

**Evaluation of outdoor insecticide-impregnated barriers:  
a new intervention for malaria control in the Solomon Islands**

Nicholas Deason

Advisor: Dr. Neil Lobo

4/1/15

## **ABSTRACT**

Malaria is a mosquito-borne disease that affects millions of people each year, with over 600,000 deaths globally in 2012. Recently, Solomon Islands set a goal of nationwide malaria elimination. Decades of long-lasting insecticidal nets and indoor residual spraying interventions have greatly reduced, but not eliminated malaria in Solomon Islands. Our project is testing the effectiveness of a novel outdoor vector control intervention called insecticide-impregnated barriers (IIBs) at reducing malaria transmission in Western Province, Solomon Islands. To monitor malaria infections, we developed a new highly sensitive and specific PCR diagnostic test targeting the *Plasmodium* coenzyme oxidase subunit 3 gene. A mass blood survey of 16 villages revealed a malaria prevalence of [REDACTED]. Incident cases will be monitored between intervention and control villages to test the protective effect of IIBs in a case-control study. Additionally, human landing catch surveys revealed that the dominant anthropophagic mosquito, *An. farauti*, has a peak biting time in the early evening, both indoors and outdoors. Mosquitoes were also tested for sporozite infection and parity status. This research forms the basis for a long-term intervention trial studying the impact of IIBs in the Solomon Islands.

## **INTRODUCTION**

### **A. Background**

#### Significance

Malaria is an infection caused by protozoan parasites that are transmitted to humans through the bite of a mosquito vector. The disease is widespread across the globe, with an estimated 207 million cases and 627,000 deaths in 2012 (World Health Organization, 2012), making it one of the leading causes of infectious disease mortality worldwide. The vast majority of malaria-related deaths are in children under the age of 5, and the disease disproportionately affects people living in developing nations. As a result, there is a significant global effort to fund malaria elimination projects and research, and the United Nations has designated malaria elimination as one of the eight Millennium Development Goals (United Nations, 2014). However, achieving malaria elimination will require researchers to continuously improve the tools used for transmission monitoring and to increase basic knowledge of the behavioral and ecological characteristics of vector populations. In this way, existing control interventions can be tailored to the specific needs of different malaria endemic regions, and new tools may be developed to combat residual transmission that is occurring outside the scope of current interventions (Alonso *et al.*, 2011; James *et al.*, 2014).

#### *Plasmodium*

The causative agent of malaria is a single-celled protozoan parasite from the genus *Plasmodium*, which enters the human bloodstream through the bite of an *Anopheles* mosquito. The infective form of *Plasmodium* is called a sporozoite. Upon injection into the bloodstream, sporozoites

travel to the liver to undergo development and replication into blood-stage merozoites. Merozoites enter red blood cells and consume globin proteins while continuing to replicate. The parasites eventually burst out of the host erythrocyte, leading to cell death and the manifestation of the clinical symptoms associated with malaria – alternating fever and chills, anemia, and in the worst cases, coma and death. Some blood-stage merozoites develop into gametocytes, which are then able to infect new mosquitoes that feed on the host. Within the mosquito midgut, gametocytes mature into gametes and sexual reproduction produces ookinetes, which eventually become new sporozoites that are able to infect secondary hosts (James & Tate, 1937; Sinden, 1983).

The two most common parasite species that cause human malaria are *Plasmodium falciparum* and *P. vivax*. Most acute infections and deaths are caused by *P. falciparum*, while *P. vivax* is less pathogenic but has a larger global distribution (Gething *et al.*, 2011; Schouseboe *et al.*, 2014). Additionally, *P. vivax* is able to lay dormant in the liver after primary illness and emerge weeks to months later to cause secondary relapse infections. This makes it particularly difficult to treat and detect *P. vivax* infections (White, 2011). The less commonly seen parasites *P. ovale* and *P. malariae* are not significant causes of global mortality but still contribute to the malaria burden (Doderer-Lang *et al.*, 2014).

### Mosquito Vectors

The parasites that cause human malaria can only be transmitted by certain species of mosquitoes in the genus *Anopheles*. Mosquitoes become infected by biting an infected human and ingesting *Plasmodium* gametocytes circulating in the bloodstream. Only female mosquitoes bite, as they

require nutrients and protein from the ingested blood for egg development, and thus only female mosquitoes can transmit malaria. After ingestion of parasites, there is a period of extrinsic incubation during which the parasites develop in the mosquito midgut and migrate to the salivary glands. Extrinsic incubation period length varies from 10–14 days or more depending on parasite species and environmental temperature. The mosquito is non-infectious during this time (Blanford *et. al*, 2011; Killeen *et. al*, 2000; Chua, 2012). The mosquito must then bite another human in order to complete the transmission cycle. Due to these temporal restrictions, mosquitoes that transmit malaria are often among the oldest in the population. Low daily survival rates mean that the proportion of sporozoite-positive females in a population are typically less than 10% and sometimes as little as <1%, even in high transmission areas (Hamad *et. al*, 2002; Shililu *et. al*, 1998; Gu, 1995). Importantly, the proportion of mosquitoes in a given population that are living long enough to transmit malaria can be estimated by the equation  $p^n$ , where  $p$  is the daily survival rate and  $n$  is the extrinsic incubation of the parasite. Daily survival rate,  $p$ , is calculated by  $\sqrt[x]{P}$ , where  $x$  is the length of the gonotrophic cycle from eclosion to oviposition, and  $P$  is the proportion of parous mosquitoes as determined by ovary dissections of collected specimens (Bugoro *et. al*, 2011). Measuring these parameters allows tracking of changes in mosquito population age structure over time.

## B. Vector Control

The field of vector control was born in 1897 when Sir Ronald Ross demonstrated the ability of mosquitoes to transmit the parasites responsible for malaria (Capanna, 2010). For many years control efforts relied on larviciding or draining mosquito breeding waters, but this strategy is complicated by the tendency of mosquitoes to utilize a diverse and scattered variety of breeding

sites that require extreme effort for control workers to access (Russel *et al.*, 2013). Altering wetland habitats and releasing larvicides directly into breeding waters also poses a threat to non-target organisms such as other macroinvertebrates, fish, and amphibians (Kamareddine, 2012). Subsequently, the discovery in 1939 that dichlorodiphenyltrichloroethane (DDT) acts as a potent insecticide led to malaria control programs implementing indoor residual spraying (IRS) of DDT to target mosquitoes that feed on humans and rest indoors (Joy, 1999). In 1955, the World Health Organization (WHO) initiated the first large-scale malaria elimination program called The Global Malaria Eradication Program (GMEP), based on a strategy of DDT-IRS and mass drug administration (MDA). The program was highly successful in some areas, eliminating endemic malaria in much of Europe and the Soviet Union, but gains were not sustainable in most tropical regions and elimination was not even attempted in sub-Saharan Africa due to the high rates of transmission, appearance of drug and insecticide resistance, and lack of long-term financial support (Trigg & Kondrachine, 1998; Mabaso *et al.*, 2004; Najera, 2001).

Fortunately, a new surge of resources is being invested toward malaria elimination programs led largely by the Roll Back Malaria Partnership and the Bill & Melinda Gates Foundation. Vector control represents a major area of research in this new era, as anti-parasite drugs alone are insufficient to achieve malaria elimination and there are still no approved and effective malaria vaccines. Currently, there are two methods of vector control in widespread use and known to reduce malaria transmission: IRS and insecticide-treated bed nets (ITNs) (Eneyati & Hemingway, 2010). Both interventions aim to reduce human-vector contact, but ITNs have become the most widely implemented strategy due to their ease of distribution and long-term effectiveness (Enayati *et al.*, 2009). Specifically, long-lasting insecticidal nets (LLINs) are ITNs

whose embedded insecticide has been formulated to last the lifetime of the net, without the need for repeated chemical applications. Both IRS and ITNs work by taking advantage of the characteristic biting behavior of many malaria vector species. Typically, female mosquitoes will enter human dwellings at night to obtain the bloodmeal necessary for completion of their egg development. Then, after ingestion of blood, the mosquitoes will rest on the inside walls of homes to digest the nutrient-rich meal. ITNs target mosquitoes that are host-seeking, while IRS is aimed at mosquitoes that rest on inside walls. Both interventions expose vectors to a lethal dose of insecticide, and have been shown to reduce human morbidity and mortality due to malaria (Pluess *et al.*, 2010; Okumu & Moore, 2011; World Health Organization, 2007.) Specifically, a review article incorporating studies from Africa, Asia, and South America has shown that ITNs can reduce malaria cases by 39%–62% and child mortality by 14%–29% (Lengeler, 2004), while IRS has been documented reducing malaria from 60–65% prevalence to 4–8% prevalence in a high transmission area of Mozambique (Pluess *et al.*, 2010).

However, IRS and ITNs alone are not sufficient to eradicate malaria (malERA Consultative Group on Vector Control, 2011). The biggest threat to the success of IRS and ITNs is the development of insecticide resistance in mosquito populations. Mosquito populations have shown physiological resistance to all major classes of insecticide compounds in use today, the most common of which are members of the pyrethroid family. Mechanisms of physiological resistance include increased rates of detoxifying metabolism, reduced insecticide-receptor activity, and thickening of the outer cuticle to reduce chemical exposure (Hemingway *et al.*, 2004). Additionally, mosquitoes may show a behavioral resistance to these interventions by shifting their feeding and resting behavior to outdoors, where ITNs and IRS offer no protection.

Thus, residual malaria transmission has persisted even in the face of these interventions (Killeen, 2014).

Two recent studies have tested the vector control potential of outdoor insecticide barriers, which may be able to target outdoor biting and resting mosquitoes. Faiman *et al.* (2009) draped deltamethrin-impregnated netting 2m high along a chain link fence surrounding an Israeli village and counted the number of sandflies caught with CDC light traps. Results showed a 60% reduction in the mean number of sand flies trapped behind the net-enclosed areas compared with adjacent untreated areas. A similar study by Faiman *et al.* three years later also found that the netting reduced mosquito numbers. However, this study only compared the number of mosquitoes caught in traps pre- and post-treatment, and did not make spatial comparisons to an untreated control section of the village. Furthermore, neither study looked at the effect of these barriers on disease transmission, which is crucial if these interventions were to be implemented on a large scale.

### C. Malaria Diagnostic Tools

In order to gauge the effectiveness of an intervention, indicators of malaria prevalence and incidence must be measured in the human and mosquito populations. Estimates of malaria prevalence are strongly affected by the diagnostic methodology used. Under field conditions, experienced microscopists can typically detect parasitemias as low as 50-100 parasites/ $\mu\text{L}$  of blood (Wongsrichanalai *et al.*, 2007), but misdiagnosis may occur with mixed infections or lower parasitemias. Rapid diagnosis tests (RDTs) have a limit of detection of 100-200 parasites/ $\mu\text{L}$  and can only distinguish *P. falciparum*, while they are panspecific for other human *Plasmodium*

species (Moody, 2002). Since the early 1990s, PCR-based diagnostic methods targeting the small subunit rRNA (18S rRNA) genes have been routinely used for the specific detection of human *Plasmodium* species (Singh *et al.*, 1999; Snounou *et al.*, 1993) and this target is considered the standard for PCR malaria diagnosis. This technique also allows for detection of sporozoites in mosquito collections (Arez *et al.*, 2000; Moreno *et al.*, 2004), though ELISAs are also widely employed (Burkot *et al.*, 1984).

The reported diagnostic sensitivity of several approaches based on the 18S rRNA target is approximately 10 parasites/ $\mu$ L (Snounou & Singh, 2002). The use of this gene, as well as other targets such as the mitochondrial cytochrome *b* gene (Steenkeste *et al.*, 2010; Steenkeste *et al.*, 2009) have allowed mass screening of field samples for epidemiological studies and characterized high prevalences of asymptomatic malaria. Still, better tools for malaria diagnosis are needed for three reasons: 1.) the threshold for fever and clinical disease for both *P. falciparum* and *P. vivax* malaria is <10 parasites/ $\mu$ L blood (Kamau *et al.*, 2011), 2.) sub-microscopic parasitemias are common in asymptomatic subjects (Gray *et al.*, 2013; Steenkeste *et al.*, 2010) and 3.) the standard 18S rRNA PCR method has come under scrutiny recently for issues with sensitivity and specificity.

#### **D. Malaria Transmission in the Solomon Islands**

The Solomon Islands are an area of high asymptomatic malaria where an IRS/ITN-induced behavioral shift is believed to have occurred in certain regions (Taylor, 1975). Prior to vector control interventions, there were three primary malaria vectors in the Solomon Islands: *An. farauti*, *An. koliensis*, and *An. punctulatus*. However, after several years of repeated DDT-IRS

applications, Taylor (1975) found that the latter two species were virtually eliminated from the landscape, while *An. faruati* had shifted its biting behavior from late and indoors to earlier and outdoors, as determined in human landing catch (HLC) surveys on San Cristobal island. Furthermore, no evidence for physiological insecticide resistance has been seen in the Solomon Islands (Bugoro *et al.*, 2011). Because *An. farauti* was able to adapt its behavior to survive in some areas, residual malaria transmission has continued in the Solomon Islands to this day, despite high coverage of ITNs and moderate usage of IRS (World Health Organization, 2012). According to the WHO (2012), over 99% of the human population in the Solomon Islands lives in a high malaria transmission area with occurrence of *P. falciparum* and *P. vivax* parasites. Nevertheless, a 66% decrease in malaria cases (from 68,107 cases to 23,202) was reported between 2000 and 2012, primarily due to the scaling up of ITN distribution. Since 2008, the Solomon Islands have been moving toward malaria elimination in two of the 10 provinces, Temotu and Santa Isabel, with expected extension to other provinces in the near future (Asia Pacific Malaria Elimination Network, 2007). In a low prevalence province like Temotu, as little as 3-4% of the population may be infected with malaria (The Pacific Malaria Initiative Survey Group, 2010).

This study seeks to characterize malaria transmission in Western Province, a previously understudied region of the Solomon Islands. First, a cross-sectional mass blood survey of participants in 16 study villages revealed the existing malaria prevalence levels. Then, we implemented the construction of a relatively new form of outdoor vector control called insecticide-impregnated barriers (IIBs) in 8 of the 16 test villages for a case-control study. Subsequent monitoring of malaria incidence in human populations and changes in the

composition and infection rate of vector populations will demonstrate whether the IIBs are effective measures of malaria control.

We expect the IIBs to increase the daily mortality rate for mosquitoes emerging from the primary breeding sites. The mosquitoes will have at least two opportunities to encounter the IIBs during one gonotrophic cycle – once when traveling from the breeding site to the village in search of a host, and once on the way back to the breeding site for oviposition. This is in contrast to IRS and LLINs, which only have one opportunity to target the vector inside of human dwellings. This project addresses several hypotheses: 1.) Villages that receive IIBs will experience lower malaria incidence over the study period as compared to control villages with no intervention, 2.) Mosquitoes will tend to bite earlier and outdoors as seen on other islands in the Solomon archipelago, 3.) Vector biting rate will not decrease as a result of the IIBs, 4.) IIBs will cause a shift in vector populations towards a younger age structure as a result of increased daily mortality rate, and 5.) IIB villages will have a lower proportion of infectious mosquitoes as compared to control villages. Additionally, in this study we present a new PCR-based malaria diagnosis protocol targeting the cytochrome oxidase subunit 3 gene. The generation of this tool will aid in our evaluation of the effect of IIBs on malaria incidence.

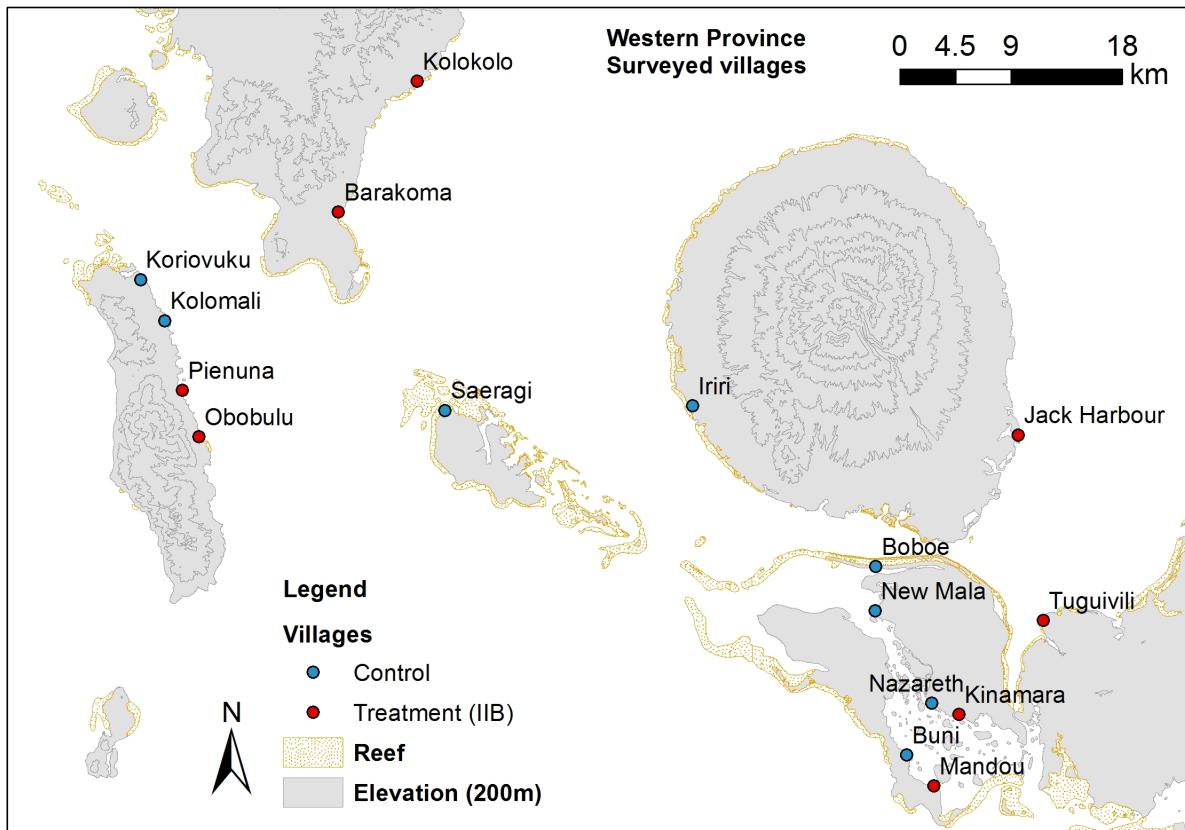
If experimental evidence shows reduced malaria incidence as well as reduced vector longevity, it may be concluded that the mechanism by which IIBs interfere with malaria transmission in human populations is by preventing vectors from becoming infectious through a reduction in vector longevity to a period shorter than the extrinsic incubation period of the parasites. In this situation, IIBs would interrupt the life cycle of the parasite, consequently rendering mosquitoes

unable to transmit malaria. If the IIBs are successful, they will have important implications for malaria control programs which have a need to target mosquitoes that bite outdoors or earlier in the day. IIBs would also be useful for areas which have few and clearly defined vector breeding habitats. IIBs could be incorporated into existing infrastructure such as fences around homes or buildings in order to add extra protection to those areas. Ultimately, IIBs may become another useful tool for vector control in the battle for worldwide malaria eradication.

## **MATERIALS AND METHODS**

### **A. Field Site Selection**

Western Province is an understudied region for malaria in the Solomon Islands. Before testing novel interventions that target changing vector behaviors, we needed basic knowledge about the malaria prevalence and epidemiology of Western Province. We chose 16 villages across five island regions in Western Province in which to work. Villages were chosen based on the presence of malaria and logistical factors, including ease of site access and a minimum population size of 100 people. Information on bednet usage was also obtained from local inhabitants. Eight villages were randomly allocated to receive IIB placements at the start of the study while the other eight served as controls with no intervention (**Fig. 1**).



**Figure 1. Map of the 16 study villages in Western Province, Solomon Islands.** Treatment villages that received IIBs are indicated with red dots while non-intervention control villages are shown with blue dots.

## B. IIB Intervention

The IIB interventions consist of a knitted polyethylene textile that has been impregnated with UV-stable deltamethrin insecticide (ZeroFly®, Vestergaard Frandsen). The barriers were placed with wooden stakes along an arc that separates the distally most extreme houses in a village from the primary mosquito breeding sites. These breeding sites are large inland lagoons or swamps located next to villages, and account for the production of high densities of *Anopheles* mosquitoes observed in Western Province (Bugoro *et al.*, 2011). The concentration of mosquito production in discrete sites makes villages in Western Province ideal to test IIBs because the barriers can be placed strategically between breeding sites and the edge of villages.

A modified EMOD epidemiological model (Eckhoff, 2011) simulating the malaria transmission cycle in the Solomon Islands indicates that 10% coverage of IIBs is enough to eliminate malaria during the two-year life of the IIB material. In order to compensate for possible inaccuracies or simplifications in the model, IIBs were constructed to cover greater than 20% of the length of the arc required to effectively separate the intervention village from the breeding site behind it. IIBs were placed in several sections of 5-10 meters each along this arc.

IIBs work by exposing mosquitoes that land on the barrier to a lethal dose of deltamethrin insecticide. Previous studies by Burkot et al. (2013) show that mosquitoes will readily land on barrier screens (without insecticide) placed in between villages and breeding sites in the Solomon Islands. The majority of mosquitoes collected on the village-side of screens were bloodfed, and were likely using the barriers as a resting site to digest bloodmeals. Mosquitoes on the opposite side of the screens were not bloodfed and instead were likely travelling towards the village in search of a bloodmeal.

### **C. Human Blood Collections**

To date, there have been three blood collections carried out in Western Province: a baseline collection in August 2014 ( $n=3,837$ ), the first follow-up collection in September/October 2014 ( $n=1,084$ ), and the second in November/December 2014 ( $n=1,077$ ). Each collection took place in all 16 of the study villages. The August 2013 collection included as many participants as possible and occurred before the construction of IIB interventions in order to form a baseline collection. The September/October 2014 and November/December 2014 collections were carried out with a

smaller cohort of individuals aged 0-18 after the construction of IIBs in order to make comparisons between intervention and control villages. All cohort members were also given a radical cure of artemisinin combination therapy and primaquine two months before the September/October 2014 bleedings to clear existing malaria infections, including liver stage hypnozoites. All blood samples were collected from a finger-prick on filter paper. The blood spots filter papers (BSFPs) were air-dried at room temperature and stored in individual bags with silica gel and shipped to the University of Notre Dame. Participants presenting with fever and possible malaria infection were checked by thick smear microscopy. Any patients subsequently testing positive for malaria were given a radical cure.

#### **D. Development of Cox3 PCR for Human Malaria Detection**

##### Cox3 PCR Protocol

The current gold standard method for PCR detection of human malaria parasites was published by Snounou *et al.* (1993). We began our analyses using this method but due to poor results with low parasitemia samples, we formulated a new protocol using a single-step PCR with a mitochondrial gene target followed by sequencing. The target for the newly generated primers was the cytochrome oxidase subunit 3 (cox3) mitochondrial gene, with a PCR product of 520bp.

For each BSFP sample, two hole punches of 1.5 mm diameter were placed in the well of a 96-well PCR microplate. Each plate included two positive controls (*P. vivax* and *P. falciparum*) and three negative controls (two samples from a known malaria-negative donor and a no-template reaction control). To remove hemoglobin from the blood, 70 µL of water were added to each well and the plate was incubated at 50°C for 5 min, 21°C for 15 sec, 50°C for 1.5 min and 21°C

for 15 sec. The water was then removed from each well and discarded. The primers short\_cox3F and short\_cox3R were used in a 30 uL final volume mastermix with 11.75 µL water, 12.5 µL 2X Phusion® Buffer (New England BioLabs), 0.25 µL each of the 100mM primers, and 0.25 µL of Phusion® High-Fidelity DNA Polymerase (New England BioLabs). The primer sequences are as follows: short\_cox3F 5'- AGCGGTTAACCTTCTTTTCCTTACG -3' and shortcox3R 5'- AGTGCATCATGTATGACAGCATGTTACA-3'. The cycle conditions were: an initial incubation at 98°C for 4 min, 60 cycles of 98°C for 1 sec, 70°C for 5 sec and 72°C for 35 sec, followed by a final step of 72°C for 10 min. PCR products were visualized on a 1% gel stained with SYBR®safe (Invitrogen, Carlsbad, CA).

#### Sequencing and Species Identification

Eight uL of PCR product from positives samples were purified using an Exonuclease 1 – Shrimp Alkaline Phosphatase (ExoSAP) method. All sequencing reactions were performed on a MicroAmp Optical 96-well plate using the BigDye® Terminator V3.1 kit (Applied biosystems). The precipitation of the BigDye® sequencing reaction was performed using 100% ethanol, 125 mM EDTA (pH 8.0), and 70% ethanol. Samples were re-suspended in 10 µL of Hi-Di Formamide and sequenced on an ABI 3730XL 96-capillary sequencer in the Genomics & Bioinformatics Core Facility at the University of Notre Dame. Chromatogram analyses and BLAST of the sequences were performed using the DNASTAR Lasergene ® 11 software (DNAstar Inc. Madison WI).

#### Reference Controls/Validation

Cultures of *P. falciparum* (HB3 strain) and *P. vivax* (Miami strain) were used for initial testing of the cox3 protocol. Serial dilutions of the parasites were blotted on filter paper in order to determine limit of detection in PCR methods with. Two human blood spots from clinically diagnosed Indonesian patients were obtained to test the protocol on field samples. Known malaria-negative donors were used for negative controls.

#### **E. Mosquito Collections**

To date, there have been three mosquito collections carried out in Western Province: one in May/June 2014 (n=3,067), one in September/October 2014 (n=2,309), and one in December 2014 (n=1,288). The May/June 2014 collections occurred in six villages (Jack Harbour, Kinamara, Nazareth, New Mala, Obobulu, Saeragi) before the construction of IIB interventions in order to form a baseline collection. The September/October 2014 collections were carried out in three intervention villages (Jack Harbour, Kinamara, Obobulu) and three control villages (Nazareth, New Mala, Saeragi). To date, only Jack Harbour collection data has been analyzed from the December 2014 collection.

All mosquitoes were collected using the human landing catch (HLC) method (Service, 1993). Each collection consisted of four nights of collection from 6 P.M. to 12 A.M., utilizing ten collectors located linearly outdoors across a village. In Jack Harbour, two collectors were also placed indoors for the September/October 2014 collection. Mosquitoes were identified morphologically on site using appropriate keys (Belkin, 1962), and all Anophelines were sent to the University of Notre Dame (USA) for molecular identification.

A subset of collected mosquitos were dissected on site to assess parity status (World Health Organization, 1975). This information can be used to find the proportion of mosquitoes in a given population that are living long enough to transmit malaria ( $p^n$ ), where  $p$  is the daily survival rate and  $n$  is the extrinsic incubation of the parasite. Daily survival rate,  $p$ , is calculated by  $x\sqrt[n]{P}$ , where  $x$  is the length of the gonotrophic cycle from eclosion to oviposition, and  $P$  is the proportion of parous mosquitoes as determined by ovary dissections of collected specimens. In these calculations, 2.5 days was used for the length of the gonotrophic cycle (Bugoro *et al.*, 2011), and 9 days and 12 days were used for the extrinsic incubation periods of *P. vivax* and *P. falciparum*, respectively (MacDonald, 1952).

## F. Molecular Identification of Mosquitoes

### PCR Amplification of ITS2 DNA

Anophelines from the collections were sequenced at the ribosomal DNA internal transcribed spacer region 2 (rDNA ITS2) region for species identification. DNA was extracted from individual specimens using a CTAB technique. The ITS2 region was amplified from genomic DNA using the ITS2A and ITS2B primers (Beebe and Saul, 1995). The primer sequences are: ITS2A 5'-TGTGAAC TG CAGGACACAT-3' and ITS2B 5'-TATGCTTAAATTCA GGGGGT-3'. The PCR mastermix contained 2.5 µl of 10X buffer, 200µM of each dNTP, 0.5 units of Taq DNA polymerase, 0.75µl of 10 pmol/µl each of forward and reverse primers, and 2 µl of DNA template. The PCR amplification was carried out under the following conditions: an initial incubation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min, with a single final extension at 72°C for 5 min (Lobo *et al.*, unpublished manuscript).

### Sequencing and Species Identification

Also according to Lobo *et al.* (unpublished manuscript), the PCR product was purified using 2U of Exonuclease 1 (USB Corporation, Cleveland, OH), 1U of Shrimp Alkaline Phosphatase (USB), and 1.8 µl of ddH<sub>2</sub>O added to 8 µl of PCR product. The mixture was incubated at 37°C for 15 min, followed by 15 min at 80°C to inactivate the enzymes. The PCR products were sequenced directly using Sanger sequencing on an ABI 3730xl DNA Analyzer platform (PE Applied Biosystems, Warrington, England).

Raw ITS2 sequences were initially aligned using the Seqman pro assembler (Lasergene v 10.1.1) with a minimum match threshold of 95%. Assembled contigs were manually examined for insertions, deletions, and repeat structures. Contigs were then further divided into sub-contigs based on consistent single nucleotide polymorphisms (SNPs). A limit of 98% identity was used to assemble ITS2 sequences into final species groups. Low quality or contaminated sequences were not included in the analysis. The consensus sequences of the ITS2 contigs were compared to the NCBI nr database for species identification.

### **G. PCR-based Sporozoite Detection in Mosquito Samples**

The head and thorax of each mosquito were ground for 20 min with a pulsating vortex mixer in 1.5 mL microfuge tubes containing two stainless steel beads and then DNA was extracted using a CTAB technique. Sporozoite DNA was amplified using the protocol described in Echeverry *et al.* (unpublished manuscript). The primers were [REDACTED]  
[REDACTED]

[REDACTED]. The PCR product was 512 bp. The PCR mastermix for a single reaction contained 3 µl of DNA template, 4.075 µl of water, 7.5 µL of 2X Phusion® Buffer (New England BioLabs), 0.15 µL each of the 100mM primers, and 0.125 µL of Phusion® High-Fidelity DNA Polymerase (New England BioLabs). The PCR amplification was carried out under the following conditions: an initial incubation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 40 sec, and extension at 72°C for 75 sec, with a single final extension at 72°C for 10 min. Five µL of the PCR product was visualized on 1% agarose gel.

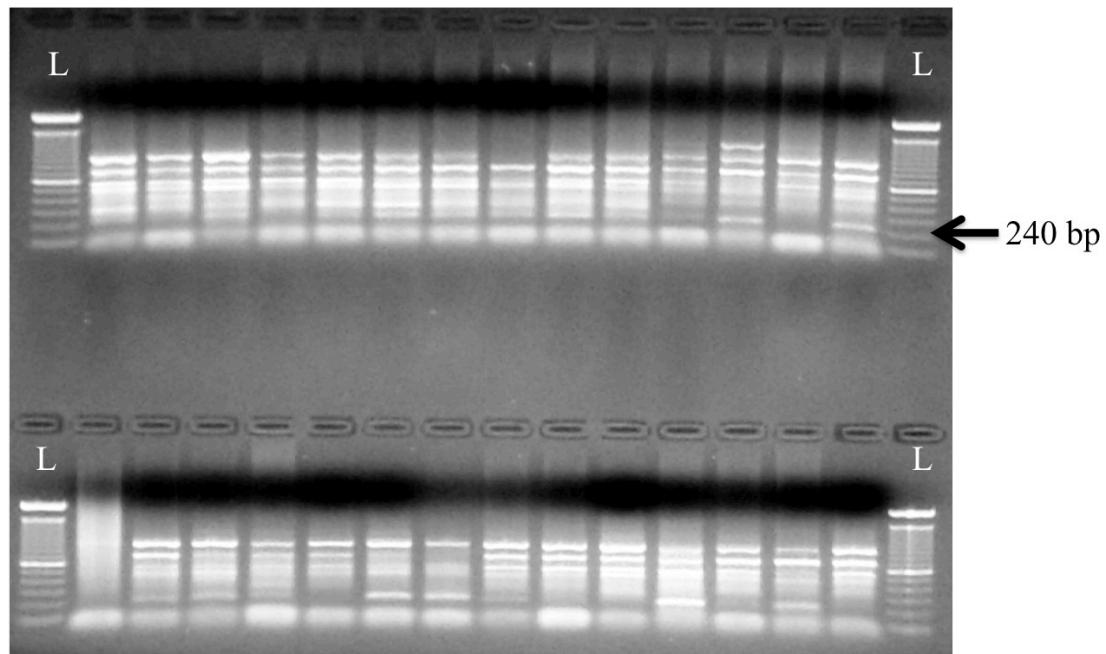
## H. Statistical Analysis

A simple linear regression analysis was performed using GraphPad Prism version 5.00 for Mac OS X, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). This software was also used to create all graphs and to calculate standard error of the mean (SEM) expressions.

## RESULTS

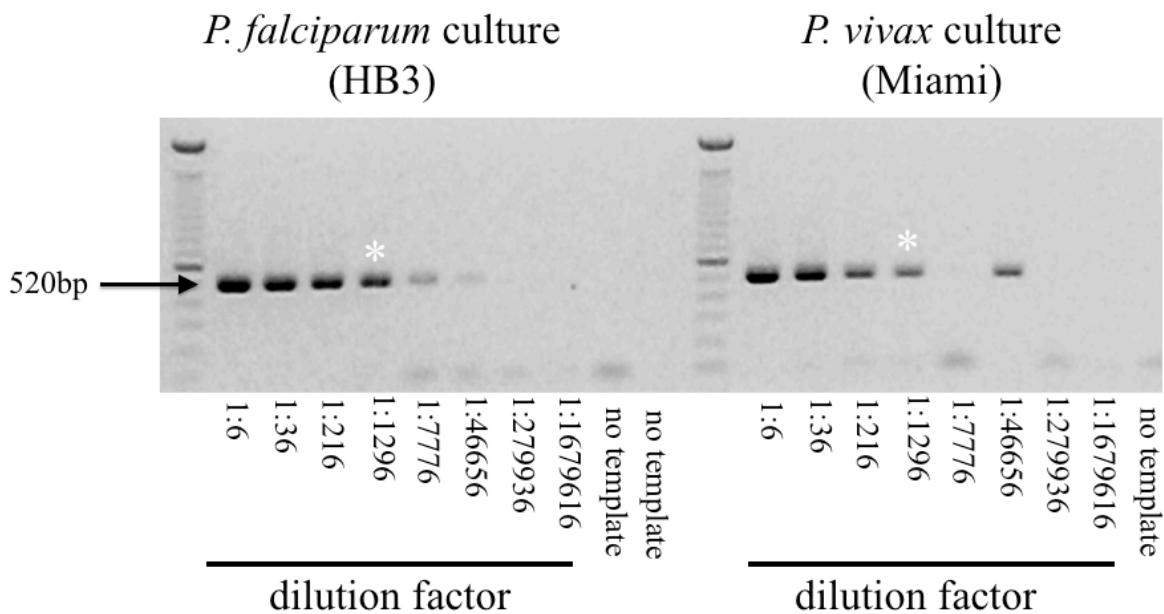
### **A. A single-step PCR followed by sequencing improves malaria diagnosis sensitivity and specificity**

Due to poor results (**Fig. 2**) and the time-consuming multi-step nature of the standard 18s rRNA-based nested PCR diagnostic method (Snounou *et al.*, 1993), we attempted to formulate a new protocol using a single-step PCR with a mitochondrial gene target followed by sequencing.



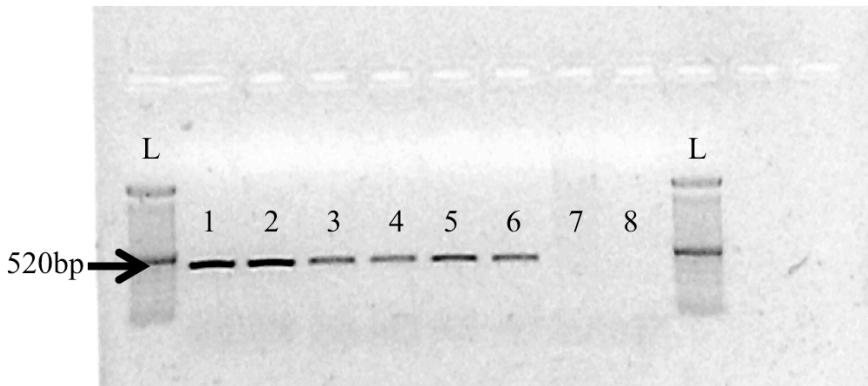
**Figure 2. Gel showing PCR products of Snounou *et al.* (1993) protocol used with Solomon Islands BSFP samples.** The arrow shows the diagnostic band size of 240 bp specific to the genus *Plasmodium*. The electrophoresis gel visualization showed a high number of non-specific amplification products that made it difficult to score positive results. Gel shows 28 of 3,837 samples, virtually all of which showed off-target amplification. L: ladder.

The target for the generated cox3 primers was the cytochrome oxidase subunit 3 (cox3) mitochondrial gene, with a PCR product of 520bp. The primers were validated with cultured strains of *P. falciparum* (HB3) and *P. vivax* (Miami strain). Primers were used with serial dilutions of parasite culture to find the limit of detection. The limit for a strong, reliable detection of both *P. falciparum* and *P. vivax* was a 1:1,296 dilution of the original culture solution, or approximately 0.6 parasites/ $\mu$ l of media (**Fig. 3**).



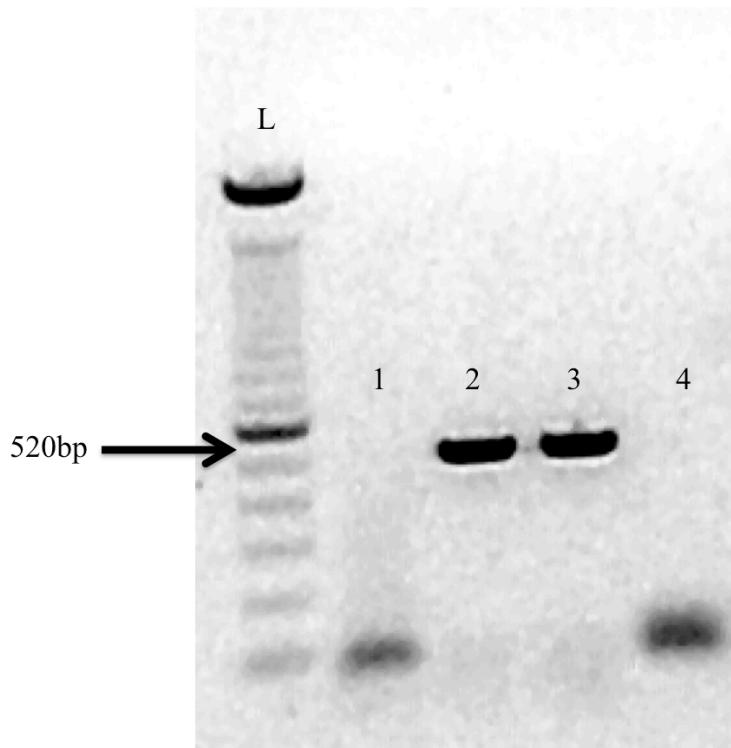
**Figure 3. Limit of detection using cox3 primers.** *P. falciparum* (HB3 strain) and *P. vivax* (Miami strain) were obtained from culture and serially diluted. The limit of strong and consistent PCR detection using the cox3 primers was a dilution of 1:1,296 in each case (indicated with white asterisk). This dilution of the original culture concentration gives a parasitemia of approximately 0.6 parasites/ $\mu$ l.

One issue observed with the current standard Snounou *et al.* (1993) PCR is non-specific amplification of off-target products, especially in low parasitemia samples. To determine whether human blood would potentially lead to non-specific amplification products in the new cox3 PCR, cultured *P. falciparum* dried on filter paper was combined with three 1.5mm diameter hole punches from non-infected human blood spots on filter paper (BSFP). The test showed no non-specific bands when the PCR product was run and visualized on an electrophoresis gel (Fig. 4).



**Figure 4. Cox3 primers do not show non-specific amplification when used with human blood.** Two BSFP punches from *P. falciparum* HB3 culture of either 6 parasites/ $\mu$ l or 0.6 parasites/ $\mu$ l were added to 3 BSFP punches of either Donor A or Donor B (non-infected blood samples). Image is representative of three replicate experiments. **Lane 1:** 6 parasites/ $\mu$ l; **Lane 2:** 0.6 parasites/ $\mu$ l; **Lane 3:** 6 parasites/ $\mu$ l + Donor A blood; **Lane 4:** 0.6 parasites/ $\mu$ l + Donor A blood; **Lane 5:** 6 parasites/ $\mu$ l + Donor B blood; **Lane 6:** 0.6 parasites/ $\mu$ l + Donor B blood; **Lane 7:** Donor A blood; **Lane 8:** Donor B blood; **L:** ladder.

The cox3 PCR method was then validated with patient blood samples collected from the field, indicating the usefulness of the method for detecting real cases of malaria infection. Two microscopy-positive blood samples from Indonesian patients were subject to the PCR reaction. Both were found positive, corroborating the clinical diagnoses (Fig. 5). Subsequent sequencing of PCR product confirmed presence of *Plasmodium* DNA. Two samples from a known uninfected donor were used as negative controls.

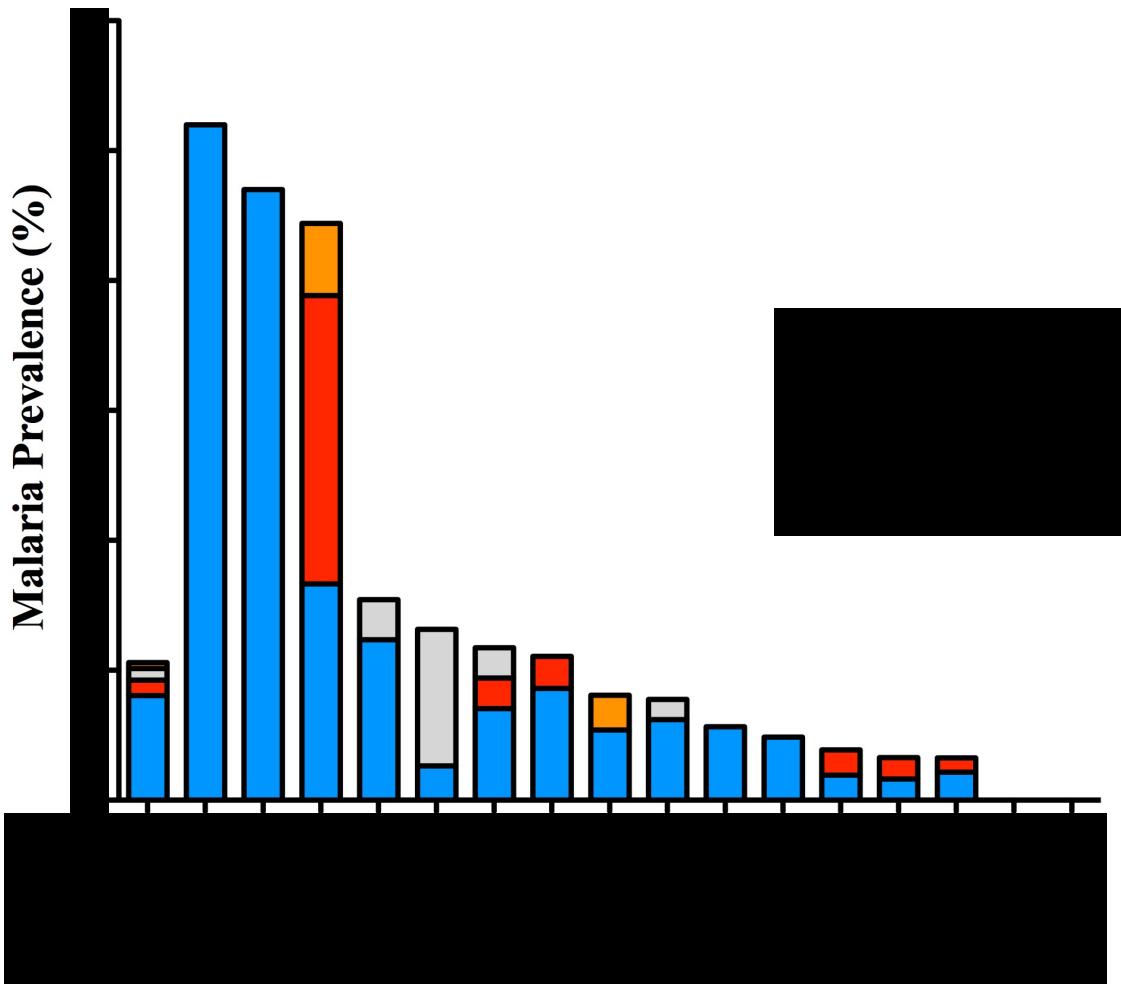


**Figure 5. Validation of cox3 PCR in patient blood samples collected from the field.** Two samples collected from clinically-positive Indonesian patients are shown. **Lane 1:** Donor B blood (uninfected patient); **lane 2:** Malaria patient 197; **lane 3:** Malaria patient 198; **lane 4:** Donor B blood; **L:** ladder.

#### B. Western Province has low malaria prevalence with a high proportion of asymptomatic cases

In the August 2013 survey, BSFP samples were collected from 3,837 individuals across Western Province, Solomon Islands. Two individuals with fevers were diagnosed with malaria based on microscopic analysis of blood smears. BSFPs of all individuals were then sent to Notre Dame where 79 samples were found positive based on the cox3 PCR protocol. This gives an estimated prevalence of [REDACTED] asymptomatic (non-febrile) malaria in Western Province. Sequencing of the PCR-positive samples revealed [REDACTED] infections. Prevalences in individual villages ranged from a high of [REDACTED] to a low of [REDACTED] (Fig. 6). Finally, a simple linear regression analysis showed

that the degree of prior bednet coverage in a village was not predictive of the malaria prevalence found in the baseline survey (slope not significantly different than 0,  $p=0.7$ ; data not shown).



**Figure 6. Malaria prevalence by species in each village.** Prevalences were determined

### C. Malaria incidence is █ in all villages.

Following the baseline malaria prevalence survey in August 2013, a cohort of 1,084 individuals aged 0-18 were enrolled in an incidence-monitoring cohort. Blood collections were performed in

September/October and November/December 2014, with 927 and 856 participants respectively.

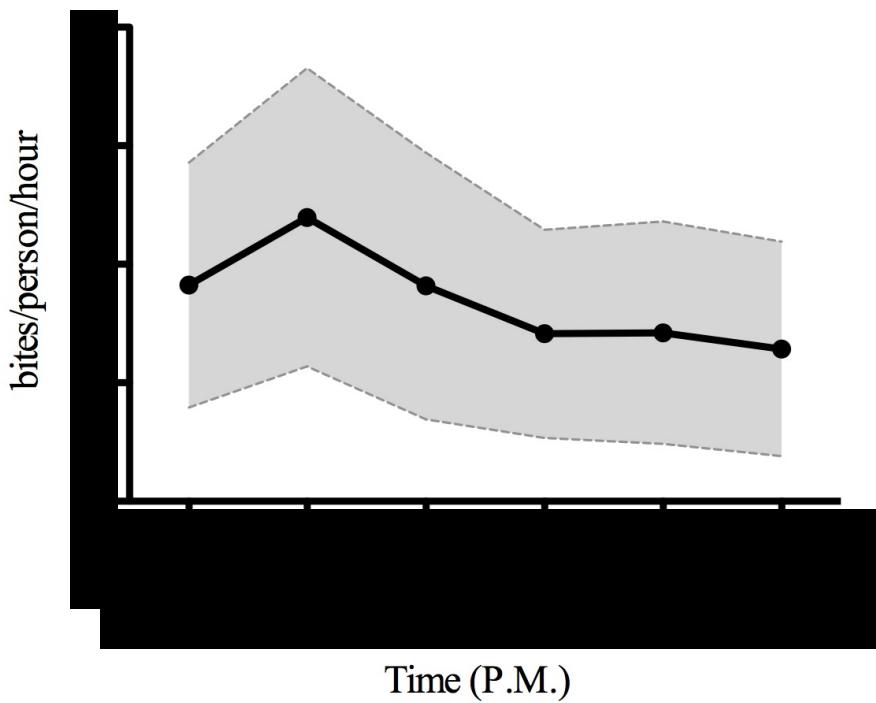
The number of new infections after the initial radical cure was [REDACTED] cases in September/October, and [REDACTED] November/December. Cases arose in [REDACTED] and [REDACTED] through the first collection. Incident cases were only found in [REDACTED] in November/December 2014.

**Table 1. New infections of malaria found in the incidence-monitoring cohorts in two follow-up blood collections.** [REDACTED] were found in the September/October [REDACTED] were found in the November/December collection.

	September/October 2014 Collection		November/December 2014 Collection	
	<u>Village</u>	<u>Spp.</u>	<u>Village</u>	<u>Spp.</u>
<b>Intervention Villages</b>	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
<b>Control Villages</b>	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]

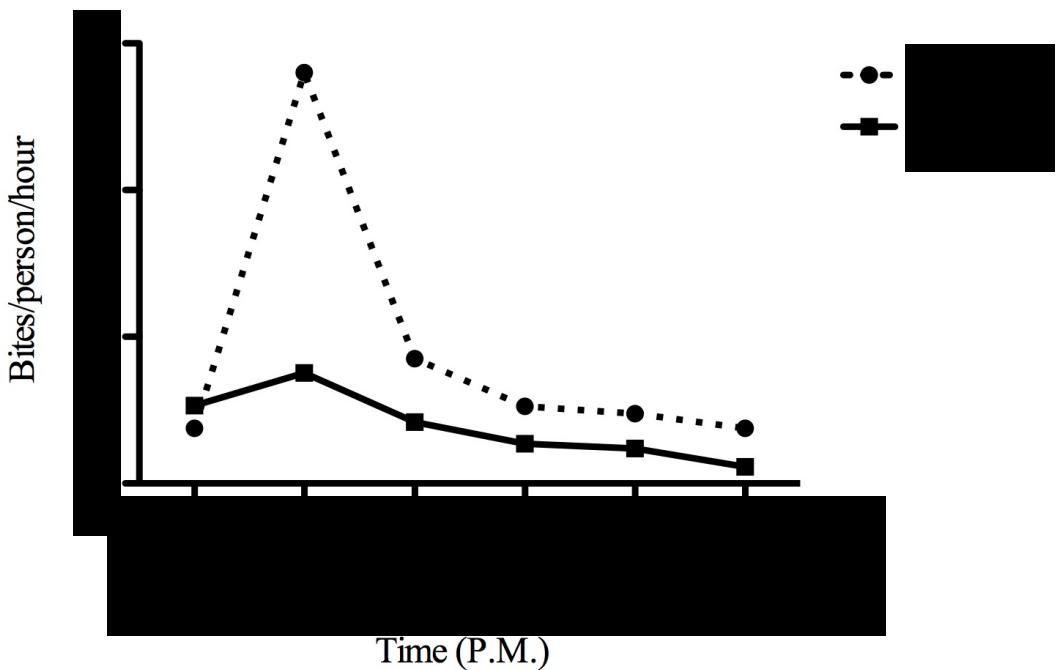
#### D. Anophelines in Western Province exhibit peak biting in the [REDACTED] [REDACTED]

In order to visualize the biting profiles of Anophelines in Western Province, HLC data was combined from all three mosquito collections spanning May-December 2014. The mean hourly biting rates from each village during each collection were averaged and plotted. The peak biting hour was [REDACTED] the majority of biting occurred [REDACTED] (Fig. 7).



**Figure 7. Biting profile of Anopheline mosquitoes in Western Province, Solomon Islands.**  
Data from all three collections was compiled and averaged to visualize a representative curve for Anopheline biting behavior in Western Province. Shaded region shows  $\pm$ SEM.

In the highest biting density village, Jack Harbour, an additional 2 collectors were stationed indoors for the September/October 2014 collection to see if there was significant biting occurring indoors. Extremely high densities of Anophelines were found biting [REDACTED] P.M. (Fig. 8).

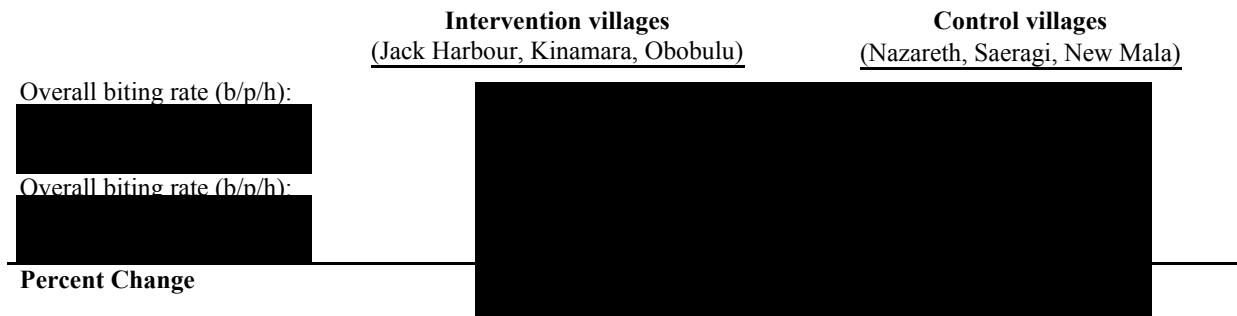


**Figure 8. Indoor and outdoor biting profiles of Anophelines in Jack Harbour during September/October 2014.** 10 collectors were stationed outdoors as usual, with an additional 2 collectors stationed in homes to measure indoor biting.

#### E. Preliminary results show [REDACTED] on human biting rate

Some initial comparisons can be made between HLCs in control and intervention villages, though more collections will be needed to draw strong conclusions about the effect of IIBs on mosquito numbers and biting rates. Nevertheless, the bites/person/hour (b/p/h) of [REDACTED] [REDACTED] intervention villages between pre- and post-intervention HLC surveys, while b/p/h [REDACTED] (Table 2). Future HLCs will add to this data, and may allow for a two sample t-test between percents to statistically compare the differences in percent change in biting rates.

**Table 2. Percent change in biting rate between May/June and Sept/Oct 2014 HLCs.**  
 Comparisons were made by averaging b/p/h from [REDACTED] for both HLC surveys.



**F. Parity dissections reveal the proportion of mosquitoes living long enough to transmit malaria**

Parity dissections were completed on a subset of caught mosquitoes in one intervention village (Jack Harbour) and one control village (Saeragi) in the September/October 2014 collections. Parity proportions were used to calculate the daily survival rates of Anophelines in these villages, as well as the maximum theoretical proportion of the mosquito populations living long enough to transmit *P. vivax* and *P. falciparum*, respectively (**Table 3**).

**Table 3. Parity data for mosquitoes in Jack Harbour and Saeragi, September/October 2014.**

	Parous	Nulliparous	Proportion Parous (P)	Daily Survival Rate ( <i>p</i> )	% of pop. able to transmit <i>P. vivax</i>	% of pop. able to transmit <i>P. falciparum</i>
Jack Harbour	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
Saeragi	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]

**G. Barcoding using ITS2 gene reveals the species diversity of Anopheline mosquitoes in Western Province**

A subset of mosquitoes from each collection village was subject to PCR amplification and sequencing of a region of the ITS2 gene to identify the species of mosquitoes biting humans in Western Province. **Table 4** shows the species identity of mosquitoes caught in each village. Analyzed mosquitoes are combined from all three collections spanning May-December 2014. Four species were found, with *An. farauti* and *An. lungae* being the most dominant.

**Table 4. Diversity of Anophelines caught in human landing catches spanning May-December 2014.** A subset of mosquitoes from each collection village were identified to species by ITS2 gene sequences.

					Total
<b>Jack Harbour</b>	321	0	0	0	321
<b>Kinamara</b>	1	4	2	1	8
<b>Nazareth</b>	1	1	32	1	35
<b>New Mala</b>	13	1	12	0	26
<b>Obobulu</b>	3	1	0	0	4
<b>Saeragi</b>	2	7	65	2	76

#### H. Sporozoite-infected mosquitoes were found

A subset of mosquitoes from each collection were subject to PCR amplification of *Plasmodium* DNA using the cox1 primers for sporozoite detection. Positive samples were then sequenced to identify parasite species. The only village found to have sporozoite-infected mosquitoes was Jack Harbour. The number of mosquitoes analyzed from each collection, along with the species of detected sporozoites is shown in **Table 5**. Analysis is still currently underway for mosquitoes captured in the November/December 2014 collection.

**Table 5. Sporozoite-positive mosquitoes.** [REDACTED]  
infected mosquitoes, all of which were *An. J.*

Village	Collection Date	Mosquitoes Analyzed	Mosquitoes Positive for Sporozoites	Sporozoite Positivity (%)	Parasite Species	Mosquito Species
<b>Jack Harbour</b>	May/June 2014	532	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
	Sept./Oct. 2014	465	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]
	Nov./Dec. 2014	94	[REDACTED]	[REDACTED]	[REDACTED]	[REDACTED]

## **DISCUSSION**

Malaria transmission in the Solomon Islands has persisted in spite of widespread IRS and LLIN use. We implemented IIB interventions in Western Province in an attempt to contribute to vector control efforts. In order to detect malaria cases throughout the study, we developed a highly sensitive and specific PCR diagnostic method targeting the *Plasmodium* cox3 gene. The cox3 PCR had a limit of detection of 0.6 parasites/ $\mu$ L blood. This is a significant improvement over the sensitivity of the standard 18s rRNA diagnostic method (Snounou *et al.*, 1993) which has a reported sensitivity of approximately 10 parasites/ $\mu$ L (Snounou & Singh, 2002). The difficulties with the 18srRNA target appear to be related to the low parasitemias associated with the Solomon Islands samples in particular. Presumably, the increased sensitivity of the cox3 PCR is due to the fact that the cox3 target is a mitochondrial gene, and thus there may be 20 or more copies of the target template genome per *Plasmodium* cell (Preiser *et al.*, 1996), as opposed to one copy per cell in the case of a single gene target in the nuclear genome. The cox3 method also has superior specificity as it does not show a tendency to produce off-target amplification products when used with human samples from the field. Additionally, our method required a single PCR step, as opposed to multiple nested PCRs in the gold standard method. This was

accomplished by directly sequencing the cox3 PCR product for species identification. This improvement is made possible by the falling cost and time required for sequencing.

Using the cox3 diagnostic method, we determined the malaria prevalence in a group of 3,878 individuals in Western Province to be [REDACTED]. This number is [REDACTED] to other provinces in the Solomon Islands such as Temotu with a prevalence of approximately 3-4% (The Pacific Malaria Initiative Survey Group, 2010), but is [REDACTED] than the pre-elimination province of Santa Isabel with a prevalence of 0.16% (Bugoro *et al.*, 2011). The observed prevalence in Western Province, while typical of the Pacific region, is still much lower than high transmission areas in sub-Saharan Africa, where prevalences can be in excess of 60% (Pluess *et al.*, 2010). Similar to Temotu and Isabel provinces, however, the majority of malaria cases in Western were caused by [REDACTED]. This complicates elimination efforts as hypnozoites may lay dormant in the liver for months where they are invisible to detection methods and untreatable with most antimalarials (White, 2011). The hypnozoites can later emerge without warning to cause illness and propagate malaria transmission.

Follow-up blood testing in the incidence-monitoring cohort revealed [REDACTED] acquired malaria infections in the cohort in the 6 months following a radical cure mass administration. [REDACTED] were in intervention villages while [REDACTED] in control villages. This is a re[REDACTED] number of cases and significant comparisons between control and intervention villages cannot be made until more time points are added to the data pool. An indication the IIBs are working would be a significantly lower number of newly acquired cases in intervention villages as compared to control villages.

HLC surveys were carried out in six villages to determine the bionomics of anthropophagous Anopheles in Western Province. Species were identified by sequencing the ITS2 gene and included [REDACTED]

The peak biting hour of mosquitoes, and in particular [REDACTED] with significant biting occurring [REDACTED] s. This result is consistent with findings from Isabel Province (Bugoro *et al.*, 2011) and Guadalcanal (Bugoro *et al.*, 2014) where the peak biting hour was also 7-8 P.M. Surprisingly, [REDACTED] has one of the highest densities of [REDACTED] reported from the Solomon Islands, with biting rates at times approaching [REDACTED]. This is likely due to the enormous breeding habitat caused by permanent flooding after a 2007 earthquake. It is also interesting to note that Jack Harbour had one of the [REDACTED] observed malaria prevalences, especially of [REDACTED], in the pre-intervention baseline survey. *P. falciparum* malaria, being acute and transient, likely requires the [REDACTED] densities in Jack Harbour to maintain persistent transmission in the human population. The other three Anopheline species found in Western villages played lesser roles in the biting densities, and are not known to support transmission of malaria. Overall, the hypothesis that Anopheles in Western Province bite early in the evening was [REDACTED], and a surprisingly large amount of [REDACTED] occurred.

At the current time, the [REDACTED] in reducing the biting rates of Anopheles in intervention villages. A comparison of the changes in biting rate between control and intervention villages before and after IIB construction showed that there was a [REDACTED] [REDACTED] respectively. The similar magnitude of these numbers suggests that the temporal reduction in biting rates from May/June to September/October 2014 are a result of seasonal or

environmental fluctuations. However, it is not crucial that IIBs reduce the mosquito biting rate to eliminate malaria. Rather, the IIBs need only target a small percentage of the population of the mosquitoes that are living long enough to transmit malaria in order to be effective. A small increase in daily mortality rate can have a huge impact on the ability of a mosquito population to support the extrinsic incubation of malaria parasites (Cook *et al.*, 2008).

The evidence of whether IIBs are increasing daily vector mortality rate will come from analysis of the parity status of caught females. A parity analysis of mosquitoes caught in September/October 2014 shows a daily survival rate of (███████████) and ██████████. These values are █████ compared to the survival rates of ~0.7 in Santa Isabel (Bugoro *et al.*, 2011), but are more █████ to the rate of 0.79 found in Guadalcanal (Bugoro *et al.*, 2014). Small changes in these values have large implications for malaria transmission as they are raised to the power of the length of the gonotrophic cycle when calculating the proportion of the mosquito population living long enough to transmit malaria. In order to fully test the hypothesis that IIBs will reduce the vector daily survival rate, more data must be collected on the parity status of caught Anophelines in control and intervention villages.

Finally, cox1 sporozoite PCRs detected █████ parasites in █████ in █████. █████ is understood to currently be the only malaria vector in the Solomon Islands. Both █████ were found in the human population in █████ corroborating the sporozoite findings. No █████ mosquitoes were found in any other of the 6 collection villages, though much fewer mosquitoes overall were collected from those locations. Sporozoite prevalence was a █████ in the analyzed mosquitoes from

Jack Harbour in September/October 2014. This is [REDACTED] than that found in Santa Isabel Province in 2011 (0.001%; Bugoro *et al.*, 2011) and may be reflective of the relatively [REDACTED] baseline malaria prevalence in Jack Harbour when compared to Santa Isabel. Comparatively, sporozoite rates may be as high as 10% in high transmission areas of sub-Saharan Africa, but are typically around 1% (Shililu *et al.*, 1998). Sporozoite rates will need be continually monitored in intervention and control villages in order to observe the any long-term effect IIBs may be having on transmission in Western Province.

This study accomplished several goals as part of a long term evaluation of IIBs on malaria transmission in Western Province, Solomon Islands. First, the generation of the new cox3 PCR diagnostic method will ensure accurate and sensitive monitoring of human malaria cases that arise in the future. The protocol also helped to us determine the baseline malaria prevalence in Western Province, which was low and consisted of mostly [REDACTED] infections. This knowledge will assist malaria control efforts going forward. Additionally, HLCs revealed the diversity of Anophelines in Western Province, as well as the peak biting times in the [REDACTED] [REDACTED]. In order that the hypotheses of this study be fully tested, it is imperative that the effect of IIBs on incident cases of human malaria, as well as sporozoite rates and daily survival rates in mosquitoes continue to be monitored in the future to increase the data available for analyses. If future evidence shows reduced malaria as well as reduced vector longevity, it may be concluded that the mechanism by which IIBs reduce malaria incidence in human populations is by preventing vectors from becoming infectious through a reduction in vector longevity to a period shorter than the extrinsic incubation period of the parasites. In this situation, IIBs would interrupt the life cycle of the parasite, consequently rendering mosquitoes unable to transmit malaria. If

the IIBs are successful, they will have important implications for malaria control programs which have a need to target mosquitoes that bite outdoors or earlier in the day, and may be added to our arsenal of effective vector control interventions in the fight for malaria eradication.

## **LITERATURE CITED**

1. Alonso, P. L., Brown, G., Arevalo-Herrera, M., Binka, F., Chitnis, C., Collins, F., ... & Tanner, M. (2011). A research agenda to underpin malaria eradication. *PLoS medicine*, 8(1), e1000406.
2. Arez, A. P., Lopes, D., Pinto, J., Franco, A. S., Snounou, G., & Do Rosário, V. E. (2000). Plasmodium sp.: Optimal Protocols for PCR Detection of Low Parasite Numbers from Mosquito (Anopheles sp.) Samples. *Experimental parasitology*, 94(4), 269-272.
3. Asia Pacific Malaria Elimination Network, (2007). Strategic Program Goals for Elimination. National Malaria Strategic Vision 2007–2016. Solomon Islands.
4. Beebe, N. W. & B. A. Saul. (1995). Discrimination of all members of the Anopheles punctulatus complex by polymerase chain reaction restriction fragment length polymorphism analysis. *Am. J. Trop. Med. Hyg.*, 53(5), 478-481.
5. Belkin, J. N. (1962). The Mosquitoes of the South Pacific (Diptera, Culicidae), Vol. 2. *The Mosquitoes of the South Pacific (Diptera, Culicidae)*, Vol. 2.
6. Blanford, J. I., Blanford, S., Crane, R. G., Mann, M. E., Paaijmans, K. P., Schreiber, K. V., & Thomas, M. B. (2013). Implications of temperature variation for malaria parasite development across Africa. *Scientific reports*, 3.
7. Bugoro, H., Cooper, R. D., Butafa, C., Iro'ofa, C., Mackenzie, D. O., Chen, C. C., & Russell, T. L. (2011). Bionomics of the malaria vector Anopheles farauti in Temotu Province, Solomon Islands: issues for malaria elimination. *Malar J*, 10, 133.
8. Bugoro, H., Hii, J. L., Butafa, C., Iro'ofa, C., Apairamo, A., Cooper, R. D., ... & Russell, T. L. (2014). The bionomics of the malaria vector Anopheles farauti in Northern Guadalcanal, Solomon Islands: issues for successful vector control. *Malaria journal*, 13(1), 56.
9. Bugoro, H., Iro'ofa, C., Mackenzie, D. O., Apairamo, A., Hevalao, W., Corcoran, S., ... & Cooper, R. D. (2011). Changes in vector species composition and current vector biology and behaviour will favour malaria elimination in Santa Isabel Province, Solomon Islands. *Malar J*, 10, 287.
10. Burkot, T. R., Russell, T. L., Reimer, L. J., Bugoro, H., Beebe, N. W., Cooper, R. D., ... & Lobo, N. F. (2013). Barrier screens: a method to sample blood-fed and host-seeking exophilic mosquitoes. *Malaria journal*, 12(1), 49.
11. Burkot, T. R., Zavala, F., Gwadz, R. W., Collins, F. H., Nussenzweig, R. S., & Roberts, D. R. (1984). Identification of malaria-infected mosquitoes by a two-site enzyme-linked

- immunosorbent assay. *The American journal of tropical medicine and hygiene*, 33(2), 227-231.
12. Capanna, E. (2010). Grassi versus Ross: who solved the riddle of malaria? *International Microbiology*, 9(1), 69-74.
  13. Chua, T. H. (2012). Modelling the effect of temperature change on the extrinsic incubation period and reproductive number of *Plasmodium falciparum* in Malaysia. *Trop Biomed*, 29, 121-128.
  14. Cook, P. E., McMeniman, C. J., & O'Neill, S. L. (2008). Modifying insect population age structure to control vector-borne disease. In *Transgenesis and the management of vector-borne disease* (pp. 126-140). Springer New York.
  15. Doderer-Lang, C., Atchade, P. S., Meckert, L., Haar, E., Perrotey, S., Filisetti, D., ... & Candolfi, E. (2014). The ears of the African elephant: unexpected high seroprevalence of *Plasmodium ovale* and *Plasmodium malariae* in healthy populations in Western Africa. *Malaria journal*, 13(1), 240.
  16. Echeverry, D.F., N.A. Deason, H. Xiao, J. Davidson, V. Makuru, J. Niedbalski, F.H. Collins, T.L. Russell, T.R. Burkot, and N.F. Lobo. (Unpublished manuscript). A fast and robust single PCR for *Plasmodium* sporozoite detection using the cytochrome oxidase I gene.
  17. Eckhoff, P. A. (2011). A malaria transmission-directed model of mosquito life cycle and ecology. *Malar J*, 10(10).
  18. Enayati, A., & Hemingway, J. (2010). Malaria management: past, present, and future. *Annual review of entomology*, 55, 569-591.
  19. Enayati, A., Lines, J., Maharaj, R., & Hemingway, J. (2009). Suppressing the vector. *Shrinking the malaria map: a prospectus on malaria elimination*, 140-154.
  20. Faiman, R., Cuno, R., & Warburg, A. (2009). Control of phlebotomine sand flies with vertical fine-mesh nets. *Journal of medical entomology*, 46(4), 820-831.
  21. Faiman, R., & Warburg, A. (2012). Insecticide-treated vertical mesh barriers reduce the number of biting mosquitoes. *Medical and veterinary entomology*, 26(1), 26-32.
  22. Gething, P. W., Patil, A. P., Smith, D. L., Guerra, C. A., Elyazar, I. R., Johnston, G. L., ... & Hay, S. I. (2011). A new world malaria map: *Plasmodium falciparum* endemicity in 2010. *Malar J*, 10(378), 1475-2875.
  23. Gray, K. A., Dowd, S., Bain, L., Bobogare, A., Wini, L., Shanks, G. D., & Cheng, Q. (2013). Population genetics of *Plasmodium falciparum* and *Plasmodium vivax* and asymptomatic malaria in Temotu Province, Solomon Islands. *Malar J*, 12, 429.

24. Gu, W. D. (1995). Estimating sporozoite rates by examining pooled samples of mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 89(4), 359-360.
25. Hamad, A. A., Nugud, A. E. H. D., Arnot, D. E., Giha, H. A., Abdel-Muhsin, A. M. A., Satti, G. M., ... & Elnaiem, D. E. A. (2002). A marked seasonality of malaria transmission in two rural sites in eastern Sudan. *Acta tropica*, 83(1), 71-82.
26. Hemingway, J., Hawkes, N. J., McCarroll, L., & Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect biochemistry and molecular biology*, 34(7), 653-665.
27. James, S., Takken, W., Collins, F. H., & Gottlieb, M. (2014). Needs for Monitoring Mosquito Transmission of Malaria in a Pre-Elimination World. *The American journal of tropical medicine and hygiene*, 90(1), 6-10.
28. James, S. P., & Tate, P. (1937). New knowledge of the life-cycle of malaria parasites. *Nature*, 139, 545.
29. Joy, R. J. (1999). Malaria in American troops in the south and southwest Pacific in World War II. *Medical history*, 43(02), 192-207.
30. Kamareddine, L. (2012). The biological control of the malaria vector. *Toxins*, 4(9), 748-767.
31. Kamau, E., Tolbert, L. S., Kortepeter, L., Pratt, M., Nyakoe, N., Muringo, L., ... & Ockenhouse, C. F. (2011). Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of Plasmodium by amplifying RNA and DNA of the 18S rRNA genes. *Journal of clinical microbiology*, 49(8), 2946-2953.
32. Killeen, G. F., McKenzie, F. E., Foy, B. D., Schieffelin, C., Billingsley, P. F., & Beier, J. C. (2000). A simplified model for predicting malaria entomologic inoculation rates based on entomologic and parasitologic parameters relevant to control. *The American journal of tropical medicine and hygiene*, 62(5), 535-544.
33. Killeen, G. F. (2014). Characterizing, controlling and eliminating residual malaria transmission. *Malaria journal*, 13(1), 330.
34. Lengeler, C. (2004). Insecticide-treated bed nets and curtains for preventing malaria. *Cochrane Database Syst Rev*, 2(2).
35. Lobo, N.F., St. Laurent, B., Sikaala, C., Seyoum, A., Hamainza, B., Chanda, J., Chinula, D., Krishnankutty S.M., Mueller, J.D., Deason. N.A., Hoang, Q.T., Boldt, H.L., Thumloup, J., Stevenson J.4, Collins, F.H. (Unpublished manuscript). Molecular

characterization reveals an unexpected diversity of Anopheles species in Eastern Zambia: implications for evaluating vector behavior and interventions.

36. Mabaso, M. L., Sharp, B., & Lengeler, C. (2004). Historical review of malarial control in southern Africa with emphasis on the use of indoor residual house-spraying. *Tropical Medicine & International Health*, 9(8), 846-856.
37. MacDonald, G. (1952). The analysis of sporozoite rate. *Trop Dis Bull.*, 49, 569-585.
38. malERA Consultative Group on Vector Control. (2011). A research agenda for malaria eradication: vector control. *PLoS medicine*, 8(1), e1000401.
39. Moody, A. (2002). Rapid diagnostic tests for malaria parasites. *Clinical microbiology reviews*, 15(1), 66-78.
40. Moreno, M., Cano, J., Nzambo, S., Bobuakasi, L., Buatiche, J. N., Ondo, M., ... & Benito, A. (2004). Malaria Panel Assay versus PCR: detection of naturally infected Anopheles melas in a coastal village of Equatorial Guinea. *Malaria journal*, 3(1), 20.
41. Nájera, J. A. (2001). Malaria control: achievements, problems and strategies. *Parassitologia*, 43(1-2), 1-89.
42. Okumu, F. O., & Moore, S. J. (2011). Combining indoor residual spraying and insecticide-treated nets for malaria control in Africa: a review of possible outcomes and an outline of suggestions for the future. *Malar J*, 10(208), 13.
43. The Pacific Malaria Initiative Survey Group (PMISG) on behalf of the Ministries of Health of Vanuatu and Solomon Islands. (2010). Malaria on isolated Melanesian islands prior to the initiation of malaria elimination activities. *Malaria journal*, 9, 218.
44. Pluess, B., Tanser, F. C., Lengeler, C., & Sharp, B. L. (2010). Indoor residual spraying for preventing malaria. *Cochrane Database Syst Rev*, 4(4).
45. Preiser, P. R., Wilson, R. J., Moore, P. W., McCready, S., Hajibagheri, M. A., Blight, K. J., ... & Williamson, D. H. (1996). Recombination associated with replication of malarial mitochondrial DNA. *The EMBO journal*, 15(3), 684.
46. Russell, T. L., Beebe, N. W., Cooper, R. D., Lobo, N. F., & Burkot, T. R. (2013). Successful malaria elimination strategies require interventions that target changing vector behaviours. *Malar J*, 12, 56.
47. Service, M. W. (1976). *Mosquito ecology: field sampling methods*. Applied science.
48. Shililu, J. I., Maier, W. A., Seitz, H. M., & Orago, A. S. (1998). Seasonal density, sporozoite rates and entomological inoculation rates of Anopheles gambiae and

- Anopheles funestus in a high altitude sugarcane growing zone in western Kenya. *Tropical Medicine and International Health*, 3(9), 706-710.
49. Sinden, R. E. (1983). Sexual development of malarial parasites. *Adv Parasitol*, 22, 153-216.
  50. Singh, B., Bobogare, A., Cox-Singh, J., Snounou, G., Abdulla, M. S., & Rahman, H. A. (1999). A genus-and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *The American journal of tropical medicine and hygiene*, 60(4), 687-692.
  51. Schousboe, M. L., Ranjitkar, S., Rajakaruna, R. S., Amerasinghe, P. H., Konradsen, F., Morales, F., ... & Roper, C. (2014). Global and local genetic diversity at two microsatellite loci in Plasmodium vivax parasites from Asia, Africa and South America. *Malaria journal*, 13(1), 392.
  52. Snounou, G., & Singh, B. (2002). Nested PCR analysis of Plasmodium parasites. In *Malaria Methods and Protocols* (pp. 189-203). Humana Press.
  53. Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., & Brown, K. N. (1993). Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molecular and biochemical parasitology*, 58(2), 283-292.
  54. Steenkeste, N., Rogers, W. O., Okell, L., Jeanne, I., Incardona, S., Duval, L., ... & Rogier, C. (2010). Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. *Malar J*, 9(1), 108.
  55. Steenkeste, N., Incardona, S., Chy, S., Duval, L., Ekala, M. T., Lim, P., ... & Ariey, F. (2009). Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers. *Malaria journal*, 8(1), 86.
  56. Taylor, B. (1975). Changes in the feeding behaviour of a malaria vector, Anopheles farauti Lav., following use of DDT as a residual spray in houses in the British Solomon Islands Protectorate. *Transactions of the Royal Entomological Society of London*, 127(3), 277-292.
  57. Trigg, P. I., & Kondrachine, A. V. (1998). Commentary: malaria control in the 1990s. *Bulletin of the World Health Organization*, 76(1), 11.
  58. United Nations, (2014). The Millennium Development Goals Report 2014.
  59. White, N. J. (2011). Determinants of relapse periodicity in Plasmodium vivax malaria. *Malaria journal*, 10(1), 297.

60. Wongsrichanalai, C., Barcus, M. J., Muth, S., Sutamihardja, A., & Wernsdorfer, W. H. (2007). A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *The American journal of tropical medicine and hygiene*, 77(6 Suppl), 119-127.
61. World Health Organization. (1995). Manual on practical entomology in malaria. Part II. Methods and techniques. WHO Offset Publication No. 13. Geneva: WHO Division of Malaria and Other Parasitic Diseases. 195, 195.
62. World Health Organization. (2007). Insecticide-treated mosquito nets: a WHO position statement. *Geneva: WHO*.
63. World Health Organization. (2012). World malaria report 2012.