and antimalarials can be tested using membrane feeding assays.

The main aim of my PhD thesis was to establish the direct membrane feeding assay (DMFA) and the direct skin feeding assay (DSF) to investigate the infectiousness of malaria parasites in symptomatic and asymptomatic individuals to *Anopheles farauti* mosquitoes in Papua New Guinea (PNG), with a focus on *Plasmodium vivax.*

I optimized the assay conditions in order to maximize the *An farauti* blood feeding rate. I then exposed *An farauti* to infected blood from symptomatic patients. I then adapted a qPCR assay for detecting blood stage parasites on mosquito stage parasites and compared two DNA extraction methods: heating and the conventional DNA extraction methods prior to qPCR detection of the parasites. I also performed serum replacement experiments, where the plasma of an infected blood sample was replaced with malaria naive serum prior to use in DMFA. Finally, I performed DSF on healthy individuals within a community to understand the proportion of asymptomatic individuals who can still transmit malaria parasites.

Varying the conditions of the different parameters associated with DMFAs increased the *An. farauti* feeding rate from 50 % to 85 %. The optimized conditions were to use 50 mosquitoes (with an approximate density per cup of 1 mosquito/6.8 cm^2) of 3-5 days old overnight starved mosquitoes per cup, before exposing to 250 – 500 µL of infected blood for 20 minutes using a Baudruche membrane in the light while maintaining the water bath temperature at 37 °C. When infecting the *An. farauti* mosquitoes with malaria parasites I observed a significantly higher infection rate with *P. vivax* asexual stages (33.1 %) and this increased to 58.3 % when gametocytes were detected by microscopy as compared to *P. falciparum*. I was able to successfully detect single oocysts and low sporozoite numbers using qPCR. There was no significant difference between the heating and conventional DNA extraction methods on single oocysts and more than one oocysts. However, heating performed significantly better than the conventional DNA extraction method

when detecting sporozoites using qPCR. Heating the mosquito gut or the head and thorax region of the mosquitoes instead of performing conventional DNA extraction significantly reduced the time to process samples. I further observed evidence of immune enhancing and immune suppressing effects in patients when performing serum replacement experiments. When performing DSF within an endemic community I observed that 31 % of the individuals screened from the community had malaria either by rapid diagnostic tests or qPCR with more than half of the infected individuals being asymptomatic. Of these, 5 % were infectious to the *An. farauti* colony mosquitoes.

In summary, DMFA successfully infected *An. farauti* colony mosquitoes with *P. vivax* parasites. This is important as it allows us to test potential transmission blocking vaccines and antimalarials as currently it is difficult to culture *P. vivax* parasites in the lab. I have also been able to show that by heating the mosquito samples prior to performing qPCR, yields similar results as when doing the conventional DNA extraction prior to performing qPCR. This will greatly reduce the time in processing the samples and also reduce the cost in processing the samples. I also adapted a qPCR assay to detect mosquito stage parasites which further allows the detection of parasites that would otherwise be missed by microscopy diagnosis following membrane feeding. I also established that variable immune responses can be expected in individuals when performing serum replacement experiments and this will be important when testing potential transmission blocking vaccines and antimalarials. I also confirmed that asymptomatic individuals were infectious and are able to transmit malaria, thus highlighting the importance of the asymptomatic reservoir of infections in maintaining malaria transmission**.**

Statement of Candidature Contribution

Nature of Assistance	**Contribution** ***(specify only those contributions that are applicable to your thesis; the list below is not exhaustive)***	**Names, Titles *(if relevant)* and Affiliations of Co-Contributors**
Intellectual support	Proposal writing Data Analysis & statistical support Editorial assistance	I wrote up my proposal and was guided by my Primary Advisor, Dr. Stephan Karl. I did the initial data analysis and was supported by Dr. Stephan Karl. Editorial assistance was provided by Dr. Stephan Karl and Prof. Tom Burkot for the Thesis but the editorials for the publications were provided by the co-authors
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Publications (3)

1 Timinao L, Vinit R, Katusele M, Schofield L, Burkot TR, Karl S**: Optimization of the feeding rate of Anopheles farauti s.s. colony mosquitoes in direct membrane feeding assays**. Parasite Vectors 2021, 14:356.

2 Timinao L, Vinit R, Katusele M, Koleala T, Nate E, Czeher C, Burkot TR, Schofield L, Felger I, Mueller I, et al: **Infectivity of Symptomatic Malaria Patients to Anopheles farauti Colony Mosquitoes in Papua New Guinea**. Front Cell Infect Microbiol 2021, 11:771233.

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Abbreviations

%	Percent
°C	Degree Celsius
µL	Microlitre
µM	Micromolar
AL	Artemether Lumifantrine
An.	*Anopheles*
AQ	Amodiaquine
CQ	Chloroquine
DDT	Dichloro-Diphenyl-Trichloroethane
DHA	Dihydroartemisinin
DMFA	Direct Membrane Feeding Assay
DMSO	Dimethyl sulfoxide
DNA	Dioxyribonucleic Acid
DSF	Direct Skin Feeding
g/mole	Grams per mole
G6PD	Glucose-6-phosphate dehydrogenase
GFATM	Global Fund to Fight AIDS, Tuberculosis and Malaria
h	Hours
HRP2	Histidine Rich Protein 2
IRB	Institutional Review Board
IRS	Indoor Residual Spray
kg	Kilogram
LLIN	Long Lasting Insecticidal Nets
mg	Milligram
min	Minutes
mL	Millilitre
mM	Millimolar
MRAC	Medical Research Advisory Committee
n	Number
N	Total Number
NDoH	National Department of Health

NMCP	National Malaria Control Program
NSR	No Serum Replacement
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium. falciparum*
pH	Potential of Hydrogen
pLDH	Parasite Lactate Dehydrogenase
PNG	Papua New Guinea
PNGIMR	Papua New Guinea Institute of Medical Research
PQ	Primaquine
PR	Principal Recipient
Pv	*Plasmodium vivax*
qPCR	Real-time Polymerase Chain Reaction
RAM	Rotarians Against Malaria
RBC	Red Blood Cell
RPMI	Roswell Park Memorial Institute Medium
SP	Sulfadoxine Pyrimethamine
SR	Serum Replacement
WHO	World Health Organization

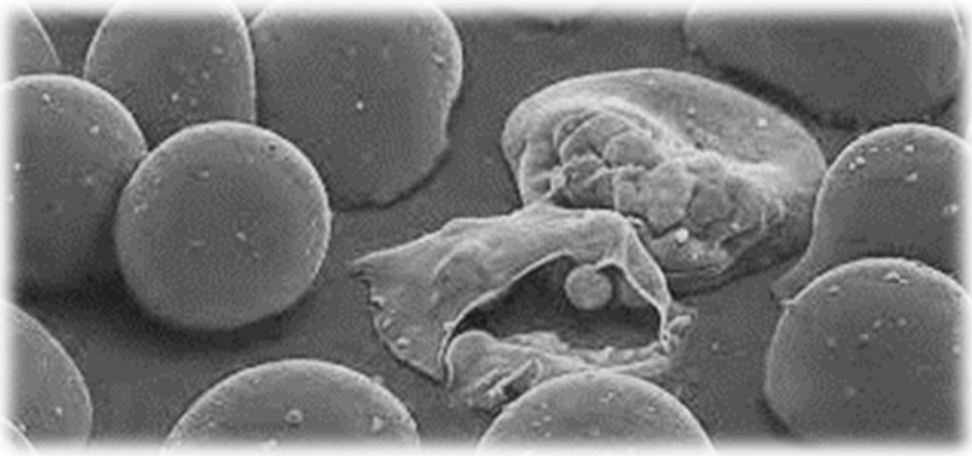
List of Tables

List of Figures

Table of Contents

CHAPTER 1

General Introduction



1.1 Brief History of Malaria

Malaria is a deadly and primarily tropical disease caused by parasitic protozoa of the genus *Plasmodium*. [1] There are 5 *Plasmodium* species that cause infections in humans; *P. falciparum, P. vivax, P. ovale* (*P. ovale curtisi* and *P. ovale wallikeri), P. malariae* and *P. knowlesi*. The first four involve human-to-human transmission, while *P. knowlesi* involves transmission between macaques and humans with limited human to mosquito transmission and is localized in the Southeast Asia region. [2] *P. falciparum* and *P. vivax* infections are responsible for most of the global malaria cases and deaths. [3]

Malaria is an ancient disease and is believed to have crossed over to humans from apes about 30,000 years ago according to the phylogenies constructed from mitochondrial DNA and parasite genomic DNA. [4] It has been hypothesized that there was a crossover of *P. falciparum* from African apes about 10 000 years ago but there has been some controversy on whether *P. vivax* parasites originated from apes in Africa or Southeast Asia about 30,000 years ago. [5-8] The earliest detections of malaria parasites were from recent immunological studies from Egyptian remains dating to 2613 – 1304 BC. [9, 10] There are a number of other earlier records which also indicated the presence of malaria (e.g., the Chinese medical records in *Nei Chin*, China in 270 BC where they described tertian (every third day) and quartian (every fourth day) fevers with enlarged spleen, a common occurrence in malaria patients. The ancient Chinese blamed the headaches, chills and fevers caused by malaria on three demons, one carrying a hammer another a pail of water and the last one a stove. [11] The term malaria was first coined by an Italian physician, Franscisco Torti in 1718 meaning 'bad air'. This stemmed from the earlier beliefs that the disease was caused by bad air and bad water of marshes which produced miasmata which affected people living near these environments. [12]

Although quite an ancient disease, the causative agent of malaria remained elusive for centuries. It was only in 1880 that Charles Louis Alphonse Laveran (1845-1922), a French army doctor who, at the end of his tenure worked in Algeria, discovered malaria parasites in human blood. He was awarded the Nobel Prize for his work in 1907. Despite the discovery of malaria parasites, the mode of malaria transmission was still unclear. It was in 1897 (17 years following the discovery of malaria parasites) that Surgeon-Major Ronald Ross (1857-1942) discovered oocysts on the stomach linings of *Anopheles* mosquitoes and deduced that malaria was transmitted by them. [11, 13, 14] This discovery contributed to the basic understanding of malaria infection and transmission

and paved way for many subsequent studies.

1.2 Current Global Situation of Malaria

There were an estimated 241 million cases of malaria worldwide in 2020 (World Malaria Report 2021), an increase from 227 million cases in 2019 attributed to disruptions caused by the COVID-19 pandemic. There was no change in the global estimated malaria cases between 2018 and 2019 however this was a drop from what was observed in 2016 and 2017 (226 & 231 million cases respectively). There was a slight increase in the malaria cases (224 million cases) between 2015 and 2016 -17. There was a steady reduction with global malaria cases observed between 2010 (244 million cases) to 2015. It can be noted that after 11 years (2010 to 2021) of combating malaria there may seem to be no substantial change in the global estimates of malaria cases however, when taking the population growth over the 11 years, this figure may represent a drop in the total estimate number of cases. [3]

There was a steady decline in the estimated number of deaths due to malaria between 2000 and 2015 from 896 000 to 562 000 and a slight decrease to 558 000 in 2019. However, in 2020 the deaths by malaria increased to an estimated 627 000 a 12 % increase from 2019. It was estimated that the 47 000 (68 %) of the additional 69 000 deaths by malaria were due to disruptions during the COVID-19 pandemic while the remaining 22 000 additional deaths represented the expected increase between 2019 and 2020. The total global estimated malaria deaths among children under 5 years has shown no significant difference from 2000 (87 %) to 2020 (77 %). The WHO African region has the highest mortality rates with 96 % (602 000) of deaths related to malaria followed by 2 % (12 300) in the WHO Eastern Mediterranean region, 1.4 % (9 000) in the WHO South-East Asian region, 0.5 % (3 200) in the WHO Western Pacific region and 0.07 % (409) in the WHO Americas region. [3]

1.3 Malaria Parasite Life Cycle

1.3.1 Mosquito vectors and the development of parasites in the mosquito

Mosquitoes of the genus *Anopheles* are the vectors of malaria. There are around 430 species of *Anopheles* mosquitoes with about 70 being known malaria vectors [11] and 41 being considered dominant vectors. [15] There are differences in vector composition and distribution between the Americas, Europe and Middle-East, Africa and Asia and also within each region. According to

Sinka and colleagues these are the number of dominant vector species and species complexes per region; Americas have 9, Europe and Middle East have 6, Africa has 7 and Asia (including Oceania) has 19. [16] Intervention strategies aiming to control mosquitoes or to reduce human-to-mosquito contact should take into consideration the differences in vector species compositions within their respective habitats and behaviors.

Briefly, the life cycle of the mosquito is one that has 4 developmental stages; the eggs, larvae, pupae and adults. The eggs are laid in a pool of water, after which they hatch and develop as larvae in the water. After 4 larval molts in 8-10 days the larvae molt to pupae where there is no feeding as they prepare to undergo metamorphism. They then emerge as mosquitoes. Both, the male and female *Anopheles* mosquitoes feed on nectar; however, only female *Anopheles* mosquitoes also blood feed, usually 1-2 days after mating. Mating usually occurs in the evening where there is a swarm of male mosquitos where females enter. During copulation spermatozoa are transferred to the female and stored in a single spermatheca. The spermatozoa are sufficient to fertilize all the egg batches the female will lay during her lifetime. [11] It is crucial for most female mosquitoes to feed on blood as it is a source of protein and amino acids required for egg maturation. While taking a blood meal, the female *Anopheles* mosquito can also transmit malaria parasites.

During a blood meal on an infectious individual, the female mosquito takes in the sexual blood stages, or gametocytes, of the malaria parasite. Only the sexual stages will develop in the mosquito gut. Gametocyte infected red blood cells (RBC), when ingested by the mosquito, rupture in the mosquito gut releasing the mature male and female gametocytes. In the mosquito gut, a temperature drop of at least 5 °C and a rise in the pH level above 8 in the presence of xanthurenic acid, a metabolite found in insects, triggers male gametocyte exflagellation. [17] During exflagellation, a single male gametocyte releases 8 motile microgametes, which fuse with a female gametocyte or macrogamete to form a fertilized diploid zygote. The zygote develops into a motile ookinete, which migrates to the internal wall linings of the gut to form an oocyst. The oocysts are visible on the dissected gut of the mosquito at 40 X magnification 7-9 days post blood meal. Each oocyst produces hundreds to thousands of sporozoites. Depending on the *Plasmodium* species it can take between 14 – 30 days before the oocysts burst and release the sporozoites, which migrate into the salivary gland of the mosquito. The sporozoites are injected into the new human host when the mosquito takes another blood meal. The developmental stages of the malaria parasite in the mosquito are illustrated in Figure 1.1. [18]

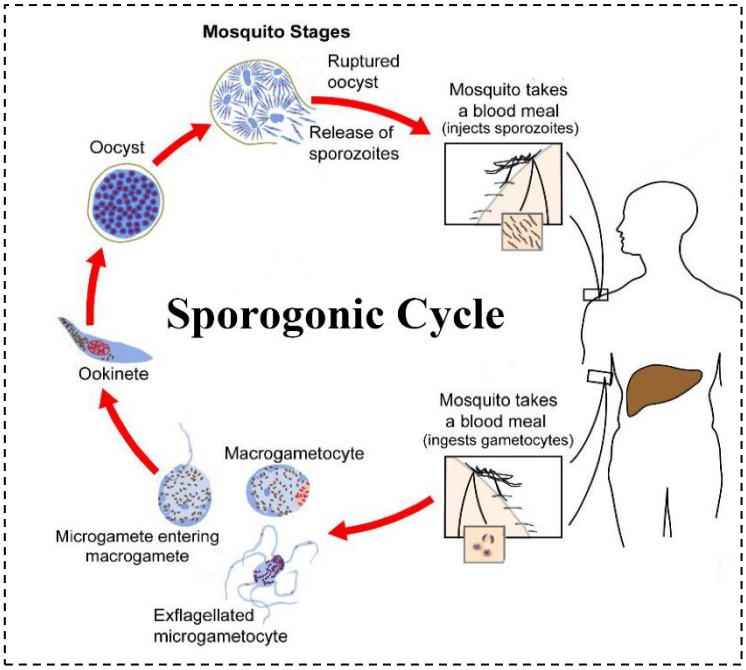


Figure 1.1 Malaria parasite development in mosquitoes. *Adapted from the US CDC.* https://www.cdc.gov/malaria/images/graphs/life_cycle/Malaria_LifeCycle_1.gif [18]

1.3.2 Development of the parasite in the human host

A human becomes infected when an anopheline mosquito inoculates sporozoites during blood feeding (Figure 1.2). Within minutes the sporozoites migrate to the liver, invade hepatocytes (liver cells) and develop into either an actively dividing tissue schizont or a dormant hypnozoite, depending on the species (only *P. vivax* and *P. ovale* develop hypnozoites). [19] The latent hypnozoite stage can remain inactive in the liver for months or even years and cause subsequent infections (relapses). [20] In the case of actively dividing tissue schizonts, (also called exoerythrocytic schizonts) thousands of merozoites develop before the infected hepatocytes rupture releasing the merozoites into the blood stream. These merozoites then invade either erythrocytes (mature RBCs) or reticulocytes (young RBCs) depending on the malaria species. [19, 21] *Plasmodium falciparum* parasites invade mature erythrocytes, with a preference for reticulocytes, while *P. vivax* parasites exclusively invade reticulocytes. Merozoites invade RBCs (usually 1 merozoite/RBC) and undergo multiple cycles of mitotic replication producing schizonts in the RBCs which rupture and release more merozoites into the blood stream leading to cycles of asexual reproduction. It is during these cycles that the person shows malaria symptoms such as the characteristic cyclic fevers and chills. Eventually, some parasites differentiate into gametocytes. For both *P. falciparum* and *P. vivax,* gametocytes can potentially appear in the blood stream after the first cycles of the asexual replication process. [22] However, *P. vivax* can also produce

gametocytes at the onset of the initial infection without the individual showing clinical signs of illness. Hence, transmission can occur before treatment. [23] There is a distinction between the development of *P. falciparum* and *P. vivax* gametocytes. While *P. falciparum* gametocytes develop slowly over up to 12 days, *P. vivax* gametocytes mature faster. There are 5 morphologically distinct developmental stages of the *P. falciparum* gametocytes. Stages 1- 4 are sequestered mostly in the bone marrow to avoid immune clearance by the spleen while the mature stage 5 gametocytes circulate in the blood where they can be ingested by mosquitoes. *P. vivax* gametocytes, on the other hand, do not have morphologically distinct developmental stages. [22]

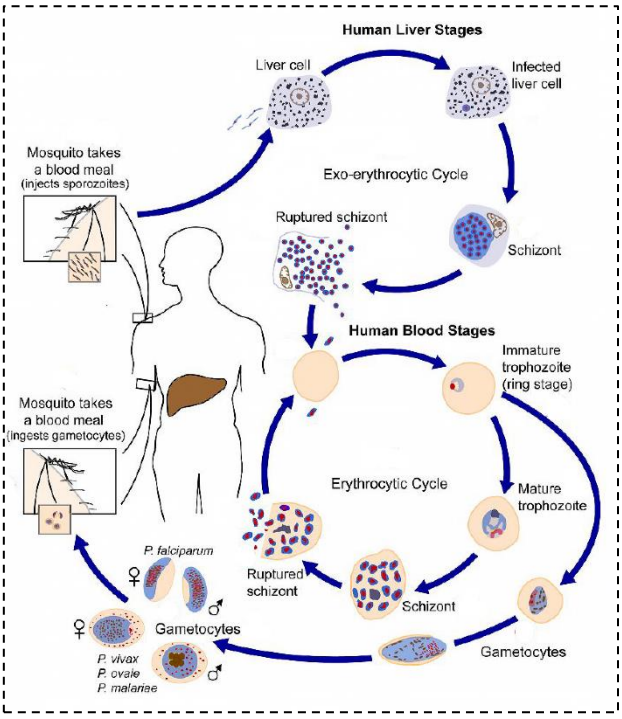


Figure 1.1 Developmental stages of the malaria parasite in the human body. *Adapted from the https://www.cdc.gov/malaria/images/graphs/life_cycle/Malaria_LifeCycle_1.gif .* [18]

1.4 Diagnosis of Malaria

Light microscopy is the gold standard for detection, identification and quantification of malaria parasites. [24] Thick and thin blood films are prepared on glass slides and stained with Giemsa stain, which enables the parasites to be viewed under a microscope at 1000 X magnification. Although this technique is comparatively low in cost and effective in diagnosing malaria, it requires well-trained technicians, a continuous stock of reagents and consumables, and constant supply of electricity to power the microscopes. Many clinics located in rural areas in developing countries do not have access to resources that support microscopy diagnosis and often must resort

to presumptive diagnosis. The advent of rapid diagnostic tests (RDTs) has greatly improved access to adequate diagnosis before treatment. RDTs are portable, can be used in almost any setting and results are available faster than microscopy. However there are some draw backs with RDTs, they can detect false positives especially when the *P. falciparum* antigen can remain in the circulation for more than 30 days leading to false positive results [25], deletions in the HRP2 and HRP3 genes have been detected which compromises the performance of the RDTs [26] and also RDTs are quite expensive and need to be subsidized to be widely accessible. While microscopy and RDTs are primarily used in routine hospital/clinic settings, more sensitive molecular methods such as the quantitative real-time polymerase chain reaction assays (qPCR) have also been developed to diagnose malaria. [27] Molecular diagnosis is much more expensive than the two preceding diagnostic tools and has been mainly used for high-throughput detection of malaria parasites in research settings. Molecular methods are currently not recommended by WHO for programmatic use.

1.5 Clinical Features of Malaria

Malaria signs and symptoms are non-specific and initially can be mistaken for flu-like symptoms. [28, 29] These symptoms comprise of headache, lassitude, fatigue, abdominal discomfort, muscle, and joint aches, usually followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise. [30] In young children it may present as lethargy, poor feeding and cough. Malaria can be classified into severe (complicated) malaria and uncomplicated malaria. Severe malaria is defined as individuals with a positive parasitological test (microscopy or RDT) together with the following clinical manifestations; impaired consciousness, respiratory distress (acidotic breathing), multiple convolutions, prostration, shock, pulmonary oedema (radiological), abnormal bleeding and jaundice. Uncomplicated malaria is defined as individuals with a positive parasitological test (microscopy or RDT) but with no features of severe malaria. [31] Laboratory indices of severe malaria include; severe anaemia, hypoglycaemia, acidosis, hyperlactataemia, renal impairment and hyperparasitaemia. [31] Cerebral malaria is a form of severe malaria that affects children under the age of 5 years which is more common in sub Saharan Africa. [3] Sequestration of cytoadherent erythrocytes containing mature forms of *P. falciparum* in the cerebral microvasculature is thought to be the primary pathophysiological cause of cerebral malaria. [32, 33]

1.6 History of Antimalarial Therapy

Malaria is treatable with a number of drugs. Conventional treatment of malaria began when quinine was first isolated from the bark of the *Cinchona* tree in 1820. [34, 35] Since then a number of drugs have become available to treat malaria primarily due to the rapid development and spread of drug resistant parasites. Briefly, Chloroquine was introduced in 1945 with the first case of resistance detected in 1957, Proguanil was introduced in 1948 with the first case of resistance detected in 1949, Sulfadoxine and Pyrimethamine were introduced in 1967 and the first case of resistance was detected in the same year, while Mefloquine was introduced in 1977 and the first case of resistance was detected in 1982 and Atovaquone was introduced in 1996 with the first case of resistance was detected the same year. [36] Malaria treatment started as monotherapy with individual drugs being used for treatment. However, parasites eventually developed resistance to monotherapies; therefore, combination therapies were introduced to counter/slow the development of resistance such as sulfadoxine pyrimethamine (SP). However, the parasites have developed resistance to combination therapies, which led to a global change to artemisinin combination therapies. Artemisinin combination therapies are the most affordable and effective drugs being used in malaria endemic countries to treat malaria. A delayed clearance of parasites indicating the development of artemisinin resistance has been observed with artemisinin derivatives in the South-East Asian region posing a threat to malaria treatment in other endemic regions. [37, 38] Also sporadic detection of the mutations associated with artemisinin resistance has been observed in Africa [39] as well as in PNG. [40, 41]

1.7 Measuring Infectiousness through Mosquito Feeding Assays

Since anopheline mosquitoes have been incriminated as responsible for the transmission of malaria by Ronald Ross [13, 14] many subsequent studies have been conducted on investigating malaria transmission using reared mosquitoes. Initial mosquito feeding experiments were performed by Muirhead - Thomson in the 1950s to identify the malaria reservoir and investigate the infectivity of humans to mosquitoes. [42, 43] A pioneering transmission study based in a West African village where asymptomatic participants were recruited at random and exposed to laboratory-reared *An. gambiae* mosquitoes using direct skin feeding assay (DSF) showed that there was a low proportion of successful mosquito infections and no association between infectivity and age, with adolescents and adults forming 30 % of the total reservoir. [42, 43]

Mosquito feeding experiments can be separated into three types*; i)* standard membrane feeding assay (SMFA), *ii)* direct membrane feeding assay (DMFA) and *iii)* direct skin feeding assay

(DSF). SMFA entail working with parasites that are cultured in the laboratory, with mosquitoes that have been reared under laboratory conditions or caught in the wild and reared in the lab until they reach adult stage. SMFAs are primarily used to study *P. falciparum* parasites as it is quite difficult and resource intensive to maintain a continuous culture of *P. vivax* parasites. [44, 45] DMFAs mainly involve infecting laboratory-reared mosquitoes with parasites taken from the blood of infected individuals. DMFAs provides an artificial method to infect mosquitoes with malaria parasites circulating in human populations, and then observe their development in the mosquito. Both the SMFA and DMFA systems involve offering blood in water-jacketed glass feeders to starved mosquitoes via membranes with the blood being kept warm by circulating warm water from a warm water bath (Figure 1.3). [46] The methodology using autoclaved, water-jacketed glass feeders was initially developed by Rutledge and colleagues in 1964. [47] Also, the Hemotek ® system has been developed to perform DMFAs as well. DSF involve exposing a part of the body usually the arm, thigh or calf muscle to the mosquitoes to feed on. [48]

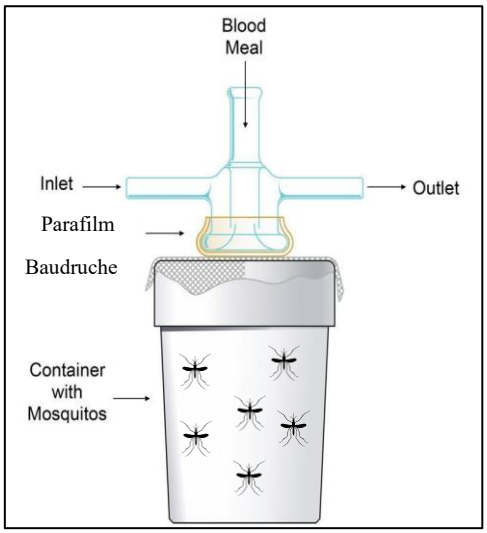


Figure 2.3 Set up for a mosquito membrane feeding experiment. The glasses are connected in a series with a hose connecting the arms. *Image taken from Chemglass Life Sciences and modified.*

1.7.1 Direct skin feeding versus membrane feeding

Few reports compared DSF and DMFA with *P. falciparum* or *P. vivax* with various vectors. DSF closely mimics natural conditions with some reports showing that direct skin feeds perform better in terms of mosquito infection rates and oocysts per midgut than direct membrane feeding experiments: 19.4 % (199/1025) of *An. gambiae* were infected in a DSF on 37 *P. falciparum* infected blood samples, compared to 12.1 % (131/1082) in DMFA. This difference was also

observed in the mean oocyst load where 5.63 oocysts per infected midgut were observed as a result from direct feeding experiments compared with 2.65 oocysts per midgut from DMFA. However, a concordance between oocysts per midgut for the DSF and the DMFA feeds was observed. [49] Other studies from Cameroon, The Gambia, Mali and Senegal also showed a significant difference between direct feeding and membrane feeding methods. [50-52] Sattabongkot and colleagues in Thailand also reported that direct feeding experiments to infect *An. dirus* with *P. vivax* parasites were more effective than the patient's whole blood or red blood cells that were reconstituted with the patient's own plasma. However, direct feeding was not significantly different to membrane feeds where the patient plasma was replaced with a *P. vivax* naïve donor. [53] In another report significant differences were not found between direct feeds and membrane feeds (where *An. arabiensis* was infected with *P. falciparum*) on the percentage of gametocyte carriers infective to one mosquito (52.4 % versus 57.1 %, $p = 0.77$), the mean infection rate of mosquitoes (10 % versus 11.3 %, $p = 0.43$), and the geometric mean oocyst number per mosquito (2.51 versus 3.83, $p = 0.16$). [54] The main advantage that membrane feeding experiments have over DSF is that a large number of mosquitoes can be included in the study hence increasing the precision of the results, enables gametocyte quantification and is the most feasible approach for studying all age groups. [55] In addition, membrane feeding assays can overcome inter-individual variation in their innate attractiveness to mosquitoes [56, 57] and allow modifications of the blood sample such as serum replacement [51, 53], addition of antibodies [58] or antimalarial compounds and heat inactivation of gametocytes for negative controls. [59]

1.8 Malaria in Papua New Guinea

Papua New Guinea (PNG) is a tropical Melanesian country that lies south of the equator [60] in the Pacific (Figure 1.3). PNG occupies the eastern half of the island of New Guinea sharing a border with West Papua, a province of Indonesia, and some 600 smaller offshore islands. It has an estimated land mass of around 462,840 km^2 [61] with an estimated population of over 8 million with a population growth of 3.1 % per year. [62] PNG experiences a tropical climate without much seasonal temperature differences.

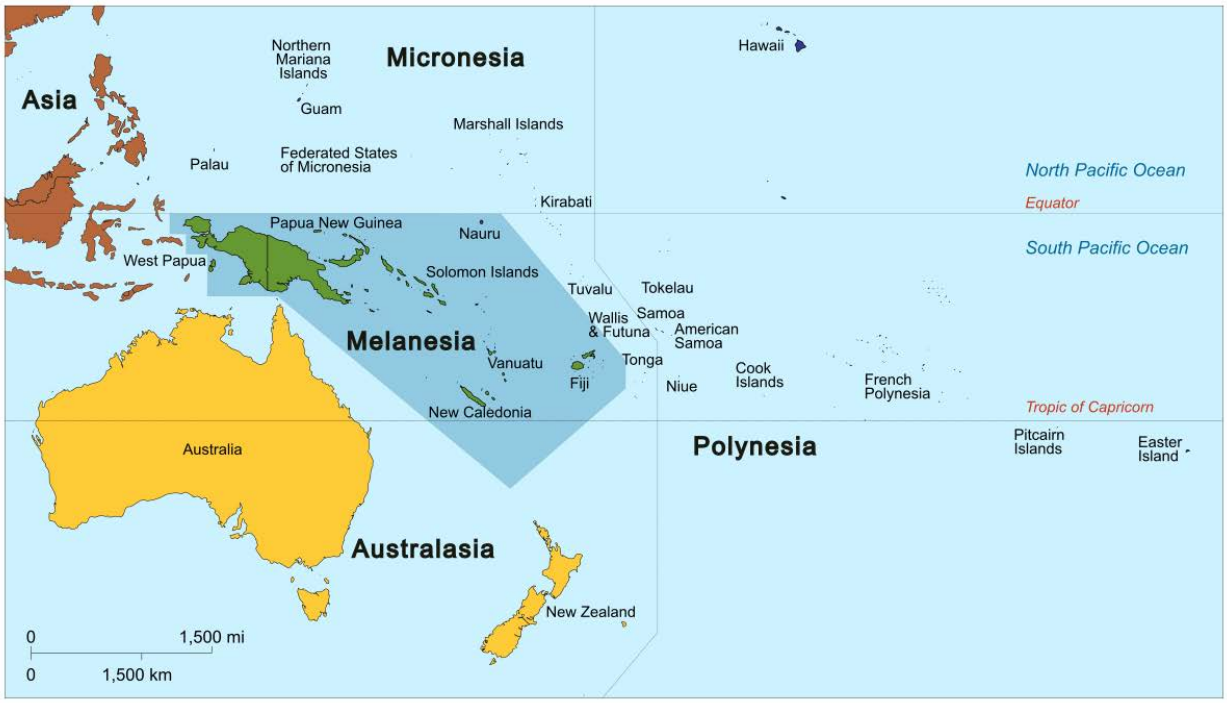


Figure 1. 2 Map showing the Melanesian countries, including Papua New Guinea. [63]

1.8.1 History of Malaria Research in PNG

PNG has a rich history of malaria research dating back more than 100 years to the initial work done by the famous microbiologist Robert Koch in the 1800s. Koch was the first to conduct systematic studies in malaria epidemiology in PNG along the north coast of the current Madang Province. His findings were published as reports to the Colonial Department of the German Foreign Office and were published in the German Medical Weekly (Deutsche Medizinische Wochenschrift) with the English translations published in the British Medical Journal. [64-67] Koch's observations were recorded in these reports and formed the basis of understanding the epidemiology of malaria and also gave insights into the acquisition of immunity against the disease. Since then, PNG has been the hub to numerous subsequent malaria studies particularly along the north coast of PNG in Madang and East Sepik Provinces.

1.8.2 Epidemiology of Malaria in PNG

Papua New Guinea carries the burden of over 80 % of the malaria cases in the WHO Western Pacific Region. [3, 68] Malaria is one of the major health problems in PNG and is the second most common cause of hospital admission with the greatest burden on pregnant women, and children

under five. [69, 70] Of the five *Plasmodium* species that cause human malaria, four are found in PNG (*P. falciparum, P. vivax, P. ovale* and *P. malariae*). *P. falciparum* and *P. vivax* are the predominant species responsible for most of the malaria cases in PNG. [68, 71] The distribution of *P. malariae* is unknown, but prevalence as high as 13 % have been observed in parts of East Sepik Province. [71] *P. ovale* is only occasionally found. [72]

The epidemiology of malaria in PNG was greatly influenced by the malaria control programs since the end of World War II. A global elimination campaign using mass drug administration with chloroquine, environmental management and indoor residual spraying (IRS) with dichloro-diphenyl-trichloroethane (DDT) and dieldrin began in 1957. [73] A coverage of 53% was achieved for DDT by 1973. [73] The average parasite prevalence was reduced to as low as < 2 % for some areas, and especially highland areas were approaching elimination while significant reductions in the parasite prevalence were observed within the coastal region. The elimination campaign was officially abandoned around 1969 due to operational challenges including difficulties with logistical support [73] and accessing large parts of the country, diminishing community support [73, 74], increases in resistance to commonly used drugs [75, 76] and changes in mosquito biting behaviour [77], which together indicated that the program was unsustainable. [78] DDT spraying continued until the mid-1980s when it was stopped when the responsibilities of conducting IRS were transferred to provincial governments. [79] Prior to the elimination program *P. vivax* was the predominant species followed by *P. falciparum* and *P. malariae*. [80, 81] The spraying of DDT initially seemed to increase *P. vivax* dominance [80] due to the shorter extrinsic cycle, rapid gametocytaemia and liver stages that can last for more than a year making it difficult to interrupt transmission. [11, 71] However, following the cessation of the elimination campaign *P. falciparum* emerged as the dominant species followed by *P. vivax* and *P. malariae*. [71, 82]

According to the PNG National Health Plan 2011-2020 malaria was ranked as the leading cause of outpatient visits and the second highest leading cause of admissions. [83] There has not been any major nation-wide efforts to control malaria since the elimination campaigns in the 1950s to 1970s until 2004 when PNG secured a grant from the Global Fund to Fight AIDS, Tuberculosis and Malaria (GFATM Round 3) which allowed for the first country-wide free distribution of long lasting insecticidal nets (LLIN). The distribution commenced in the following year. The PNG National Department of Health (NDoH) was the initial principal recipient (PR) of the Global Fund grant from 2004 - 2009 (Round 3). NDoH was responsible for the purchase and delivery of LLINs,

(ii) Training of staff in microscopy and use of RDTs, (iii) Purchase and delivery of microscopes and RDTs, (iv) Diagnosis of patients, and (v) Purchase and delivery of Artemisinin based Combination Therapies (ACT). Following reports of mismanagement of funds, NDoH relinquished its position to be the PR of the Global Fund grant. [84] In 2009 the Rotarians Against Malaria PNG (RAM), a non-profit organization dedicated to combating malaria in PNG became the PR of the Global Fund grant 2009 - 2014 (Round 8). RAM initially focused on the training of health staff in the new diagnostic and treatment protocols, LLIN procurement, LLIN distribution and monitoring and evaluation. [84] LLINs were supplied to the provincial or district headquarters for distribution. The PNG Institute of Medical Research (PNGIMR) was engaged to evaluate the outcome and impact of the GFATM funded malaria control program since 2008. [85] This grant round also supported the up-scaling of malaria diagnosis in health facilities by improving microscopy and free distribution of rapid diagnostic test (RDT) kits to enable diagnosis before treatment.

Between the years 2004 - 2009, 2.3 million LLINs were distributed to households across PNG on a district-by-district basis resulting in 65 % household ownership with 33 % LLIN use countrywide. [86] The subsequent Global Fund grant (Round 8) received in 2009 allowed the continuation of the free distribution of LLINs and also the roll-out of RDTs and ACTs. A change in malaria policy to a test-and-treat policy began in 2009 [87] and was not being fully implemented in late 2011. [88] Additional funding from Global Fund has been received by RAM in 2015 and lasted till 2020. [89] Further funding was received in 2021 by RAM for the current free distribution of LLINs together with RDTs and ACTs. Over 13.3 million nets were cumulatively distributed by Global Fund grants. [90] A total of 80 % coverage was achieved over all with 72 % of people using an LLIN. [90]

These efforts to control malaria coincided with an overall decrease in the prevalence of malaria in PNG from 15.7 % in 2008/2009 (prior to LLIN distribution) to 4.8 % 2010/2011 to < 1 % in 2013/2014. [91-93] However, sustaining intervention programs to curb malaria is a great challenge. PNG has been experiencing a resurgence of malaria to 7 % prevalence by 2016/2017. [94, 95] The WHO World Malaria Report 2018/2019 also points out that there has been an increase in malaria case incidence between 2015 and 2018 in PNG. [68, 96] The cause of this upsurge is not well understood but is likely due to multiple factors.

PNG experienced prolonged stock-outs of antimalarial drugs and RDTs during that period but this is unlikely to be the main cause of the malaria resurgence as > 90 % of infections are asymptomatic and would remain untreated. [97] In addition, there were reports of behavioral resistance or the change in the biting behavior of the mosquitoes after the distribution of LLINs which could also have contributed in the rise in malaria prevalence. [98] There was a shift in the biting times as mosquitoes tend to bite earlier in the evening before people retire into their houses where they would be protected by the LLINs. There have been reports of nets performing sub-optimally which could have also potentially contributed to the resurgence of malaria during that period. Essentially, the nets distributed were not performing according to the WHO recommended standards of having a 60 minute knock down rate of ≥ 95 % and a 24 hour mortality rate of ≥ 80 % in the bioassays with pyrethroid susceptible *An. farauti* mosquitoes. [99, 100] The increase in malaria prevalence came in spite of the declared goal of the 2014 East Asia Summit to eliminate malaria by 2030 in the Asia-Pacific region. [101]

1.8.3 Malaria Vectors and Transmission in PNG

Members of the *An. punctulatus* group are major vectors of malaria in the southwest Pacific including PNG. The group has a total of 13 species which can have, different degrees of exophily, anthropophily, peak biting times, seasonality and larval habitat preferences. There are 5 major vectors in this group defined by their widespread distribution and high abundance: *An. punctulatus s.s., An, farauti s.s., An koliensis, An. hinesorum* and *An. farauti 4*. [102] In addition other minor vector species include *An. bancroftii, An. longirostris, An. karwari* and *An. subpictus.*

Both *P. falciparum* and *P. vivax* have been identified in the 5 major species by various studies; *An. punctulatus,* [102-104] *An, farauti s.s*, [102, 104, 105] *An. koliensis,*[102-104] *An. hinesorum,* [102, 104, 105] and *An. farauti 4.* [102, 104] Also, both plasmodium species were found to be transmitted by the minor vector species as well. [102, 103, 106, 107]

The *An. punctulatus* group of mosquitoes have adapted to unique oviposition settings according to their species. [108] *An. farauti s.s* is found along the entire coastline of PNG and is the dominant species. [108] It can tolerate saline conditions and thrives in brackish water but is found in fresh water as well. The other vector species are found primarily inland, with *An. punctulatus* being the dominating species followed by *An. koliensis* and the others. [108, 109]

Malaria transmission in PNG is highly variable ranging from intense perennial transmission in the northern coastal lowlands to seasonal moderate transmission in the south coastal regions to unstable transmission in the higher altitude regions. [71]

1.8.4 Malaria Treatment in PNG

It is challenging to clearly map out the exact dates in which the different antimalarials were introduced to PNG however; the reports on the use of these drugs provide an indication of the antimalarials to which the parasites in PNG were exposed. Quinine, the first conventional antimalarial drug, was initially used by researchers during Koch's visits in late 1800 to early 1900 in PNG. [65-67] There were also records stating that the seeds of the cinchona tree was brought and grown in PNG in the early 1900s. [110] By 1979 the parasites in PNG had been exposed to a number of antimalarial drugs particularly 4-aminoquinolines (chloroquine, CQ, and amodiaquine, AQ), 8-aminoquinolines (primaquine, PQ), proguanil, pyrimethamine, sulphonamides, dapsone and tetracycline. [111-115]

1.8.5 First change in antimalarial treatment

PNG has undergone two major changes in the antimalarial treatment regimen due to the rapid spread of drug resistance. The first major change in malaria treatment in PNG was from using monotherapy to a combination treatment regimen as the first line treatment of uncomplicated malaria. CQ was the first line treatment for malaria since its use during the elimination efforts in the early 1950s. [78, 79] Resistance to CQ by *P. falciparum* was first detected in 1976 [116] while resistance of *P. vivax* was detected over a decade later in 1989. [117, 118] The spread of resistance to CQ [115, 119-121] prompted the National Department of Health (NDoH) to revise the treatment guidelines in 1997. The review recommended that the monotherapy treatment regimen be changed to a combination therapy of sulfadoxine-pyrimethamine (SP) plus 4-aminoquinolines. The decision to use this combination therapy was based on a study conducted in 1998 -1999, where the treatment failure rates of the new regimen were below 5 % [122] which was consistent with WHO recommendations. The NDoH officially changed the treatment guidelines in 2000 to AQ with SP for young children and CQ with SP for adolescents and adults. There were subsequent studies that also supported this change in treatment regimen. [123]

The first reports of treatment failure of SP plus CQ or AQ came just 3 years after the change in

the treatment guidelines. The report described treatment failures to *P. falciparum* and *P. vivax* in three sites over a period of 3 years. The treatment failure rates for *P. falciparum* ranged between 10.3 % and 28.8 % for AQ and SP and between 5.6 % and 28.6 % for CQ and SP. The overall treatment failure rate of *P. vivax* to SP plus CQ or AQ was 12 %. [124] The rapid development and spread of drug resistant parasites is not surprising as there were already reports of reduced efficacy to SP prior to its implementation as the first line treatment therapy in 2000. *Plasmodium* parasites have been exposed to SP or its components since the 1970s [111] and there were records of treatment failures already documented by the late 1970s to early 1980s. A clinical trial conducted in 1980 reported a 10.3 % treatment failure rate of SP against *P. falciparum* with a longer parasite clearance time in *P. vivax* almost 2 decades before it was nominated as the first line treatment. [112-114] There were other reports of *P. vivax* resistance to pyrimethamine which was used as a prophylaxis in combination with dapsone. [125, 126]

1.8.6 Second change in antimalarial treatment

In light of the rapid spread and selection of the 4-aminoquinolines and SP-resistant parasites, a review of the National Malaria Control Program (NMCP) was conducted in 2007 which resulted in a second major change in the standard antimalarial treatment by 2008. A combination therapy consisting of artemether-lumifantrine (AL) was selected as the new first line treatment for uncomplicated malaria. AL was chosen because of its high efficacy against *P. falciparum* malaria in a study conducted between 2005 and 2007. [127] The study compared four different drug combinations CQ-SP, artesunate-SP, dihydroartemisinin (DHA) - piperquine and AL in 2 endemic regions of PNG. Of the patients diagnosed with *P. falciparum* AL performed slightly better in clearing the parasites and curing clinical symptoms while DHA-piperaquine had similar effect with *P. vivax*. [127] AL was selected over DHA-piperquine firstly, because it addressed 60 -70 % of all malaria cases in PNG which are caused by *P. falciparum* and secondly it met the WHO guidelines for adapting a new treatment therapy of having > 95 % adequate clinical and parasitological response (ACPR). [128] AL plus PQ was recommended for *P. vivax* malaria. However, this treatment may not be ideal for *P. vivax* according to a recent systematic review which looked at AL and DHA-piperquine with and without PQ in a total of 19 studies from 2000 – 2018. The study noted a 12 fold difference in the risk of recurrence at day 42 when treated with just AL as compared to DHA-piperquine. Also when co-administered with PQ the reduced risk of recurrence at day 42 after being treated with AL was 80 % and 92 % at 63 days after being treated with DHA-piperquine. [129]

While PQ has been the only drug available, with the recently approved Tafenoquine [130] (which is in the same 8-aminoquinlines class of antimalarials as PQ,) to treat *P. vivax* hypnozoites, they both can cause haemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals. Currently there are no point-of-care tests available in public hospitals and clinics in PNG to determine the G6PD status of individuals before treating with PQ. Patients are only cautioned to stop PQ treatment and seek out a medical officer upon observing darkening of their urine, a sign of haemolysis. Due to these potential side effects, PNG is currently implementing the conservative dosage level of 0.25mg/kg of body weight (in a single daily dose) as recommended by WHO. [87] Also, there are adherence issues with taking PQ for 14 days following the 3 days of receiving AL after being diagnosed as having *P. vivax* infection. The patients will have recovered as the blood stage parasites will have been cleared hence, they tend to ignore the need to take PQ for the full 14 days.

There are increasing reports of *P. falciparum* parasites resistant to artemisinin in Southeast Asia [131, 132] and there is mounting evidence of resistance to artemisinin in PNG also developing, with studies detecting the presence of a mutation (C580**Y**) which is strongly associated with artemisinin resistance in Southeast Asia in some individuals. [40, 41] However, more monitoring needs to be done to know how widespread the mutation is within PNG and also to observe any signs of treatment failure.

1.8.7 Papua New Guinea Institute of Medical Research

The Papua New Guinea Institute of Medical Research (PNGIMR) was established in 1968. The main focus of PNGIMR is to conduct research on major medical health problems in PNG and to provide advice to the National Government. Since its inception PNGIMR has been involved in diseases research such as kuru, pigbel, cretinism, syphilis, pneumonia, enteric diseases, lymphatic filariasis, malnutrition and malaria. Research at PNGIMR is internationally recognized and highly collaborative. Most studies include collaborations with investigators from institutions in other countries and many studies are funded through internationally competitive research grants.

Over the years, PNGIMR has been involved in numerous malaria research studies including epidemiological, entomological, parasitological, vaccine, antimalarial safety and efficacy, and clinical studies primarily around its sites in East Sepik and Madang Provinces. PNGIMR has

contributed much to our understanding of malaria parasites and how the efforts by the National Government to curb malaria have performed. For example; PNGIMR was involved in the research that lead to the second change in the antimalarial treatment policy and was further involved in the study to identify AL as the appropriate treatment following the treatment failure of SP and CQ or AQ. PNGIMR was also involved in monitoring the coverage, usage and the efficacy of long lasting insecticidal nets (LLINs) after distribution. Currently PNGIMR is involved in trialing out the indoor residual spraying as a means to reduce the malaria vectors within homes in PNG.

1.8.8 Key knowledge gap

The transmission of malaria parasites from humans to mosquitoes represents an important part of the malaria life cycle. It is a bottleneck where the parasite numbers shrink from millions in the human body to less than a hundred in the mosquito gut where only the sexual stages can further develop. [133] This is a vulnerable point in the parasite life cycle. Hence, it is important to understand this process and the tools that can be used to inhibit or block malaria transmission.

Currently, research into the transmission of *P. falciparum* parasites to mosquitoes can be done in the laboratory, as there are established protocols for culturing the parasites since it was first established in 1976 by Trager and Jensen. [134-136] This has allowed researchers to grow parasites in a lab setting which can be used to successfully infect mosquitoes and test potential transmission blocking vaccines [137-140] and antimalarials. [141] However, this is not the case with *P. vivax*. It is still very difficult to maintain a continuous culture of this parasite in the lab as it prefers reticulocytes. [142] Hence, any transmission studies with *P. vivax* needs access to infected patients.

PNG offers a rare opportunity to study and test transmission blocking vaccines and antimalarials in a context where multiple malaria parasite species co-exist, especially when considering that it is extremely difficult and resource intensive to culture *P. vivax* in vitro. The first mosquito infection study in PNG was conducted in the 1980s where Graves and colleagues performed mosquito infection studies using DSF on volunteers within the communities in the Madang area, and also DMFA on individuals who presented with malaria infection at two clinics. [143] In their studies, they observed that the *An. farauti* mosquitoes were infected by both, *P. falciparum* and *P. vivax* parasites using both, DSF and DMFA. Since then, there have not been any reports on mosquito infection studies being conducted in PNG. PNGIMR has the research capacity and

provides a suitable research environment for such studies. Currently, PNGIMR has a well-established insectary where *An. farauti* mosquitoes are maintained in colony, and can be used to perform experiments such as the DSF or DMFA to measure the impacts on transmission by drugs and antibodies. This PhD thesis seeks to understand the transmission of both symptomatic and asymptomatic individuals to mosquitoes and by doing so create a platform to test potential vaccine and antimalarial candidates that can inhibit transmission especially for *P. vivax* infections.

1.9 Aims of this Thesis

1.9.1 Overall Aim

To investigate the transmission of malaria parasites from humans to *An. farauti* mosquito in symptomatic and asymptomatic individuals within a malaria endemic setting by performing direct membrane feeding and direct skin feeding assays.

1.9.2 Specific Aims

The specific aims of this thesis are to;

1. Establish and optimize the direct membrane feeding assay to increase mosquito feeding rates.
2. Adapt and optimize the membrane feeding assay for *Plasmodium vivax.*
3. Adapt a qPCR assay for detecting the mosquito stages of *P. vivax* parasites.
4. Investigate transmission-blocking or enhancing effects of plasma replacement
5. Determine the importance of asymptomatic individuals in the transmission of malaria in PNG.

1.9.3 Scope of Studies in this Thesis

This thesis includes some published chapters and unpublished work. The studies are presented in chronological order with the main findings presented as summary points at the end of each chapter followed by bridging statements introducing the following chapter.

Chapter 1 provides a general introduction to malaria and identifies the key knowledge gap that this research addresses. Chapter 2 outlines the general methods used in the different chapters of the thesis. Chapter 3 describes the optimization of the direct membrane feeding assay to increase

mosquito feeding rates. This chapter has been published. [144] Chapter 4 demonstrates that it is still possible to infect the *An. farauti* colony mosquitoes with *P. vivax* and *P. falciparum* parasites and also was published. [145] In Chapter 5, a qPCR assay to detect *P. vivax* parasite stages in the mosquito is described (manuscript submitted). Chapter 6 describes serum replacement experiments to determine the natural immunity of individuals in regards to transmission and blocking immunity. Chapter 7 investigates the malaria infectivity of asymptomatic individuals to mosquitoes. Chapter 8 summarizes the key findings and highlights the limitations and strengths of the studies and the practical and logistic issues from a PNG perspective and raises further research questions arising from the work presented in this thesis.

Bridge

The next chapter describes the insectary set up that we currently have at the PNG Institute of Medical Research (PNGIMR), the direct membrane feeding assay setup, the patient recruitment and sample collection procedures, microscopy and qPCR diagnosis of the blood samples collected from the participants and mosquito dissection for oocysts. These methods are being used in each chapter, and to avoid repetition, I have explained them in detail here and only briefly mention them in the results chapters containing unpublished work (Chapters 6 and 7), with references made back to this chapter.

CHAPTER 2

General Methodology

2.1 Insectary Setup

2.1.1 Rearing *Anopheles farauti sensu stricto* in Papua New Guinea

Despite the operational challenges resulting from resource limitations and lack of infrastructure in PNG, PNGIMR maintains an insectary at the vector-borne diseases unit in Madang, where *An. farauti s.s* mosquitos are reared. The insectary is maintained at 28 ± 8 °C and 68 ± 25 % relative humidity. The light cycle is 11 h dark and 12 h light with 30 minutes dusk and 30 minutes dawn by making use of a timer, which dims the light and then turns it off or on completely. The larvae is fed ground fish food (Tropical Fish Flake, Marine Master, Australia) and the adult mosquitoes are provided with 10% sugar solution (Ramu Sugar, PNG). The colony was established more than 50 years ago. The initial ancestors of these *An. farauti* colony mosquitoes were collected in Rabaul, East New Britain Province and were brought to the PNGIMR, Madang Province, in 1968. The *An. farauti* mosquitoes were then transported to Australia and were reared by the Australian Army Institute. The colony was reestablished at PNGIMR Madang Province 2009 - 2010 from eggs received from the Australian Army Institute. Initially the colony mosquitoes were not susceptible to malaria infection so a back cross with the wild *An. farauti* species was done in 2012 however, we have yet to confirm if the backcross was maintained by the colony species.

2.1.2 Direct membrane feeding assay setup

The DMFA set up is shown in Figure 2.1; Water is circulated through polypropylene tubing between a laboratory water bath (Ratek, Australia) maintained at ~38 °C and a series of water jacketed glass mosquito feeders (Somnunk Scientific, Thailand) using an aquarium pump (Eheim compact 1000, Australia).

Blood (250 – 500 uL) is inserted using a 1 mL pipette (Eppendorf, Australia) through the upper opening of the feeders. The feeders are fitted with a membrane (e.g., Baudruche or Parafilm) which covers the bottom opening of the feeder through which the mosquitoes are able to penetrate to feed on the blood. The total number of glass feeders that are connected in a series to feed the mosquitoes in each cup depends on the experiment that is being conducted.

Figure 2.1. Direct membrane feeding assay (DMFA) set up. Cups connected in a series by tubes to a mini aquarium pump within the water bath which is maintained at ~38 ºC.

2.1.3 Recruitment of symptomatic patients and sample collection

Nursing officers enroll patients presenting at Yagaum clinic and Madang Town clinic with malaria symptoms and who test positive with a rapid diagnostic test (RDT) for malaria. If malaria infection is confirmed, patients are approached to provide informed consent to participate in the study. Patients between the ages of 5 to adults were recruited to the study. After providing a brief description of the study to the participants or their parents or guardians they were then asked if they would be willing to be part of the study. After agreeing to participate, they were asked to sign the informed consent form. For those under the age of 16 parents or guardians were approached to consent on behalf of them. Blood samples (5-6 mLwere collected from consented patients into BD Vacutainer ® vacuum sample collection tubes (BD, Australia) coated with lithium heparin as anticoagulant. Hemoglobin concentration (HemoCue, Australia) and Hemoglobin levels, and temperature were also measured using a digital thermometer (Buzzel, China). Thick and thin blood smears were prepared on microscopy glass slides (Livingstone, Australia) for subsequent malaria

diagnosis. The BD Vacutainer ® was then immediately stored in a beverage cooler flask (Coleman Company Inc, USA) filled with water warmed to ~38 °C. A digital thermometer was attached to the flask to monitor the temperature. The blood sample was then transported to the insectary for use in DMFAs.

2.1.4 Microscopy and qPCR diagnosis

The diagnosis by microscopy and qPCR were done retrospectively. Thick and thin blood films were prepared using WHO standard methods and were stained with 4 % Giemsa stain for 30 min. Slides were read by two WHO certified microscopists according to WHO standards. Parasite density was calculated by multiplying the parasite count per white blood cell (WBC) as counted in a total of 200 WBCs count (or 500 WBCs if the parasite count is <100 after counting 200 WBCs) by 8000 leukocytes. [146] The final parasite density was calculated by taking the geometric mean of the densities obtained from reads by two expert microscopists. Discrepancies in the presence or absence of parasites, parasite density (i.e., if they differed by a factor of 10) and parasite species between the two microscopists was resolved by a third expert microscopist, where the two most similar readings is taken. [146]

DNA extraction was performed on 250 µL of red blood pellets using Favorgen DNA extraction kits (Favorgen Biotech Corp, Taiwan) according to the protocol for blood genomic DNA extraction provided by the manufacturer. Following DNA extraction a Taqman based qPCR assay was performed to quantify the infection and determine the parasite species as described elsewhere. Briefly, the Taqman qPCR assay targets a conserved region of the 18s rRNA gene of the respective parasite species. [147]

2.1.5 Mosquito dissections

Following blood feeding, fully fed colony *An farauti* mosquitoes were separated from the unfed mosquitoes and kept in a secondary cage with a damp cotton containing 10 % sugar solution (Ramu Sugar, PNG) placed on top of the cups for subsequent feeding by the mosquitoes (Figure 2.2 A). The reason for the fully fed mosquitoes being kept in a secondary cage is that if the infected mosquitoes escaped from the cups they will be trapped by the secondary cage and won't pose a threat to staff in the insectary. The mosquitoes were kept until day 7 when they were dissected for oocysts using a dissecting microscope (Leica, USA); (Figure 2.2 B). The mosquitoes were placed in the fridge at 4 °C for 10 minutes until they were knocked down and then they were placed on a

petri dish, on an ice pack with a tissue placed between the ice pack and the petri dish. The tissue prevents frosting on the surface of the petri dish. The ice pack with the petri dish were placed in a beverage cooler box (Coleman Company Inc, USA) and the petri dish was covered with the petri dish lid to prevent any mosquito who recovered from the knock down to fly away. The mosquitoes were then placed in a drop of 1X PBS on a microscopy slide where they were dissected. The guts were removed using small fine-tipped forceps and dissecting needle probes. Once the guts were successfully dissected from the mosquito they were then placed in a pool of 0.2 % mercurochrome solution for 15 minutes. After the staining, the guts were taken and placed on microscopy slides and cover slips were placed over the top of the guts per slide. The guts were then viewed under a slide microscope (Zeiss, Australia) at 10 X magnification to check for the presence of oocysts. The oocysts were counted and the infected guts were stored for molecular confirmation of the oocysts. The mosquitoes were kept for 14 days prior to dissecting the head and thorax area for the salivary glands in order to identify whether the mosquito had sporozoites or not. The salivary glands were removed using small fine-tipped forceps and dissecting needle probes. Once the salivary glands were removed, they were then placed in a pool of PBS on glass slides with cover slips placed over the top of the salivary gland on each glass slide and viewed under a slide microscope.

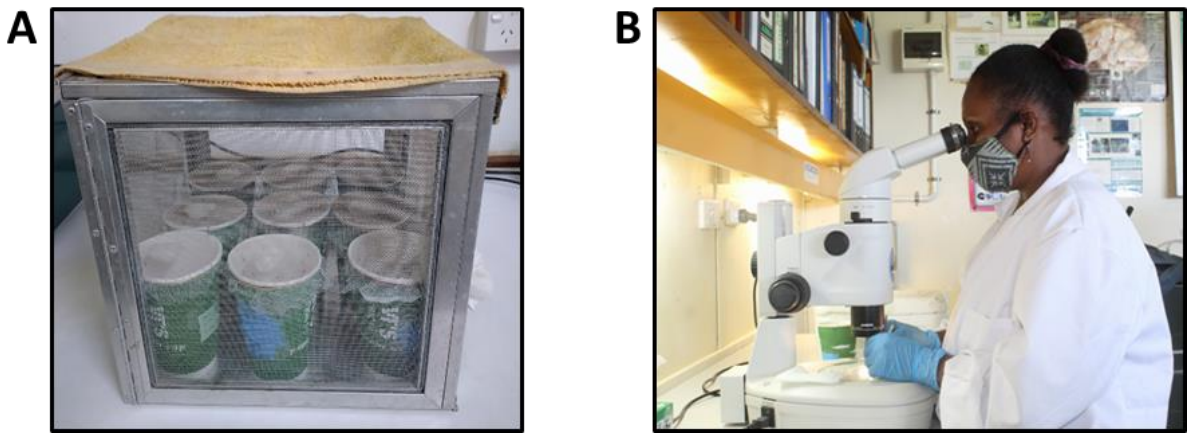


Figure 2.2. Post feeding storage and mosquito dissection. **Panel A** shows the blood fed cups placed in a cage with damp cotton containing 10 % sugar solution on the mesh net on top of the cups with a damp towel on top of the cage to maintain the relative humidity at ~80 %. **Panel B** shows the dissection of mosquitoes on day 7 post-feed by a staff member.

2.1.6 Ethics

The studies presented in this thesis were based in Papua New Guinea and had the ethical approval of the Papua New Guinea Institute of Medical Research Institutional Review Board (PNGIMR IRB) and the Papua New Guinea Medical Review Board (MRAC) before commencing. The respective ethical approvals are presented in the proceeding chapters. James Cook University has acknowledge the ethical approvals received for these studies as well.

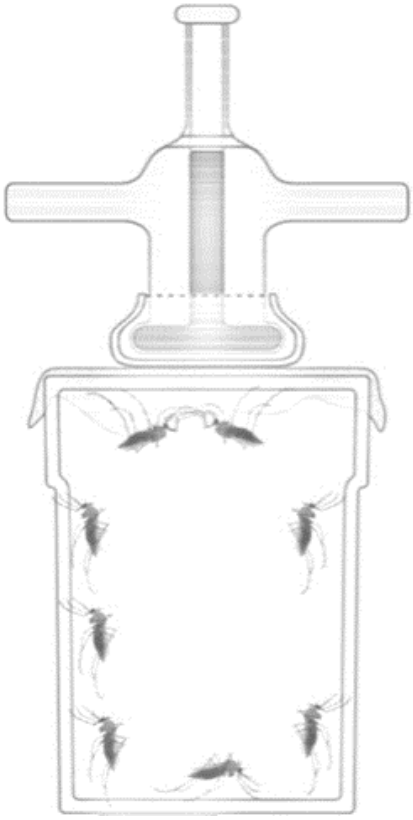
Bridge

Chapter 3 describes the steps taken to optimize mosquito feeding conditions to maximize feeding rates in Direct Membrane Feeding Assays (DMFA). The conditions screened were the mosquito starving time, type of starving, membrane type used, exposure time, mosquito age, illumination when feeding, volume of blood, mosquito density, and water bath temperature. This chapter was published in *Parasites & Vectors* (doi.org/10.1186/s13071-021-04842-y). The published paper has been modified to flow with the thesis formatting and referencing.

Timinao L, Vinit R, Katusele M, Schofield L, Burkot TR, Karl S**: Optimization of the feeding rate of Anopheles farauti s.s. colony mosquitoes in direct membrane feeding assays**. Parasite Vectors 2021, 14:356.

CHAPTER 3

Optimization of the feeding rate of *Anopheles farauti s.s.* Colony Mosquitoes in Direct Membrane Feeding Assays



3.1 Introduction

In the malaria parasite life cycle transmission through the mosquito vector represents a bottleneck where parasite populations shrink from millions in the human body to as few as one in the mosquito. [133] Thus, malaria transmission is vulnerable to interruption when transiting from the human to the mosquito host. [148] This transition can be studied by direct membrane feeding assays (DMFA). During DMFA, mosquitoes feed through a membrane on blood kept warm via water-jacketed glass feeders [47] including blood harvested from humans with circulating malaria parasites, [143] to either study parasite development in the mosquito [143] or to test interventions that disrupt parasite development hence, interrupting transmission. [149]

The mosquito blood feeding rate, i.e., the proportion of mosquitoes that successfully ingest blood is an important determinant of overall infection success. The success rate of ingesting blood from a membrane feeder can vary depending on the mosquito species, whether the mosquitoes were collected in the wild [150] or reared in a colony [151], as well as the level of adaptation of the colony.

Blood feeding rates also depend on the experimental conditions under which the DMFAs are conducted including i) the duration of starvation before exposure, ii) the starving conditions (access to water or no access to water) , iii) the type of membrane used, iv) the amount of time mosquitoes are allowed to feed v) the mosquito age, vi) feeding in the light or in the dark, vii) the blood volume in the feeder, viii) the density of mosquitoes attempting to feed and ix) water bath temperature during DMFA (Figure 3.1). Other parameters which also potentially influence blood-feeding rates in DMFA but were not investigated here include the blood meal source [152], the hemocrit level [153] and phagostimulants such as sodium chloride and sodium bicarbonate for Anopheles species. [154]

However, membrane feeding studies have been conducted with a range of conditions and with varied feeding success. [155, 156] Thus, there is a need to optimize DMFA conditions for each colony mosquito species.

Starving conditions are a key component that greatly impact mosquito feeding rates and a balance needs to be established between starving the mosquitoes for too long, thereby increasing mosquito mortality or affecting their fitness [52], and not starving for long enough so mosquitoes only partly feed or not at all.

Most studies describe dry starving for durations from 5-36 h [156-159] while other studies performed starving where the mosquitoes had access to water for 12 h. [53, 160] A study conducted

by Coulibaly and colleagues compared the feeding rates of mosquitoes dry starved 8 h, 14 h and 20 h and concluded that mosquitoes starved 8 – 14 h yielded significantly higher feeding rates than mosquitoes starved 20 h. [52] However, most studies did not directly report the impact of starving on the feeding rate.

Membranes take the role of an artificial skin in the feeding experiments. An ideal membrane will yield the highest feeding rates in the shortest period of time. Parafilm and natural membranes such as Baudruche, sausage casing, chicken skin or rat skin have been used. [150, 161] Natural membranes which closely mimic the skin resulted in the highest feeding rates followed by Baudruche membrane which is derived from bovine cecum and finally Parafilm, a wax synthetic membrane. [47] Most studies reported using Baudruche membrane [54, 155, 162] while others used Parafilm membrane. [49, 159] Interestingly, a study done by Coulibaly and colleagues showed that there was no significant difference between the feeding rates, survival and infection rates from feeding experiments with either Baudruche or Parafilm membranes, for *Anopheles coluzzii* mosquitoes. [52]

Mosquitoes 2-8 days post-emergence have been used in different studies. [51, 52, 150, 151, 156, 159, 160, 163, 164] The main consideration in this is that mosquitoes are fed at an early age so that they survive for the required duration for either oocysts [155, 159, 165-167] or sporozoites [155, 166, 167] to develop. The study by Coulibaly and colleagues is so far the only one that compared the feeding rate of *An. coluzzii* mosquitoes between 3 days and 9 days post emergence. The authors determined that 3 day old mosquitoes had a significantly higher feeding rate compared to 6 and 9 day old mosquitoes. [52]

Mosquito density is another factor that may influence the mosquito feeding rate. Rutledge and colleagues observed that having more mosquitoes per cage can result in lower feeding rates [47] and crowding, making handling, especially removing of unfed mosquitoes, difficult. Vallejo and colleagues observed that 100 *An. coluzzii* mosquitoes per cage (or 1 mosquito per 5 cm^2) resulted in the highest *P. vivax* infection prevalence after DMFA. [167] However, the study did not report on the feeding rate of the different mosquito densities in relation to infection success.

Not much has been reported also with respect to the impact of the other parameters listed above on the feeding rates. Much of the focus is on the infection rates. As such, the focus of this study was to determine the optimal feeding conditions for *Anopheles farauti sensu stricto* colony mosquitoes in order to maximise their feeding rates in DMFA.

3.2 Methods

3.2.1 Mosquito colony maintenance

The *An. farauti* mosquito colony was derived from a colony established in 1968 in Rabaul, East New Britain province. [168] The laboratory colony was maintained at 28 ± 8 ºC and 68 ± 25 % relative humidity. The light cycle is approximately 11 h dark and 12 h light including a 30 min dusk and 30 min dawn period. The larvae were fed ground fish food (Marine Master, Tropical Fish Flake) while the adults were provided 10 % sucrose (Ramu Sugar) solution available as soaked cotton wool balls placed on top of the mosquito cages. To maintain the colony, uninfected blood (no malaria) was obtained from donors following informed consent procedure. Occasionally, direct skin feeding is used to maintain the colony.

3.2.1.1 Direct Membrane Feeding Assays

Water-jacketed glass membrane feeders were connected in series by rubber hoses to a mini aquarium pump placed inside a 37-38 ºC water bath (Figure 3.2). Unless otherwise stated, all trials used an average of 5 day old mosquitoes with 50 female *An. farauti* placed in a cup (surface area of ~340 cm^2 with a total volume of ~476 cm^3) and offered blood meals from feeders with a diameter of 2.5 cm (a surface area of ~5 cm^2 with a maximum blood volume capacity of 1 mL). The duration of overnight starving ranged between 18-21 h.

The experiments were done sequentially with a single parameter being varied and tested, incorporating the optimal conditions of the preceding tests. Following the feeding experiments, unfed mosquitoes were separated from the fully fed mosquitoes and the feeding rate calculated. Data were transformed using Acrsine (p) [169] prior to performing paired t-tests to test the significance of the difference observed between groups. ANOVA, followed by t-tests, was used to test for significant variation between more than two groups. A flow chart summarizing the parameters tested is provided in the Appendix 1 Table A1.

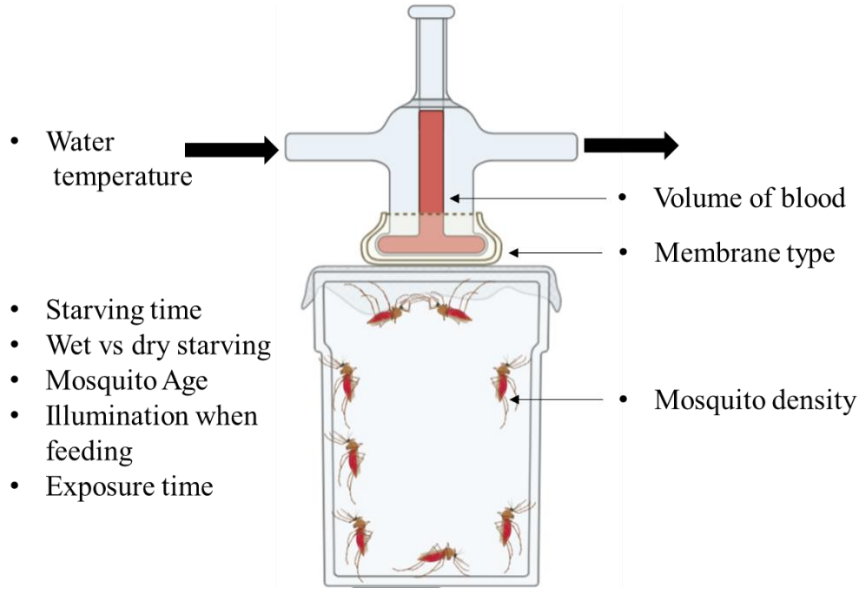


Figure 3.1. Membrane feeding assay set up with the parameters impacting the feeding success of mosquitoes on the direct membrane feeders (figure created using BioRender.com).

3.2.1.1.1 Starving time

An. farauti were dry starved (no access to sugar or water) for 2 h, 4 h, 6 h, and overnight (~21 h) and compared to a control of mosquitoes exposed without starving. Mosquitoes were exposed to ~750 µL of blood for 30 min in the dark (with a black piece of blanket draped over the membrane feeder) following starvation. Parafilm membrane (cut into 3 cm x 3 cm and stretched to ~5 cm x ~5 cm) was used to feed the mosquitoes. The parafilm was not exposed to human odor prior to feeding. Ten replicates were performed.

3.2.1.1.2 Type of starving; access to water versus dry (no access to water)

An. farauti mosquitoes were starved overnight with one cup of mosquitoes having access to cotton soaked in water while the other did not have access to water (dry starved). The mosquitoes were then given access to ~750 µL of blood using a Parafilm membrane for 30 min in the dark. Five replicates were performed.

3.2.1.1.3 Membrane type

Two membrane types were tested, namely Parafilm and Baudruche membranes. The Parafilm membrane was standardized by cutting it into 3 cm x 3 cm pieces and stretching to ~5 cm x ~5 cm. Mosquitoes were dry starved overnight. The mosquitoes were given access to ~750 µL of blood in a membrane feeder for 30 min in the dark. Eight replicate experiments were performed.

3.2.1.1.4 Exposure time

Exposure times of 10 min, 20 min and 30 min were evaluated following dry overnight starving and the mosquitoes were then exposed to ~750 µL of blood for the specified time. Baudruche membrane was used to feed the mosquitoes. Eight replicate experiments were performed.

3.2.1.1.5 Mosquito age

Mosquitoes aged 3, 5 and 7 days were tested in nine replicates. The mosquitoes were starved overnight before exposure to ~750 µL of blood via a Baudruche membrane for 20 min in the dark.

3.2.1.1.6 Feeding in the light or in the dark

A total of 2 cups of 5 days old mosquitoes were prepared and starved overnight. One cup of mosquitoes was fed with the net top exposed to ambient room lighting while the second cup had a black blanket placed over it while they were exposed to ~750 µL of blood via Baudruche membrane for 20 min. Seven replicate experiments were performed.

3.2.1.1.7 Volume of blood

The following blood volumes were tested to determine the minimum blood volume, which could yield high feeding rates; 125 µL, 250 µL and 500 µL in a water-jacketed glass feeder of 1 mL maximum capacity. Three cups of 6 day old (mean age) *An. farauti* were prepared, starved overnight and allowed 20 min to feed in the dark at each blood volume via a Baudruche membrane. Six replicate experiments were performed.

3.2.1.1.8 Mosquito Density

Three different mosquito numbers 20, 50 and 100 per cup were tested. A mean of 4 day old mosquitoes were dry-starved overnight. The mosquitoes were allowed to feed on ~500 µL of blood for 20 min under illuminated conditions via a Baudruche membrane. Six replicates were performed for this test.

3.2.1.1.9 Water Temperature

Four different water bath temperatures were tested 34 °C, 38 °C, 42 °C and 46 °C. Mosquitoes were dry-starved overnight before being allowed to feed on ~500 µL of blood for 20 min under illuminated conditions via a Baudruche membrane. Seven replicates were performed for this test.

Figure 3.2. **Direct membrane feeding assay (DMFA) set up.** Cups connected in a series by tubes to a mini aquarium pump within the water bath which is maintained at ~38 ºC.

3.3 Results

The volunteers who donated blood for membrane feeding were adults with a median age of 46 (range of 28-52) and a median hemoglobin level of 15.5 g/dL with the range of 11.1 -17.7 g/dL. The median room temperature was 28.05 °C (range of 22.2 – 30.34 °C) with a relative humidity of 78.38 % (range of 56-89.3 %). The baseline parameters to which subsequent tests were compared were: 50 female *An. farauti* per cup, aged between 3-5 days, dry starved between 0-4 h, and fed in the dark using Parafilm as the membrane for ~30 min, with 750 µL of blood at a water bath temperature of ~38 °C.

Table 3.1 summarizes the results of the analysis for the starving duration, type of starving, membrane type, feeding duration, mosquito age, feeding in the light versus in the dark, volume of blood, mosquito density and water bath temperature.

Table 3.1. Mosquito feeding rate according the feeding parameters being tested.

Feeding Parameters		Total number of mosquitoes in cups	Total number fed	Total unfed	*Empirical average feeding rate (%)	Range (%)	**P value
Starving time	0 h	916	240	676	27	4-45	<0.01
	2 h	864	288	576	34	15-56	<0.01
	4 h	933	368	565	40	20-54	<0.05
	6 h	954	405	549	45	23-82	<0.01
	Over Night (~21 h)	841	492	349	60	19-97	Ref
Type of starving	Access to water	217	134	83	62	43-89	0.47
	Dry starving	234	167	67	71	31-92	Ref
Membrane type	Baudruche	476	389	87	85	70-100	<0.05
	Parafilm	457	268	189	53	42-76	Ref
Exposure time	10 min	426	326	100	77	63-98	0.63
	20 min	469	368	101	80	43-100	0.61
	30 min	386	314	72	81	72-89	Ref
Mosquito Age	3 days old	631	447	184	75	39-96	0.38
	5 days old	606	473	133	81	50-93	Ref
	7 days old	613	449	164	75	55-90	0.08
Light/Dark	Light	317	274	43	85	64-96	0.88
	Dark	326	276	50	84	63-96	Ref
Volume of blood	125 uL	289	190	99	65	50-88	<0.05
	250 uL	272	229	43	84	67-96	0.54
	500 uL	295	256	39	87	77-98	Ref
Mosquito density	20 mosquitoes	194	148	46	76	64-79	0.13
	50 mosquitoes	480	410	70	85	79-87	Ref
	100 mosquitoes	927	696	231	75	67-74	<0.01
Water bath	34 °C	330	271	59	82	51-95	0.23

temperature	38 °C	335	299	36	89	81-100	Ref
	42 °C	320	277	43	86	72-98	0.61
	46 °C	305	244	61	79	58-96	0.09

Ref: reference group for the calculation of the *P* values.

*Empirical averages were calculated as the average feeding rate of all replicates obtained for a specific condition.

**Significantly different from the reference when $p < 0.05$. Data was transformed using Acrsine (p) prior to paired t-test.

There was a statistically significant difference observed between the feeding rates of the different starving times as determined by one-way ANOVA ($F(3, 24) = 8.982$, $p < 0.001$). When performing t tests for the different paired groups, I found that there were significant differences between the overnight starving and the other starving times (Figure 3.3) with feeding rates approximately doubled when comparing from 0 h (27 %) to ~21 h (60 %). The differences in the feeding rates after starving the mosquitoes for 6 h, 4 h and 2 h were not statistically significant ($p = 0.51$, $p = 0.10$, $p = 0.18$). I observed a mortality rate of 7 % with the overnight starvation compared to the other groups, which exhibited an average mortality of 1 %. The observed difference between the mortality rates at starving times of 0 h, 2 h, 4 h, 6 h and overnight is statistically significant as determined by the one-way ANOVA ($F(3, 19) = 4.000$, $p = 0.03$). There was no significant difference observed between the type of starving, i.e., whether the mosquitoes were dry-starved or allowed to feed on water during the starving period ($p = 0.47$).

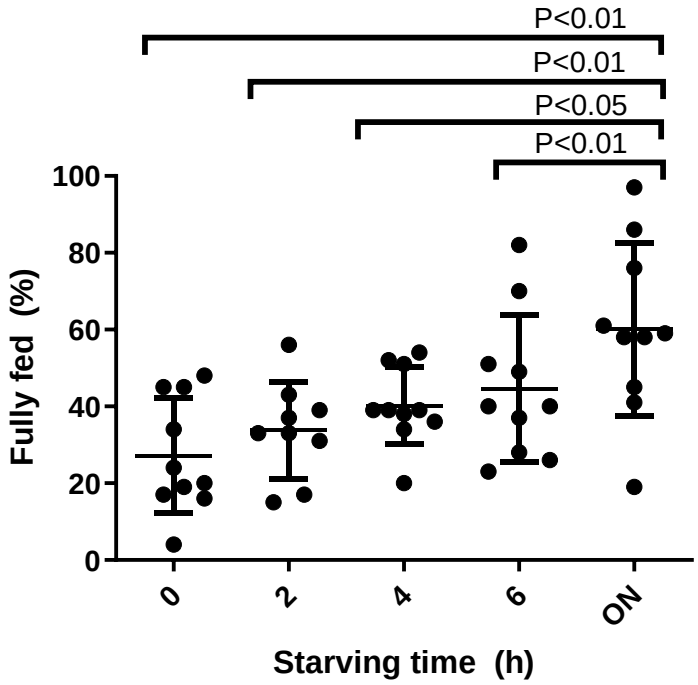


Figure 3.3. Proportion of mosquitoes that fed following the different starving times. The differences between the starving times of 0-4 h and overnight (ON) starving were statistically significant. The error bars denote means and standard deviations. P-values denotes an arcsine transformation of the data.

There was a statistically significant difference in the feeding rates for the two types of membranes tested, Baudruche and Parafilm ($p < 0.05$) (Figure 3.4). Feeding rate increased to 85 % when the Baudruche membrane was used.

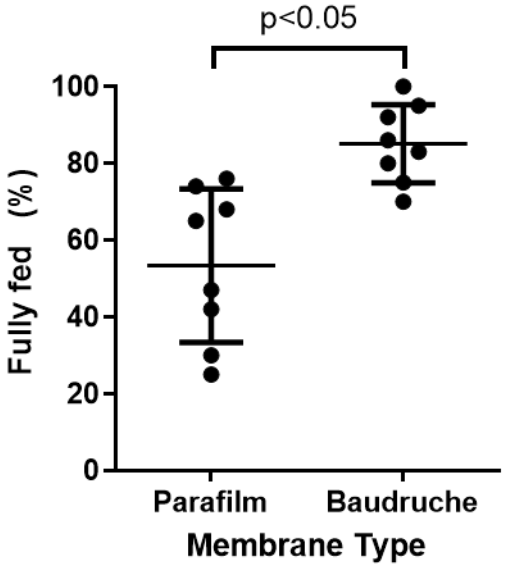


Figure 3.4. Proportion of mosquitoes that were fully fed when using Parafilm and Baudruche membrane types. The observed difference in the performance of Parafilm and Baudruche membrane is statistically significant ($p < 0.05$). The error bars represent means and standard deviations.

Exposure times, mosquito age or feeding in the light versus in the dark were not observed to significantly influence membrane feeding rates (Table 3.1). However, there was a statistically significant difference in the feeding rates for the different volumes of blood as determined by one-way ANOVA ($F(2, 10) = 13.70$, $p < 0.01$). I observed that there was an increase in the feeding rate when the blood volume was increased from 125 µL to anything above 250 µL. The difference was statistically significant ($p < 0.01$) (Figure 3.5). The differences in the feeding rates at different mosquito densities approached significance as determined by one-way ANOVA ($F(2, 15) = 3.861$, $p = 0.052$). When comparing different mosquito density groups, I observed that feeding rates were higher for cups with a mosquito density of 50 per cup as compared to 100 per cup ($p < 0.01$) (Figure 3.6). However, there was no significant difference between 50 and 20 mosquitoes per cup ($p = 0.13$) indicating that 50 mosquitoes per cup was closer to the optimum mosquito density. Interestingly, I did not observe any statistically significant difference in the feeding rate between 20 mosquitoes and 100 mosquitoes.

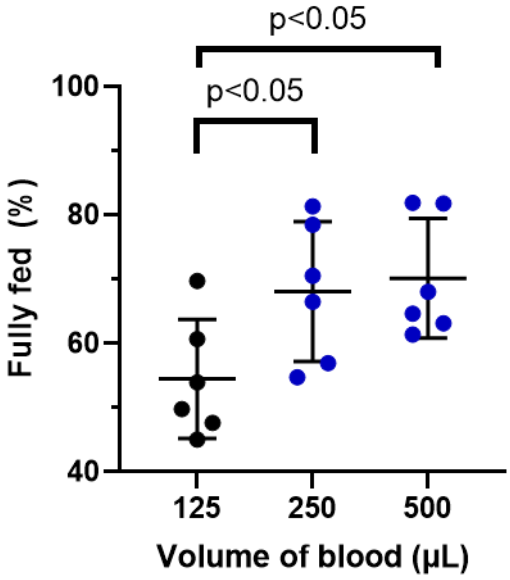


Figure 3.5. Feeding rates for varying blood volume. A significantly higher proportion of mosquitoes fed on 250 µL and 500 µL of blood compared to the feeding rate of 125 µL of blood ($p < 0.05$). The error bars are means with standard deviations. The groups represented by black dots.

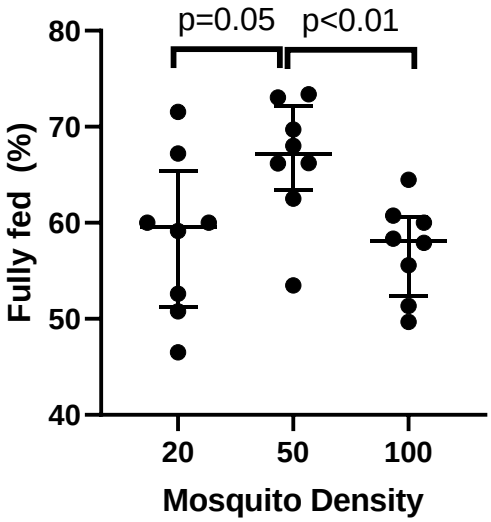


Figure 3.6. Feeding rates for different mosquito densities. The feeding rates for 50 mosquitoes per cup were significantly higher than for 100 mosquitoes per cup ($p < 0.01$). The error bars represent means and standard deviations.

There was no statistically significant difference between the feeding rates at different exposure

times (10, 20 and 30 min), the different mosquito ages (3, 5 and 7 day old) and the different water bath temperatures (34, 38, 42 and 46 °C) as determined by one-way ANOVA ($F(1, 9) = 0.3522$, $p = 0.63$, $F(2, 13) = 1.197$, $p = 0.32$, $F(1, 9) = 0.3522$, $p = 0.63$), respectively.

3.4 Discussion

In this study, a selection of parameters that could potentially influence the feeding rate of *An. farauti* colony mosquitoes in DMFAs were investigated to identify the optimal conditions to enable high feeding rates. By systematically varying individual parameters sequentially, the baseline feeding rate of ~50 % was increased to ~85 %.

Two parameters in particular were associated with improved feeding rates: the starving duration prior to membrane feeding and the membrane type used during the feed. Increasing the starving duration to overnight (~21 h) resulted in a statistically significant difference in the feeding rate (60 %) in comparison to the other starving durations (27 % - 45 %) I selected overnight starving as there were no significant differences between the 6 h and 4 h or 2 h ($p = 0.51$, $p = 0.10$) which had lower feeding rates. However, I did observe a significantly higher mortality rate with overnight starving (7 %) as compared to the other starving times (1 %). This may be due to the long hours of starving. However, the high feeding rate compensates for the increased mortality rate. When compared to the average feeding rate of the other starving times (2 h, 4 h and 6 h) of 39 %, overnight starving resulted in a higher feeding rate by a factor of 1.5. Overnight starving was used in several previous studies [49, 50, 52, 159, 167] while some studies used a minimum of 5 h of starvation with *Anopheles* mosquito species. [54, 165] Use of Baudruche membrane together with overnight starving increased the feeding rate further to 85 %. Previous studies have used either Baudruche membrane or Parafilm for performing DMFAs [49, 54, 155, 159, 162] with different mosquito species. A study by Coulibaly and colleagues comparing the two membrane types using *An. coluzzii* mosquitoes showed that there was no significant difference between the Baudruche and Parafilm membranes when adjusting for other covariates. [52] The superior performance of Baudruche membrane over Parafilm observed here may be particularly due to the fact that Baudruche membrane is made from a natural material as previous evidence has shown that natural membranes have better feeding performances. [170] Another possibility is that the natural membrane was favored as it resembled more closely the direct skin feeding which is occasionally used to feed the mosquitoes for colony maintenance. Varying the conditions of the other feeding parameters (e.g., feeding in the light versus in the dark, mosquito age, blood volume, feeding

duration and water bath temperature) did not significantly increase the feeding rate further.

Even though there was no significant increase in the feeding rates when testing the other parameters, the chosen selection contributed towards the economical use of resources. It was observed that feeding mosquitoes for 10 min yielded similar feeding rates as feeding for 20-30 min. This is within the range of feeding times between 10 min to 30 min that have been used in different studies, and represents a significant time saving. [50, 155, 162, 171] I chose to use 10 - 20 min depending on the feeding schedule. The optimal volume of blood used per glass feeder is important especially when working with limited amounts of infected blood. A volume of 350 µL was recommended for the size (2.5 cm in diameter, surface area of ~5 cm^2) of the glass feeders used in the present study [155] while another study used a total of 1.5 mL [54] using the same size feeders. However, volumes between 250 µL to 500 µL yielded similar high feeding rates. As such, I chose to use any volume within the range of 250 µL – 500 µL depending on the total volume of blood available to feed the mosquitoes. This represents a 3 fold decrease in the volume of blood used compared to the original volume of 750 µL.

With respect to mosquito density, it was observed that 50 mosquitoes (approximate density per cup of 1 mosquito/6.8 cm^2) feeding on a ~5 cm^2 membrane surface area yielded a high feeding rate while 100 mosquitoes per cup (i.e., 1 mosquito/3.4 cm^2) resulted in lower feeding rates. The difference was statistically significant ($p < 0.01$). There was no significant difference in the feeding rates when comparing densities of 20 mosquitoes and 50 mosquitoes per cup. Interestingly there was also no significant difference between 20 mosquitoes and 100 mosquitoes per cup. This observation could be indicative of there being really no significant difference between the three mosquito densities as indicated by the ANOVA test ($F(2 , 15) = 3.861$, $p = 0.052$). However, the p value observed here is approaching statistical significance indicating that more tests need to be conducted in order to conclude if the difference observed between the mosquito densities are significant. Established protocols recommend using 50 - 100 mosquitoes for similar sized cups depending on the size of the feeder and the feeding rate. [155, 165] Based on these results, I chose to use 50 mosquitoes per cup in order to maximize on the number of mosquitoes exposed to blood and reduce the risk of a crowding effect during feeding Although various groups preferred using either of the two types of starving conditions dry or exposing the mosquitoes to water prior to feeding [53, 156-160], I did not observe any significant difference between the two. It may be that *An. farauti* mosquitoes do not feed on water as efficiently as they would on sugar solution as the mosquitoes have specialized sensory organs that detect the presence of blood or nectar triggering

them to feed. [172] Hence, I observed no significant difference between the feeding rates of the starving conditions. I opted for dry starving.

I also did not observe any significant difference between feeding the mosquitoes when exposed to light or in the dark. However, other studies indicated that their membrane feeding experiments were performed in the dark. [49, 54] Also, a protocol by Ouedraogo and colleagues indicated that DMFA should be performed in the dark to mimic the natural feeding conditions during the night, while membrane feeding experiments are commonly undertaken during the day. [165] The contrasting observation may be because this mosquito species has been colonized for over 50 years and feeds well regardless of the light condition. Usually in the wild the *An. farauti* mosquito would feed in the evening starting at 6 pm and peak between 10 and 11 pm when it is dark. [109] Here I chose to feed the mosquitoes while exposing them to the light as it is easier to monitor the progress of the mosquitoes feeding.

Furthermore, I did not detect any significant differences in the feeding rates between age groups 3, 5 and 7 day old mosquitoes. In contrast, Coulibaly and colleagues noted a significant difference in the feeding rates between 3 days and 6 - 9 day old *An. coluzzi*. It could be that this age dependent behavior change is species or colony specific. Collectively, studies on various Anopheles mosquito species reported using mosquitoes aged between 2-8 days. [51, 52, 150, 151, 156, 159, 160, 163, 164] Here I chose to work with 3-5 day old mosquitoes to ensure that I achieve high survival rates on day 7 for the dissection for oocysts and day 14 for the dissection for sporozoites. Finally I did not observe any significant difference between the water bath temperatures of 34 °C, 38 °C, 42 °C and 46 °C. While most studies have indicated using a water bath at 37 °C [50, 53] , this parameter has not been investigated before. The results show that mosquitoes were able to feed efficiently regardless of fluctuations in the water bath temperatures. I chose to use a water bath temperature of 37-38 °C as it closely resembles the human body temperature and will be most conducive for malaria parasite survival.

Most of the conditions tested here will contribute to either improving the efficiency of blood feeding or effective use of our time and resources when performing DMFA with infected blood. These conditions can indirectly influence the transmission of the parasites to the mosquitoes however, only water temperature directly influences the infectivity of the parasites to the

mosquitoes as the parasite viability is influenced by the temperature. All these conditions need to be tested with infected blood to ensure that the ideal conditions for an efficient DMFA with infected blood is selected. There are very few studies which have tested some of these parameters with infected blood. Coulibaly MB and colleagues observed that the optimal parameters for infection are the use of Baudruche membrane, starvation times between 12 and 15 hours, and a mosquito age of 3 days, [52] while Vallejo AF and colleagues observed that the optimal mosquito infectivity occurs with mosquitoes four days after emergence and at a cage density of 100. [167]

These optimized conditions are specific for *An. farauti* mosquitoes and tailored to the operational settings that are currently feasible at the PNGIMR. It may be used as a guide to set up of similar DMFAs with other species under different settings.

3.5 Conclusion

By sequentially and systematically varying individual membrane feeding parameters, the blood feeding rate of *Anopheles farauti s.s.* colony mosquitoes was increased significantly to 85 %. This highlights the importance of parameter selection and optimization in direct membrane feeding assays. Further work will need to be performed with infected blood to ensure that these parameters result in high infection and survival rates for *An. farauti* colony mosquitoes using DMFA.

Summary

- The feeding rate of the DMFA with *An. farauti* was increased to 85 %.
- Baudruche membrane and overnight starving maximized feeding success rate.
- The optimized feeding conditions were: exposing 3-5 day old overnight starved *An farauti* to 250-500 µL of blood at 38 °C via a Baudruche membrane for 10 – 20 minutes under illuminated conditions.

Bridge

Having identified the optimal feeding conditions in Chapter 3, in Chapter 4 I describe the use of the DMFA to infect *An. farauti* colony mosquitoes with *P. falciparum* and *P. vivax* parasites from infected individuals. This chapter describes how I have successfully infected the colony mosquitoes with malaria parasites. This chapter was published in *Frontiers in Cellular and Infection Microbiology* (doi:10.3389/fcimb.2021.771233). The published paper has been modified to flow with the thesis formatting and referencing.

Timinao L, Vinit R, Katusele M, Koleala T, Nate E, Czeher C, Burkot TR, Schofield L, Felger I, Mueller I, et al: **Infectivity of Symptomatic Malaria Patients to Anopheles farauti Colony Mosquitoes in Papua New Guinea**. Front Cell Infect Microbiol 2021, 11:771233.

CHAPTER 4

Infectivity of symptomatic malaria patients to *Anopheles farauti* colony mosquitoes in Papua New Guinea

4.1 Introduction

Transmission between the human host and the mosquito vector is a crucial step in the malaria parasite life cycle. It represents a bottleneck where parasite numbers shrink from billions in the human body to less than a hundred in the mosquito vector. [133] Transmission through the mosquito is thus vulnerable to interruption and is a key focus of malaria research [148, 173], with research tools including membrane feeding assays designed to explore this transitioning phase of the parasite. DMFAs were initially developed by Rutledge and colleagues in 1964 in which malaria parasites were exposed to mosquitoes via a membrane feeding apparatus. [47]

Direct Membrane Feeding Assays provide a means to investigate the still poorly understood process of human to mosquito transmission and the resulting mosquito infection. For example, DMFAs can be used to study the infectiousness of different human malaria reservoirs, and estimate their contribution towards transmission. [50, 143] This can include symptomatic, patent infections as in the present study and asymptomatic, often low-density infections. [162] In addition, DMFAs can be used to study the effect of drugs, vaccine candidates and immune factors on the development of the mosquito stages of the *Plasmodium* parasites. [155, 167, 174, 175] Also, DMFAs provide an opportunity for circumventing some of the operational and ethical complicating factors associated with feeding mosquitoes directly on the skin of malaria infected individuals. Finally, there is evidence that there is no clustering of gametocytes in the skin as initially perceived thus making DMFAs a reliable tool for infection studies. [176, 177]

Despite these advantages, DMFAs are resource intensive, require an insectary and rely on stringent logistics for sample collection, handling, rapid transportation and processing as it has been shown that the time between blood collection and performance of the DMFA can impact assay outcome, most likely due to premature gametocyte activation. [155, 178, 179] As a further complication, conducting DMFAs with *P. vivax* requires proximity to endemic areas in order to access infected samples as continuous culture of this parasite species remains elusive. [180] Papua New Guinea (PNG) is amongst the countries with the highest *P. vivax* burden in the world, thus *P. vivax* is a research priority for the country and infected blood samples can still easily be obtained. [68, 71, 181-183] Establishing DMFAs with *P. vivax* provides a tool to study *P. vivax* transmission that is of potentially global relevance.

DMFAs were performed in PNG previously in 1983 - 1985 in village-based malaria surveys, prior

to diagnosis and on known gametocyte carriers in clinical outpatient populations in Madang and Goroka. [143] In the present study, I investigated the infectivity of blood samples obtained from symptomatic, rapid diagnostic test (RDT)-positive individuals to *Anopheles farauti* colony mosquitoes.

4.2 Methods

4.2.1 Sample collection

This study was conducted at the PNG Institute of Medical Research (PNGIMR) in Madang Province, PNG, between May 2014 and November 2018. Study participants were recruited from Madang Town Clinic and Yagaum Rural Health Centre. Ethical approval was received from the PNGIMR Institutional Review Board (IRB #1516) and the PNG Medical Research Advisory Committee (MRAC #16.01). Written informed consent was received from all individuals enrolled in the study. Individuals presenting with malaria symptoms were tested with a malaria rapid diagnostic tests (RDT). In the present study, CareStart Malaria Pf/PAN (HRP2/pLDH) Ag Combo RDTs kits (Access Bio, Cat No. RMRM-02571CB) were used. From RDT-positive individuals venous blood samples (3 - 5 mL) were collected in BD Vacutainer ® which contain spray-coated lithium heparin (BD, North Ryde, NSW, Australia) and immediately stored in a beverage cooler flask (Coleman Company Inc, Kansas, USA) filled with warm water (~37.0 ˚C, measured by a digital thermometer attached to the flask). I also measured their Hemoglobin level using a HemoCue machine (HemoCue ®, Mt Waverley, VIC, Australia), their temperature using a digital thermometer and their weight using a bathroom scale.

In the present study, the time between sampling and feeding was approximately 20 - 30 min for samples collected at Yagaum clinic, located in a 10 min walking distance from the insectary. Transport of blood samples collected in Madang town clinic took about 1.5 - 2 h and involved a 30 - 40 min drive.

4.2.2 Mosquito colony maintenance, membrane feeding assays and mosquito dissection

The present study used an *An. farauti sensu stricto* colony, which was first adapted in Rabaul, East New Britain province of PNG in 1968. In 1984 females from Agan village, Madang were added

to the Rabaul colony in an attempt to back cross. The colony was subsequently used in several studies. [143, 168, 184, 185] The colony was maintained using established methods. [186] To conduct DMFAs, 3 - 5 day old female mosquitoes were put into paper cups (50 -100 per cup). One mL of human blood sample was inserted into the water jacketed glass feeder where I initially exposed up to 400 mosquitoes with 100 mosquitoes per cup (X 4 cups) and then changed with up to 200 with 50 mosquitoes per cup (X 4 cups) as it was less crowded and the feeding rate was much improved. [144]

The feeding cups and water jacketed glass feeder were set up in the laboratory prior to the arrival of blood samples to minimise the time between blood sample collection and direct membrane feeding. The light in the insectary was dimmed and the glass feeder with the cups were covered with a dark cloth for the period of feeding. After ~15 - 20 min, the cups of *An. farauti* were removed from under the glass feeders and any unfed mosquitoes were removed. The cups containing the fully fed mosquitoes were kept for 7 - 9 days before dissecting for oocysts. [155, 165] Dissection was performed as described elsewhere. [165] Briefly, mosquito guts were stained with 0.2 % mercurochrome for 10 - 15 min and oocysts were counted under a light microscope at 10 X magnification. Oocysts per midgut were counted once by an experienced microscopist.

4.2.3 Light microscopy and PCR detection of malaria parasites

Retrospective diagnosis of the malaria parasites was performed by light microscopy and quantitative real-time PCR (qPCR). Thick and thin blood films were prepared using standard WHO methodology. The blood films were stained with 4 % Giemsa stain for 30 min. [146] Slides were read according to WHO standards and by WHO certified microscopists. Parasite density was calculated by multiplying the parasite count/200 WBC count (or 500 WBC count if the parasite count is < 100) by 8000 leukocytes. [146] The final parasite density was calculated by taking the geometric mean of the densities obtained from reads by two expert microscopists. Discrepancies in the presence or absence of parasites, parasite density (i.e. if they differed by a factor of 10) and parasite species between the two microscopists was resolved by a third expert microscopist. DNA extraction was performed on 250 µL of red blood cell pellets using Favogen DNA extraction kits (Favogen Biotech Corp, Ping Tung, Taiwan) and performed according to the protocol for extraction of genomic DNA from blood. Following DNA extraction, a qPCR assay was performed to quantify the infection and determined the parasite species as described elsewhere. [147] Briefly, this is a probe based qPCR assay where a conserved region of the 18s rRNA gene was amplified

for both *P. falciparum* and *P. vivax*.

4.2.4 Statistical analyses

Prism 6.01 (GraphPad Software, La Jolla, CA USA) and Stata 13 (StataCorp, College Station, TX, USA) were used to analyse data. To compare proportions, two-sample tests of proportions were used. To test the influence of a continuous variable (such as parasite density) on a binary outcome variable (such as DMFA success rate), logistic regression was used. To test the association between two continuous variables such as infection rate in the successfully infected mosquitoes versus gametocyte density I used non-parametric correlation analysis (Spearman's rank correlation).

4.3 Results

4.3.1 Study population

Selection of patients relied on RDT diagnosis. Subsequent light microscopy examinations of the corresponding blood slides and molecular diagnosis by qPCR were conducted for 182 RDT-positive participants. A total of 45 patients were recruited from Madang town clinic while 137 were recruited from Yagaum clinic. Table 4.1 shows the characteristics of the study population and Table 4.2 shows the results from RDT, light microscopy examination and molecular diagnosis by qPCR.

Table 4.1. Characteristics of the study population. Values are presented as proportions (n/N) and percentage or median and range.

Demography	**Median (range) or n/N (%)**
Age in years (N=182[a])	17 (5-55)
Female (N=182)	91/182 (50.0 %)
Weight, kg, (N=175[b])	47 (14-96)
Hemoglobin, g/dl, (N=118[b])	9.1 (4.2-13.7)
Temperature, ˚C , (N=161[b])	36.6 (34.1-40)
Fever, >37.5 ˚C, (N=161)	48/161 (29.8 %)

[a] Eight individuals with unknown age so were considered as adults

[b]These data were not collected from all 182 patients

4.3.2 Malaria diagnosis

The largest proportion of individuals (49.5 %; 95 % CI 41.97 - 56.95 %) was RDT positive for both, HRP2 and pLDH tests while 30.2 % (95 % CI 23.65 - 37.45 %) and 20.3 % (95 % CI 14.74 - 26.92 %) of patients were positive only for HRP2 or pLDH-based tests, respectively. Light microscopy revealed that the largest proportion of symptomatic patients in this study population were infected with *P. falciparum* (47.8 %; 95 % CI 40.90 - 55.86 %) followed by *P. vivax* (28.6 %; 95 % CI 22.13 - 35.72). Median (range) parasite density was 6423 (110 - 51,040) parasites/µL for *P. falciparum* and 4240 (136-32,480) parasites/µL for *P. vivax.* There were 3 mixed infections (1.6 %; 95 % CI 0.3 - 4.74 %) containing both, *P. falciparum* and *P. vivax*. The qPCR results revealed a slightly higher proportion of *P. falciparum* infections (40 %; 95 % CI 36.62 - 51.49 %) than *P. vivax* infections (30.2 %; 95 % CI 23.65 - 37.45 %). A higher proportion of the samples were diagnosed as mixed infections by qPCR as compared to microscopy (11% vs 1.6 %). Over all I observed a higher proportion of patients who were diagnosed as *P. falciparum* positive by microscopy and qPCR as compared to RDT diagnosis (Table 4. 2).

Results of the diagnosis by RDT, microscopy and qPCR, N = 182 samples in each case.

Table 4.2. RDT, Microscopy and qPCR diagnosis of malaria parasites.

Diagnosis		n	n/N (%)	95% CI
RDT	HRP2	55	30.2	23.65 - 37.45
	pLDH	37	20.3	14.74 - 26.92
	HRP2 & pLDH	90	49.5	41.97 - 56.95
Microscopy	*P. falciparum* asexual only	68	37.4	23.65 - 37.45
	P. falciparum asexual with gametocytes	20	11.0	6.84 - 16.46
	P. vivax asexual only	28	15.4	10.47 - 21.46
	P. vivax asexual with gametocytes	24	13.2	8.63 - 18.98
	P.falciparum with gametocytes & *P.vivax* with gametocytes	2	1.1	0.13 - 3.91
	P.falciparum asexual & *P.vivax* with gametocytes	1	0.5	0.01 - 3.02
	Microscopy negative	37	20.3	15.70 - 28.11
qPCR	*P. falciparum*	80	44.0	36.62 - 51.49
	P. vivax	55	30.2	23.65 - 37.45
	P.falciparum & P.vivax mix	20	11.0	6.84 - 16.46
	PCR negative	27	14.8	10.01 - 20.85

n – the number of individuals diagnosed for malaria by either RDT, microscopy or qPCR. N = 182

A total of 154/182 (85 %) of the samples were concordant between qPCR and microscopy diagnosis. It was also observed that 19/182 (10.4 %) patients were negative by microscopy but were positive by qPCR for malaria parasites while 9/182 (5 %) were positive by microscopy but negative for qPCR (Table 4.3). A sensitivity of 94 % was observed for qPCR diagnosis with a specificity of 49 %. The positive predictive value (PPV) of 88 % and a negative predictive value (NPV) of 67 % was observed for qPCR. I also observed that there was no correlation between the microscopy diagnosis and qPCR for the following groups; *P. vivax, P. vivax* with gametocytes and *P. falciparum* with gametocytes. However, there was a significant but weak correlation observed

with *P. falciparum* (Spearman's rank correlation coefficient R=0.4, p<0.001).

Table 4.3. Comparison of malaria parasite detection in patients' blood by microscopic examination and qPCR.

	Microscopy (Gold standard)			
	Test Result	**+**	**-**	**Total**
qPCR	+	136	19	155 (85 %)
	-	9	18	27 (15 %)
	Total	**145 (80 %)**	**37 (20 %)**	**182 (100 %)**

4.3.3 Mosquito infection

Overall, 38/182 (20.9 %) of blood samples in DMFAs infected mosquitoes with 36/38 (94.7 %) of the patients being recruited from Yagaum clinic while the remaining 2/38 (5.3 %) were Madang town clinic. Figure 4.1 shows an example of an *An. farauti* midgut infected with *P. vivax* oocysts 7 days post infection.

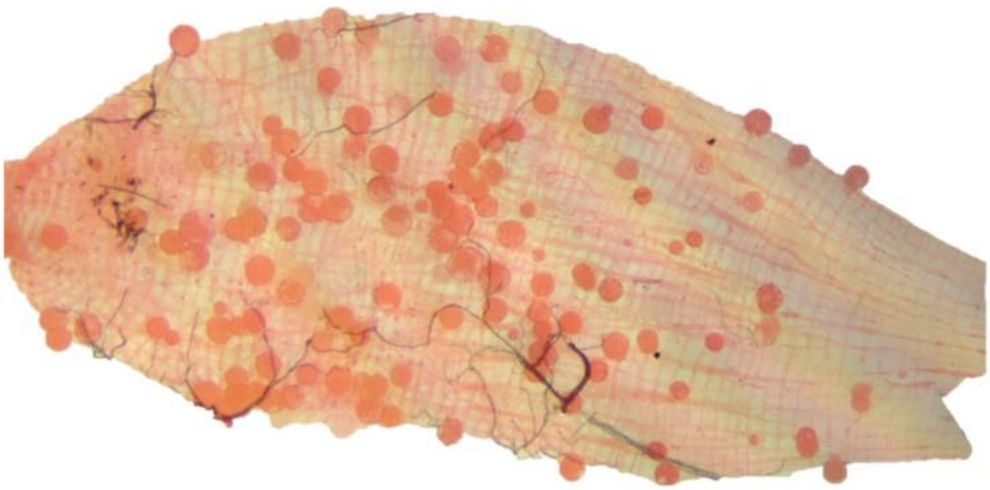


Figure 4.1. *P. vivax* infected midgut from *An. farauti* mosquito dissected in the present study. The image was taken on a Zeiss Primostar microscope equipped with an Axiocam 105 Color camera (Carl Zeiss Pty. Ltd.) at 10x magnification. The image was then edited using PowerPoint, Microsoft office 2010 and Adobe Photoshop CS6.

Although not significant I did observe a higher proportion of infections by individuals diagnosed with RDT as pLDH positive than those diagnosed as HRP2 positive (35.1 % vs 27.3 %, p = 0.43) (Table 4.4). Interestingly, *P. vivax* infections diagnosed by light microscopy were significantly more infectious to mosquitoes compared to *P. falciparum* infections (44.2 % vs. 11.4 %, p < 0.01). Similar observations were made with qPCR diagnosis (43.6 % vs 10 %, p < 0.001). Within the *P. vivax* samples, a higher proportion of blood samples were infectious to mosquitoes when *P. vivax* gametocytes were detected by microscopy (58.3 %). I noted that 32 % (9/28) and 10.3 % (7/68) of the *P. vivax* and *P. falciparum* infections that infected mosquitoes were gametocytaemic by microscopy. In addition, all the mixed infections (3/3) by microscopy gave rise to mosquito infections.

Table 4.4. Mean oocyst counts from DMFAs in *An. farauti* according to RDT, microscopy and qPCR. All samples were collected from symptomatic RDT positive patients. Values are presented either as proportion (n/N) and percent, or as average and minimum to maximum range.

RDT, microscopy & qPCR results	Proportion of DMFAs resulting in mosquito infection		Proportion of mosquitoes infected*		Oocyst number
	n/N	% (95% CI)	n/N	% (95% CI)	average (range)
RDT					
HRP2	15/55	**27.3** (15.5 - 39.1)[a]	564/966	**58.4** (55.2 - 61.5)	**6** (1-106)
pLDH	13/37	**35.1** (19.7 - 50.5)[b]	349/863	**40.4** (37.2 - 43.8)	**27** (1-534)
HRP2 & pLDH	10/90	**11.1** (4.6 - 17.6)[c]	60/415	**14.5** (11.2 - 18.2)	**3** (1-17)
Microscopy					
P. f. asexual only	7/68	**10.3** (3.1 - 17.6)[d]	66/376	**17.6** (13.8 - 21.8)	**5** (1-16)
P. f. + gametocytes	3/20	**15** (0 - 30.6[§])	47/94	**50.0** (39.5 - 60.5)	**3** (1-9)
P. v. asexual only	9/28	**32.1**	218/627	**34.8**	**9**

		(14.8 - 49.3)[e]		(31 - 38.6)	(1-93)
P. v. + gametocytes	14/24	**58.3** (38.6 - 78)[f]	424/749	**55.9** (52.8 - 60.2)	**19** (1-534)
P. f. + gametocytes & *P. v.* + gametocytes	2/2	**100** (NA)	13/69	**18.8** (10.4 - 30.1)	**3** (1-13)
P. f. asexual only & *P. vivax gametocytes*	1/1	**100** (NA)	83/89	**93.3** (85.9 - 97.5)	**7** (1-36)
Microscopy Negative	2/39	**5.1** (0 - 12[§])	13/38	**34.2** (19.6 - 51.4)	**9** (1-29)
qPCR					
P. falciparum	8/80	**10** (3.4 - 16.6)	135/481	**28** (24.1 - 32.3)	**3** (1-43)
P. vivax	24/55	**43.6** (30.5 - 56.7)	735/1501	**49** (46.4 - 51.5)	**12** (1-534)
P. falciparum & *P. vivax*	4/20	**20** (2.5 - 37.5)	96/248	**38.7** (32.6 - 45.1)	**8** (1-106)
qPCR Negative	2/27	**7.4** (0 - 17.3)[§]	7/14	**50.0** (23.0 – 77)	**7** (1-24)

Note: [*]only infected mosquitoes were considered (i.e., uninfected mosquitoes were not included into this calculation); significant differences were observed in the proportions *a vs. c*; *b* vs. *c*, *d* vs. *e* and *d* vs. *f*. No significant difference was observed between a vs. b, p = 0.43

[§]95 % confidence interval includes negative values.

There was a weak correlation between the proportion of infected mosquitoes and *P. vivax* density by microscopy with the correlation approaching significance (p = 0.08, Spearman's rank correlation coefficient R = 0.4) as shown in Figure 4.2 A. The proportion of infected mosquitoes was significantly correlated with *P. vivax* gametocyte density (p < 0.05, Spearman's rank correlation coefficient R = 0.6) as shown in Figure 4.2 B. However, the considerable scatter and correlation coefficient of R = 0.6 indicated that the correlation was not very strong. There was no correlation between the mosquito infection rate and the copy numbers of *P. falciparum* or *P. vivax* by qPCR.

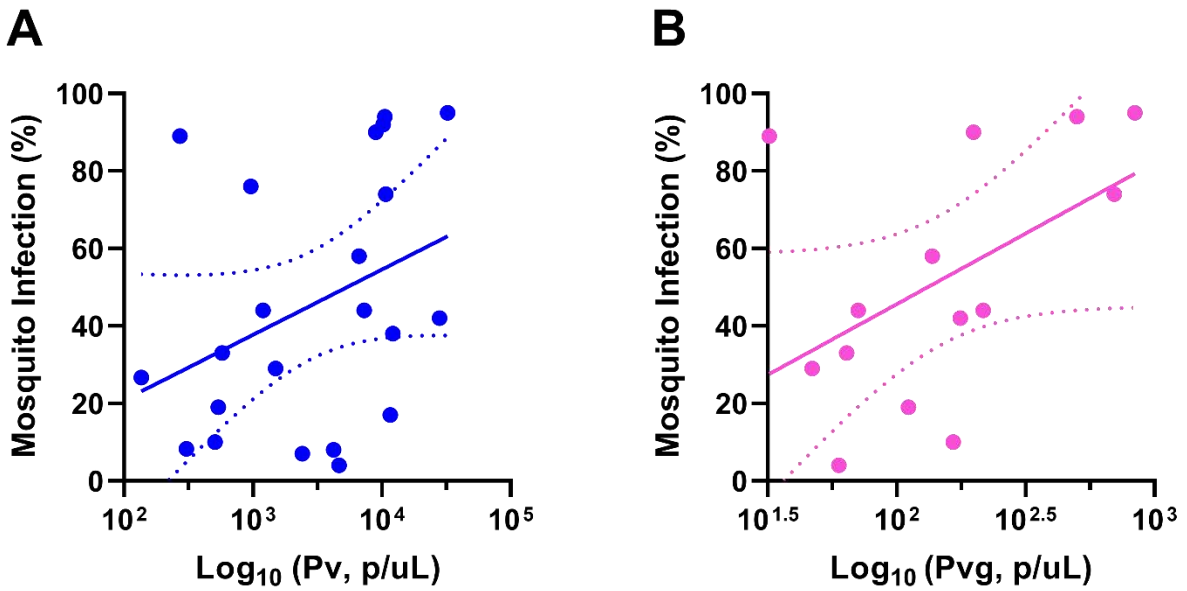


Figure 4.2. Correlation between mosquito infection rate and parasite burden of infected humans. Correlation between mosquito infection and **Panel A**: *P. vivax* parasites (R = 0.4, p = 0.08), **Panel B**: *P. vivax* gametocytes (R = 0.6, p = 0.04). The trend lines are linear regression while the area between the dotted lines represents the 95% confidence interval. *Pv: Plasmodium vivax, Pvg: P. vivax* gametocytes. Each dot represents a mosquito that was infected with 1 or more oocyst. Panel A has 22 while Panel B has 14 successful infections. Infection success, i.e., DMFAs resulting in at least 1 infected mosquito, was not significantly correlated with parasite or gametocyte density when tested using logistic regression in any of these groups; *P. vivax*, *P. falciparum*, *P vivax* with gametocytes.

I observed moderate and significant correlations between the number of oocysts per infected mosquito midgut and the proportion of infected mosquitoes per DMFA according to microscopy diagnosis for the following; *P. vivax* (Spearman's rank correlation coefficient R = 0.7, $p < 0.0001$), *P. vivax* with gametocytes (Spearman's rank correlation coefficient R = 0.7, $p < 0.01$,) and *P. falciparum* (Spearman's rank correlation coefficient R = 0.7, $p < 0.05$) as shown in Figures 4.3 A-C. I also observed a moderate and significant correlation between oocysts per infected mosquitoes and mosquito infection by qPCR for *P. vivax* according to qPCR diagnosis (R = 0.7, $p < 0.001$) as shown in Figure 4.3 D. There was no correlation observed between the proportion of infected mosquitoes and the copy numbers of *P. vivax* or *P. falciparum* by qPCR.

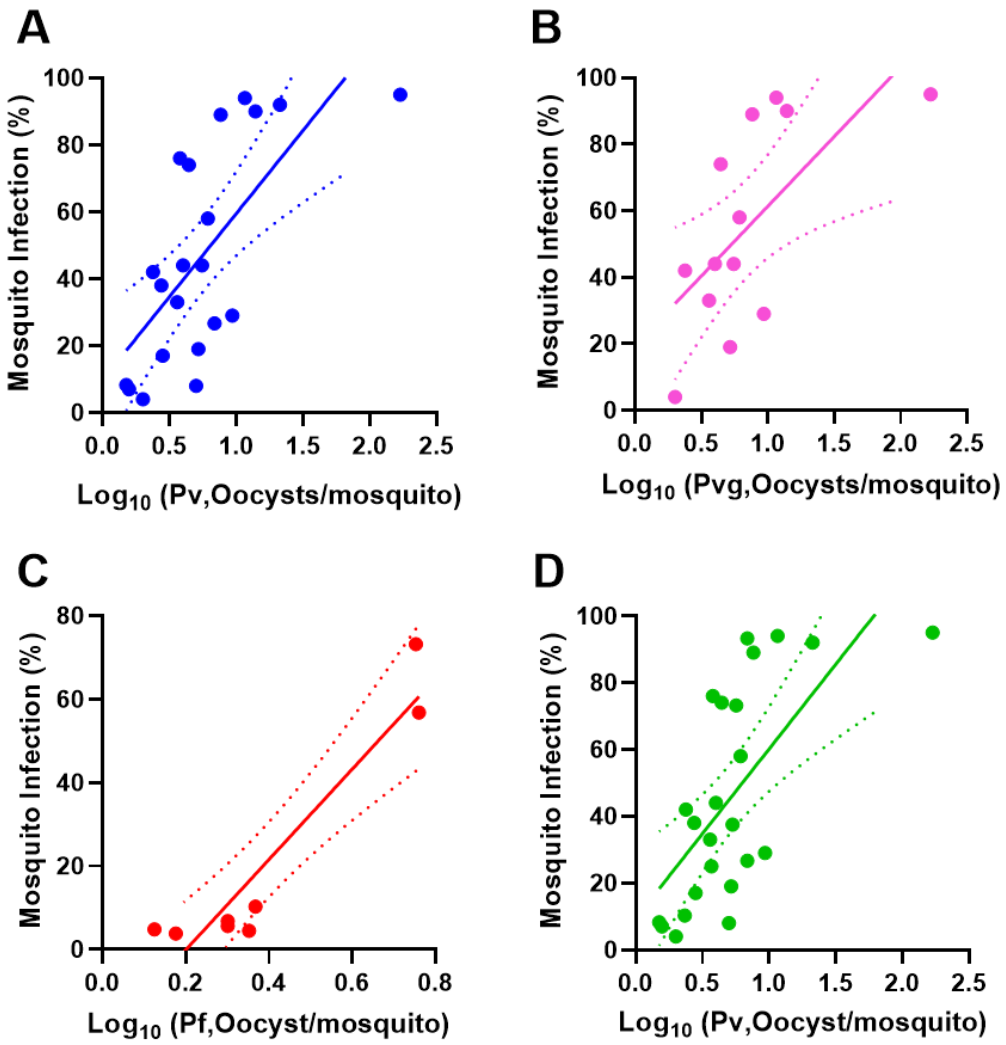


Figure 4.3. Correlation between proportion of infected mosquitoes and the mean oocyst number per infected mosquito. The oocysts per infected mosquito for **Panel A:** *P. vivax* ($R = 0.7$, $p < 0.001$), **Panel B:** *P. vivax* with gametocytes ($R = 0.7$, $p < 0.01$), **Panel C:** *P. falciparum* ($R = 0.8$, $p < 0.05$) and **Panel D:** *P. vivax* by qPCR ($R = 0.7$, $p < 0.001$). The trend lines are liner regression while the area between the dotted lines represents the 95 % confidence interval. *Pv: Plasmodium vivax, Pvg: P. viv*ax gametocytes, *Pf: P. falciparum*.

4.4 Discussion

Currently the limitation with doing research with *P. vivax* is it is still difficult to maintain a continuous culture of *P. vivax*, which necessitates access to naturally acquired infections in field settings, often associated with additional cost and operational constraints. As such, a reliable *P. vivax* DMFA setup in an endemic setting can be of great value.

In the present study, I investigated the infectiousness of symptomatic, RDT-positive malaria cases obtained from local health facilities. In a resource constrained situation where diagnosis by microscopy is not readily available and where the primary diagnosis of malaria is performed by RDT, it is important to assess which RDT result will most likely lead to a mosquito infection. As commonly known, RDT results are not reliable in distinguishing between *Plasmodium* species in co-endemic settings, however, the present study shows that they can be used to prioritize samples selected for DMFAs to maximize the probability of a specific species being present in the sample and to increase infection success (Table 4.2). [187] I observed that in the group of samples positive for only pLDH with the CareStart RDT the proportion of successful DMFAs was highest (35.1 %) compared to HRP2 (27.3 %) or when positive for both antigens (11.1%). This difference in proportions was statistically significant between pLDH and both antigens ($p < 0.01$) but not between pLDH and HRP2 ($p = 0.43$). In PNG where both *P. falciparum* and *P. vivax* are present in roughly equal proportions, *P. vivax* infections were more likely when the RDT is positive for only the pLDH antigen. [188] I therefore decided that by selecting samples only positive for pLDH over HRP2 (or both antigens) the likelihood of the sample being *P. vivax* would be significantly increased and DMFA success can be increased up to 3-fold. There is a sound biological explanation as to why acute *P. vivax* infections result in mosquito infections more frequently. It has been shown that *P. vivax* gametocytes develop faster, and are present and infectious at the onset of an infection while *P. falciparum* gametocytes can take 10 days to mature. [22] Consequently, lower infectiousness in symptomatic *P. falciparum* patients as compared to *P. vivax* patients is expected since people are likely to seek treatment before *P. falciparum* gametocytes have matured. [162] Furthermore, HRP2 based RDTs can remain positive for 35-42 days after treatment and clearance of parasitaemia, while for pLDH it takes only 2 days before the antigen is cleared from circulation giving a more reliable result. [189]

I observed that the proportion of samples that infect mosquitoes was higher for *P. vivax* (44.2 %) compared to *P. falciparum* (11.4 %) according to microscopy. Interestingly, I observed a higher mosquito infection rate (58.3 %) for samples with *P. vivax* gametocytes detectable by light microscopy while the mosquito infection rate with *P. falciparum* gametocytes was low (15 %). Although the findings are in contrast to what was observed previously by Graves and colleagues in *An. farauti* where they showed a 37.5 % (6/16) infectivity with *P. vivax,* 18.8 % (3/16) infectivity in *P. vivax* with gametocytes and a 48.1 % (13/27) infectivity in *P. falciparum* with gametocytes, this may be due to the difference in sample sizes used. [143] The current results show

that DMFA with *P. vivax* is about 4 times more successful than with *P. falciparum* especially when considering samples with gametocytes. Although I am uncertain as to why I observed low *P. falciparum* infections a possible explanation is that I did not evaluate the immunity-related factors. This could be further studied by comparing, in parallel, DMFAs conducted with autologous plasma (i.e., replacement of patient's plasma with the individual's own plasma), whole blood (directly added to the feeder) and malaria-naïve plasma (i.e., replacement of patient plasma with plasma from a *P. falciparum* naive donor). Furthermore, a possible explanation could be that this strain of mosquitoes may have become refractory to wild-type *P. falciparum* infection. This was observed with cultured gametocytes of *P. falciparum* which were fed to this strain of *An. farauti* mosquitoes via standard membrane feeding which resulted in an extremely low mosquito infection rate. [190] This indicated that the *An. farauti* strain was refractory to cultured gametocytes and that could also be the case with wild-type parasites as well. Interestingly, my observations that this does not apply to *P. vivax* may be the basis for further studies into species-specific mechanisms of mosquito infection.

In the present study, I observed an 85 % concordance between the microscopy diagnosis and qPCR diagnosis. I observed a 94 % sensitivity and a 49 % specificity when comparing qPCR with light microscopy as the reference method. This is a result of the lower limit of detection of the qPCR method, which is able to detect many more infections as compared to light microscopy. As qPCR is able to detect these sub-microscopic infections, the proportion of false positive is overestimated when compared to light microscopy leading to an apparently low specificity. The possibility of an infection (or no infection) by microscopy being confirmed by qPCR is expressed by a moderate PPV and NPV (88 % and 67 %). I note that the lack of having microscopy diagnosis being done prior to bleeding was a limitation in this study and light microscopy results were only obtained retrospectively by highly trained microscopists. I found that species and parasite stage determination by light microscopy was a very good predictor of infection success, as *P. vivax* with gametocyte infections resulted in approximately 4-fold increased infection success in the mosquitoes as compared to *P. falciparum*. Based on the results I estimate that light microscopy diagnosis before bleeding would enable a further increase of DMFA success rate with *P. vivax* to around 60 % if suitable *P. vivax* samples (those with gametocytes by light microscopy) were selected. Similar infectivity rates (45-60 %) were measured in *Anopheles aquasalis, Anopheles albitarsis* in Brazilian Amazon, *Anopheles albamanus* in Colombia, *An. dirus* in Thailand and *Anopheles arabiensis* in Ethiopia. [53, 159, 167, 191, 192] I did also observe a significant but

moderate correlation between *P. vivax* gametocytes and mosquito infection (Figure 4.2B). Other studies observed similar but often stronger positive associations between *P. vivax* gametocyte densities and the proportion of infected mosquitoes in *An. dirus* in Thailand and *An. arabiensis* in Ethiopia. [162, 192] However, there are other studies which describe the relationship between *P. vivax* gametocytemia and mosquito infection as weak with *An. dirus* in Thailand. [53, 193]

The observed correlation between mosquito infection prevalence and oocyst density was moderate but significant for both *P. falciparum* and *P. vivax* (Figure 4.3 A-D). That is, the more mosquitoes are infected during a DMFA, the higher the average number of oocysts in the infected mosquitoes. These findings are in contrast with a previous study where a strong correlation was observed between the mosquito infection rate and the oocyst rates for *An. dirus* with *P. vivax.* [162]

I noted that only 2/45 (4.4 %) of samples resulted in mosquito infections from DMFA using blood from the Madang Town Clinic while 36/137 (26.2 %) of samples from Yagaum clinic infected mosquitoes. The low infection rate from Madang Town Clinic was mainly because most of the samples were without gametocytes especially *P. vivax* gametocytes. Of the 45 samples 3 samples had only *P. falciparum* gametocytes while 2 had only *P. vivax* gametocytes and one with both *P. falciparum* and *P. vivax* gametocytes. Of the 2 samples that led to successful infections, one had only *P. vivax* gametocytes while the other had both *P. falciparum* and *P. vivax* gametocytes. Another factor which could have influenced the infectivity of the mosquitoes but was not investigated here is impact of temperature fluctuations of the thermal flask while transporting it from Madang to the laboratory, and the longer duration between collection of the sample and the DMFA. It has been shown elsewhere that temperature of thermal flask does influence the infectivity of the mosquitoes. [179]

4.5 Conclusion

This study provides important insights into the infectivity of symptomatic malaria cases to *An. farauti* in PNG. Overall, I show that symptomatic *P. vivax* infections are more likely to be infectious to mosquitoes as compared to symptomatic *P. falciparum* infections. This may be a result of the differences in gametocyte dynamics that exist between *P. falciparum* and *P. vivax*. I have re-established a DMFA set up in PNG, where frequent access to *P. vivax* infections is provided. This could serve as a platform to test potential transmission blocking vaccines and antimalarials, which act on gametocytes or the mosquito developmental stages of *P. vivax*.

Summary

- 44.2 % and 11.4 % of *An. farauti* mosquitoes were infected with *P. vivax* and *P. falciparum*, respectively.
- 58.3 % of *An farauti* were infected when *P. vivax* gametocytes were observed by microscopy.
- A significant correlation was observed between *P. vivax* gametocytes and mosquito infection.
- A significant correlation was observed between mosquito infection prevalence and oocyst density for both *P. falciparum* and *P. vivax.*

Bridge

Having established an optimal DMFA for infecting laboratory *An. farauti* with malaria, a qPCR assay to detect oocysts and sporozoites is described in Chapter 5 including a comparison of two parasite DNA extraction methods. The manuscript was submitted to *Frontiers in Parasitology* and comments from the first reviewer have been included in this chapter. The submitted paper has been modified to flow with the thesis formatting and referencing.

Timinao L, Esther W. Jamea, Katusele M, Burkot TR, Karl S: **Using qPCR to compare the detection of Plasmodium vivax oocysts and sporozoites in Anopheles farauti mosquitoes between two DNA extraction methods.** *Manuscript submitted to Frontiers in Parasitology*

CHAPTER 5

Using qPCR to compare the detection of *Plasmodium vivax* oocysts and sporozoites in *Anopheles farauti* mosquitoes between two DNA extraction methods

5.1 Introduction

Malaria is a significant health problem in 85 countries and nearly half of the world's population is living in areas with risk of malaria transmission. [3] Despite the efforts to curb malaria globally, it has proven difficult to achieve a steady decrease in malaria cases over the years, highlighting the need for additional interventions. Transmission blocking interventions such as vaccines and antimalarials can be effective tools used to prevent the spread of malaria parasites. [194]

Human-to-Mosquito transmission, and the activity of potential transmission-blocking compounds, can be investigated using artificial systems such as membrane feeding set ups. Membrane feeding assays (MFAs) were initially developed by Rutledge and others in the 1960s. [195] In MFAs malaria parasites (whether cultured *in vitro* in the laboratory or from infected patients) are fed to the mosquitoes. [196, 197] Transmission success can be evaluated by the observation of various parasite developmental stages in the mosquito in particular, the oocysts in the midgut and sporozoites in the salivary glands using light microscopy. Traditionally, light microscopy (LM) was used for assessing the presence or absence of the oocysts or sporozoites in the mosquito however, there are inherent limitations with LM detection of parasite mosquito stages. These include labor intensiveness, the requirement for trained personnel and the resulting low throughput. In addition, low-level infections can easily be missed or misdiagnosed, and the differentiation between parasite species in co-endemic settings is not possible.

MFAs can be operationally challenging particularly in resource-limited settings. Since there is no continuous *P. vivax* culture, access to infected individuals is currently the only option. [45] This comes with inherent issues, including in some instances the lack of correlation between the gametocyte densities in natural infections and either the oocyst density or the frequency of mosquito infection. [193, 198] In order to study transmission of malaria parasites derived from infected individuals, a high-throughput method to detect oocysts and sporozoites with high sensitivity is beneficial.

To overcome the limitations of microscopy a number of assays have been developed to enable high throughput detection of parasites in the mosquito gut and salivary glands. These assays include ELISA to detect the circumsporozoite protein (CSP) in mosquito lysates (CSP-ELISA) [199-201], bioluminescence assays to detect transgenic parasites with the green fluorescence protein (GFP) [202-204], near-infrared spectroscopy (NIRS) to detect parasites within mosquitoes

[205, 206], enhanced chemiluminescent slot blot (ECL-SB) for detecting *Pf*CSP in mosquito samples [207, 208] and molecular detection of *Plasmodium* DNA. [209-211] Although the CSP-ELISA is relatively robust and cost effective it is only semi quantitative. [199-201] An assay that is quantitative will enable us to know density of the malaria parasites in the mosquito infection. Bioluminescence GFP assays allow for high-through-put but it cannot be used with wild parasites. [202-204] NIRS has been successfully used to detect *P. falciparum* parasites in lab reared mosquitoes with relatively high accuracy but it is still semi quantitative. [205, 206] ECL-SB assays can potentially be used to screen large numbers of mosquitoes for oocysts with high sensitivity and specificity. [207, 208] However, this assay is not quantitative. Various qPCR-based methods have been successfully developed and used to detect blood stage and mosquito infection. However, some qPCR are still semi quantitative mainly due to the design of the qPCR where nonspecific SYBR-green or EVA-green fluorescent dyes were used. [209-212] Taqman assays are an alternative to SYBR-based real time assays. Taqman assays utilize hydrolysis probes that bind to the target sequence and provides a means to quantify the parasite DNA. The Taqman hydrolysis probes have been used to detect blood stage parasites by targeting the 18s ribosomal RNA gene. [147, 213, 214] Taqman assays are able to detect parasites at levels 4-5 fold lower than expert thick film microscopy. [215, 216] Taqman assays detect *P. falciparum* [209, 213, 217, 218] and *P. vivax* parasites in mosquitos using minor grove binding (MGB) probes. [217-220] Minor groove binding probes increase the specificity of the probe binding to the target DNA sequence as compared to unmodified probes and limits cross-hybridization of primers and probes in duplexes. [221]

Bass and colleagues established a qPCR assay where they evaluated field caught mosquitoes for the presence of *P. vivax* sporozoites in the head and thorax of individual mosquitoes. They did not investigate the qPCR detection of oocysts or the intensity of sporozoite infections. [217] Rao and colleagues established a multiplex qPCR to detect *Wuchereria bancrofti, P. falciparum*, and *P. vivax* in pools up to 23 field caught mosquitos but did not distinguish between potential sporozoite or oocyst infections. [219] Bickersmith and colleagues also established a qPCR assay on individual field caught mosquitoes but did not distinguish between the oocyst and sporozoite stages as it was not part of the study design. [220] Graumans and colleagues also established a qPCR assay where they successfully detected *P. vivax* oocysts stages in mosquitoes but did not investigate the detection of a single *P. vivax* oocyst as it was not part of the study design. [218] Also they did not investigate the qPCR detection of *P. vivax* sporozoites.

Sample processing time is an important aspect to consider when setting up an MFA or when processing field collected samples. This includes extracting DNA through to qPCR detection of the parasites in the mosquito. DNA extraction using commercially available kits can usually takes several hours depending on the number of samples that are being processed. In a study by Bass and colleagues they heated the mosquito samples for 10 minutes at 95 °C, cooled the sample and directly performed qPCR after thus reducing the sample processing time. [217] However, they did not evaluate the heating technique against the conventional DNA extraction method. This study addresses the key knowledge gap that exists in setting up a sensitive Taqman qPCR assay for both oocysts and sporozoites with known infection densities and compare the mosquito preparation methods of conventional DNA extraction versus heating.

5.2 Methods

5.2.1 Mosquito rearing

Anopheles farauti mosquitoes were reared at 28 ± 8 ºC and 68 ± 25 % relative humidity (RH) on an 11 h dark and 12 h light including a 30 min dusk and 30 min dawn period. The larvae were fed ground fish food (Marine Master, Tropical Fish Flake) while the adults were provided with 10 % sucrose (Ramu Sugar) solution available as soaked cotton wool balls placed on top of the mosquito cages as previously described. [144] Individuals who provided informed consent performed direct skin feeding to maintain the colony mosquitoes.

5.2.2 Sample Collection

This study was conducted at the Papua New Guinea Institute of Medical Research (PNGIMR). Ethical approval was received from the PNG Medical Research Advisory Committee (MRAC #16.01). Patients at Yagaum Clinic in Madang Province of PNG, who consented to participate in the study were recruited. Patients were tested with malaria rapid diagnostic tests (RDTs). In the current study the CareStart Malaria Pf/PAN (HRP2/pLDH) Ag Combo RDT kits (Access Bio, Cat No. RMRM-02571CB) were used. Thick and thin blood films were prepared according to WHO methods for evaluation by a certified microscopist. The blood slides were then stained for 30 minutes using 4 % Giemsa (Sigma-Aldrich, Australia) stain. [146] Slides were read by the microscopist to identify the presence of the parasites, the species and stages of the parasite in the

blood. Parasite density was calculated using the assumption that one microliter of blood contains 8000 white blood cells (WBC). [146] Venous blood samples (5-6 mL) were collected from microscopy positive patients in BD Vacutainer ® sampling tubes coated with lithium heparin (BD, Australia). Hemoglobin was measured using a HemoCue® hemoglobin analyzer (HemoCue, Australia). Axillary temperature was taken using a digital thermometer and weight was measured with a bathroom scale (precision ± 0.1 g). After collection of the blood sample, the BD Vacutainer ® was then immediately stored in a beverage cooler flask (Coleman Company Inc, USA) filled with water adjusted to a temperature of 38 °C. A digital thermometer was used to monitor the temperature of the cooler flask. The blood sample was then transported to the insectary for membrane feeding. Transportation time between health facility and laboratory was around 10 minutes.

5.2.3 Infecting mosquitoes

At the insectary 3-5 day old *Anopheles farauti* colony mosquitoes were prepared the previous day and dry starved (i.e., without any sugar or water) overnight. A total of 2 paper cups of 50 mosquitoes per cup were prepared. Baudruche membrane was used to feed the mosquitoes through a water-jacketed glass feeder as described previously. [144] Once a blood sample arrived at the insectary it was immediately fed to the mosquitoes for 20 minutes. Unfed mosquitoes were removed and only the fully fed were kept until day 7 post feed when one cup was dissected for oocysts as previously described. [145] The mosquito guts with oocysts were then stored in PBS in Eppendorf tubes at -20 °C and then the samples were selected for the thermal treatment and DNA extraction arms. The second cup was held until day 14 post feed for detection of sporozoites. The dissections of salivary glands were done by trained microscopists. The salivary glands that were infected with sporozoites were then stored in 100 – 200 µL of PBS buffer together with head and thorax. The total number of mosquitoes with single oocyst infections together with those with more than one oocyst per mosquito were down-selected for DNA extraction and heating (Appendix 2 Table A1). The sporozoites were classed as low infection (1-20 sporozoites) moderate (21-100) and high (> 101) and were also split between the DNA extraction and heating method (Appendix 2 Table A2).

At the insectary 3-5 days-old *Anopheles farauti* colony mosquitoes were prepared the previous day and dry starved (i.e., without any sugar or water) overnight. A total of 2 paper cups of 50 mosquitoes per cup were prepared for each feed. Baudruche membrane (Wilco Biotech, USA) was

used to feed the mosquitoes through a water-jacketed glass feeder as described previously. [144] Once a blood sample arrived at the insectary it was immediately fed to the mosquitoes for 20 minutes. Unfed mosquitoes were removed and only the fully fed mosquitoes were kept until day 7 post feed when one cup was dissected for oocysts as previously described. [145] The mosquito guts with oocysts were then stored in phosphate-buffered saline (pH ~7.4) solution (PBS) in 2 mL Eppendorf tubes at -20 °C and then the samples were selected for the thermal treatment and DNA extraction arms. The total number of mosquitoes with single oocyst infections together with those with more than one oocyst per mosquito were down-selected for DNA extraction and heating (Appendix 2 Table A1). The second cup was held until day 14 post feed for detection of sporozoites. The dissections of salivary glands were done by trained microscopists. The dissection for salivary glands were done in a pool of PBS solution. Once the salivary glands were removed from the thorax they were placed on a microscopy slide with a cover slip placed on top. The salivary glands were then taken and viewed under a microscope at 40 X magnification to identify the presence of sporozoites. An estimation of the sporozoite infection was made by classifying them into the following categories; low (1-20 sporozoites), moderate (21-100) and high (> 100). The salivary glands that were infected with sporozoites were then carefully transferred from the slides to 2 mL Eppendorf tubes and then stored in 100 – 200 µL of PBS solution together with the head and thorax. The stored salivary glands were then split between the DNA extraction and the heating method (Appendix 2 Table A2).The salivary glands that were infected with sporozoites were then carefully transferred from the slides to 2mL Eppendorf tubes and then stored in 100 – 200 µL of PBS solution together with the head and thorax.

5.2.4 Heating and DNA extraction

Parasite DNA was extracted using two methods, the conventional DNA extraction with a commercial kit and heating. In this study we used the FavorPrep® DNA extraction kits (Favorgen Biotech Corp, Ping Tung, Taiwan) and performed DNA extraction according to the protocol for extraction of genomic DNA from tissues and for red blood cells and the DNA was eluted in a final volume of 50 µL of elution buffer. The mosquito samples were taken out of the freezer and allowed to defrost on the bench. The samples were vortexed for 30 seconds and then centrifuged for 10 seconds prior to DNA extraction. In the heating method, the down-selected samples with oocyst/s and sporozoites were then vortexed for 30 s and then centrifuged for 10 s at 500 g and then heated

at 99 °C for 10 min and then cooled on ice prior to performing qPCR. [217]

5.2.5 Quantitative real-time PCR (qPCR)

Following heating and DNA extraction of the samples, an established Taqman qPCR assay that utilizes MGB probes was performed to quantify the infection and determine the parasite species. [147] This Taqman qPCR assay was used to detect blood stage parasites. Briefly, this qPCR assay targets the conserved region of the 18s rRNA gene for both *P. falciparum* and *P. vivax*. The quantification of parasite copy numbers is derived from synthetic plasmid DNA of known concentrations that are included in each run.

The plasmid concentrations are as follows; $10,10^2,10^3$ and 10^4 copies. The plasmid concentrations of $10\text{-}10^3$ were run in duplicates. The Cq values (number of cycles that were needed for the fluorescence signal to reach a quantification threshold) of the plasmids of known concentrations were then plotted on a graph against the log starting quantity. A line of best fit (standard curve) was then constructed. The Cq values of the samples were then used to derive the starting quantity from the line of best fit. Figure A1 in Appendix 2 illustrates this.

The qPCR was performed on a CFX96 Touch Real-Time Detection System (Bio-Rad, Australia).The primer and probe sequences together with the reaction mix and the thermo profile are shown in Appendix 3 Tables A1- A4.

5.2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (ver. 8.0) and Stata 13 (StataCorp, College Station, TX, USA). The Mann-Whitney test was used to compare the DNA copy numbers between the heating and DNA extraction of mosquito guts with known oocysts counts. The Mann Whitney test was also used to compare the DNA copy numbers between the DNA extraction and the heating method for the sporozoites. The two sample test of proportions was used to compare the proportions of microscopy positive samples that were confirmed by qPCR in the heating and DNA extraction arms.

5.3 Results

A total of 68 patients were recruited. (Table 5.1). Table 5.2 shows the results from the three diagnostic methods used.

Table 5. 1 Demographic and clinical data for the study population.

Demography	Median or n/N	IQR (Q1,Q3)	Range	%
Age in years (N=68)	14	11 (10, 21)	4-56	
Female (N=68)	24/68			35.3
Weight, kg, (N=68)	40.5	30 (25, 77)	15 - 77	
Haemoglobin, g/dl, (N=65)*	9.9	2.9 (8.6 , 11.5)	5.6 - 15.5	
Temperature, ˚C , (N=68)	36.5	0.725 (36.2, 36.9)	35.2 - 39.9	
Fever, >37.5 ˚C, (N=68)	9/68			13.2

* Data were not collected for all 68 patients

Table 5. 2 Diagnostic results by RDT, microscopy and qPCR. The number of positive samples per test is n. The total number of samples is N = 68. Population averages (n/N (%)) and 95 % confidence intervals of proportions (95 % CI) are also provided.

Diagnosis		n	n/N (%)	95% CI
RDT	HRP2	1	1/68 (1.5)	0.04 - 7.9
	pLDH	60	60/68 (88.2)	78.1 - 95.8
	HRP2 & pLDH	7	7/68 (10.3)	4.2 - 20.1
Microscopy*	*P. falciparum* asexual with gametocytes	5	5/68 (7.4)	2.4 - 16.3
	P. vivax asexual only	10	10/68 (14.7)	7.3 - 25.4
	P. vivax asexual with gametocytes	43	43/68 (63.2)	50.7 - 74.3
	P.falciparum & P.vivax	7	7/68 (10.3)	4.2 - 20.1
	Microscopy negative	3	3/68 (4.4)	0.9 - 12.4
qPCR	*P. falciparum*	4	4/68 (5.9)	1.6 - 14.4

P. vivax	43	43/68 (63.2)	50.7 - 74.6
PCR negative	21	21/68 (30.9)	20.2 - 43.3

*I did not detect any infection with only *P. falciparum* asexual by microscopy or §both *P. falciparum* and *P. vivax* by qPCR.

I detected *P. vivax* oocyst and sporozoite stages of the malaria parasites in the mosquitoes using an established protocol. Figure 1 shows exemplary amplification curves from a qPCR run.

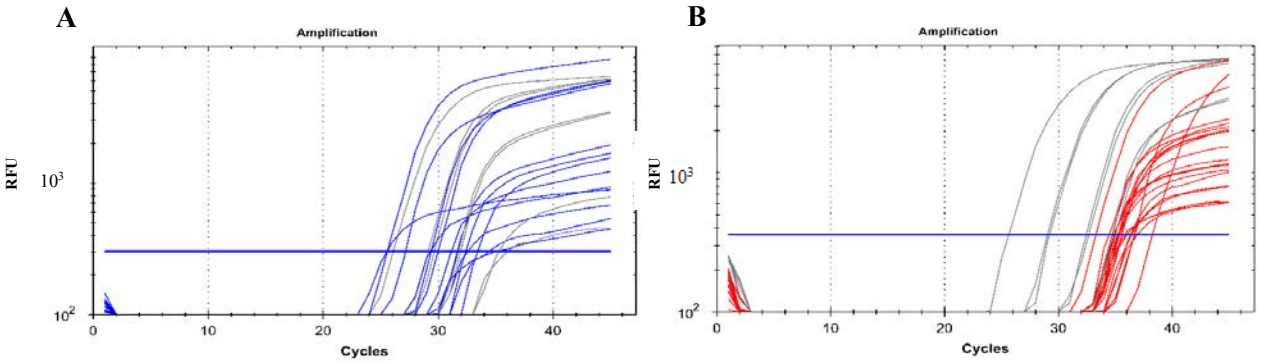


Figure 5. 1 A qPCR amplification plot showing successful amplification of malaria parasite DNA from oocysts and sporozoites. **Panel A** represents the amplification of parasite DNA from oocysts with the blue curves being the parasite DNA from mosquito gut samples and the grey lines representing DNA from plasmids of known concentrations which were used as positive controls starting from 10, 10^2, 10^3 and 10^4 copies. **Panel B** represents the amplification of parasite DNA from sporozoites from the mosquito salivary glands with the red curves being the parasite DNA while the grey lines representing DNA from plasmids of known concentrations which were used as positive controls starting from 10, 10^2, 10^3 and 10^4 copies. The blue horizontal line represents the threshold value; any curve above this is considered an infection. *RFU - relative fluorescence unit*

A total of 73 and 72 mosquito samples had at least one oocyst in the mosquito gut which was detected by microscopy for the heating and DNA extraction arms respectively. I observed a significantly higher proportion of mosquito samples that were confirmed by qPCR in the heating arm 78 % (57/73) as compared to the DNA extraction arm, 39 % (28/72) ($p < 0.0001$).

A total of 17 mosquitoes with single oocysts according to microscopy were processed in both the heating and the DNA extraction arm (Table 5.3). I observed a statistically significant difference with the detection of oocysts by qPCR between the heating arm with a sensitivity of 88 % (15/17) and the DNA extraction arm with a sensitivity of 29 % (5/17) ($p = 0.0019$).

Table 5. 3 Comparison of microscopy positive and qPCR positive oocysts.

	Microscopy		qPCR		
	No. of mosquitoes	**No. of oocysts/ mosquito**	**No. of mosquitoes**	**Sensitivity %, (n/N)**	**95 % CI**
Heating	17	1	15	88 (15/17)	63 - 99
	20	2 - 10	14	70 (14/20)	46 - 88
	36*	> 1	33	92 (33/36)	78 - 98
Total	73	>1	62	85 (62/73)	75 - 92
DNA Extraction	17	1	5	29 (5/17)	10 - 65
	16	2 - 10	6	38 (6/16)	15 - 65
	39*	> 1	18	46 (18/39)	30 - 63
Total	72	>1	29	40 (29/72)	29 - 53

*This represents pools of midguts with oocysts and not mosquitoes.

When comparing only the oocysts that were successfully detected by qPCR I observed no significant difference between the copy numbers when comparing the detection of parasites from both arms for single oocysts. The observed mean of the $\log_{10}$ transformed copy number data was 2.3 (SD, ± 0.82) for the heating and 1.7 (SD, ±1.1) for the conventional DNA extraction (Figure 5.2 Panel A). Also there was no significant difference in the DNA copy numbers between the two arms with all mosquitoes with oocysts. I observed that the $\log_{10}$ transformed copy number data mean was 2.4 (SD, ± 1.3) for the heating and 2.8 (SD, ± 1.1) for the conventional DNA extraction (Figure 5.2 Panel B). I also did not observe any correlation with the DNA copy numbers and the oocyst numbers (Figure 5.2 Panel B). I also did not observe any correlation with the DNA copy numbers and the oocyst numbers.

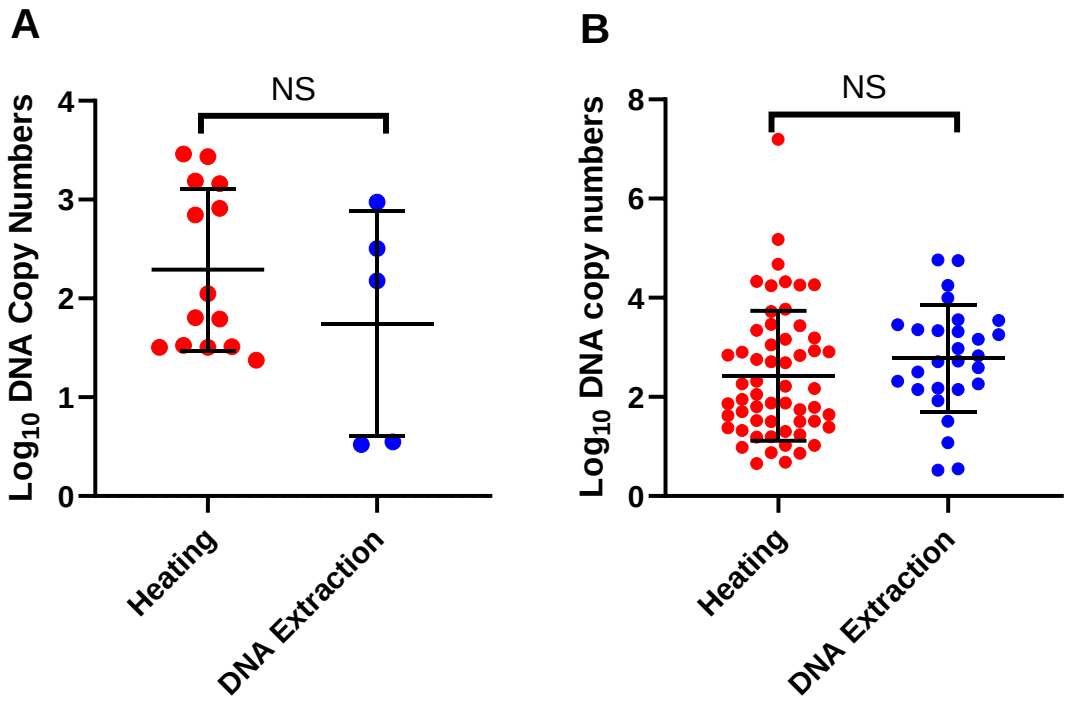


Figure 5. 2 Detection of oocysts using qPCR. Panel A shows the DNA copy numbers from the single oocysts that were detected by qPCR in the two arms. **Panel B** shows DNA copy numbers of all the mosquito samples with one or more oocysts that were detected by qPCR in the two arms. The error bars show the mean and the standard deviation. The dots are mosquitoes. NS – Not significant.

A total of 60 mosquito samples positive for sporozoites by microscopy underwent heating (n=30) and DNA extraction (n = 30) (Table 5.4). I observed no significant difference with the detection of sporozoites by qPCR between the heating arm with a sensitivity of 40 % (12/30) and the DNA extraction arm with a sensitivity of 60 % (18/30) (p = 0.121).

Table 5. 4 Comparison of microscopy positive and qPCR positive sporozoites.

Microscopy		**qPCR**	
Sporozoite classification*	**No. of mosquitoes**	**No. of mosquitoes**	**Sensitivity %, (n/N)**

Heating	High	17	4	23.5 (4/17)
	Moderate	9	5	55.6 (5/9)
	Low	4	3	75 (3/4)
Total		30	12	40 (12/30)
DNA Extraction	High	17	13	76.5 (13/17)
	Moderate	9	4	44.4 (4/9)
	Low	4	1	25 (1/4)
Total		30	18	60 (18/30)

*Sporozoite classification: High - >100, Moderate - 21-100, Low - 1-20

I observed significantly higher DNA copy numbers (p = 0.0126) in the qPCR detection of sporozoites in the heating arm as compared to the DNA extraction arm (Figure 5.3). I noted that there was a gradual increase in the mean DNA copy number from Low to High sporozoite count (Low: 12.78 (SD, ± 19.38), Moderate: 29.85 (SD, ± 28.08) and high: 187.29 (SD, ± 772.95).

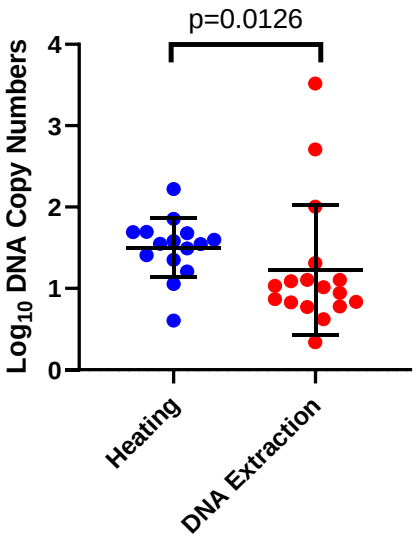


Figure 5. 3 Detection of sporozoites by qPCR showing the DNA copy numbers of the mosquito samples with sporozoites that were detected by qPCR. The error bars show the mean and the standard deviation. Each dot represents a mosquito.

5.4 Discussion

This study describes the adaptation of a high-through-put qPCR based technique for detecting low levels of oocysts and sporozoites and the evaluation of the conventional DNA extraction method versus heating. The qPCR assay is sensitive enough to detect midgut infections with single oocysts. Furthermore, this assay was able to detect low sporozoite infections by microscopy.

Here I have established a qPCR assay that utilizes the Taqman hydrolysis MGB-probe with increased sensitivity in detecting the *P. vivax* parasite target gene and can potentially enable increased through-put for large scale transmission studies. A number of studies have validated TaqMan qPCR assays for detecting *P. vivax* oocysts and/or sporozoites [217-220] but have not investigated the limit of detection. I have shown that this qPCR assay is sensitive in detecting low *P. vivax* oocyst and sporozoites infections in mosquitoes.

I show that there is a higher chance of detecting single oocyst infections when heating the dissected midgut compared to the common method of performing DNA extraction. I also show that there is no significant difference between the detection of the parasite's DNA copy numbers between heating and DNA extraction especially with low infections indicating that heating has a similar DNA output as the common DNA extraction method. I further observe that there is no significant difference between the DNA copy numbers between the two arms with one or more oocysts.

The current study revealed no significant difference in the qPCR detection of sporozoites between the two techniques used to extract DNA from the microscopy positive salivary glands together with the head and thorax. However, heating yielded significantly higher quantities of DNA copies demonstrating the superior performance of heating over the DNA extraction method.

To my knowledge, this is the first research evaluating heating of mosquito guts and salivary gland (with head and thorax). I show that heating is the better option for releasing oocyst and sporozoite DNA and significantly reduces sample processing time and ensures that samples are processed with high efficiency. It also reduces the cost of processing a sample by skipping DNA extraction step using a conventional DNA extraction kit. Bass and colleagues did use heat to free their *P. falciparum* sporozoite DNA prior to performing qPCR but did not evaluate the sensitivity of the technique. [217] Although similar studies have not been done on mosquitoes, I found that similar comparisons were made with bacteria where they evaluated heating the samples versus using commercially available DNA extraction kits. They found no significant difference between the

PCR output from both techniques and suggested that heating was efficient, simple, cheap and suitable for high-through-put. [222, 223] Similar to what was seen in the case of bacteria, heating the mosquito midguts and salivary glands yielded similar qPCR detection rates for sporozoites while higher detection rates with oocysts as compared to DNA extraction.

There are limitations to the present study. Firstly, I noted lower overall sensitivities of qPCR as compared to microscopy for both oocyst and sporozoite detection. This could have resulted from DNA degradation as the samples were not stored in preservatives or that the samples were lost while transferring the dissected guts or salivary glands from the glass slides to the tubes for heating or DNA extraction. Also the total number of samples used were low mainly due to the duration of the study and the rate at which infected individuals were recruited, thus limiting the strength of the statistical analysis. Furthermore, we would need to do bisections to distinguish between the developmental stages of the parasites within the mosquitoes as it would be difficult to distinguish between oocysts and sporozoites on wild caught mosquitoes as we would not know when the mosquitoes were infected with the malaria parasite and that sporozoites traverse all over the mosquito once they are released from the oocysts.

5.5 Conclusion

In summary, I show that a qPCR assay can be used to detect very low numbers of mosquito stage *P. vivax* parasites. Furthermore, I show that by heating the mosquito guts and the head and thorax we save on costs and reduce the time taken to process the samples. I believe that this high-through-put setup will be a valuable tool in evaluating potential transmission blocking vaccines or antimalarials or for evaluating the infection status of field caught mosquitoes.

Summary

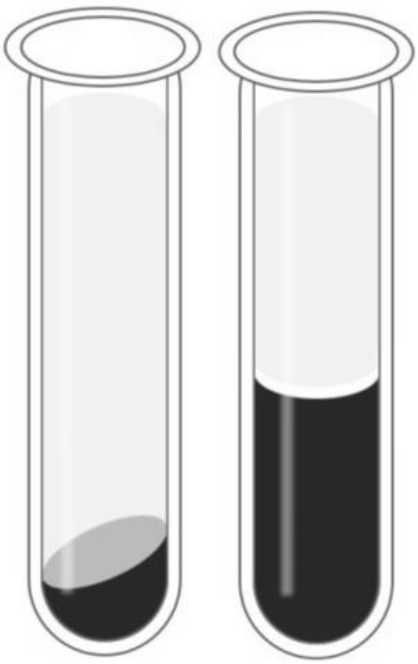
- A qPCR to detect *P. vivax* oocysts and sporozoites was established.
- The qPCR assay can detect single oocysts and low numbers of sporozoites.
- A significantly higher proportion of microscopy positive samples with oocysts were confirmed with qPCR when heating the mosquito guts compared to conventional DNA extraction.
- There was no significant difference between the qPCR confirmations of microscopy positive sporozoite samples of the two DNA extraction methods.
- There was no significant difference between the DNA copy numbers of the two DNA extraction methods for oocysts while significantly higher DNA copy numbers were observed in the heating arm vs the DNA extraction arm for sporozoites.
- Heating the mosquito samples prior to performing qPCR saves time and significantly reduces the cost of processing the samples than the conventional DNA extraction method.
-

Bridge

After establishing a successful DMFA for *P. vivax* with high feeding rates and also having a qPCR assay set up to detect the mosquito stages of the parasite, the next focus was to investigate the effect of an infected individual's plasma components on mosquito infection. The focus of Chapter 6 was on investigating the impact of replacing the plasma of a malaria infected person with a malaria naïve serum on mosquito infection.

CHAPTER 6

Evaluating the effect of plasma on *P. vivax* infectivity to *An. farauti* in PNG



6.1 Introduction

Transmission of Plasmodium parasites from humans to mosquitoes is achieved by gametocytes - the sexual stages of the parasites - when ingested by mosquitoes during a blood meal. [18] The intraerythrocytic gametocytes emerge when the erythrocytes are digested releasing the male and female gametes that fuse to form a zygote that subsequently develops into an ookinete which penetrates the mosquito gut wall to form an oocyst. [22] During the parasites' extracellular phase in the mosquito gut, the gametes and zygotes are exposed to human antibodies co-ingested with the gametocytes in the blood meal which can affect the transmission success, either by blocking or enhancing infectivity. [224] Initial studies in birds, rodents, monkeys and humans have shown that antibodies against gametes and zygotes can inhibit transmission of malaria parasites to mosquitoes. [225-228]

Individuals exposed to multiple malaria infections develop protective immunity. [229] Numerous studies demonstrated that humoral responses to gametocyte proteins can inhibit parasite development inside mosquitoes. [224, 226, 230-235] The development of the direct membrane feeding assay (DMFA) by Rutledge and others in the 1960s enabled replacing the patient's plasma with a malaria naive serum prior to feeding the mosquitoes thus answering questions surrounding the impact of antibodies on transmission of the parasites from humans to mosquitoes. [195]

There are two possible outcomes that have been reported when plasma from the patients' blood is replaced with a malaria naïve plasma or serum. It is expected that the infectivity of the parasites to the mosquitoes may either increase as anti-parasite antibodies will have been removed or the effect of the antibody is enhanced leading to lower mosquito infection or oocyst densities. For example, a study comparing the infectiousness of naturally infected *P. falciparum* gametocyte carriers to *An. gambiae* in Africa found that replacing the plasma of the gametocyte donor with a malaria naïve control serum led to higher mosquito infection rates compared to replacing the blood sample with the individuals own plasma (OR 1.92, 95 % CI 1.68 – 2.19). [174] A study in Thailand with *P. vivax* and *An. dirus* also observed similar findings. [53] Other studies have observed similar results with either replaced plasma or inactivated plasma. [159, 231, 236] In contrast, there was a report which observed that replacing the patients plasma had little impact on the proportion of infected mosquitoes as compared to mosquitoes which fed on the blood with the individuals own plasma. [162]

A report in Papua New Guinea (PNG) suggested that humoral immune responses in individuals

from malaria endemic areas may prevent the development of gametocytes of cultured *P. falciparum* in *An. freeborni.* [224] Whilst there is some data on *P. falciparum*, there is no data on the impact of innate humoral responses on gametocyte transmission of *P. vivax* parasites in PNG. Here, the impact on mosquito infection and oocyst development when blood plasma was replaced autologously, i.e., plasma was removed and then added back to the patient's blood and heterologously, i.e., blood plasma replaced by a malaria naïve serum was investigated using *An. farauti* mosquitoes and *P. vivax* parasites from infected individuals.

6.2 Methods

6.2.1 Patient recruitment and collection of blood samples

Patients were screened at Yagaum Clinic located in the Madang Province of PNG between 2019 and 2021. A nursing officer was stationed at the clinic and performed a malaria rapid diagnostic test (RDT) on individuals who presented with malaria symptoms. The CareStart Malaria Pf/PAN (HRP2/pLDH, India) Ag Combo RDTs kits (Access Bio, Cat No. RMRM-02571CB) was used. The nurse recruited only those individuals who consented to be part of the study and were positive for malaria by RDT. The age range was from 5 years old to >20 years. Parents or guardians were asked to consent on behalf of the patients aged between 5 - 16 years. More specifically, a selection was made mainly for individuals who had a positive result for pLDH by RDT. Following recruitment, the nurse measured the patient's weight using a bathroom scale, temperature using a digital thermometer, and their hemoglobin level using a HemoCue® machine (HemoCue, Australia). Approximately 6 mL of venous blood was collected using BG butterfly needle in BD Vacutainer ® containing spray-coated lithium heparin (BD, Australia) and immediately stored in a beverage cooler flask (Coleman Company Inc, USA) filled with warm water (at ~37.0 ˚C, measured by a digital thermometer attached to the flask). Chapter 2 describes this in more detail.

6.2.2 Microscopy and qPCR diagnosis

Retrospective diagnosis of the malaria parasites was performed by light microscopy (LM) and quantitative real-time PCR (qPCR). Thick and thin blood films were prepared using standard WHO methodology. [146] The blood films were stained with 4 % Giemsa stain for 30 min. [180] Slides were read according to WHO standards and by WHO certified microscopists. [146] Parasite density was calculated using the assumption that one microliter of blood contains 8000 white blood cells (WBC). [146] Slides were read by two expert microscopists and if there were discrepancies between the two, a third read is done by a third expert microscopists and the two similar readings

are taken. Chapter 2 discusses this in detail.

DNA extraction was performed on 250 µL of red blood cell pellets using Favorgen DNA extraction kits (Favorgen Biotech Corp, Taiwan) and performed according to the kit protocol for extraction of genomic DNA from blood. Following DNA extraction, a qPCR assay was performed to quantify the infection and determined the parasite species as described elsewhere. [140] Briefly, this is a probe-based qPCR assay where a conserved region of the 18s rRNA gene was amplified for both *P. falciparum* and *P. vivax*. Chapter 2 discusses DNA extraction and the qPCR assay in detail.

6.2.3 Study design

Infected blood samples from patients recruited into the study were divided into 3 arms: plasma replaced (PR) or heterologous replacement, no plasma replaced (NPR) or autologous replacement and control (normal feed) (Figure 6.1). One mL of blood was prepared for each treatment arm. In the PR and the NPR arms, the blood plasma of the patients was removed following centrifugation at 200 RCF for 3 min. The plasma was kept in Eppendorf ® tubes warmed to ~39 °C. The centrifuge rotor was also kept warm at ~39 °C. Red blood cell pellets were washed with 500 µL of incomplete RPMI pre-warmed to 38 °C and mixed by pipetting it up and down 3 times and then centrifuged at 200 RCF for 3 min before removing the supernatant. For the NPR arm the original plasma from the participant was added back to the red blood cell pellets. In the PR arm the blood plasma of the study participant was replaced with a sterile filtered malaria naïve AB serum (Sigma-Aldrich, USA) to the 1 mL total volume. Three groups of 50 3–5-day old *An. farauti* mosquitoes, dry-starved overnight, were allowed to feed on the three study conditions via Baudruche membrane for 20 minutes. Fully fed mosquitoes were separated from unfed and partial feds and kept in a secondary cage until day 7-day post feed before they were dissected.

6.2.4 Ethical Approval

Ethical approval for this study was received from the Papua New Guinea Institute of Medical Research Institutional Review Board (IRB #1516) and The Papua New Guinea Medical Research Advisory Committee (MRAC #16.01). These ethics approvals were acknowledged by the JCU Human Research Ethics Committee.

6.2.5 Statistical Analysis

The mosquito infection rates were calculated as the proportion of dissected mosquitoes with at least one oocyst divided by the total number of dissected mosquitoes from DMFAs where a minimum of 1 oocyst was found in any of the three study arms. The average oocyst number per infected mosquito was calculated by dividing the total number of oocysts by the total number of infected dissected mosquitoes. The arithmetic mean on a logarithmic scale was used to assess the mosquito infection rates and the oocyst numbers as described elsewhere. [237] The following formula was used;

$$\bar{Y} = \log(x + 1) / n$$

where x is the sum of the infected mosquitoes or oocysts in one assay and n is the total number of mosquitoes in the assay.

A reduction in the mosquito infection rate or the oocyst number was measured by an R value as described elsewhere, which is calculated from the number of infected mosquitoes or the oocysts level from the serum replaced arm (T_{SR}) and the no serum replaced (T_{NSR}). The R value was calculated using the following formula:

$$R = (T_{NSR} - T_{SR}) / T_{NSR}$$

For evaluation of transmission reduction, the R value then assumes a value between zero (no reductions $T_{NSR} = T_{SR}$) and one (maximal reduction, $T_{SR} = 0$). [237] Prism 8.4.2 (GraphPad Software, USA) and Stata 13 (StataCorp, USA) were used for the analysis.

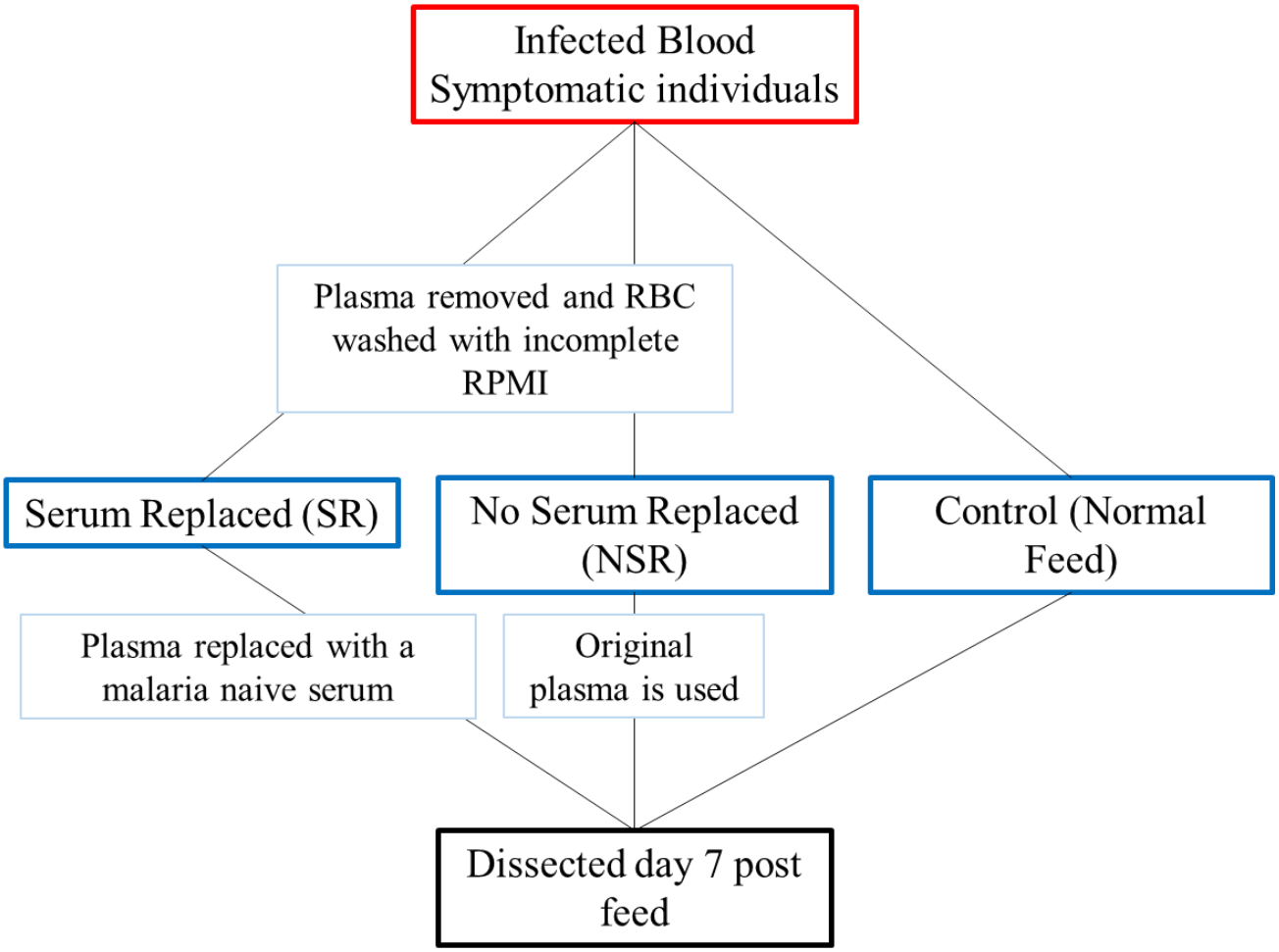


Figure 6.1 Flow chart for the feeding experiments with serum replacement.

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6.3 Results.

Seventy-seven individuals (49.4 % female; median age of 11) were recruited into the study (Table 6.1). The malaria diagnosis results by RDT, microscopy and qPCR are presented in Table 6.2.

Table 6. 1 Characteristics of the study population.

Demography	**Median (range) or n/N (%)**
Age (n=77)	11 (4-59)
Female (n=77)	38/77 (49.4%)
Weight, kg, (n=74) [a]	27.5 (12-87)
Haemoglobin, g/dl ((n=69) [a]	9.85 (6 -16.8)
Temperature, °C, (n=74) [a]	36.3 (35.3-39.2)

Fever, °C, (n=74) [a]	12/74 (16.2%)

[a]These data were not collected from all 78 patients

A high proportion of those individuals that were detected as pLDH (81.8 %) by RDT were confirmed by microscopy as *P. vivax* positive (20.7 %), *P. vivax* with gametocytes (49.4 %) and by qPCR (63.6 %) as *P. vivax*.

Table 6. 2 Malaria diagnosis by RDT, microscopy and qPCR.

Malaria diagnosis		**n/N (%)**	**95% CI (%)**
RDT	HRP2	5/77 (6.5)	2.1 - 14.3
	pLDH	63/77 (81.8)	70.3 - 88.8
	HRP2 & pLDH	9/77 (11.7)	5.4 - 20.8
Microscopy	*P.falciparum*	1/77 (1.3)	0.03 - 6.9
	P.falciparum + gametocytes	8/77 (10.4)	4.5 - 19.2
	P. vivax	16/77 (20.7)	12.2 - 31.2
	P. vivax + gametocytes	38/77 (49.4)	38.5 - 61.5
	P. falciparum & P. vivax	9/77 (11.7)	5.4 - 20.8
	Negative	5/77 (6.5)	2.1 - 14.3
qPCR	*P. falciparum*	9/77 (11.7)	5.4 - 20.8
	P. vivax	49/77 (63.6)	52.4 -74.7
	Negative	19/77 (24.7)	15.4 - 35.4

I observed that 41.6 % (29/77) of the DMFAs infected mosquitoes in at least one of the three arms (Normal, NSR and SR). The proportions of the successful mosquito infections per feed are shown in Table A1 in Appendix 4. I observed no significant difference between the Normal arm and the NSR arm ($p = 0.73$) however I did note a significant difference between the Normal arm and the SR arm ($p = 0.02$) and the NSR arm and the SR arm ($p = 0.02$) (Wilcoxon matched-pairs signed-rank test). These findings are shown in Figure 6.2. Only mosquito feeds which had 5 or more mosquitoes surviving until day seven in any of the three arms were considered for analysis. A total of 509 mosquitoes were dissected for the Normal arm, 542 in the NSR arm and 492 in the SR arm.

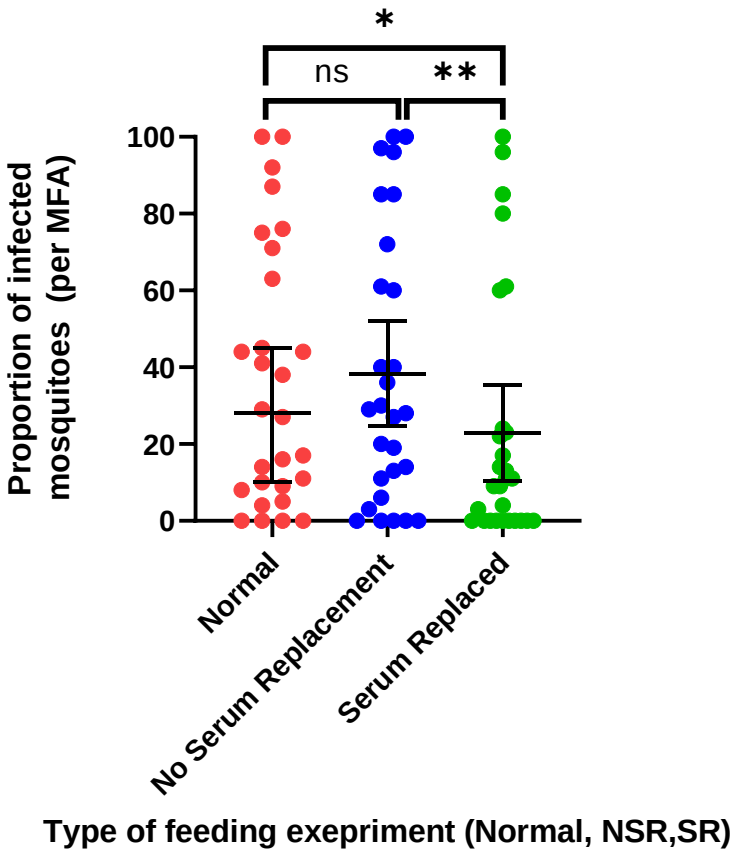


Figure 6.2 Proportion of mosquitos successfully infected in each of the arms. Normal, NSR and SR. The dots represent individual mosquito feeds with the error bars representing the median and interquartile range. *ns - not significant, * p = 0.02, ** p = 0.02.*

Figure 6.3 shows the agreement between the proportion of successful mosquito infection between Normal versus NSR, Normal versus SR and the NSR versus SR arms. The bias (mean difference between the two arms tested) between the Normal and the NSR arm is -1.7 (95 % CI; -62.02, 58.61). The bias for the Normal and the SR arm is 13.6 (95 % CI; -36.0, 63.3) while the bias for the NSR and the SR arm is 15.3 (CI; -51.2, 81.9).

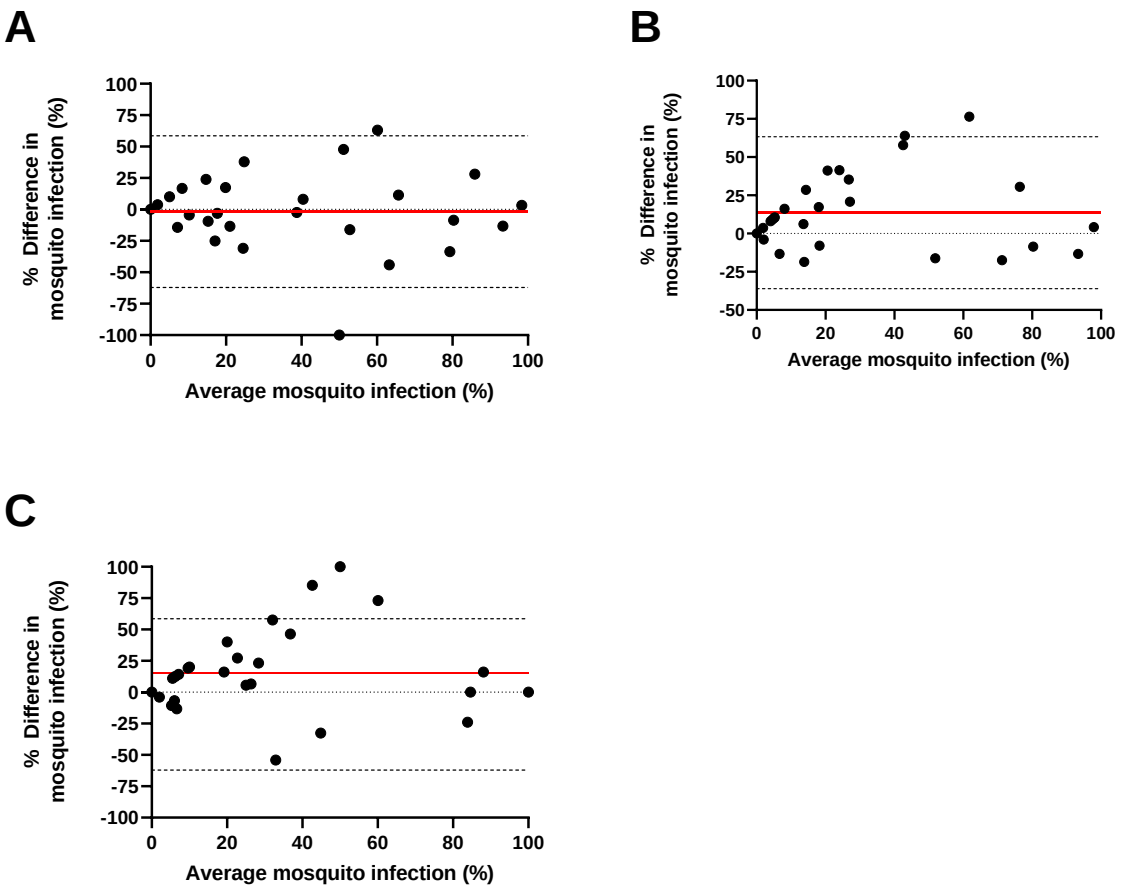


Figure 6. 3 The Bland-Altman plot showing the agreement between the proportion of successful mosquito infections of Normal and NSR (Panel A), Normal and SR (Panel B) and NSR and SR (Panel C) arms. The red line shows the bias while the dotted lines show the 95% confidence intervals.

The reduction of mosquito infection rates were classified as either immune enhancing, reducing or suppressing where enhancing occurs when there is no transmission at all ($R > 0.9$), while reducing classifies some decrease in transmission ($0.9 > R > 0.3$) and suppressing was used to describe cases where there is high transmission in the SR arm ($R < 0.3$).

I observed that 38 % (9/24) of the assays resulted in enhancing, 21 % (5/24) resulted in having a reducing effect while 42 % (10/24) were seen as having a suppressing effect (Appendix 6.3).

Table 6. 3 Immune enhancing, reducing and suppressing effects on mosquito infection.

Infection rate (%) NSR	Infection rate (%) SR	R value	Status
100	0	1.00	Enhancing
14	0	1.00	Enhancing
20	0	1.00	Enhancing
71	0	1.00	Enhancing
11	0	1.00	Enhancing
13	0	1.00	Enhancing
40	0	1.00	Enhancing
85	0	1.00	Enhancing
19	0	1.00	Enhancing
61	3	0.80	Reducing
60	14	0.68	Reducing
36	9	0.57	Reducing
97	24	0.43	Reducing
27	11	0.39	Reducing
40	17	0.28	Suppressing
29	61	0.12	Suppressing
30	23	0.08	Suppressing
100	100	0.04	Suppressing
85	85	0.00	Suppressing
28	22	-0.23	Suppressing
72	96	-0.33	Suppressing
96	80	-0.36	Suppressing
3	9	-1.31	Suppressing
6	60	-2.15	Suppressing

*5 assays were discarded as either there was no oocysts observed in both the SR and the NSR arms or that there were oocysts in the SR arm while there was no oocysts recorded in the NSR arm.

There was no significant difference between the mean oocyst number per infected mosquito between treatment arms: Normal vs. NSR ($p = 0.674$), Normal vs. SR ($p = 0.296$) and NSR vs. SR ($p = 0.919$) according to the Wilcoxon matched-pairs signed-rank test. (Figure 6.4) Table A1 in Appendix 5 shows the mean number of oocysts per mosquito in each arm.

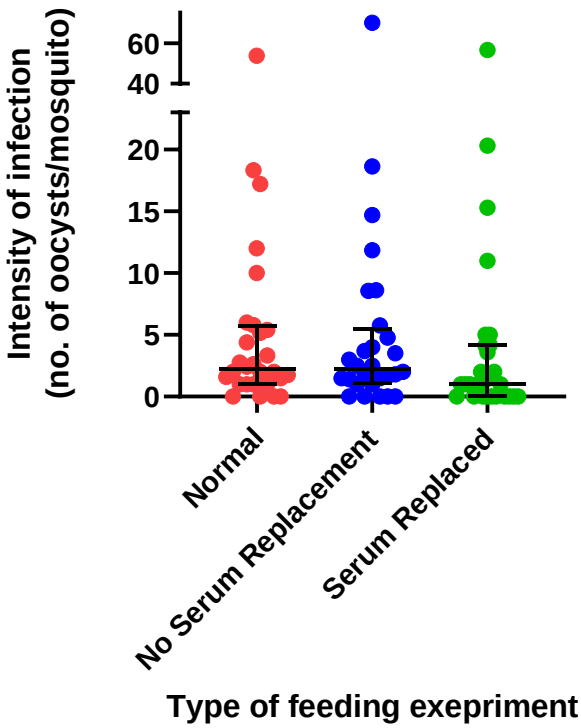


Figure 6.4 Mean number of oocysts per mosquito gut in each arm Normal, NSR and SR. Each dot represents the mean number of oocysts per mosquito during a successful feed. The error bars represent the median and interquartile range. There was no significant difference between each pair of the three different test conditions.

The reduction of oocysts was classified as either immune enhancing, reducing or suppressing where enhancing occurs when there is no transmission at all ($R > 0.9$), while reducing classifies some decrease in transmission ($0.9 > R > 0.3$) and suppressing was used to describe cases where the SR arm had a higher oocyst level than the Normal arm ($R < 0.3$). I observed that 35 % (8/23) of the assays resulted in and enhanced immune effect, 17 % (4/23) resulted in having a reducing effect while 48 % (11/23) were seen as having a suppressing effect.

Table 6. 4 Showing the transmission immune enhancing, reducing and suppressing effects.

Oocyst density NSR	Oocyst density SR	R	Status
8.60	0.00	1.00	Enhancing
3.00	0.00	1.00	Enhancing
1.00	0.00	1.00	Enhancing
1.50	0.00	1.00	Enhancing
5.75	0.00	1.00	Enhancing

2.50	0.00	1.00	Enhancing
8.55	0.00	1.00	Enhancing
1.80	0.00	1.00	Enhancing
14.71	1.00	0.90	Reducing
4.00	1.00	0.80	Reducing
3.50	1.00	0.74	Reducing
70.10	4.13	0.59	Reducing
1.67	2.00	0.25	Suppressing
1.88	1.33	0.18	Suppressing
2.50	4.18	0.16	Suppressing
4.79	3.60	0.11	Suppressing
18.64	56.64	-0.21	Suppressing
1.50	5.00	-0.53	Suppressing
11.84	20.33	-0.67	Suppressing
3.70	15.30	-0.76	Suppressing
2.00	1.67	-0.89	Suppressing
1.40	11.00	-2.02	Suppressing
1.00	5.00	-4.04	Suppressing

*6 assays were discarded as either there was no oocysts observed in both the SR and the NSR arms or that there were oocysts in the SR arm while there was no oocysts recorded in the NSR arm.

I observed no significant correlation between the *P. vivax* gametocyte density and the mean number of oocysts per infected mosquito in both the NSR and SR arms when considering only the successful infections for the immune enhancing and suppressing effects (Immune enhancing effect: NSR; R = 0.7, p = 0.052, SR; R = 0.7, p = 0.073. Immune suppressing effect: NSR; R = 1, p = 0.08, SR; R = 0.9, p = 0.17) (Figure 6.7). No correlation was observed between the asexual parasite density and the oocyst numbers per infected mosquito that had immune enhancing and immune suppressing effects in both arms as well.

There was significant and strong correlation between the mosquito infection rates and the mean oocyst counts per mosquito for the three arms: Normal ($R = 0.74$, $p < 0.0001$), NSR $R = 0.82$, $p < 0.0001$) and SR ($R = 0.93$, $p < 0.0001$). Figures 6.5 illustrates the correlation between the mosquito infection rates and the mean oocyst counts for the respective arms.

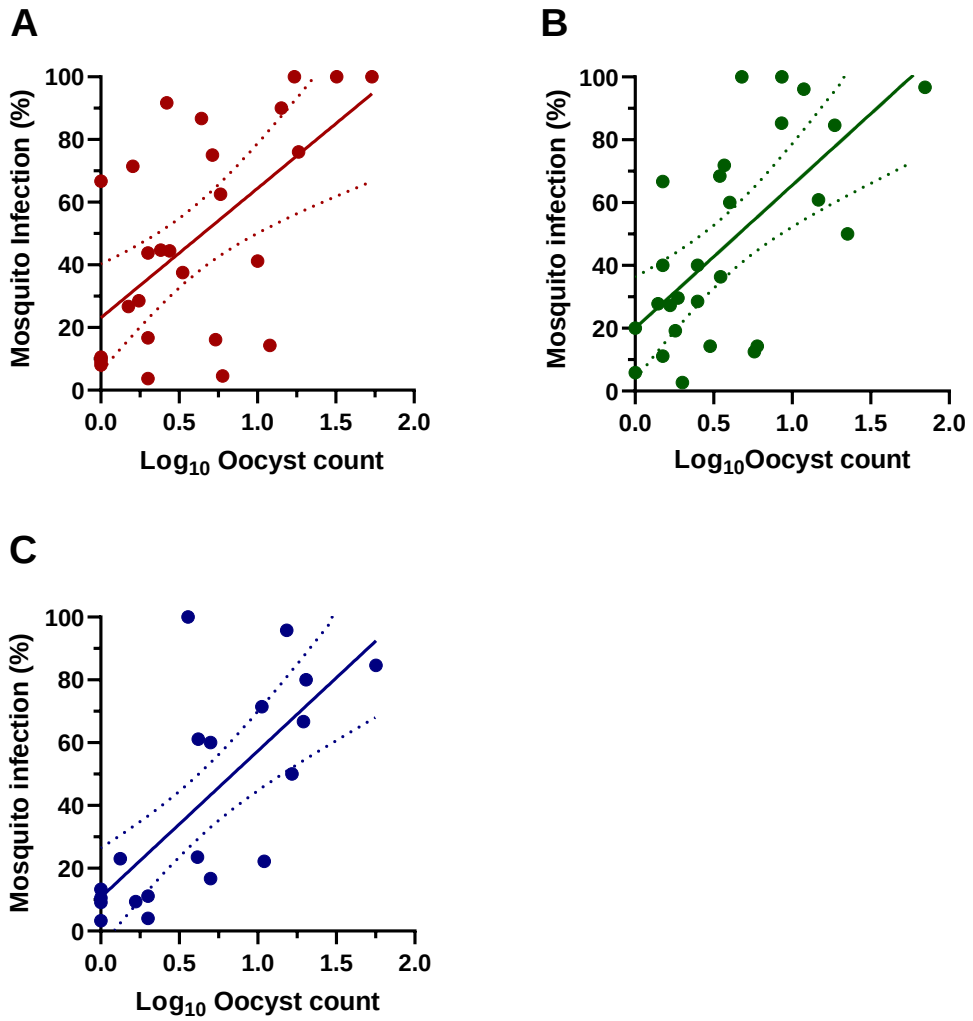


Figure 6. 5 Correlation between the proportion of successful mosquito infections and the mean oocyst count per infected mosquito in the three arms; Panel A Normal, **Panel B** No Serum Replacement and **Panel C** Serum Replacement. The linear line represents the line of best fit.

6.4 Discussion

Most of the individuals that were diagnosed by RDT as pLDH were confirmed by microscopy and qPCR as *P. vivax*. Almost all the successful mosquito infections had *P. vivax* gametocytes detectable by microscopy while a few had submicroscopic gametocytes.

The SR arm was expected to perform better as the antibodies against the sexual stages will have been removed before exposing to mosquitoes with the malaria parasites. This was noted by

previous studies where serum replacement resulted in higher mosquito infection rates and/or higher oocyst density compared to the NSR and the Normal arms while there was no significant difference observed between the NSR and the Normal arm. [174] This trend was observed in studies where they performed DMFAs with *P. falciparum* and *An. gambiae* and *P. vivax* with *An dirus*. [53, 238] However, this was not observed here, instead, I observed that the NSR and Normal arms had significantly higher proportion of mosquitos infected as compared to the SR arm. According the Bland-Altman plot it was observed that the bias between the Normal and NSR arms was small meaning that there was almost no difference between proportions of infected mosquitoes between the two arms. This was also confirmed where there was no significant difference observed between the proportion of successful mosquito infections in the Normal and the NSR arm. However, it was noted that on average the Normal arm would have a 13.6 % and the NSR arm would have a 15.3 % higher successful infection rate as compared to the SR arm when performing an assay. This was also confirmed by the significant differences observed between the Normal ($p = 0.02$), NSR ($p = 0.02$) and the SR arms in the proportion of successful mosquito infections. There was no significant difference in the oocyst density between the three different arms.

Moreover, I observed variable effects when replacing the individual's plasma with a malaria naïve serum in the mosquito infection rate and the oocyst density. I noted that in some individuals the malaria naïve serum enhanced the antibodies thus leading to lower mosquito infection rates and the mean oocyst densities per infected mosquito while in other individuals it had a suppressing effect leading to higher mosquito infection rates and mean oocyst densities. A contributing factor to the variable immune responses observed could be the quality of the naïve serum used. The serum was aliquoted into individual sterile tubes so as to reduce the number of times the serum is defrosted before using and to reduce contamination. While every effort was taken to maintain sterility, the possibility of contamination cannot be excluded entirely. In order to eliminate this as a contributing factor various naïve sera would need to be tested. Also the extended handling of the blood samples and the possibility of temperature fluctuations can lead to premature exflagellation of male gametes thus leading to a reduction of infectivity. It is also clear that individual immune responses vary in how they affect transmission to mosquitoes. Similar findings were observed with *P. vivax* and *An. dirus* where increases in the infection rate (% mosquito infected) and /or the mean oocyst per mosquito were observed in some samples while not in other samples. [162] My findings also concur with a previous study in PNG which revealed that there was variability in the antibody responses to different gamete surface antigens with different individuals. [224]

Despite observing variable effects in the mosquito infection rates as well as the oocyst density with replacing the patient's plasma with the malaria naïve serum, I noted strong correlations in the SR arm between the *P. vivax* gametocyte density and the proportion of infected mosquitoes. Furthermore, I observed strong correlations between *P. vivax* gametocyte density and the mean oocyst density per infected mosquito for both NSR and SR arms. These results indicates that gametocyte density influences the mosquito infection rate and oocyst density. [198] However, there are contradicting reports stating that even though there may be an association between gametocyte density and mosquito infection rate, the association is not that strong especially when considering very low gametocyte infections. [22, 239] Also high gametocyte densities do not always result in mosquito infections. [22, 143]

I also noted strong correlations between the proportion of mosquito infections and the oocyst density for all three arms. My findings show that the more mosquitoes that are successfully infected the higher the chances of having more oocysts present as well.

A concern which was noted was that I observed a higher number of qPCR negatives as compared to the microscopy and RDT positives. It may be that they are actually false positives or that the qPCR assays may have missed them due to errors that may have occurred during processing of the samples. This requires further investigation.

6.5 Conclusion

The findings reveal that I can expect to see both the immune suppressing and immune enhancing effects when performing serum replacement experiments in PNG. I also show that I can expect a higher proportion of immune suppressing effects in both the mosquito infection success and oocyst density as compared to immune enhancing effects.

Summary

- The Normal and No Serum Replaced treatment arm had significantly higher mosquito infection rates than the Serum Replaced treatment arm suggesting high levels of transmission enhancing immune effect
- No significant difference was observed in the mean oocyst counts between the three arms
- Strong and significant correlations were observed between the mosquito infections and the mean oocyst counts.
- Variable responses were observed in the SR arm; where an enhancing effect was noted in some individuals with higher proportions of successful mosquito infections and the mean oocysts per gut while a suppressing effect was noted in other individuals where lower proportions of successful mosquito infections and mean oocysts per gut were observed as compared to the NSR arm.

Bridge

Chapters 3-6 discussed mosquito infection on symptomatic individuals. Chapter 7 investigates the infectivity of mosquito infections on asymptomatic individuals. The focus of this chapter was to determine the proportion of asymptomatic individuals within a malaria endemic community and the proportion of the asymptomatic individuals who were infectious to mosquitoes.

CHAPTER 7

Asymptomatic Transmission of Malaria Parasites by Direct Skin Feeding Assay

7.1 Introduction

Malaria remains a serious global health problem with an estimated 241 million cases in 2020. [3] Individuals can be symptomatic or asymptomatic with malaria infections. Malaria parasites can be transmitted by both symptomatic and asymptomatic individuals. Asymptomatic transmission of malaria parasites poses a threat to malaria elimination as undetected individuals harboring malaria parasites are not treated. These asymptomatic infections contribute to the human infectious reservoir and ensure that transmission is maintained. [240]

Asymptomatic individuals remain oblivious that they harbor the malaria parasites and may act as a parasite reservoir as they don't feel sick and therefore do not seek treatment. It is known, however, that not all individuals with blood-stage parasitaemia are infectious to mosquitoes and that only a fraction of all the parasites within the human host develop into gametocytes which can be taken up by the mosquito, and then complete their lifecycle and become transmissible. [22] Gametocytes account for a small proportion of all the parasites in peripheral blood so they are more challenging to detect than asexual stages using microscopy. The development of molecular methods to detect gametocyte-specific mRNA transcripts by nucleic acid sequence-based amplification (NASBA) [241] or reverse-transcriptase quantitative PCR (RT-qPCR) [242] has greatly improved the sensitivity with which gametocytes can be detected. There are a relatively small number of studies conducted outside of Africa but detection of *P. vivax* gametocyctes has been reported from asymptomatic individuals, in Thailand, the Solomon Islands and PNG. [97, 243-245] A recent study has also reported detectable *P. falciparum* and *P. vivax* gametocytes in 44-94 %, and 23-72 % of asymptomatic humans who were screened by RT- qPCR in Brazil, Thailand, Papua New Guinea and Solomon Islands. [246] Furthermore, a meta-analysis of the diagnostic accuracy of RDTs and microscopy in Asian countries on asymptomatic individuals revealed a pooled sensitivity by RDT (59 %, 95 % CI: 16–91 %) or microscopy (55 %, 95 % CI: 25–82 %) for *P. falcipaurm*. For detection of *Plasmodium vivax*, a pooled sensitivity of RDT (51 %, 95 % CI: 7–94 %) had also the comparable accuracy of microscopy (54 %, 95 % CI, 11–92 %). [247] A review of studies conducted in Brazil and Peru from 2002 – 2015 revealed the presence of asymptomatic *P. vivax* infections to be from 58.5 % to 92.6 %. [248]

Infection studies involving direct membrane feeding assays (DMFA) or direct skin feeding assays (DSFA) have been used to determine the potential of individuals to transmit malaria parasites to

mosquitoes. [155, 162, 192, 249, 250] However, few studies have reported on DSFA studies with asymptomatic individuals in non-African settings, particularly with *P. vivax*. [143] At present, the relative contributions of asymptomatic populations to transmission remains unclear. Information on asymptomatic transmission is required in order to understand and improve the current disease control and elimination programs. A substantial contribution by asymptomatic individuals to transmission will require that interventions target these carriers.

Asymptomatic malaria infections are usually submicroscopic; low-density Plasmodium infections detected only by molecular methods, and may represent 20 % of all infections in areas of high transmission intensity (where the prevalence of community infections by microscopy is ≥ 75 %). However, it may increase to 70-80 % of infections in areas of low transmission (where the prevalence of community infections by microscopy is < 10 %). [251] A possible explanation for these observations is that individuals have had past and/or current exposure to parasites and have developed immunity. The high proportion of asymptomatic infections has been observed in regions where malaria control efforts were successful in reducing transmission from high to low. [252, 253] A high prevalence of submicroscopic infection has been observed in regions of Africa where there has been a significant decline in transmission. [252, 253] Furthermore, low transmission intensity areas in Asia and the Americas are associated with the efficient acquisition and maintenance of memory B cells and antibodies. [254, 255] Such immunity is persistent, with stable levels of Plasmodium-specific memory B cells [256, 257] and antibodies [258, 259] reported in adults with historical, but not current, exposure to malaria.

Only a few studies in PNG have determined the proportion of asymptomatic malaria infections in the population [260-263] and even fewer studies have investigated the potential of asymptomatic transmission based on their gametocyte carriage. [97, 245, 246] One PNG study reported that between 2006 and 2014, the proportion of *P. falciparum* submicroscopic infections was between 36.2 % - 72.1 %, with 43.3 % - 60.6 % having gametocytes. They also reported 48.2 % - 86.7 % *P. vivax* submicroscopic infections of which 22.6 % - 48.9 % had gametocytes. [97] A survey conducted in 2010 and 2014 in PNG described a drop in the prevalence of both the asexual and sexual stages of the malarial parasites. They reported a drop in the prevalence of *P. falciparum* parasites by qPCR from 18.5 % - 8.98 % and *P. falciparum* gametocyte by and RT-qPCR where the prevalence also dropped from 11.19% - 3.9%. They however, reported a slight increase in the prevalence of *P. vivax* from 13% - 19.7 % but still observed a decrease in the gametocyte

prevalence from 6.3 % - 4.5 %. [246]

There is only one study which performed DSFA on individuals in malaria endemic communities within the Madang Province of PNG. They reported that 4 % of the individuals successfully infected at least one mosquito with malaria parasites with *P. falciparum* being responsible for 0.5 %, *P. vivax* being responsible for 2.5 % and *P. malariae* being responsible for 1 % of the successful mosquito infections. [143]

Currently the proportion of asymptomatic individuals within PNG communities and their contribution to transmission remains unclear. Here, I investigated the prevalence of asymptomatic individuals within a malaria endemic community and their ability to transmit malaria to mosquitoes using DSFA.

7.2 Methods

7.2.1 Study site

The cross-sectional study took place along the North Coast of Madang Province, PNG (Figure 7.1) in February and August of 2021. Participants 5 years and older were recruited into the study after obtaining informed consent (Figure 7.2 A). A guardian consented on behalf of those who were below the age of 16 years or who could not sign the consent form. Following enrolment, the participants' temperature was taken and they were interviewed for clinical signs of malaria (axillary temperature ≥ 37.5 °C, headache, chills, myalgia, dizziness, nausea and diarrhea). Individuals with clinical evidence of malaria, pregnancy or puerperium were excluded. Malaria rapid diagnostic tests (First Response Malaria Ag.pLDH/HRP2 Combo Card Test, India) were performed on participants. Hemoglobin levels were checked, thick and thin blood films were prepared on microscopy slides and 250 µL of blood was collected in lithium heparin microtainers (BD, Australia) for further microscopy and qPCR detection of malaria parasites.

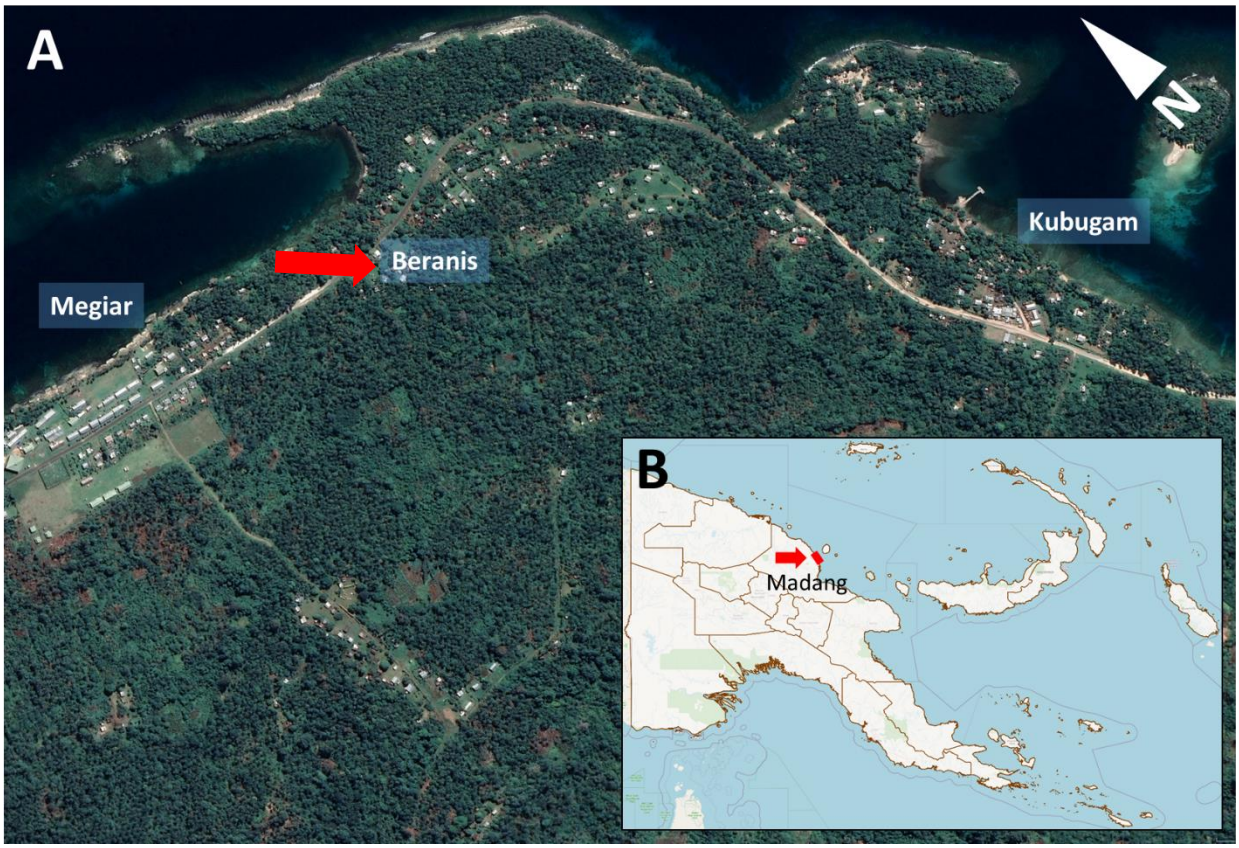


Figure 7. 1 Map of PNG showing where the study was conducted along the north coast of the island (red dot and red arrow pointing to the study area). *Map generated using the QGIS Software 3.18.*

7.2.2 Mosquito preparation

The field site where the DFSA's were conducted was about 1 h 30 min drive from the laboratory where the *Anopheles farauti* mosquitoes were reared as described in Chapter 2. The mosquitoes used in the feeding assays were 3-5 day old. Thirty females were placed in each feeding cup, using a mesh net lid secured with elastic bands (Figure 7.2 B). Mosquitoes were starved overnight and cups were placed in a large box cooler (Coleman Company Inc, USA) with an ice pack to keep the mosquitoes cool while being transported. Mosquitoes were allowed to acclimatize for 10 min at the field site before being allowed to feed for 10 minutes on the arms of RDT-positive participants through the mesh lids of the cups (Figure 7.2 C). Individuals positive by molecular detection were asked to feed mosquitoes the following day. All individuals testing positive by RDT or molecular diagnosis were treated immediately after they had fed mosquitoes. Treatment was administered according to the national malaria treatment guidelines. Malaria positive individuals who declined to feed mosquitoes were also treated.

Once fed, mosquitoes were transported to the lab where unengorged mosquitoes were removed. Blood-fed individuals were maintained on 10 % sugar solution until their guts were dissected on day 7 post feed. Experienced microscopist then dissected the mosquitoes and read them.

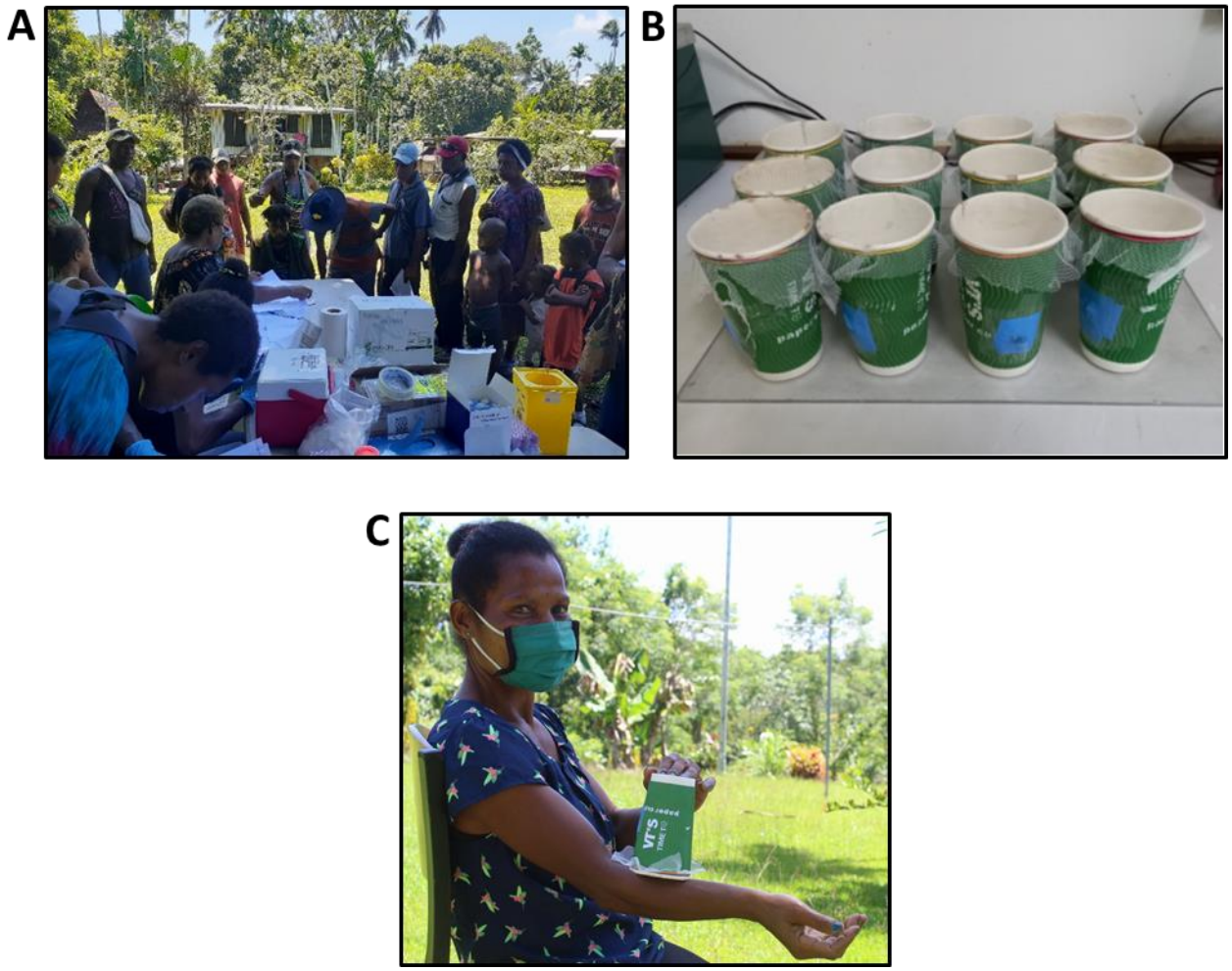


Figure 7.2 Pictures from the field study. Panel A shows the eagerness of the community members gathering around to participate in the study. **Panel B** shows how the cups are prepared and **Panel C** shows an individual allowing mosquitoes in the cup to feed on her arm for 10 minutes.

7.2.3 Molecular analysis of blood

The 250 µL of blood was spun at 3000 RCF for 10 minutes to separate plasma from red blood cells (RBC). DNA extraction was performed on RBC pellets (Favogen Extraction Kit, Favogen Biotech Corp, Taiwan). An established real time qPCR assay for detecting *P. falciparum* and *P. vivax* was performed on the extracted DNA samples. [147]

7.2.4 Microscopy

Retrospective diagnosis of malaria parasites was performed on the thick and thin blood films using standard methods following staining with 4 % Giemsa stain for 30 mins. Each microscopy slide was read by two WHO certified microscopists. Any disagreement between microscopists was resolved by a third expert. Parasite density was calculated using the assumption that one microliter of blood contains 8000 white blood cells (WBC). [146]

7.2.5 Ethical Approval

The study received ethical approval from the PNG Institutional Review Board (IRB no.1911) and the PNG Medical Research Advisory Committee (MRAC no.2013). These ethics approvals were acknowledged by the JCU Human Research Ethics Committee.

7.2.6 Data analysis

Asymptomatic individuals were identified using RDT and qPCR diagnostics. The proportion of asymptomatic individuals were calculated by taking the total number of malaria positive individuals over the total number of individuals that met the inclusion criteria. The successful human-mosquito transmission rate was calculated by taking the total number of individuals who successfully infected ≥ 1 mosquito over the total number of individuals that were exposed to mosquitoes. The mosquito infection rate was calculated by taking the total number of infected mosquitoes over the total number of mosquitoes dissected. The oocyst rate per infected mosquito was calculated by taking the total number of oocysts per mosquito over the total number of infected mosquitoes dissected.

7.3 Results

A total of 269 individuals were screened in the study. The demographic data describing the population is presented in Table 7.1.

Table 7. 1 Demographic data for the study population.

Demography	**median (range) or n/N (%)**

Age in years (N=269)	24 (5-75)
Female (N=269)	128/269 (48%)
Weight, kg, (N=269)	48 (10 - 88)
Hb, g/dl, (N=264)[a]	11 (3.4 - 17.3)
Temperature, ˚ C, (N=253)[a]	36.4 (33-40)
Fever, >37.5 ˚C, (N=253)[a]	13/253 5%)

n - number of individuals screened according to the demographic measurement. N - total number of individuals screened. [a] Not all data were collected for these.

Table 7. 2 Malaria diagnosis by RDT, microscopy and qPCR.

Diagnosis	n/N	(%)	95% CI
RDT[a]			
HRP2 (P.f.)	23	9.02	5.8 - 13.2
pLDH (PAN)	13	5.10	2.7 - 8.6
HRP2 & pLDH	16	6.27	3.6 - 1.0
RDT Negative	203	79.61	74.7 - 84.6
Microscopy [b]			
P. falciparum	22	9.13	5.8 - 13.5
P. falciparum with gametocytes	2	0.83	0.1 - 3.0
P. vivax	7	2.90	1.2 - 5.9
P. vivax with gametocytes	8	3.32	1.4 - 6.4
P.falciparum & P.vivax mix	6	2.49	0.9 - 5.3
Microscopy negative	196	81.33	75.8 - 86.0
qPCR			
P. falciparum	28	10.4	7.0 - 14.7
P. vivax	25	9.3	6.1 - 13.4
P.falciparum & P.vivax mix	5	1.9	0.6 - 4.3
PCR negative	211	78.4	73.0 - 83.2

n - number of individuals according to the diagnosis. N- total number of individuals tested. [a] N = 255 as 14 individuals were not screened with RDT. [b] N =241 as microscopy slides were not prepared for 28 individuals.

Figure 7.3 is a flow chart of the selection criteria that was used to screen through the recruited individuals and then down-select for the appropriate individuals. The selection of the individuals were based on the RDT and qPCR diagnosis.

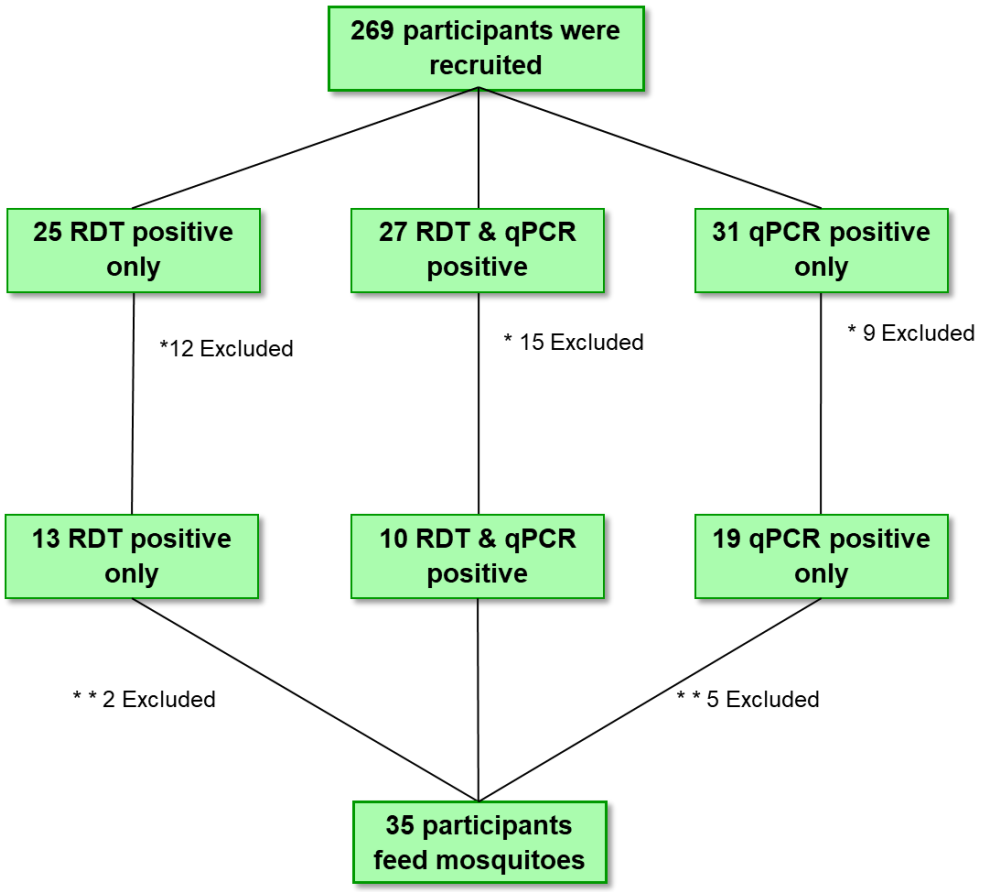


Figure 7. 3 Flow chart illustrating the selection of individuals that were asymptomatic and those that were willing to feed the mosquitoes via DSF. . *Excluded because they had clinical signs of infections. **Excluded because they did not consent to feeding the mosquitoes.

Thirty one percent (83/269) were positive for malaria by RDT or qPCR. Individuals were excluded if they had symptoms of malaria leaving 57 % (47/83) malaria positive asymptomatic individuals. Eighty five percent (40/47) of those asymptomatic individuals were willing to feed the mosquitoes and 5 % (2/40) successfully infected *An. farauti* mosquitoes. The two individuals that had successfully infected the mosquitoes had *P. vivax* gametocytes by microscopy. The mosquito infection rate of these two individuals were 7 % (1/14) and 42 % (5/12) respectively with an oocyst density of 1 and 2 oocysts per mosquito.

I observed that 9 % (4/47) of the asymptomatic individuals that were detected by RDT as HRP2

were confirmed by qPCR as *P. falciparum*. I also observed that 11 % (5/47) of the asymptomatic individuals that were positive by RDT as pLDH were confirmed by qPCR as *P. vivax*. Over all qPCR detected 47 % (22/47) of the asymptomatic individuals that were missed by RDT while 28 % (13/47) where detected as positive by RDT but negative by qPCR. (Table 7.3)

Table 7.3 Positivity agreement between RDT and qPCR for asymptomatic individuals.

		qPCR % (n/N)				
		Pf	**Pv**	**Pf & Pv**	**Negative**	**Total**
RDT	**HRP2**	9 (4/47)	0	0	11 (5/47)	19 (9/47)
	pLDH	0	11 (5/47)	2 (1/47)	2(1/47)	15 (7/47)
	HRP2 & pLDH	4 (2/47)	0	0	15 (7/47)	19 (9/47)
	Negative	19 (9/47)	26 (12/47)	2 (1/47)	0	47 (22/47)
	Total	32 (15/47)	36 (17/47)	4 (2/47)	28 (13/47)	100 (47/47)

n - number of individuals according to the diagnosis. N= 47. Pf, *P. falciparum*, Pv, *P. vivax*

I observed that 44.7 % (21/47) of the asymptomatic individuals were microscopy positive with a mean parasitaemia of 1285.76 parasites per µL (31.4 - 5890.5 parasites per µL) for *P. falciparum* and 639.84 parasites per µL (47.2 - 4098.5 parasites per µL) for *P. vivax.* We noted that 31.9 % (15/47) of the malaria infections were submicroscopic.

I did observe a strong and significant correlation between the parasitaemia by microscopy and the DNA copy numbers by qPCR for both *P. falciparum* ($R = 0.72$, $p = 0.004$) and *P. vivax* ($R = 0.84$, $p < 0.001$) when all the malaria infections were considered (Figure 7.4 A & B). However, I observed no significant correlation between the asymptomatic parasitaemia by microscopy and qPCR in *P. falciparum* ($R = 0.15$, $p = 0.78$) and in *P. vivax* ($R = 0.52$, $p = 0.16$). (Figure 7.4 C & D)

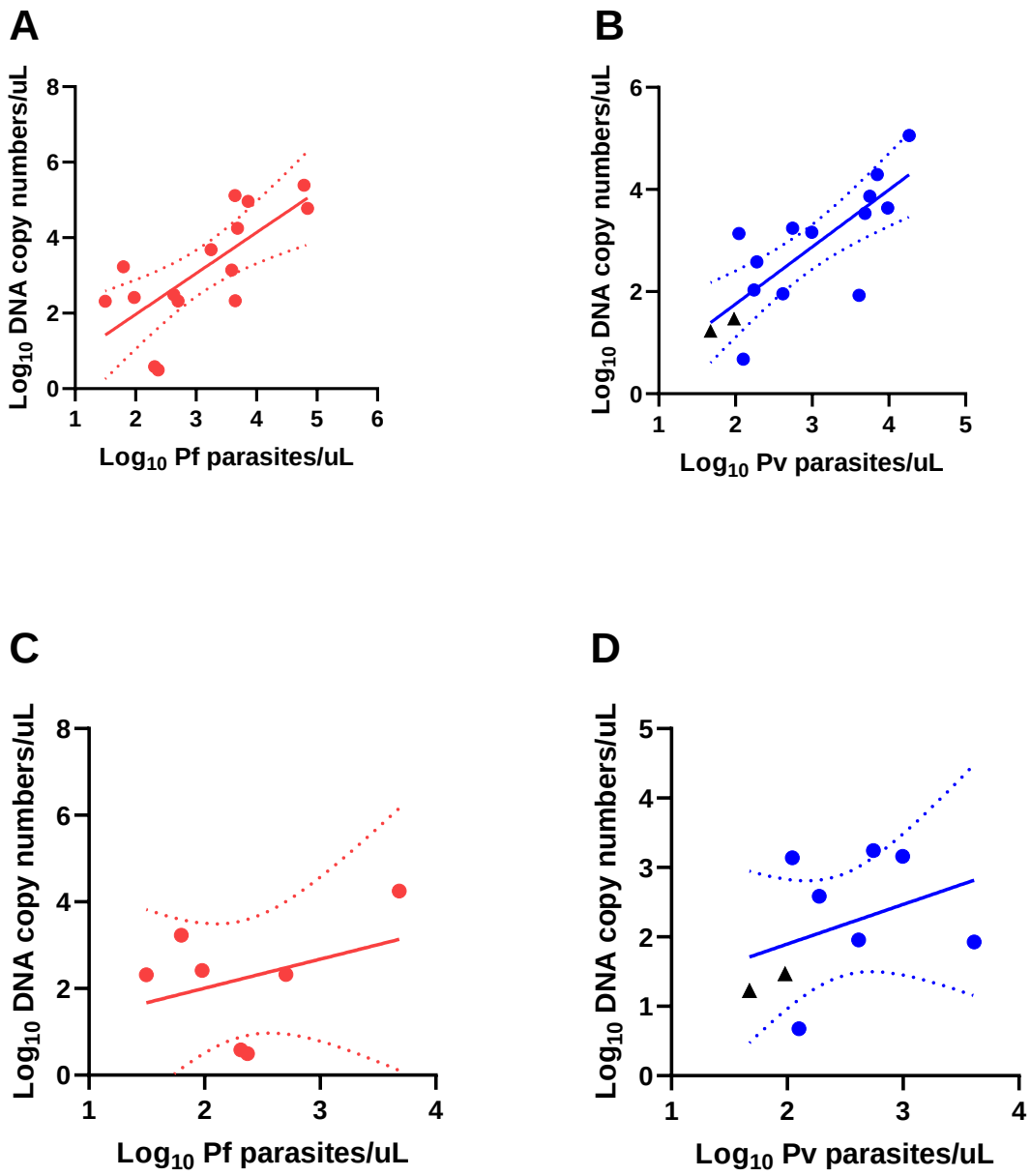


Figure 7.4 Correlation between microscopy densities and DNA copy numbers by qPCR. **Panel A** shows the correlation for all the *P. falciparum* cases while **Panel B** shows the correlation for *P. vivax* for all the malarial positive cases. **Panel C** shows the correlation for only the asymptomatic individuals in *P. falciparum* while **Panel D** shows the correlation for only the asymptomatic *P. vivax* individuals. The dots represent individuals while the linear line is the line of best fit with 95 % confidence intervals. The black triangles represents the two cases that successfully infected the mosquitoes.

I did also observe a significantly moderate correlation between microscopy and qPCR diagnosis when considering all the malaria positive and negative individuals in both *P. falciparum* (R = 0.4,

p < 0.0001) and *P. vivax* (R = 0.6, p < 0.0001). However, I did not observe any significant correlations between the two diagnostic methods when considering malaria positive and negative individuals for only asymptomatic infections for *P. falciparum*. I did observe a significantly moderate correlation between the two diagnostic methods with asymptomatic infections for *P. vivax* (R = 0.4, p = 0.01). Graphs shown in Appendix 6 Figure A1.

When grouping the asymptomatic individuals into age groups I observed that there was drop in the DNA copy numbers starting at ages 10 - 20 years or more for *P. falciparum* and for *P. vivax* there was a consistent drop in the DNA copy numbers from ages 3 – 20 years or more. (Figure 7.5). There was also no significant difference between the genders in the asymptomatic infections.

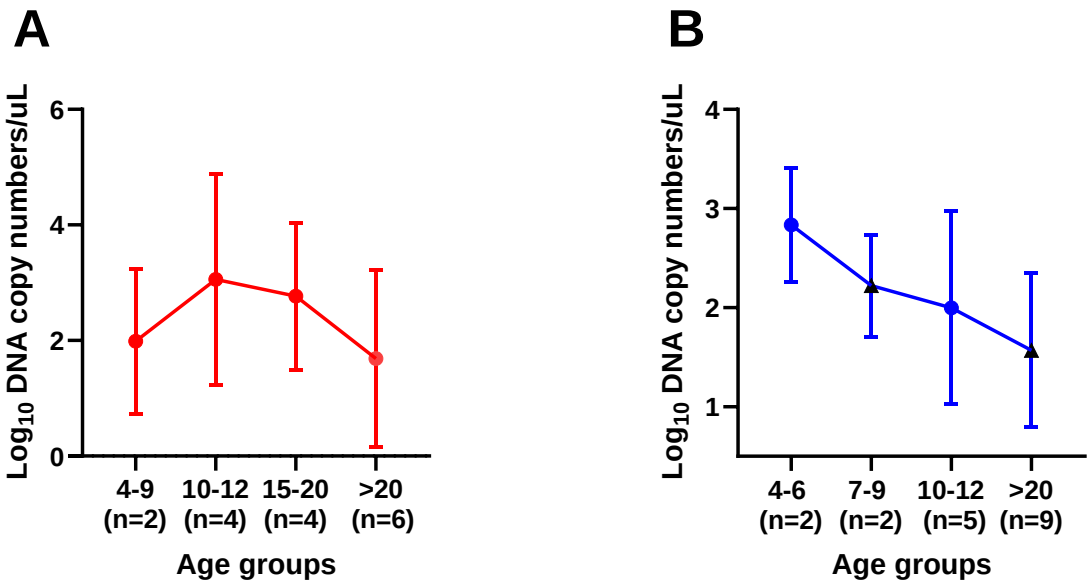


Figure 7. 5 Age distribution of the asymptomatic infections. Mean copy numbers for *P. falciparum* (**Panel A**) and *P. vivax* (**Panel B**) across age groups of asymptomatic individuals. Error bars show the standard deviations. The two black triangles represents the age group which the two successful mosquito infections were in. n = number of individuals in the respective age group.

7.4 Discussion

Understanding the contribution of asymptomatic malaria infections to transmission is critical when gearing up towards eliminating malaria as these infections will fall under the radar of the current malaria surveillance programs. The asymptomatic individuals contributes to over 50 % of the malaria infected individuals within the community as shown in this study and that they were still able to transmit malaria parasites to mosquitoes

Asymptomatic individuals were defined as individuals positive by RDT or qPCR with no fever (< 37.5 °C), headaches, chills, myalgia, dizziness, nausea or diarrhea at the time of recruitment. We observed that 45 % of the recruited asymptomatic individuals were malaria positive by microscopy with low to moderate parasitaemia levels. Only 30 % were submicroscopic. This is in contrast to a study done in Thailand which reported that all asymptomatic infections were submicroscopic. [162] However, the low microscopic and submicroscopic levels detected by this study is in agreement with observations of both submicroscopic and microscopic asymptomatic infections in the malaria endemic Amele region of PNG [263] and elsewhere. [97, 260, 264, 265]

I observed that qPCR confirmed that 9 % of HRP2 positives were *P. falciparum* and 11 % of pLDH positives were *P. vivax*. We also observed that qPCR was sensitive enough to pick up 47 % of the asymptomatic individuals missed by RDT. We also observed that 28 % of asymptomatic individuals were positive by RDT but negative by qPCR. This could be because the RDT picked up the circulating antigens from previous infections. We noted that most of the qPCR negative and RDT positives were HRP2 (11 %) and mixed (both HRP2 and pLDH) (15%) while only 2 % were pLDH. A study revealed that HRP2 antigens can remain in circulation for 35 days or more while pLDH antigen can remain up to 2 days in the body. [189] It appears that individuals that had malaria previously still had circulating HRP2 antigens which were picked up by the RDT, giving a false positive result.

I observed that generally the parasite densities of asymptomatic individuals decline with age in both *P falciparum* and *P vivax*. The decrease in *P. vivax* parasite densities was more pronounced than that was observed in *P. falciparum*. This indicates that individuals with low levels of immunity are expected to present with higher parasites densities and the risk of developing clinical malaria is also higher. Koepfli and colleagues also observed that parasite densities decreased with age in both species in asymptomatic individuals where samples were collected over three different years in a malaria endemic region of PNG. [97]

The strong correlation between parasite density derived from microcopy and the DNA copy numbers for both species is expected. However, the correlation not significant with asymptomatic *P. vivax* and *P. falciparum* infections. This is in contrast with a study done in Kenya on submicroscopic infections where significant correlations between microscopy and DNA copy

numbers ($R = 0.66$, $p < 0.01$) was observed. [266] My observation may be due to the low sample numbers.

I observed no significant distinction between the proportions of asymptomatic individuals being infected with *P. falciparum* or *P. vivax* by qPCR indicating that both species contribute equally to the asymptomatic malaria reservoir. Similar observations have been made for Solomon Islands, Thailand, Brazil, Ethiopia and PNG. [246, 267]

I reported that 5 % (2/40) of asymptomatic individuals, both with *P. vivax*, were infectious to the mosquitoes in the community survey. The findings are similar to those reported by Graves et al [143] where 2.5 % of the individuals in their community survey were infected with *P. vivax* were infectious. The study indicates ongoing transmission from asymptomatic individuals but much more needs to be done to fully understand the extent of the contribution of asymptomatic infections to malaria transmission.

7.5 Conclusion

Asymptomatic malaria infections pose a huge problem to eliminating malaria as these individuals are not being treated. I noted the presence of microscopic and submicroscopic asymptomatic infections. I showed that parasitaemia levels are generally higher in younger individuals while there is decline with increasing age. I also demonstrated that malaria transmission by asymptomatic individuals is ongoing and that more needs to be done to understand their role in maintaining transmission.

Summary

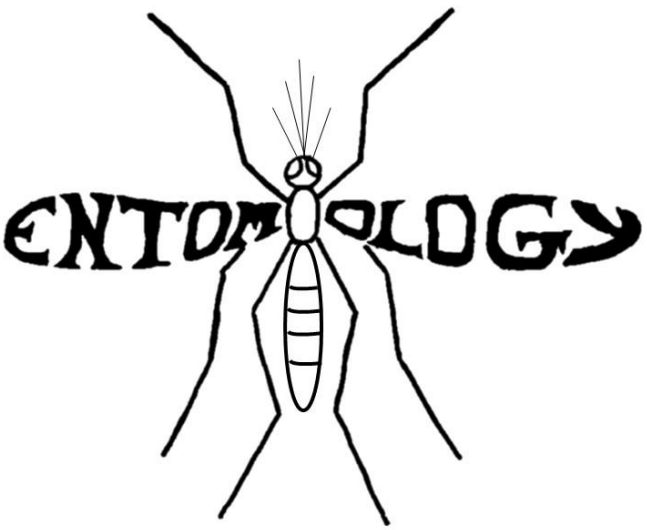
- There is ongoing asymptomatic malaria transmission within PNG.
- The asymptomatic infections can be microscopic or submicroscopic.
- Parasitaemia levels are generally higher in younger individuals declining with age.

Bridge

Chapter 8 discusses the overall aim of this PhD and how the chapters have addressed this aim. This chapter also presents possible future studies from this work and the challenges that affected this PhD and concludes with the major contributions of this PhD thesis to address general knowledge gaps.

CHAPTER 8

Discussion & Conclusion



8.1 Discussion

Malaria research in PNG dates back to early studies by Robert Koch in his malaria expedition in 1899-1990. [65-67] He was the first to carry out systematic malaria epidemiology studies in New Guinea during his visit to Madang and Rabaul. Since Koch, a number of studies have been carried out on various aspects of the malaria parasite infections.

This thesis was focused on understanding the transmission of malaria parasites from humans to mosquitoes by both symptomatic and asymptomatic individuals especially with *P. vivax*. Transmission studies on *P. vivax* are hampered by the lack of a continuous culture technique. Currently, *P. vivax* cannot be grown in a laboratory. Hence, any studies involving *P. vivax* parasites needs access to parasites from infected individuals. As a result only a few labs with access to *P. vivax* infections globally can investigate the transmission of *P. vivax* parasites from humans to mosquitoes.

Here, I have successfully established a DMFA to investigate the transmission of *P. vivax* from humans to mosquitoes. [145] using optimal feeding conditions to maximize mosquito feeding rates. [144] This DMFA setup is valuable to malaria research and puts us in a unique position where we can be able to test potential malaria transmission blocking vaccines and antimalarials against *P. vivax* parasites. I have also adapted a qPCR assay to detect the mosquito stages of the malaria parasites, reducing the mosquito processing time by heating instead of performing the conventional DNA extraction method prior to performing qPCR. I also observed that individuals have varying immunological responses from the serum replacement experiments. However, the possibility that these originated from contamination of the malaria naïve serum cannot entirely be excluded. Extended handling time and potential fluctuations in temperature can also lead to premature gametogenesis resulting in reduced infectivity. This is a complication that needs to be considered when performing serum replacement experiments in resource-constrained setting such as in PNG.

I also observed that 17 % of individuals within a malaria endemic community in Madang were asymptomatic. Only 5 % of the asymptomatic individuals transmitted malaria parasites to mosquitoes via direct feeds. These findings indicate the need to monitor the prevalence of asymptomatic infections within the communities as discussions to eliminate malaria are being made. Asymptomatic infections poses a problem as these infections remain undetected and

contribute to the malaria parasite reservoir which sustains transmission.

8.1.1 Challenges

Logistic and general operational challenges are ongoing in PNG and there was no exception to that with this work. I was able to mitigate these challenges. For example, the study had initially been recruiting patients from two clinics but decided to focus on a clinic that was close to the insectary where I could perform the DMFA within 30 minutes. It was logistically easier to organize that as well. There would be times where the clinic would be closed due to various reasons and it would affect the patient recruitment. This just added to the delay of patient recruitment. Also there would be power outage or water outage where I would depend of the backup generator or fetch water from nearby tanks. Through this PhD I have also learnt to be prepared for anything unexpected that may come be it in the logistic and operational challenges or a drastic adjustment in the project aim. Covid 19 was also another challenge that affected the progress of my study where I had to suspend patient recruitment for some time and had to stay at home for some time as well. I had requested for an extension on my PhD to cater for the loss in time through the various challenges that was faced.

8.2 Conclusion

This PhD thesis contributes to our understanding of the different contributions of asymptomatic and symptomatic *P. vivax* infections to transmission in PNG. I have improved the feeding rates of the *An. farauti* mosquitoes by identifying the optimal feeding conditions and have further successfully set up a DMFA to infect the mosquitoes with *P. vivax* parasites. I have adapted a qPCR assay to detect mosquito stages of the malaria parasites and have reduced the time taken to process the mosquito samples by heating instead of doing the conventional DNA extraction method prior to performing qPCR. Also I have shown that there is variable immune responses expected when performing serum replacement experiments in PNG. Finally, this PhD thesis has indicated that onward transmission of asymptomatic infections is ongoing and more needs to be done to fully understand the contribution of asymptomatic infections to the malaria reservoir.

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Appendix 1

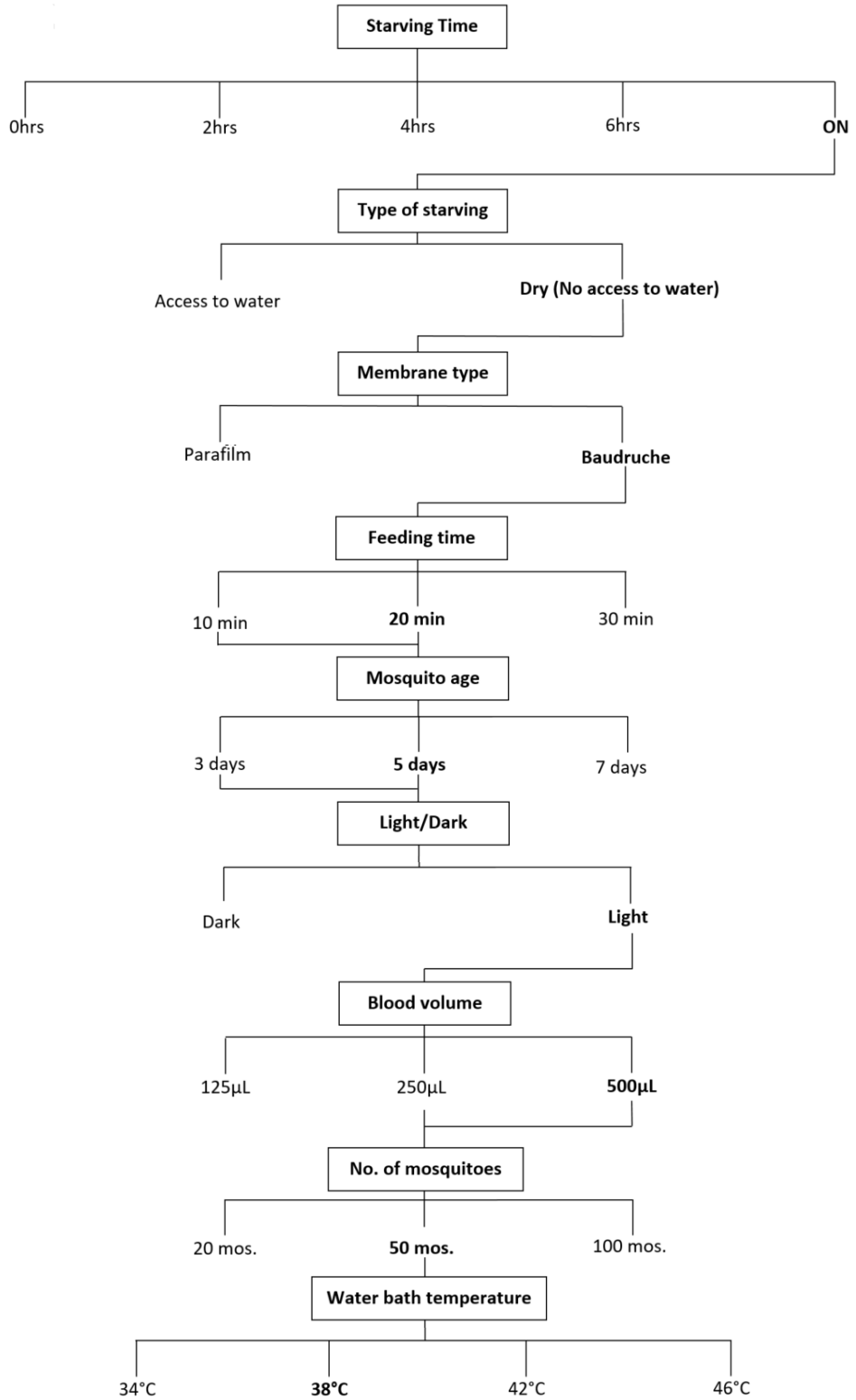

Figure A1. Flow chart of the feeding parameters that were tested progressively. The selected parameters are in bold and were used in the subsequent tests.

Appendix 2

Table A1. Oocyst counts and whether they were processed by heating or DNA extraction.

Number of oocyst/mosquito	Number of Mosquitoes	Heating / DNA extraction
1	17	Heating
2	5	Heating
3	6	Heating
4	4	Heating
5	2	Heating
6	1	Heating
7	1	Heating
10	1	Heating
Pools	36	Heating
1	17	DNA extraction
2	4	DNA extraction
3	4	DNA extraction
4	5	DNA extraction
6	1	DNA extraction
8	1	DNA extraction
13	1	DNA extraction
14	1	DNA extraction
46	1	DNA extraction
Pools	36	DNA extraction

Table A2. Sporozoites classification and whether they were processed by heating or DNA extraction.

Sporozoite classification*	Number of Mosquitoes	Heating / DNA extraction
High	17	Heating
Moderate	9	Heating
Low	4	Heating
Negative	8	Heating

High	17	DNA extraction
Moderate	9	DNA extraction
Low	4	DNA extraction
Negative	8	DNA extraction

* High >100 sporozoites, Moderate 20 -100, Low 1-20 sporozoites

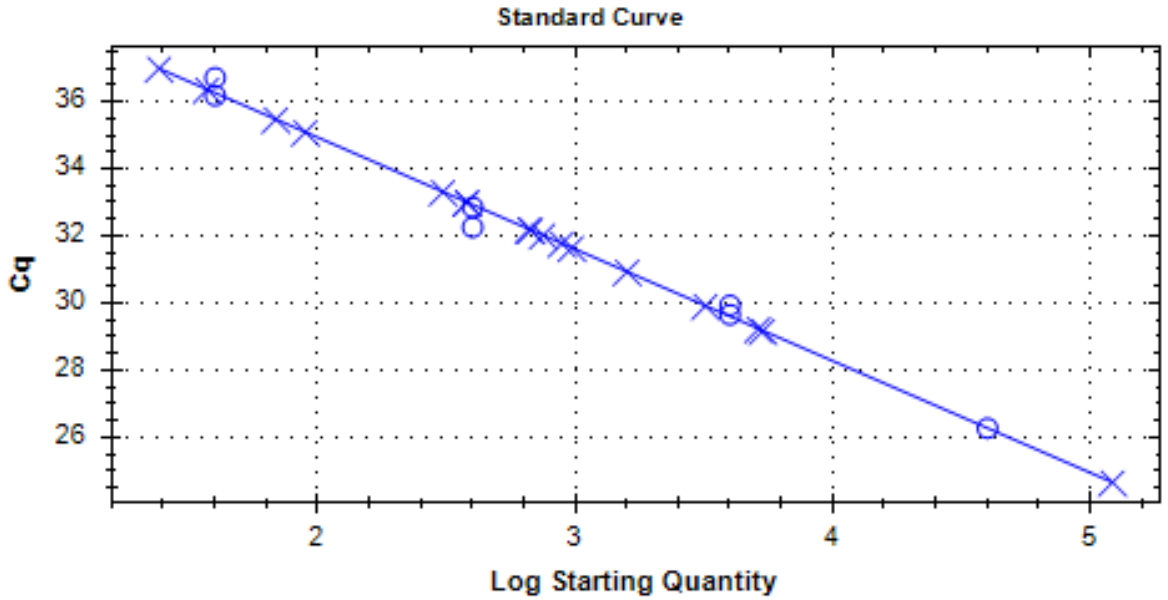

Figure A1 Standard curve derived from known concentrations of plasmids. The plasmid concentrations ($10,10^2,10^3$ and 10^4) are represented by the clear circles while the samples are represented by the cross.

Appendix 3

Table A1. Primer sequences of for the qPCR assay to detect *P. falciparum* and *P. vivax* parasites.

Species	Primer[1]	Sequence (5' - 3')
P. falcipaurm	**Pf_fwd**	TATTGCTTTTGAGAGGTTTTGTTACTTTG
	Pf_rev	ACCTCTGACATCTGAATACGAATGC
P. vivax	**Pv_fwd**	GCTTTGTAATTGGAATGATGGGAAT
	Pv_rev	ATGCGCACAAAGTCGATACGAAG

Table A2. Probe sequences for the qPCR assay to detect *P. falciparum* and *P. vivax* parasites.

Species	Probe[2]	Sequence (5' - 3')
P. falciparum	**Pf probe**	**6FAM**-ACGGGTAGTCATGATTGAGTT-**MGBNFQ**
P. vivax	**Pv probe**	**VIC**-AGCAACGCTTCTAGCTTA -**MGBNFQ**

Table A3. The reaction mix for the qPCR

qPCR Reaction mix
Total volume 14µL
2X Roche Master mix[3]
350nM per primer (forward and reverse)
350nM per probe (forward and reverse)
4µL of DNA

Table A4. The cycling conditions for the qPCR.

Thermo profile			
Hold	50°C	2min	
Hold	95°C	15min	
Denaturation	95°C	15sec	X 45
Annealing	60°C	1min	

[1.] Integrated DNA Technologies (IDT), New Zealand

2. LifeScience Roche, NSW, Australia

3. TaqMan MGB Probes ThermoFisher Scientific.,Auckland, New Zealand

Appendix 4

Table A1 Proportion of successful mosquito infection in each are (Normal, NSR and SR).

Normal	No Serum Replacement	Serum Replaced
86.67	100	100
0	100	0
44.44	36.36	9.09
0	14.29	0
37.5	40	16.67
10	0	0
10.53	20	0
75	27.27	11.11
0	0	4
76	84.62	84.62
3.7	0	0
14.29	27.78	22.22
0	0	13.33
62.5	96.15	80
28.57	11.11	0
43.75	5.88	60
91.67	28.57	61.11
16.67	0	10.53
4.55	29.63	23.08
8	12.5	0
9.09	40	0
100	71.88	95.83
100	96.67	23.53
41.18	85.29	0
16.13	19.23	0
71.43	60	13.64
26.67	2.7	9.38
44.74	60.87	3.33

Appendix 5

Table A1 Mean number of oocysts per infected mosquito per feed according to the three arms; Normal, NSR and SR.

Normal	No Serum Replacement	Serum Replaced
4.38	4.79	3.60
0.00	8.60	0.00
2.75	3.50	1.00
0.00	3.00	0.00
3.33	1.50	5.00
1.00	0.00	0.00
1.00	1.00	0.00
5.17	1.67	2.00
0.00	0.00	2.00
18.32	18.64	56.64
0.00	0.00	0.00
2.00	0.00	0.00
12.00	1.40	11.00
0.00	0.00	1.00
5.80	11.84	20.33
1.75	1.50	0.00
2.00	1.00	5.00
2.64	2.50	4.18
2.00	0.00	1.00
6.00	1.88	1.33
1.00	5.75	0.00
1.00	2.50	0.00
17.20	3.70	15.30
53.79	70.10	4.13
10.00	8.55	0.00
5.40	1.80	0.00
1.60	4.00	1.00

1.50	2.00	1.67
2.41	14.71	1.00

Appendix 6

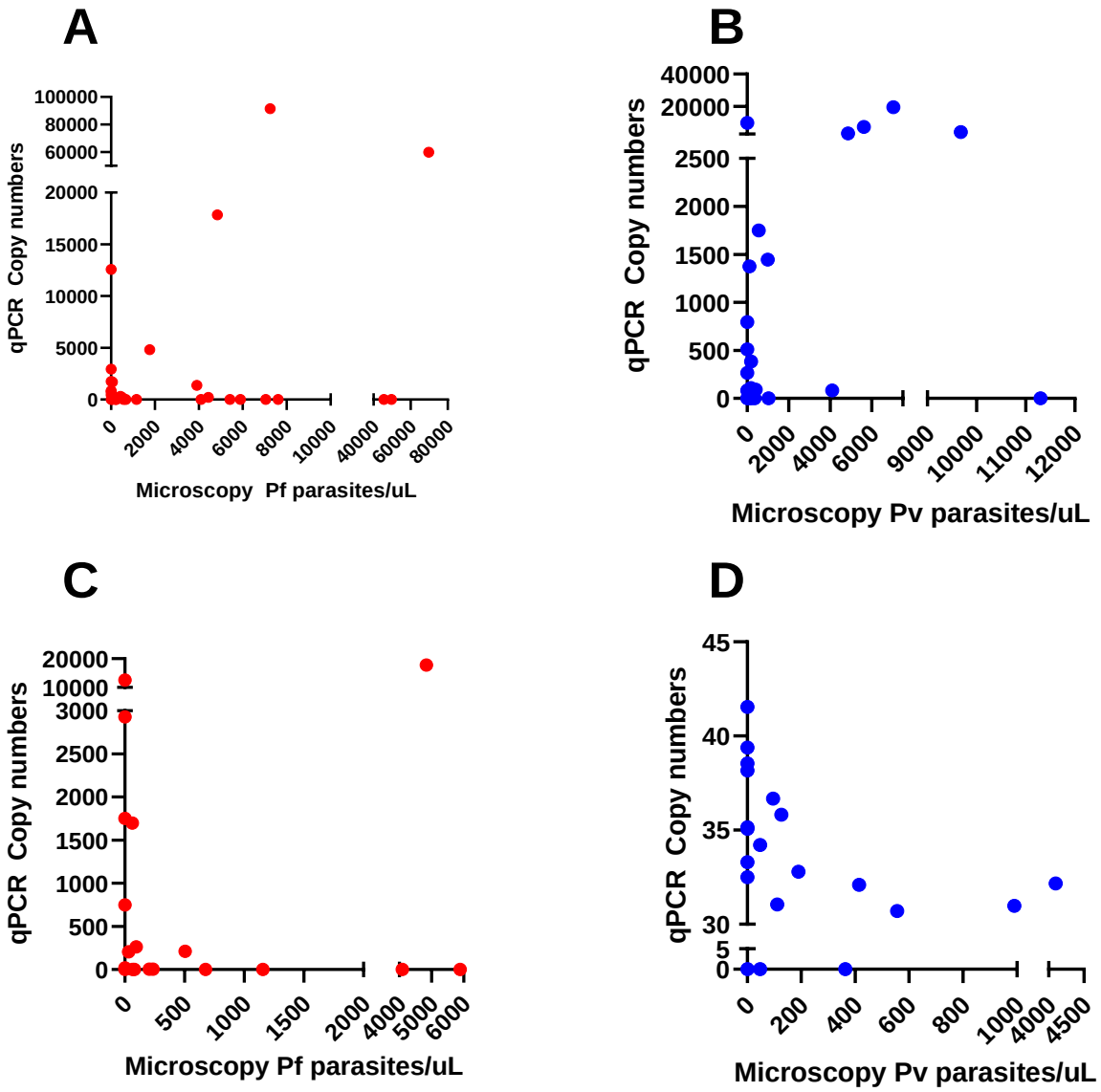

Figure A1. Graphs showing the parasitaemia (parasites/µL) versus the copy numbers (from qPCR) for the malaria positive individuals during the screening including those that were negative by either or both diagnostic methods. **Panel A** shows all the individuals that were diagnosed as *P. falciparum* positive or negative with a significantly moderate correlation between the two diagnostic methods (R= 0.4, p<0.0001). **Panel B** shows all the individuals that were diagnosed as *P. vivax* positive or negative with a significantly moderate correlation between the two diagnostic methods (R=0.6, p<0.0001). **Panel C** shows the asymptomatic individuals that were diagnosed as *P. falciparum* positive or negative. There was no significant correlation between the two diagnostic methods. **Panel D** shows the asymptomatic individuals that were diagnosed as *P. vivax* positive or negative with a significantly moderate correlation between the two diagnostic methods (R=0.4,

p=0.01)