Study Title: Characterizing the humoral immune response against salivary antigens of Southeast Asian mosquito vectors of malaria and dengue with a human challenge model

Short title: Characterizing humoral immune response to mosquito bites

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The investigators declare no conflict of interest.

Confidentiality Statement

This document contains confidential information that must not be disclosed to anyone other than the authorised individuals from the University of Oxford, the Investigator Team and members of the Oxford Tropical Research Ethics Committee (OxTREC), Ethics committee at the Faculty of Tropical medicine, Mahidol University, Alfred Hospital Ethics Committee and Tak Province Community Ethics Advisory Board (T-CAB), unless authorised to do so.

TABLE OF CONTENTS

1. SYNOPSIS

Study Title	**Characterizing the humoral immune response against salivary antigens of Southeast Asian mosquito vectors of malaria and dengue with a human challenge model**	
Internal ref. no.	MAL19009	
Study Design	This study is a human challenge model with five arms corresponding to controlled exposure to bites of uninfected laboratory-adapted colonies of *Anopheles minimus*, *An. maculatus*, *An. dirus, Aedes. aegypti* or *Ae. albopictus*.	
Study Participants	Healthy volunteers ≥ 18 and ≤60 years old	
Planned Sample Size	150 participants followed-up for 16 weeks (total of 1 venous blood sample of 2 mL, 14 venous blood samples of 8.6 mL each, 3 venous blood samples of 18.6 mL each, and 17 capillary blood samples of 300 uL each, yielding 178.2 mL of venous blood and 5.1 mL of capillary blood per study participant)	
Planned Study Period	February 2020 to December 2023 (Study duration is approximately 3 years after ethical approval to December 2023)	
	Objectives	**Outcome measures**
Primary	**To identify biomarkers of exposure to bites of Southeast Asian mosquito vectors of malaria and dengue.**	1) Levels and kinetics of specific antibody titers against candidate peptides before and during repeated exposure to bites of laboratory-reared *Anopheles minimus, An. maculatus, An. dirus*, *Aedes aegypti* and *Ae. albopictus* determined by ELISA and mesoscale screening. 2) Sequence of saliva antigens determined with an immuno-proteomic method (2D gel electrophoresis of salivary gland protein extracts, Western blot and mass spectrometry).
Secondary	**1) To characterize the relationship between levels of mosquito exposure and humoral response.to bites of mosquito vectors of malaria and dengue.**	Comparison of the antibody titers determined in subgroups corresponding to different level of exposure.
	2) To assess the feasibility of using dry blood spots made from capillary blood for the monitoring of exposure	Comparison of the antibody titers determined in DBS and venous blood samples.

EC: ethic committee; **DBS**: dry blood spots; **ELISA**: enzyme-linked immunosorbent assay.

2. ABBREVIATIONS

AE	Adverse event
CRF	case report form
CTCAE	Common Terminology Criteria for Adverse Events
DBS	dry blood spots
EC	Ethics Committee
ELISA	enzyme-linked immunosorbent assay
MORU	Mahidol-Oxford Tropical Medicine Research Unit
OD	Optical density
OxTREC	Oxford Tropical Research Ethics Committee
PBS	Phosphate buffered saline
SAE	serious adverse event
SMRU	Shoklo Malaria Research Unit
T-CAB	Tak Province Community Ethics Advisory Board
TMB	Tetramethylbenzidine

3. BACKGROUND AND RATIONALE

According to World Health Organization, vector-borne diseases account for 17% of all infectious diseases, causing 700,000 deaths annually. Nearly half of the world population is at risk of contracting dengue or malaria. Dengue is responsible for 96 million estimated cases and 10,000 deaths every years, and the corresponding figure for malaria is 218 million cases and 400,000 deaths (1). The Thailand-Myanmar border stretches almost 2000 km from Laos to the North down to Malaysia in the South. An estimated 5 million people live in this area, including Thai and Burmese citizens, ethnic minorities, refugees and migrant workers.

3.1. Epidemiology of malaria and dengue on the Thailand-Myanmar border

Vector-borne diseases are a major cause of morbidity and mortality for people living on the Thailand-Myanmar border. In 2010, it was estimated that febrile episodes occurred in 5% of the pregnant women and that arthropod-borne (malaria, *Rickettsia* infections, and dengue) and zoonotic disease (leptospirosis) accounted for nearly half of all febrile illnesses in this population group. Malaria and dengue were identified as the major causes of morbidity and mortality (2).

3.1.1. Malaria

In this region, malaria transmission is low, seasonal and unstable (3). *Plasmodium falciparum* was eliminated from most endemic villages with widespread deployment of community-wide access to early diagnosis and treatment with artemisinin-based combination therapies, and mass-drug administration campaigns in places where submicroscopic malaria prevalence was high (4). Although the endemicity of *vivax* malaria has also declined in recent years in this region (5), it has remained much more difficult to tackle than *falciparum* malaria because of some features in the biology of *Plasmodium vivax* (6-8). In this area, the primary vectors are *Anopheles minimus* (Minimus Complex, Funestus Group), *An. maculatus*, *An. sawadwongporni* (Maculatus Group), *An. dirus* and *An. baimaii* (Dirus Complex, Leucosphyrus Group). *Anopheles pseudowillmori* (Maculatus Group), *An. aconitus* (Aconitus Subgroup, Funestus Group) and some members in the Annularis and Barbirostris Groups also play a secondary role in the transmission (9, 10). Biting rate can be very high, thereby playing a disproportionate role in driving transmission intensity in this setting where *Plasmodium*-infection rates in mosquito populations are low (11, 12). Bed-nets and indoor residual spraying fail to prevent most of malaria infections (13-15) because of the ecology and biology of relevant anopheline species, including exophily and exophagy, zoophagy and opportunistic blood type selection, and activity peaks at dusk and dawn (11, 16, 17). Larval source management is difficult to implement because of the diverse and fragmented nature of larval habitats (18), and because incredibly high densities of vector larvae can be found over large areas covered with paddy fields (19). Several vector species pullulate in a variety of biotopes and at different time in the year, adding another layer of complexity to the dynamics of entomological indices (9).

3.1.2. Dengue and other arboviruses transmitted by Aedes mosquitoes

Dengue is a viral infection caused by four types of viruses (DENV-1, DENV-2, DENV-3, DENV-4) belonging to the *Flaviviridae* family. The spectrum of diseases severity is broad, the infection usually manifest as a mild fever but severe and deadly cases can occur (20, 21). The viruses are transmitted through the bite of infected *Aedes aegypti* and *Ae. albopictus* female mosquitoes that feed both indoors and outdoors during the daytime (22, 23), but the diversity and competence of other aedine mosquito species on the Thailand-Myanmar border is not known precisely. These mosquitoes thrive in areas with standing water, including

puddles, water tanks, containers and old tires. Lack of reliable sanitation and regular garbage collection also contribute to the spread of the mosquitoes. Cases of Dengue have been increasingly reported from Thailand over the past two decades (2, 24-26). The most affected provinces include Chiang Rai, Chiang Mai, Mae Hong Son, Nakhon Pathom, Phra Nakhon Si Ayutthaya, Phetchabun, Lamphun and Phatthalung. Dengue is present in both urban and rural areas, with elevated risk in northeastern parts of the country. Peak transmission typically occurs during the rainy season, from April to December. Other arboviruses transmitted by *Aedes* mosquitoes such as Zika and Chikungunya are emerging issues in this area (22, 27-30). Although case are rarely fatal, morbidity is high and an important proportion of the population can be affected at the same time during outbreaks.

3.2. The natural course of reactions to mosquito bites

The saliva of blood feeding arthropods is a complex mixture of biomolecules, including dozens of proteins, many of which have unknown function. Some of these proteins have been shown to facilitate ingestion of blood and pathogen transmission though tight interaction with the immune system of the host being bitten. The clinical and biological features of the immune reaction to mosquito saliva has been studied in animal and humans, both in controlled laboratory challenges and natural settings.

3.2.1. Typical features

Allergic reactions to mosquito bites are due to specific sensitization to the mosquito salivary proteins. Mosquito saliva-specific IgE and IgG antibodies as well as lymphocyte proliferation appear to be involved in the pathogenesis of allergic reactions to mosquito bites (31). Typical reactions to mosquito bites include immediate wheals 2 to 10 mm in diameter, with surrounding erythema peaking in 20 to 30 minutes (immediate reaction) and similarly sized pruritic papules peaking at 24 to 36 hours and diminishing in several days (delayed reaction). Based on clinical observations, the natural history of sensitization and subsequent desensitization to the saliva injected by mosquito bites has been classified into five stages (32, 33). Individuals who have never been exposed to a specific species of mosquito, do not get a reaction from the initial bites (stage 1), but following subsequent bites, delayed cutaneous lesions appear (stage 2). After repeated bites, immediate wheals develop (stage 3). With further exposure, delayed reactions are no longer observed, and only immediate wheals are noted (stage 4). Individuals repeatedly exposed to thousands of bites from the same species of mosquito eventually lose the immediate reactions (stage 5). Most of the population at any given time will have some reactivity to mosquito bites: immediate reactions occur in 70% to 90% and delayed reactions in 55% to 65% of patients subjected to bites of common *Ae sp.* and *Cx sp.* mosquito species (34-36). Peng *et al.* further documented the correlation of skin reactions with specific IgE and IgG antibodies and lymphocyte proliferation response to mosquito antigens and reported that it took 21 weeks of repeated exposure with 100 *Ae. aegypti* bites per week for desensitization to happen (34, 37). In natural setting, desensitization occurs during infancy (38) and individuals remain at an intermediary stage (most often stage 3 and 4) for years before eventually progressing to the next stage (39).

3.2.2. Severe reactions

In atopic individuals, large local reactions can develop including erythematous pruritic swellings, often more than 3 cm, occurring in minutes to hours at the site of a bite. Delayed large local reactions may be papular, ecchymotic, vesicular, blistering, or bullous and persist for days or weeks (36, 40). The incidence of self-reported large local reactions in one study was 2.5% (8/12 cases were children less than 10 years old) (40). Skeeter syndrome describes patients with mosquito bite-induced large local reactions

accompanied by fever. This syndrome typically occurs in otherwise healthy children, and the large local reactions may mimic cellulitis (hot, swollen, red, and painful) but may be differentiated by their occurrence within hours of mosquito bites. These symptoms resolve in 3 to 10 days (41). Anaphylactic reactions to mosquito bites are extremely rare but have occurred in patients with underlying indolent systemic mastocytose (42, 43). Persons at increased risk for severe reactions include those with high exposure (outdoor workers) and those lacking acquired immunity (young children and immigrants) (31). In addition, patients with primary or acquired immunodeficiencies and those with Epstein-Barr virus (EBV) associated lymphoproliferative diseases are also at higher risk for severe reactions (44-46).

3.3. Biomarkers of human exposure to mosquito bites

Measuring exposure to mosquito bites is informative to assess the risk of disease transmission and the efficacy of vector-control interventions. However, direct measurement of exposure is difficult because diverse mosquito species pullulate in different environments at different times in the year (9). In order to be meaningful, estimates must also take into account the behavioural interactions between mosquito and human populations (47). Finally, it is difficult to scale-up entomological investigations because of the logistic, financial and technical constrains it implies. There is an urgent need for more efficient and sensitive complementary tools to improve the impact of vector control strategies and predict the risk of transmission of mosquito-borne diseases. The use of immunological markers of human exposure to bites of mosquito vectors has been proposed in this context (48, 49).

3.3.1. Characteristics of the biomarkers

Individuals repeatedly bitten have detectable levels of circulating antibodies directed against salivary antigens (50, 51). Those antibodies have been proposed as biomarkers of human exposure to mosquito bites (48, 52). The antibody response measured in naturally exposed individuals is highly heterogeneous (49, 53). This can come from varying level of exposure as some antibody titers have been shown to correlate with the entomological indices of exposure in specific settings (54-57), and other factors might also have an effect (e.g. genetic background, concomitant infection, nutritional status, history of previous exposure). Another important aspect of biomarker validation is the assessment of the cross-reactivity with the saliva proteins of other blood feeding arthropods. The immune responses elicited by mosquito bites have a spectrum of specificity, with some antibodies sharing reactivity with saliva from other mosquito species (or even phylogenetically more distant blood feeding arthropods) while some others give a species-specific signal (53). Interestingly, transcriptomic and proteomic studies have identified groups of genus specific salivary proteins that are ideal candidate markers of host exposure to mosquito vectors (58-61). Some of those may be conserved enough to elicit a specific response specific to the mosquito genus, and others may exhibit enough variations in the amino-acid sequence to identify highly specific markers of a single mosquito species (58). Noteworthy, both genus-specific and species-specific biomarkers would be valuable in the context of entomological surveillance and assessment of the efficacy of vector control interventions.

In order to be valid, the biomarker should fulfil four fundamental criteria: (i) to discriminate individuals exposed to mosquito bites from those who are not exposed, (ii) to evaluate the density and fluctuations of vector populations that can bite human populations, (iii) to classify individuals according to their level of exposure, and (iv) to be usable at a population level (48). Studies that aim at discovering biomarkers of exposure must be design carefully in order not to be confounded by uncontrolled factors. In this regard, human challenge studies with laboratory mosquitoes can achieve a very fine level of control in the

exposure (species, dose and duration) and therefore are expected to yield invaluable information for biomarker validation. In order to discriminate individuals exposed to mosquito bites from those who are not exposed, appropriate negative controls must be used to define the positivity threshold of the assay. Typically, the positivity threshold of a given assay is defined as three times the standard deviation of the signal measured in a population of individuals who have not been exposed to the mosquito species of interest (49, 62, 63). Moreover, it is possible to ensure the specificity of the signal during prospective laboratory challenge studies by performing multiple baseline assessment of serum reactivity, by including a follow-up after the exposure to observe the decay of specific antibodies after the exposure ceases, and avoid or limit the exposure that may happen independently of the study (64). Competition ELISA can also be informative to assess the cross reactivity of the antibodies between several antigens (65). In order to evaluate the density and fluctuations of vector populations, and to classify individuals according to their level of exposure, validation of the biomarker must assess the kinetic and the dose-response relationship between the number of bites received by a given individual and the titer of specific antibodies produced in response to exposure (49). Field studies are usually limited to answer these questions because the exposure cannot be well controlled. The kinetic and dose response relationship can be better assessed by conducting laboratory studies with controlled exposure to insectary mosquitoes, including several doses and a post-exposure follow-up in the study design. Finally, in order to support the use of a given biomarker at the population level, assessment must be performed on a sufficient number of individual in order to assess inter-individual variations in the development of the immune responses against saliva antigens.

3.3.2. Malaria vectors (Anopheles mosquitoes)

Regarding exposure to *Anopheles* mosquito bites, two *An. gambiae* salivary proteins have been tested so far, namely the gSG6 protein and the antithrombin cE5. The gSG6 was the first immunogenic *Anopheles* saliva protein identified and the gSG6-P1 appeared as the best candidate peptide to assess anti-SG6 humoral response in Africa (55). Anti-gSG6-P1 antibodies were shown to be labile (66), sensitive enough to detect low-level exposure (63), and to cross-react with *An. gambiae*, *An. arabiensis* and *An. funestus* (54, 67). Since, gSG6-P1 has been used in a wide variety of African transmission settings to assess malaria transmission dynamics (68-72) and to monitor the efficacy of vector-control interventions (66, 73-75). The studies conducted with gSG6-P1 outside Africa (76-79) must be interpreted cautiously because the SG6 protein is absent from the genome of some dominant vector species or shares only a moderate identity with its orthologues in phylogenetically diverse *Anopheles* species (58). Antithrombin cE5 was reported to be more immunogenic, to induce long lasting antibodies and less immune tolerance than the SG6 protein (80).

3.3.3. Dengue vectors (Aedes mosquitoes)

The development of biomarker of exposure to *Aedes* mosquito is more recent (48). Antigenic epitopes were identified using sera from naturally exposed individuals and 2D gel electrophoresis of salivary gland extracts (81, 82). The Nterm-34kDa salivary peptide was validated as a species-specific biomarker of human exposure to bites of several *Aedes* species usable for monitoring transmission risk and vector-control intervention in a wide variety of settings and geographic area (62, 83-89). More recently, the use of protein D7 was also investigated as a marker of risk of dengue infection and as a vaccine to block the transmission of dengue virus (90-92).

3.4. Study objectives

The characteristics of the humoral response directed against mosquito saliva antigens are not known precisely (31). This is a major limitation for using immunological markers as an outcome in epidemiological trials and as an indicator for operational deployment of interventions. Recent advances in the assembly of the genome of some *Anopheles* and *Aedes* mosquito vector species (93-95) has facilitated the identification of new candidate peptides *in silico*, using the sequences of orthologous salivary gland proteins and B-cell prediction algorithms. The objective of this study is to assess the humoral immune response directed against candidate peptides following controlled exposure to laboratory-adapted colonies of *An. minimus, An. maculatus* and *An. dirus*, *Ae. aegypti* and *Ae. albopictus*.

3.5. Interest of the research

This research will provide essential information to identify and validate immunological markers of human exposure to malaria and dengue mosquito vectors in Southeast Asia. Immunological markers would be useful to understand transmission dynamics and predict the risk of transmission as part of a surveillance system, and to assess the efficacy of vector-control interventions in entomological trials or during operational deployment of interventions in the region.

4. OBJECTIVES AND OUTCOME MEASURES

Objectives	Outcome Measures	Timepoint(s) of evaluation of this outcome measure [a]
Primary Objective **To identify biomarkers of exposure to bites of Southeast Asian mosquito vectors of malaria and dengue.**	1) Levels and kinetics of specific antibody titers against candidate peptides before and during repeated exposure to bites of laboratory-reared *Anopheles minimus, An. maculatus, An. dirus*, *Aedes aegypti* and *Ae. albopictus* determined by ELISA and mesoscale screening. 2) Sequence of saliva antigens determined with an immuno-proteomic method (2D gel electrophoresis of salivary gland protein extracts, Western blot and mass spectrometry).	1) Day 0, 7 (baseline), 14, 21, 28, 35, 42, 49, 56 (exposure period), 63, 70, 77, 84, 91, 98, 105 and 112 (post-exposure period). 2) Day 14 (baseline), 63 and 112 (post-exposure period).
Secondary Objectives **1) To characterize the relationship between levels of mosquito exposure and humoral response.**	Comparison of the antibody titers determined in subgroups corresponding to different level of exposure.	Day 0, 7 (baseline), 14, 21, 28, 35, 42, 49, 56 (exposure period), 63, 70, 77, 84, 91, 98, 105 and 112 (post-exposure period).
2) To assess the feasibility of using dry blood spots made from capillary blood for the monitoring of exposure to bites of mosquito vectors of malaria and dengue.	Comparison of the antibody titers determined in DBS and venous blood samples.	Day 0, 7 (baseline), 14, 21, 28, 35, 42, 49, 56 (exposure period), 63, 70, 77, 84, 91, 98, 105 and 112 (post-exposure period).

[a] See Appendix C.

DBS: dry blood spots; **ELISA**: enzyme-linked immunosorbent assay.

5. STUDY DESIGN

A schematic diagram detailing the study design, procedures and stages step-by-step is presented in the Appendix A. This study is a human challenge model with five arms corresponding to controlled exposure to bites of uninfected laboratory-adapted colonies of *Anopheles minimus*, *An. maculatus*, *An. dirus, Aedes. aegypti* or *Ae. albopictus* (see sections 8 and 13.5.1). Following screening and eligibility assessment, 150 participants will be enrolled in the study using a block-randomized design stratified on the mosquito species and number of bites (35 or 305 bites in total) such as to constitute 10 groups of 15 individuals for

each of the study condition (Appendix B). Participants will be in the study for 112 days (Appendix C). The baseline will consist of 2 visits over 2 weeks (day 0 and day 7). In the low-exposure groups, participants will be exposed to 5 mosquito bites at weekly intervals from day 14 to day 56 (seven challenges with 5 mosquito bites/challenge over six weeks, yielding a total of 35 mosquito bites). In the high-exposure group, participants will be exposed to 5 mosquito bites on day 14 and then to 50 mosquito bites at weekly intervals from day 21 to day 56 (one challenge with 5 mosquito bites and six challenges with 50 mosquito bites/challenge over 6 weeks, yielding a total of 305 mosquito bites). The number of bites and modalities of the follow up used in this study were chosen based on previous entomological investigation conducted in this area (10, 11, 96), published reports of human challenge with mosquito bites (37, 97-99) and current knowledge on the characteristic of the humoral response against saliva antigens (31, 37, 52). Both immediate and delayed skin reactions will be recorded after day 14 and day 21 challenges, requiring additional visits at day 15 and day 22. Participants with hypersensitivity to mosquito bites will be withdrawn from the study. Both capillary and venous blood samples will be collected weekly from each study participant (Appendix D). Eighteen venous blood samples will be collected including one sample of 2 mL drawn during screening visit, 14 samples of 8.6 mL during visits 2, 3, 6, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18 and 19, and 3 samples of 18.6 mL during visits 4, 13 and 20 (178.2 mL of venous blood per participant in total). Seventeen dry blood spot (DBS) samples will be made from of 300 uL of capillary blood collected during visits 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 (5.1 mL of capillary blood per participant in total). Candidate peptides will be identified *in silico* using publicly available genomes sequences and B-cell epitope prediction algorithms. The kinetic of antibody titers against candidate biomarkers will be assessed by enzyme-linked immunosorbent assay (ELISA) and mesoscale screening performed with serum and DBS specimens (100). Briefly, the positivity threshold of the assay will be determined using reference sera specimens from individuals not exposed to mosquito bites, as described previously (62). The ELISA signal measured in 60 reference sera obtained from unexposed individuals (30 Thai sera obtained from the Thai Red Cross and 30 Australian sera obtained from the Burnett Intstitute) will be used to define the positivity threshold of the assay. The positivity threshold of the assay will be defined as three standard deviation (SD) above the mean optic density (ΔOD) measured for the unexposed control population. A test in the study population with a ΔOD above this cut-off will be considered positive (i.e. an immune response). The specific antibody titer of study samples will be determined by performing serial dilution experiments (101). Serial dilution will be made from study samples and the ΔOD will be determined for each dilution. The antibody titer will be defined as the highest dilution giving a positive signal. Results obtained with DBS and serum specimens will be compared in order to assess the feasibility of using DBS samples to monitor exposure to bites of mosquitoes during epidemiological studies. Serum specimens collected during visits 4 (baseline), 13 (early after the end of exposure) and 20 (post-exposure) will also be analysed with immuno-proteomic approach (2D gel electrophoresis of salivary gland protein extracts, Western blot and mass spectrometry) yielding additional information on the antigenic properties of mosquito salivary gland proteome (102).

6. PARTICIPANT IDENTIFICATION

Study participants are defined as healthy participants aged between 18 and 60 years old and willing to participate in the study. This study will take place at the Shoklo Malaria Research Unit (SMRU) research centre in Mae Sot.

6.1. Inclusion criteria

- Healthy male or female participant judged by a responsible physician with no abnormality identified on a medical evaluation;
- Thai, Burmese or Karen ethnicity;
- Aged ≥ 18 to <60 years old;
- Living in Mae Sot city for the last 12 months;
- Willing to participate in the activity and able to give informed consent for participating in the study;
- Able to tolerate direct mosquito exposure.

6.2. Exclusion criteria

- History of travel in a malaria endemic are (rural village) in the last 12 months, or plan to do so during the study;
- Medication or condition deemed to interfere with the outcome measure by a responsible physician;
- Moderate and severe anemia (haemoglobin concentration less than 110 g/L of blood);
- Hypersensitivity or anaphylaxis to mosquito bites;
- Pregnant women;
- Breastfeeding women.

7. STUDY PROCEDURES

7.1. Recruitment and informed consent of study participants

The study will be spread through word of mouth in Mae Sot by the study team and then interested participants will contact SMRU. The persons interested in participating in the study will be invited to individual and group discussions with the study team during which more details of the study will be presented and potential participants will be given the opportunity to ask questions. The persons willing to take part in the study will be appointed for the screening visit. Participants' presenting to the screening visit must personally and voluntarily sign and date the latest approved version of the informed consent form before any study specific procedures are performed. The Participant Information and Informed Consent Form will be presented to the participants detailing no less than: the exact nature of the study; what it will involve for the participant; the implications and constraints of the protocol and any risks and benefits involved in taking part. It will be clearly stated that the participant is free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal. The participant will be allowed as much time as they wish to consider the information, and the opportunity to question the Investigator or other independent parties to decide whether they will participate in the study. The informed consent and procedures visit will take approximatively one hour.

Written Informed Consent will be obtained by asking the participant to sign and date the informed consent form. If the participant is illiterate, they will be asked to give a thumb print, leaving the date field blank and a literate impartial witness will be asked to sign and date the consent form, confirming that the participant understands what they are being asked to do and have given consent. The form will also be signed and dated by the person who presented and obtained the Informed Consent. The person who obtained the consent must be suitably qualified and experienced, and authorised to do so by the Principal

Investigator. A copy of the signed Informed Consent will be given to the participant. The original signed form will be retained at the study site.

7.2. Screening visit

Subjects will be screened to assess eligibility (visit 1). Informed consent will be obtained before any screening procedures are conducted. The following screening procedures will be carried out no longer than 30 days before the first baseline visit:

- Personal data collection including name, age, date of birth, sex, ethnicity, address and telephone number;
- A comprehensive physical examination including measurement of height, body weight and vital signs (blood pressure, respiratory rate, body temperature and heart rate) and medical history assessment including travel history outside Maesot town during the past 3 months;
- A pregnancy test if indicated (cost of the test will be covered by the study);
- Venous blood collection (2 mL) in order to perform complete blood count and measure haemoglobin concentration.

Participants will also be informed about the importance of avoiding being bitten by mosquitoes independently of the study challenges during the follow-up. They will be provided with insecticide-impregnated mosquito bed-nets and skin repellent (N,N-diethyl-meta-toluamide 20%). Participants who will be allocated to the *Aedes* groups will receive counselling about larval source management in order to limit exposure to mosquito bites that may happen independently of the study challenges. Sand Abate granules (temephos 1%) will be provided free of charge to participants who need it.

Data collected during the screening and subsequent visits will be recorded in the Case Report Form (CRF) and stored in the study database. The CRF will be pseudonymized using the participant identification code, date of enrolment, initials, sex and date of birth. Personal data (name, telephone number and address) will be recorded and stored separately, allowing linkage of study data with participant's details by the study team.

7.3. Baseline visits

One hundred and fifty healthy subjects who fulfil the eligibility criteria will be recruited to the study. Baseline visits will be scheduled at day 0 and day 7 (visit 2 and 3). Baseline visits will include capillary blood and venous blood collection (Appendix C). Randomization will be performed during visit 2.

For baseline and subsequent visits, in case participant fails to come on the exact day a visit is scheduled, he/she will be allowed to come for a retake any other working day of the study team until the next scheduled visit.

7.4. Randomization

A block randomization schedule will be generated using the block.random() function of the R package *psych* version 1.8.12 using variables species (*An. minimus*, *An. maculatus*, *An. dirus, Ae. aegypti* and *Ae. albopictus*) and dose (35 or 305 bites in total) yielding a list of 15 blocks with 10 participants each (total of 150 participants). Following screening and eligibility assessment, participant will be assigned a study arm during visit 2 through the randomization schedule. Individual, sealed and sequentially numbered envelopes will be provided for the study site with one envelope per participant, indicating the allocation.

7.5. Subsequent visits

From day 14 to day 56, participant will be exposed to bites of laboratory-adapted colonies of *Anopheles minimus*, *An. maculatus* and *An. dirus*, *Aedes aegypti* or *Ae. albopictus* reared at the SMRU. Appropriate quality control measures will be implemented such as to guarantee the absence of human pathogen in the mosquito batches used in this study and withdraw any participant with hypersensitivity to mosquito bites (see section 13.5.1. Risk of mosquito exposure). Participants will receive a total of either 35 or 305 mosquito bites per participant in 7 challenges split over 6 weeks (see mosquito intervention section). Each visit will include physical examination, vital signs assessment, mosquito exposure, capillary blood and venous blood collection according to Appendix C. Immediate skin reactions (20-30 min after mosquito bites) will be recorded after each mosquito feeding assay. Delayed skin reactions (24-36 hours after mosquito bites) will be recorded after the day 14, 21 challenges, requiring additional visits at day 15, 22. After day 56, each visit will include capillary blood and venous blood collection according to Appendix C. The study procedure will take approximatively 1 hours for medical history assessment, physical examination and vital sign assessment, 15 minutes for blood sampling and 30-90 minutes for the challenge with mosquito bites (depending on the number of repeats needed to reach the target number of mosquito bites). During the exposure visits, the participants will be kept in observation for an additional hour after the challenge before being allowed to leave the study site. Participants will be requested not to apply any lotions, ointments, creams, powders, perfumes, antiperspirants, repellents, or attractants prior to each exposure visit. Participants will be provided palliative antipruritic medication consisting in 1% diphenhydramine hydrochloride cream and oral cetirizine at the dose of 10 mg per day in order to relieve itching from mosquito bites (103, 104). In addition, participants will be provided with a mosquito bed net and skin repellent in order to minimize uncontrolled natural exposure to mosquito bites that can happen independently of the study challenges.

7.6. Blood collection

Both capillary blood and venous blood will be collected weekly from each study participant over 112 days with an additional venous sample collected during the screening visit, yielding 17 capillary blood samples (5.1 mL of capillary blood in total) and 18 venous blood samples (178.2 mL of venous blood in total) per study participant (Appendix C). Venous blood samples will be collected by venepuncture. In addition, participants will undergo a finger-prick using a single usage sterile lancet, from which capillary blood will be collected on filter paper in order to constitute three DBS. All blood samples will be collected on site by SMRU study teams (trained and qualifies medics, nurses and medical doctors). The volume of blood collected in this study is justified based on the technical requirement to perform the assays in the different collaborative centres (see details below) (53, 101).

7.7. Specimen/sample handling

Sample pre-treatment, storage and shipment:

Venous blood samples will be spun down to separate the serum. Three serum aliquots of 2 mL, 2 mL and 0.1 mL each will be made during visits 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20. Moreover, an additional 5 mL serum aliquot be made during visits 4, 13 and 20. Hence, 8.6 mL of venous blood will be collected from participants during visits 2, 3, 6, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18 and 19 (4.1 mL of serum in total), and 18.6 mL of venous blood will be collected during visits 4, 13, 20 (9.1 mL of serum in total). The volume of blood was calculated considering a 50% hematocrit (upper limit of the reference range) and an additional 0.2 mL of buffer volume of serum (0.4 mL of blood) to avoid contamination with

the buffy coat when preparing the serum aliquots. Spotted capillary blood will be dried at room temperature. Dry blood spot specimens will be put into a zip lock bag containing silica gel. DBS and serum aliquots will be stored at -20°C until shipment to the collaborative centres. Coded specimen will be shipped to the Burnet Institute (Melbourne, Australia) for ELISA and immuno-proteomic analysis, the Singapore Immunology Network (Agency for Science, Technology, and Research, Singapore) for ELISA and to the Walter Reed Army Institute of Research (Silver Spring, United States of America) for mesoscale screening. No personally identifiable information will be transferred. Samples will be kept accessible only by authorised researchers.

A 2-mL serum aliquot and the DBS samples collected during visits 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 will be shipped to the Burnet Institute (Melbourne, Australia) for doing ELISA screening. A 2-mL serum aliquot collected during visits 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 will be shipped to the Singapore Immunology Network (Agency for Science, Technology, and Research, Singapore) for doing ELISA screening. A 0.1-mL serum aliquot collected during visits 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 will be shipped to the Walter Reed Army Institute of Research (Silver Spring, United States of America) for doing mesoscale screening. The 5-mL serum aliquots collected during visits 4, 13 and 20 will be shipped to the Burnet Institute (Melbourne, Australia) for being analysed with immuno-proteomic methods.

Enzyme-linked immunosorbent assay procedures:

Antibody titer against a broad panel of candidate peptides will be determined on the serum specimens. This panel will include, but will not be limited to, five peptides specific to each mosquito species, in addition to the *Anopheles gambiae salivary* peptide SG6-P1 (target of 10 peptides per panel). Serial dilution of the serum specimens will be carried out in order to determine antibody titer (the titer is defined as the last dilution giving a positive response; a positive response is defined as the mean + 3 standard deviation of naive serum used as negative control) (101). Each sample will be tested in duplicate with a reaction volume of 100 uL. Therefore, the volume of serum needed for each peptide is 200 uL of serum /peptide (2 mL of serum for 10 peptides). Dry blood spots will be eluted separately in 200 uL of PBS such as to have 3 technical replicates and analysed using the same ELISA protocol. Samples will also undergo further serological analyses for antigen and antibody characterisation, including but not limited to competition ELISAs (to quantify the antibody/antigen interaction) (100), functional antibody assays (*e.g.* complement fixation assays) (101).

Mesoscale assay procedures:

The serum specimens collected will be diluted 250 folds and applied to test plates. The testing will require 750 uL diluted sample (3 uL of neat serum) per test panel. A minimum volume of 6 uL of neat serum is needed in case technical issue happens and rerun is needed. It is impossible to ship such small volumes (evaporation/drying up) and therefore a minimum of 100 uL will be sent.

Immuno-proteomic procedure:

Immuno-proteomic analysis will be carried out using a procedure adapted from previously published studies (53, 102, 107). This analysis will be performed with the 5 mL serum aliquots collected from study participants during visit 4 (baseline), visit 13 (early after exposure period) and visit 20 (end of the post-exposure period). Single serum specimens will be used in order to assess inter-individual variation of the antibody response against salivary gland proteome.

Two-dimensional gel electrophoresis will be performed with protein extracts made from the dissected salivary glands of 200 female adult mosquito specimens. In order to obtain a sufficient resolution, protein separation will be done on an 11 cm × 20 cm gel format. The gel proteins will be then transferred to a membrane. The membrane will be incubated with 50 mL of serum diluted 10 folds (5mL of neat serum) in order to assess the reactivity of serum IgG by Western blot (53). Reactive spots of interest will be detected by comparing the staining patterns from baseline, end of exposure and post-exposure samples. Spots of interest will be excised from the 2D gel and used to perform in gel digestion with trypsin. The protein digest will be analysed with liquid chromatography-mass spectrometry. Immunogenic proteins will be identified by searching the mass spectra with Mascot (Matrix Science Ltd.).

7.8. Storage of Specimens/samples

Left over specimens will be stored no longer than 10 years using codes assigned by the investigators or their designee(s). Access to research samples will be limited using either a locked room or a locked freezer. Only investigators or their designee(s) will have access to the samples.

In the future, investigators from this protocol and other investigators besides those listed in this protocol may wish to study these samples. A proposal of the planned research will be submitted to the IRB for their consideration.

7.9. Discontinuation/Withdrawal of Participants from Study

Each participant has the right to withdraw from the study at any time.

In addition, the Investigator may discontinue a participant from the study at any time if the Investigator considers it necessary for any reason including:

- Ineligibility (either arising during the study or retrospectively having been overlooked at screening)
- Significant protocol deviation
- Significant non-compliance with study requirements
- Loss to follow up
- Hypersensitivity to mosquito bites or anaphylaxis

The data collected until the point of withdrawal will be included in the study and this is stated in the participant information sheet unless participant refuse to do so. The participant will be replaced with another person and the replacement will be assigned to the same study arm.

7.9. Definition of End of Study

The end of the study is defined as the last day of sample collection from the last participant.

8. MOSQUITO INTERVENTION

From day 14 to day 56, participant will be exposed to bites of laboratory-adapted colonies of *An. minimus, An. maculatus* and *An. dirus*, *Ae. aegypti* or *Ae. albopictus* reared at the SMRU. Appropriate quality control measures will be implemented such as to guarantee the absence of human pathogen in the mosquito batches used in this study and withdraw any participant with hypersensitivity to mosquito bites (see section 13.5.1. Risk of mosquito exposure). In the low-exposure groups, participants will be exposed to 5 mosquito bites at weekly intervals from day 14 to day 56 (seven challenges with 5 mosquito bites/challenge over six weeks, yielding a total of 35 mosquito bites). In the high-exposure group,

participants will be exposed to 5 mosquito bites on day 14 and then to 50 mosquito bites at weekly intervals from day 21 to day 56 (one challenge with 5 mosquito bites and six challenges with 50 mosquito bites/challenge over 6 weeks, yielding a total of 305 mosquito bites).

For the low-dose challenge, five 5 to 7-day-old nulliparous female imagoes (i.e. that have never blood fed before) will be introduced individual into 50 mL plastic tube covered with netting material. For high-dose challenge, 47 mosquitoes will be split into four plastic cups of 10 cm in diameter covered with netting material (three cups with 12 mosquitoes and one cup with 11 mosquitoes), and 3 mosquitoes will be introduced individually into 50 mL plastic tubes. At first, three mosquito bites from individual 50 mL tubes will be administered on participant's arm. Then, the remaining mosquito containers will be placed on participant's left arm, calf, thigh or back skin, and the lights will be dimmed to encourage mosquito blood feeding. Bite site will be chosen such as potential skin reactions do not overlap. Mosquito bites administered on participant's arm will be used to assess immediate and delayed skin reactions as described previously (37). Immediate and delayed skin reactions will be recorded respectively 20-30 min and 24-36 hours after the day 14 and day 21 challenges, requiring additional visits at day 15 and 22.

Mosquitoes will be given the opportunity to feed on participant's skin for 30 min. The number of bites actually received by participants will be assessed by counting the number of engorged mosquitoes at the end of the 30-minute exposure time. If not all mosquitoes would have successfully engorged, participant will be exposed to additional mosquitoes using the same procedure in order to reach the target number of bites (participant may be exposed to mosquito bites up to three times at each visit). Partially engorged mosquitoes will not be discarded and we be counted as fully engorged specimens. In case the challenge will be carried out in the morning, mosquitoes will be starved overnight. In case the challenge will be carried out in the afternoon, mosquitoes will be staved since 9 am. Mosquitoes used to challenge each study participant will be immediately killed at the end of the challenge using 70% ethanol. The mosquito exposure will be performed by study staff under supervision of a medical doctor. The challenge with mosquito bites will take 30 minutes and may be repeated twice in case not all mosquito engorge during the first challenge. The participants will be kept in observation for an additional hour before being allowed to leave the study site. During that time, participants will be provided with a snack and refreshment.

9. SAFETY REPORTING

9.1. Definition of Serious Adverse Events

A serious adverse event (SAE) is any untoward medical occurrence that:

- results in death;
- is life-threatening;
- requires inpatient hospitalisation or prolongation of existing hospitalisation;
- results in persistent or significant disability/incapacity.

The term "life-threatening" in the definition of "serious" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

9.2. Reporting Procedures for Serious Adverse Events

A SAE occurring to a participant should be reported to ethic committee at the Faculty of Tropical Medicine, Mahidol University and to the Safety Review Board within 5 working days of investigator's awareness.

9.3. Adverse event (AE)

At each visit, a CRF and an AE form will be completed. AEs will be documented according to standard definitions and procedures following the Common Terminology Criteria for Adverse Events version 5.0 guidelines (108). Pre-specified AEs include blood and lymphatic system disorders (leucocytosis and eosinophilia), general disorders and administration site conditions (chills, fatigue, fever, challenge site reaction, malaise, pain, challenge site lymphadenopathy), immune system disorders (allergic reaction, anaphylaxis), infections and infestations (papulopustular rash, rash pustular, sepsis, skin infection), injury, poisoning and procedural complications (bruising, venous injury), skin and subcutaneous tissue disorders (bullous dermatitis, eczema, erythema multiforme, pain of skin, pruritus, rash acneiform, rash maculo-papular, skin induration, skin ulceration, urticaria, skin atrophy, skin hyperpigmentation, skin hypopigmentation). Definition and detailed grading procedure of pre-specified adverse events are given in the Appendix E. Any unexpected AEs will be recorded according to the same guidelines.

9.4. Safety review board

All data from the study participant will be submitted to a safety review board after days 15 and 22 in order to review the adverse events (AEs) and decide whether the participant should be withdrawn from the study. Reactions that will exclude the participant include immediate or delayed local reactions greater than 30 mm diameter, any ecchymotic, vesicular, blistering or bullous manifestation, Skeeter syndrome or any systemic symptom (e.g. generalized urticaria, angioedema or anaphylaxis).

10. STATISTICS AND ANALYSIS

10.1. The Number of Participants

There is no preliminary data to calculate *a priori* a required sample size. The groups are 15 participants (15 in the low-exposure and 15 in the high-exposure groups), and a comparison between these groups in expected. Because there is such variation in individual's antibody responses, and the specificity and immunogenicity of the antigens of interest is not known at this stage, it is important to ensure sure that any measured responses are not due to chance. Therefore, 15 participants per study condition was deemed appropriate for this study.

10.2. Analysis of Outcome Measures

Antibody titers will be analysed as (i) a continuous outcome measure and (ii) used to define a binary responder status. Briefly, the positivity threshold of the assay will be determined using reference sera specimens from individuals not exposed to mosquito bites, as described previously (62). The ELISA signal measured in 60 reference sera obtained from unexposed individuals (30 Thai sera obtained from the Thai Red Cross and 30 Australian sera obtained from the Burnett Intstitute) will be used to define the positivity threshold of the assay. The positivity threshold of the assay will be defined as three standard deviation (SD) above the mean optic density (ΔOD) measured for the unexposed control population. A test in the study population with a ΔOD above this cut-off will be considered positive (i.e. an immune response). The specific antibody titer of study samples will be determined by performing serial dilution experiments. Serial dilution will be made from study samples and the ΔOD will be determined for each dilution. The antibody titer will be defined as the highest dilution giving a positive signal. The kinetics and longevity of antibody titers will be assessed using generalised linear mixed-effects linear modelling (family = binomial, link = logit for binary variables) showing changes in antibody titre over time, adjusting for variables of interest

(including age (continuous), sex (binary) and biting exposure group (binary)) and with the inclusion of random intercepts for individual volunteers. Interaction terms between variables and time will be investigated to determine whether kinetics and longevity vary according to variables of interest. Estimates from these equations will be used to calculate the half-life of Ig-responses to gSG6-P1 overall and according to variables of interest. Results obtained with DBS and serum specimens will be compared in order to assess the feasibility of using DBS samples to monitor exposure to bites of mosquitoes during epidemiological studies.

11. DATA MANAGEMENT

11.1. Access to Data

Direct access will be granted to authorised representatives from sponsor, the Burnet Institute, the Singapore Immunology Network, the Walter Reed Army Institute of Research, the Shoklo Malaria Research Unit/Mahidol Oxford Research Unit, and any host institution, ethics committee and regulatory authorities for monitoring, audits and/or inspections of the study to ensure compliance with regulations.

11.2. Data Handling and Record Keeping

All data collected specifically for the study will be recorded on paper forms, and entered to a study database. The database will be built in MACRO and managed by MORU data management team. Laboratory samples will be labelled using unique participant identifiers and date, which will be used to merge laboratory results with other study data. Participant identifiable information such as participant's names and telephone numbers will be stored separately from other study data and will only be accessible by authorised members of the research team.

Non-identifiable data will be shared with researchers via secure data transfer platforms for analysis. Analysis will be conducted on anonymized datasets. In accordance with regulations, study staff will retain all study records on site for at least 5 years after study closure. Data will be stored indefinitely in secure MORU servers and on secure Burnet Institute servers for a minimum of 7 years.

Volunteer's data and results from blood analyses stored in our database may be shared with other researchers to use in the future. However, the other researchers will not be given any information that could identify the subject.

12. QUALITY CONTROL AND QUALITY ASSURANCE PROCEDURES

The study will be conducted in accordance with relevant regulations and standard operating procedures.

13. ETHICAL AND REGULATORY CONSIDERATIONS

13.1. Declaration of Helsinki

The Investigator will ensure that this study is conducted in accordance with the principles of the declaration of Helsinki version 2013.

13.2. Guidelines for Good Clinical Practice

The Investigator will ensure that this study is conducted in accordance with relevant regulations and with Good Clinical Practice.

13.3. Approvals

The protocol, informed consent form, and participant information sheet and consent form will be submitted to the Oxford Tropical Research Ethics Committee (OXTREC), the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University and the Alfred Hospital Ethics Committee for written approval. Furthermore, the protocol, informed consent form, and participant information sheet will be reviewed by the Tak Community Advisory Board, a community-based committee assembling members of the communities in which the study will be performed.

The Investigator will submit and, where necessary, obtain approval from the above parties for all amendments to the original approved documents.

13.4. Participant Confidentiality

The study staff will ensure that the participants' anonymity is maintained. The participants will be identified only by a participant ID number on all study documents and any electronic database. All documents will be stored securely and only accessible by study staff and authorised personnel. The study will comply with the Data Protection Act, which requires data to be anonymised as soon as it is practical to do so.

13.5. Risks

13.5.1. Risk of mosquito exposure

The main risk associated with mosquito feeding experiments are allergic reactions, skin infections at the site of the bites and accidental transmission of vector-borne disease causative agents.

Allergic reactions to mosquito bites:

Most of the population at any given time will have some reactivity to mosquito bites. Typical reactions to mosquito bites include immediate wheals 2 to 10 mm in diameter, with surrounding erythema peaking in 20 to 30 minutes (immediate reaction) and similarly sized pruritic papules peaking at 24 to 36 hours and diminishing in several days (delayed reaction). Immediate reactions occur in 70% to 90% and delayed reactions in 55% to 65% of patients subjected to bites of common *Ae sp.* and *Cx sp.* mosquito species (34-36).

In atopic individuals, large local reactions can develop including erythematous pruritic swellings, often more than 3 cm, occurring in minutes to hours at the site of a bite. Delayed large local reactions may be papular, ecchymotic, vesicular, blistering, or bullous and persist for days or weeks (36, 40). The incidence of self-reported large local reactions in one study was 2.5% (8/12 cases were children less than 10 years old) (40). Skeeter syndrome describes patients with mosquito bite-induced large local reactions accompanied by fever. This syndrome typically occurs in otherwise healthy children, and the large local reactions may mimic cellulitis (hot, swollen, red, and painful) but may be differentiated by their occurrence within hours of mosquito bites. These symptoms resolve in 3 to 10 days (41). Anaphylactic reactions to mosquito bites are extremely rare but have occurred in patients with underlying indolent systemic mastocytose (42, 43). Persons at increased risk for severe reactions include those with high exposure (outdoor workers) and those lacking acquired immunity (young children and immigrants) (31). In addition, patients with primary or acquired immunodeficiencies and those with Epstein-Barr virus (EBV) associated lymphoproliferative diseases are also at higher risk for severe reactions (44-46).

A few previous studies reported the safety of human challenges performed with large numbers of (infected) malaria mosquito bites and no serious adverse event was observed (97-99). Immediate skin reactions (20-30 min after mosquito bites) will be recorded after each mosquito feeding assay. Delayed skin reactions (24-36 hours after mosquito bites) will be recorded after the day 14, 21, requiring additional visits at day 15, 22. All data from study participant will be submitted to a safety review board after day 15, 22 for reviewing potential adverse events (AEs) and eventually request withdrawal of the participants from the study. Reactions that will exclude the participant include immediate or delayed local reactions greater than 30 mm diameter, any ecchymotic, vesicular, blistering or bullous manifestation, Skeeter syndrome or any systemic symptom (e.g. generalized urticaria, angioedema or anaphylaxis). In addition, all mosquito feeding experiments will be conducted under the supervision of a medical doctor and resuscitation material will be available at the site of the study. In case SAE happen and cannot be managed on site, the participants will be referred to Mae Sot general hospital (located at 5 min from the study site).

Skin infection at the challenge site:

Secondary development of a skin infection may develop at the site of the challenge, especially if severe allergic skin reactions develop (36). Participants with severe reaction to mosquito bites will not be enrolled or will be withdrawn from the study, therefore limiting the risk of skin infection. In addition, the skin will be disinfected with 70% alcohol before the challenge, and appropriate diagnosis and treatment of eventual infection will be ensured during participant follow-up.

Accidental transmission of vector-borne disease causative agents:

The mosquito strains used in this study were colonized decades ago, and reared under laboratory conditions since. All feeds will be performed with nulliparous females (i.e., that never blood fed) so it is not possible for these mosquitoes to be infected with pathogen transmitted only “horizontally” (i.e. from one host to another) such as *Plasmodium* malaria parasites and lymphatic filaria. For pathogens that can be “vertically” transmitted in the mosquito (i.e. transmitted from the female mosquito to its eggs), such as dengue, Zika and Chikungunya viruses, there is a very small risk that nulliparous females inherit the pathogen from the previous generation, if the colony is accidentally fed on blood containing viable viral particles during routine maintenance procedures. This is very unlikely because colonies are maintained on human blood procured from authorized blood banks that screen donors for relevant infections including dengue, Zika and Chikungunya viruses. In addition, *Aedes* mosquito colonies will be screened for dengue, Zika and Chikungunya before the study. All mosquitoes used for challenging participants will be immediately killed and disposed at the end of the experiment.

All participants will be followed up and observed from the visit 1 until the visit 20 which corresponds to a total period of 112 to 142 days (according to the timing of the screening visit), including 56 days after the last challenge, in order to make sure that they do not get malaria or dengue fever from being bitten by mosquitoes during study procedure.

13.5.2. Risk of blood collection

Blood sampling is associated with a low risk of bruising, bleeding, swelling, fainting. Infection is very rare but may occur. Blood sampling and mosquito feeds will be made by trained and qualified SMRU staff using single-usage sterile materials.

13.6. Benefits

There is no individual benefit in participating to this study for participants. This study will benefit to the population exposed to mosquito-borne diseases transmitted by *An. minimus*, *An. maculatus*, *An. dirus, Ae. aegypti* and/or *Ae. albopictus* as a whole, by providing tools for improving entomological surveillance in the context of malaria elimination in the Greater Mekong Subregion.

13.7. Compensation

Study participants will be compensated for loss of work and transportation costs. We have determined that 350 THB (USD 11) per visit is locally appropriate. As the study consists of 20 visits the maximum compensation per participant is 7000 THB (USD 220).

13.8. Reporting

The chief investigator shall submit an Annual Progress Report to OxTREC and local EC on the anniversary of the date of approval of the study. In addition, the CI shall submit an End of Study Report to OxTREC and local EC.

14. FINANCE AND INSURANCE

14.1. Funding

This project is funded by The Australian National Health and Medical Research Council, the Burnet Institute (Melbourne, Australia) and the Singapore Immunology Network (Agency for Science, Technology, and Research, Singapore) via Oxford University and the Mahidol Oxford Tropical Medicine Research Unit (MORU).

14.2. Insurance

The project is covered under the Oxford University sponsorship.

The University has a specialist insurance policy in place which would operate in the event of any participant suffering harm as a result of their involvement in the research (Newline Underwriting Management Ltd, at Lloyd's of London).

15. PUBLICATION POLICY

All publications will abide by the International Committee of Medical Journal Editors (ICMJE) recommendations of the role of authors and contributors.

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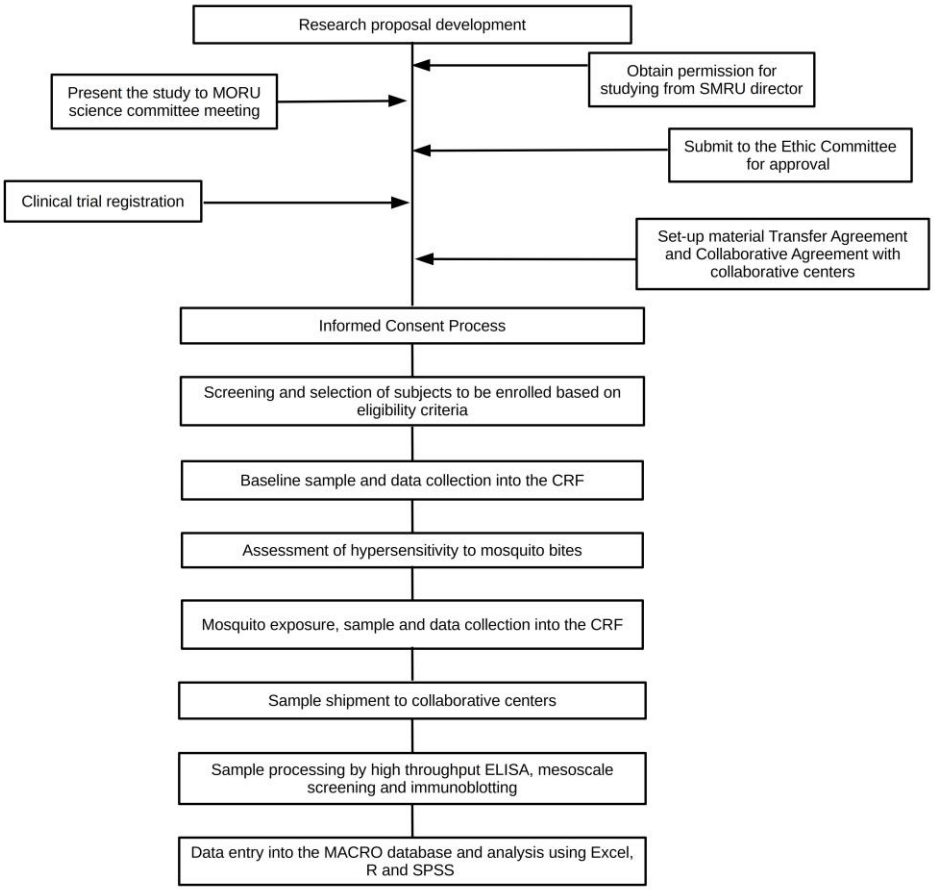
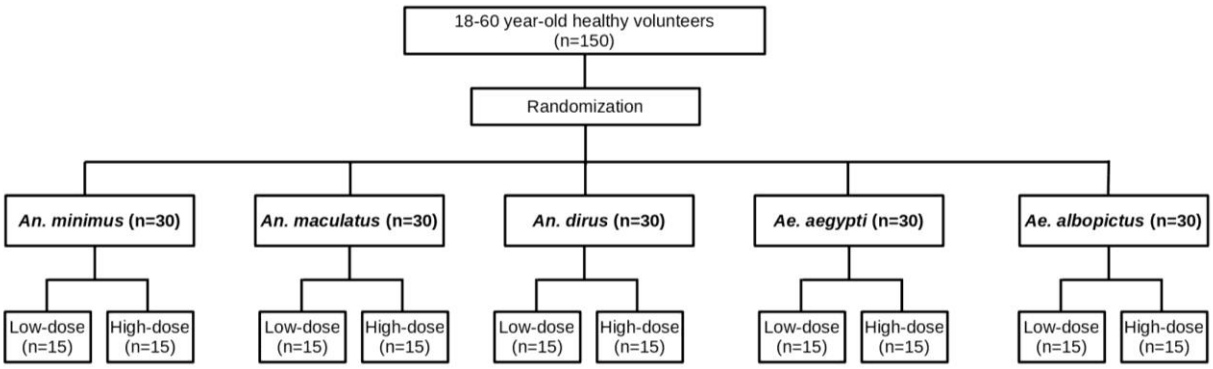
17. APPENDIX A: Schematic diagram of study design, procedures and stages, step-by-step



18. APPENDIX B: Study design and exposure randomization



Low-dose: total of 35 bites split into 7 direct feeding assays over 6 weeks (5 bites/visit);

High-dose: total of 305 bites split into 7 direct feeding assays over 6 weeks (5 bites for the screening of hypersensitivity to mosquito bites during visit 4, then 50 bites/visit).

19. APPENDIX C: Schedule of participant's visits including informed consent blood sampling and exposure to mosquito bites

Visit	Study period	Day [a]	Informed consent obtained	Medical History	PE and VS	Randomization	Mosquito exposure [b]	DBS Collection [c]	VP collection [c,d]
1	screening	30 days or less before enrolment	Yes	Yes	Yes	No	No	No	Yes
2	baseline	0	No	No	No	Yes	No	Yes	Yes
3		7	No	No	No	No	No	Yes	Yes
4	exposure	14	No	No	Yes	No	Yes	Yes [c]	Yes [c]
5		15	No	No	Yes	No	No	No	No
6		21	No	No	Yes	No	Yes	Yes	Yes
7		22	No	No	Yes	No	No	No	No
8		28	No	No	Yes	No	Yes	Yes	Yes
9		35	No	No	Yes	No	Yes	Yes	Yes
10		42	No	No	Yes	No	Yes	Yes	Yes
11		49	No	No	Yes	No	Yes	Yes	Yes
12		56	No	No	Yes	No	Yes	Yes	Yes
13	post-exposure	63	No	No	No	No	No	Yes	Yes
14		70	No	No	No	No	No	Yes	Yes
15		77	No	No	No	No	No	Yes	Yes
16		84	No	No	No	No	No	Yes	Yes
17		91	No	No	No	No	No	Yes	Yes
18		98	No	No	No	No	No	Yes	Yes
19		105	No	No	No	No	No	Yes	Yes
20		112	No	No	No	No	No	Yes	Yes

[a] In case participant fail to come on the exact day a visit is scheduled, he/she will be allowed to come for a retake any other working day of the study team until the next scheduled visit;

[b] 5 bites for the screening of hypersensitivity to mosquito bites during visit 4, then 5 or 50 bites/visit according to the randomisation schedule (total of 35 and 305 bites/participant in low- and high-exposure groups respectively, split into 7 direct feeding assays);

[c] before exposure to mosquito bites.

[d] 2 mL during the screening visit, 8.6 mL during visits 2, 3, 6, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18 and 19 and 18.6 mL during visits 4, 13 and 20.

20. APPENDIX D: Summary of blood volumes drawn from participants

Period	Visit no.	Day	Venous blood (ml)	Total volume of serum (ml)	Volume of serum sent to BI (ml) [a]	Volume of serum sent to A*STAR (ml) [b]	Volume of serum sent to WRAIR (ml) [c]	Capillary blood (ml)
Screening	1	30 days or less before enrolment	2	NA	NA	NA	NA	0
Baseline	2	0	8.6	4.1	2	2	0.1	0.3
	3	7	8.6	4.1	2	2	0.1	0.3
Exposure	4	14	18.6	9.1	7	2	0.1	0.3
	5	15	0	NA	NA	NA	NA	0
	6	21	8.6	4.1	2	2	0.1	0.3
	7	22	0	NA	NA	NA	NA	0
	8	28	8.6	4.1	2	2	0.1	0.3
	9	35	8.6	4.1	2	2	0.1	0.3
	10	42	8.6	4.1	2	2	0.1	0.3
	11	49	8.6	4.1	2	2	0.1	0.3
	12	56	8.6	4.1	2	2	0.1	0.3
Post-exposure	13	63	18.6	9.1	7	2	0.1	0.3
	14	70	8.6	4.1	2	2	0.1	0.3
	15	77	8.6	4.1	2	2	0.1	0.3
	16	84	8.6	4.1	2	2	0.1	0.3
	17	91	8.6	4.1	2	2	0.1	0.3
	18	98	8.6	4.1	2	2	0.1	0.3
	19	105	8.6	4.1	2	2	0.1	0.3
	20	112	18.6	9.1	7	2	0.1	0.3
Total			**178.2**					**5.1**
		Grand Total for Blood collection = 183.3 mL						

[a] Samples sent to the Burnet Institute (BI) will be used for ELISA screening and immuno-proteomic analysis;
[b] Samples sent to the Singapore Immunology Network, Agency for Science, Technology, and Research, Singapore (A*STAR) will be used for ELISA screening;
[c] Samples sent to the Walter Reed Army Institute of Research (WRAIR) will be used for mesoscale screening.

Abbreviations: NA, not applicable.

21. APPENDIX E: Classification, definition and grading of pre-specified adverse events (adapted from the Common Terminology for Adverse Events (CTAE) guidelines version 5.0

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Blood and lymphatic system disorders	**Leucocytosis**	-	-	>100,000/mm^3	Clinical manifestations of leucostasis; urgent intervention indicated	Death
	Definition: A disorder characterized by laboratory test results that indicate an increased number of white blood cells in the blood.					
	Eosinophilia	>ULN and >Baseline	-	Steroids initiated	-	-
	Definition: A disorder characterized by a sensation of marked discomfort in a lymph node.					
General disorders and administration site conditions	**Chills**	Mild sensation of cold; shivering; chattering of teeth	Moderate tremor of the entire body; narcotics indicated	Severe or prolonged, not responsive to narcotics	-	-
	Definition: A disorder characterized by a sensation of cold that often marks a physiologic response to sweating after a fever.					
	Fatigue	Fatigue relieved by rest	Fatigue not relieved by rest; limiting instrumental ADL	Fatigue not relieved by rest, limiting self-care ADL	-	-
	Definition: A disorder characterized by a state of generalized weakness with a pronounced inability to summon sufficient energy to accomplish daily activities.					
	Fever	38.0 -39.0 degrees C (100.4 -102.2 degrees F)	>39.0 -40.0 degrees C (102.3 -104.0 degrees F)	>40.0 degrees C (>104.0 degrees F) for <=24 hrs	>40.0 degrees C (>104.0 degrees F) for >24 hrs	Death

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
	Definition: A disorder characterized by elevation of the body's temperature above the upper limit of normal.					
	Challenge site reaction	Tenderness with or without associated symptoms (e.g., warmth, erythema, itching)	Pain; lipodystrophy; edema; phlebitis	Ulceration or necrosis; severe tissue damage; operative intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
	Definition: A disorder characterized by an intense adverse reaction (usually immunologic) developing at the site of an injection.					
	Malaise	Uneasiness or lack of well being	Uneasiness or lack of wellbeing limiting instrumental ADL	Uneasiness or lack of wellbeing limiting self-care ADL	-	-
	Definition: A disorder characterized by a feeling of general discomfort or uneasiness, an out-of-sorts feeling.					
	Pain	Mild pain	Moderate pain; limiting instrumental ADL	Severe pain; limiting self-care ADL	-	-
	Definition: A disorder characterized by the sensation of marked discomfort, distress or agony.					
	Challenge site lymphadenopathy	Local lymph node enlargement	Localized ulceration; generalized lymph node enlargement	-	-	-
	Definition: A disorder characterized by lymph node enlargement after vaccination.					
Immune system disorder	**Allergic reaction**	Systemic intervention not indicated	Oral intervention indicated	Bronchospasm; hospitalization indicated for clinical sequelae; intravenous	Life-threatening consequences; urgent intervention indicated	Death

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
				intervention indicated		
	Definition: A disorder characterized by an adverse local or general response from exposure to an allergen.					
	Anaphylaxis	-	-	Symptomatic bronchospasm, with or without urticaria; parenteral intervention indicated; allergy-related edema/angioedema; hypotension	Life-threatening consequences; urgent intervention indicated	Death
	Definition: A disorder characterized by an acute inflammatory reaction resulting from the release of histamine and histamine-like substances from mast cells, causing a hypersensitivity immune response. Clinically, it presents with breathing difficulty, dizziness, hypotension, cyanosis and loss of consciousness and may lead to death.					
Infections and infestations	**Papulopustular rash**	Papules and/or pustules covering <10% BSA, which may or may not be associated with symptoms of pruritus or tenderness	Papules and/or pustules covering 10-30% BSA, which may or may not be associated with symptoms of pruritus or tenderness; associated with psychosocial impact; limiting instrumental ADL; papules and/or pustules covering > 30% BSA with or	Papules and/or pustules covering >30% BSA with moderate or severe symptoms; limiting self-care ADL; IV antibiotics indicated	Life-threatening consequences	Death

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
			without mild symptoms			
	Definition: A disorder characterized by an eruption consisting of papules (a small, raised pimple) and pustules (a small pus filled blister), typically appearing in face, scalp, and upper chest and back. Unlike acne, this rash does not present with whiteheads or blackheads, and can be symptomatic, with itchy or tender lesions.					
	Rash pustular	-	Localized; local intervention indicated (e.g., topical antibiotic, antifungal, or antiviral)	IV antibiotic, antifungal, or antiviral intervention indicated; invasive intervention indicated	-	-
	Definition: A disorder characterized by a circumscribed and elevated skin lesion filled with pus.					
	Sepsis			Blood culture positive with signs or symptoms; treatment indicated	Life-threatening consequences; urgent intervention indicated	Death
	Definition: A disorder characterized by the presence of pathogenic microorganisms in the blood stream that cause a rapidly progressing systemic reaction that may lead to shock.					
	Skin infection	Localized, local intervention indicated	Oral intervention indicated (e.g., antibiotic, antifungal, or antiviral)	IV antibiotic, antifungal, or antiviral intervention indicated; invasive intervention indicated	Life-threatening consequences; urgent intervention indicated	Death
	Definition: A disorder characterized by an infectious process involving the skin such as cellulitis.					
Injury, poisoning and procedural complications	**Bruising**	Localized or in a dependent area	Generalized	-	-	-
	Definition: A finding of injury of the soft tissues or bone characterized by leakage of blood into surrounding tissues.					

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
	Venous injury	Asymptomatic diagnostic finding; intervention not indicated	Symptomatic (e.g., claudication); repair or revision not indicated	Severe symptoms; limiting self-care ADL; repair or revision indicated	Life-threatening consequences; evidence of end organ damage; urgent operative intervention indicated	Death
	Definition: A finding of damage to a vein.					
Skin and subcutaneous tissue disorders	**Bullous dermatitis**	Asymptomatic; blisters covering <10% BSA	Blisters covering 10 -30% BSA; painful blisters; limiting instrumental ADL	Blisters covering >30% BSA; limiting self-care ADL	Blisters covering >30% BSA; associated with fluid or electrolyte abnormalities; ICU care or burn unit indicated	Death
	Definition: A disorder characterized by inflammation of the skin characterized by the presence of bullae which are filled with fluid.					
	Eczema	Asymptomatic or mild symptoms; additional medical intervention over baseline not indicated	Moderate; topical or oral intervention indicated; additional medical intervention over baseline indicated	Severe or medically significant but not immediately life-threatening; IV intervention indicated	-	-
	Definition: A disorder characterized by skin which becomes itchy, red, inflamed, crusty, thick, scaly, and/or forms blisters.					
	Erythema multiforme	Target lesions covering <10% BSA and not associated with skin tenderness	Target lesions covering 10 -30% BSA and associated with skin tenderness	Target lesions covering >30% BSA and associated with oral or genital erosions	Target lesions covering >30% BSA; associated with fluid or electrolyte abnormalities; ICU care or burn unit indicated	Death

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
	Definition: A disorder characterized by target lesions (a pink-red ring around a pale center).					
	Pain of skin	Mild pain	Moderate pain; limiting instrumental ADL	Severe pain; limiting self-care ADL	-	-
	Definition: A disorder characterized by a sensation of marked discomfort in the skin.					
	Pruritus	Mild or localized; topical intervention indicated	Widespread and intermittent; skin changes from scratching (e.g., edema, papulation, excoriations, lichenification, oozing/crusts); oral intervention indicated; limiting instrumental ADL	Widespread and constant; limiting self-care ADL or sleep; systemic corticosteroid or immunosuppressive therapy indicated	-	-
	Definition: A disorder characterized by an intense itching sensation.					
	Rash acneiform	Papules and/or pustules covering <10% BSA, which may or may not be associated with symptoms of pruritus or tenderness	Papules and/or pustules covering 10 - 30% BSA, which may or may not be associated with symptoms of pruritus or tenderness; associated with psychosocial impact; limiting instrumental ADL; papules and/or	Papules and/or pustules covering >30% BSA with moderate or severe symptoms; limiting self-care ADL; associated with local superinfection with oral antibiotics indicated	Life-threatening consequences; papules and/or pustules covering any % BSA, which may or may not be associated with symptoms of pruritus or tenderness and are associated with extensive superinfection with	Death

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
			pustules covering > 30% BSA with or without mild symptoms		IV antibiotics indicated	
	Definition: A disorder characterized by an eruption of papules and pustules, typically appearing in face, scalp, upper chest and back.					
	Rash maculo-papular	Macules/papules covering <10% BSA with or without symptoms (e.g., pruritus, burning, tightness)	Macules/papules covering 10 -30% BSA with or without symptoms (e.g., pruritus, burning, tightness); limiting instrumental ADL; rash covering > 30% BSA with or without mild symptoms	Macules/papules covering >30% BSA with moderate or severe symptoms; limiting self-care ADL	-	-
	Definition: A disorder characterized by the presence of macules (flat) and papules (elevated). Also known as morbilliform rash, it is one of the most common cutaneous adverse events, frequently affecting the upper trunk, spreading centripetally and associated with pruritis.					
	Skin induration	Mild induration, able to move skin parallel to plane (sliding) and perpendicular to skin (pinching up)	Moderate induration, able to slide skin, unable to pinch skin; limiting instrumental ADL	Severe induration; unable to slide or pinch skin; limiting joint or orifice movement (e.g., mouth, anus); limiting self-care ADL	Generalized; associated with signs or symptoms of impaired breathing or feeding	Death
	Definition: A disorder characterized by an area of hardness in the skin.					
	Skin ulceration	Combined area of ulcers <1 cm;	Combined area of ulcers 1 -2 cm;	Combined area of ulcers >2 cm; full-	Any size ulcer with extensive	Death

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
		nonblanchable erythema of intact skin with associated warmth or edema	partial thickness skin loss involving skin or subcutaneous fat	thickness skin loss involving damage to or necrosis of subcutaneous tissue that may extend down to fascia	destruction, tissue necrosis, or damage to muscle, bone, or supporting structures with or without full thickness skin loss	
	Definition: A disorder characterized by a circumscribed, erosive lesion on the skin.					
	Urticaria	Urticarial lesions covering <10% BSA; topical intervention indicated	Urticarial lesions covering 10 -30% BSA; oral intervention indicated	Urticarial lesions covering >30% BSA; IV intervention indicated	-	-
	Definition: A disorder characterized by an itchy skin eruption characterized by wheals with pale interiors and well-defined red margins.					
	Skin atrophy	Covering <10% BSA; associated with telangiectasias or changes in skin color	Covering 10 -30% BSA; associated with striae or adnexal structure loss	Covering >30% BSA; associated with ulceration	-	-
	Definition: A disorder characterized by the degeneration and thinning of the epidermis and dermis.					
	Skin hyperpigmentation	Hyperpigmentation covering <10% BSA; no psychosocial impact	Hyperpigmentation covering >10% BSA; associated psychosocial impact	-	-	-
	Definition: A disorder characterized by darkening of the skin due to excessive melanin deposition.					

SOC	CTCAE Term	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
	Skin hypopigmentation	Hypopigmentation or depigmentation covering <10% BSA; no psychosocial impact	Hypopigmentation or depigmentation covering >10% BSA; associated psychosocial impact	-	-	-
	Definition: A disorder characterized by loss of skin pigment (e.g., vitiligo).					

ADL: activities of daily living; BSA: body surface area; CTCAE: Common Terminology Criteria for Adverse Events; ICU: intensive care unit; IV: intra-venous; SOC: system organ class; ULN: upper limit of the normal.

22. APPENDIX F: Amendment history

Amendment No.	Protocol Version No.	Date issued	Author(s) of changes	Details of Changes made

List details of all protocol amendments here whenever a new version of the protocol is produced. This is not necessary prior to initial Ethics Committee submission.