STUDY PROTOCOL

REVISED Characterizing antibody responses to mosquito salivary antigens of the Southeast Asian vectors of malaria and dengue with a human challenge model of controlled exposure: a protocol [version 2; peer review: 1 approved, 2 approved with reservations]

Sunisa Sawasdichai[1], Victor Chaumeau[1,2], Ellen Kearney[3,4], Praphan Wasisakun[1], Julie A. Simpson[4], David J. Price[4,5], Sadudee Chotirat[6], Laurent Rénia[7,8], Elke Bergmann-Leitner[9], Freya Fowkes[3,4,10], François Nosten[1,2]

[1]Shoklo Malaria Research Unit, Mahidol-Oxford Research Unit, Faculty of Tropical Medicine, Mahidol University, Mae Ramat, Tak, 63140, Thailand
[2]Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, England, OX3 7BN, UK
[3]Burnet Institute, Melbourne, VIC 3004, Australia
[4]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Victoria, VIC 3010, Australia
[5]Department of Infectious Diseases, University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Victoria, VIC 3000, Australia
[6]Malaria Vivax Research Unit, Faculty of Tropical medicine, Mahidol University, Bangkok, 10400, Thailand
[7]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore, 308232, Singapore
[8]A*STAR Infectious Diseases Labs, Agency for Science, Technology, and Research, Singapore, 138648, Singapore
[9]The Walter Reed Army Institute of Research, Silver Spring, Maryland, MD 20910, USA
[10]Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Victoria, VIC 3052, Australia

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Abstract

Background: Measurement of antibody titers directed against mosquito salivary antigens in blood samples has been proposed as an outcome measure to assess human exposure to vector bites. However, only a handful of antigens have been identified and the specificity and longitudinal dynamics of antibody responses are not well known. We report the protocol of a clinical trial of controlled exposure to mosquito bites that aims to identify and validate biomarkers of exposure to bites of mosquito vector species that transmit malaria and dengue in Southeast Asia and some other parts of the world.

Open Peer Review

Approval Status ✓ ? ?

	1	2	3
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version 1 23 Mar 2023	? view	? view	? view

1. **Julien Pompon**, Universite de Montpellier,

Methods: This study is an exploratory factorial randomized control trial of controlled exposure to mosquito bites with 10 arms corresponding to different species (*Aedes aegypti*, *Ae. albopictus*, *Anopheles dirus*, *An. maculatus* and *An. minimus*) and numbers of bites (35 or 305 bites in total over 6 weeks). Blood samples will be collected from study participants before, during and after mosquito biting challenges. Candidate peptides will be identified from published literature with antigen prediction algorithms using mosquito DNA sequence data and with immunoblotting assays carried out using protein extracts of dissected mosquito salivary glands and participants samples. Antibody titers against candidate peptides will be determined in participants samples with high-throughput cutting-edge immuno-assays. Quantification of the antibody response profile over time (including an estimate of the decay rate) and the effect of the number of bites on the antibody response will be determined using linear and logistic mixed-effects models for the continuous and the binary response, respectively.
Conclusion: This research is expected to generate important knowledge for vector sero-surveillance and evaluation of vector-control interventions against malaria and dengue in the Greater Mekong Subregion.
Registration: This study is registered with clinicaltrials.gov (NCT04478370) on July 20th, 2020.

Keywords
malaria, dengue, Southeast Asia, Anopheles, Aedes, exposure, saliva, clinical trial

This article is included in the Mahidol Oxford Tropical Medicine Research Unit (MORU) gateway.

Montpellier, France

2. **Cassandra Modahl**, Liverpool Institute of Tropical Medicine, Liverpool, UK

3. **Paolo Gabrieli**, Universita degli Studi di Milano, Milan, Italy

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Victor Chaumeau (victor@shoklo-unit.com)

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REVISED **Amendments from Version 1**

The manuscript was revised to address reviewers' comments. A sentence on vaccines against dengue viruses was added to the introduction. Participant exposure to *Aedes* sp. mosquito bites not related to the study challenges was clearly identified as a study limitation, and assessment of antibody responses to *Aedes* sp. salivary proteins was clearly defined as a secondary outcome. Details on the rationale for exposing participants to 35 and 305 mosquito bites during the biting challenges and references to the literature were added. The reasons why choosing electrochemiluminescence and ELISA immunoassays, the measurements of different antibody isotypes and subclasses and the screening strategies were explained into more details. The statistical methods for analyzing antibody responses to *Aedes* sp. bites were also clarified.

Any further responses from the reviewers can be found at the end of the article

Introduction

Background and rationale

Mosquito-borne diseases cause significant burden in populations exposed to bites of vector species across Southeast Asia[1]. Malaria and dengue are endemic and pose the greatest challenges. Malaria is transmitted in rural areas and multi-drug resistant falciparum malaria has been identified as a major threat to public health in these areas[2]. Consequently, considerable investment has been made to eliminate *Plasmodium falciparum* in the Greater Mekong Subregion, the epicenter of antimalarial drug resistance[3]. In this area, the main vectors are *Anopheles dirus*, *An. maculatus* and *An. minimus*; several other species also contribute to the transmission[4]. The efficacy of conventional vector-control measures is low[5–7] because of the ecology and biology of relevant vector species[8–10], and is particularly difficult to evaluate due to the complex transmission dynamics and low rates of disease incidence[8]. Dengue viruses are transmitted by aedine mosquitoes; the main vectors are *Aedes aegypti* and *Ae. albopictus*[11]. Infection can cause death and overall disease burden has drastically increased over the past decades[12]. As there is no specific treatment for dengue, prevention of infection is critical to reduce morbidity and mortality. Two vaccines against dengue viruses were licenced but the uptake among patients is limited: one is indicated in children with previous infection and the other is licenced only in Europe[13]. Integrated management strategies and active involvement of homeowners is necessary to control *Aedes* mosquitoes in urban and semi-urban environments. In rural areas where vector breeding sites also include a variety of natural water bodies (rock pools, tree holes and bamboo stumps), control is particularly challenging and personal protection with long-sleeve clothes and skin repellents is often the only option available[14].

Exposure to mosquito bites is a key parameter of the vectorial capacity equation[15] and its assessment is extremely informative for disease surveillance and trials of vector-control interventions. Exposure to mosquito bites results from a combination of parameters including mosquito population density, aggressivity to humans, people movements and sleeping habits, and personal protection conferred by vector-control interventions[16]. It is currently not possible to measure exposure to mosquito bites accurately; mosquito biting-rate estimates are sometimes combined with data on human behaviors and vector-control to produce elusive estimates, but the cost and challenges associated with data collection are often prohibitive[16].

When blood feeding, mosquitoes inject saliva into the skin of vertebrates[17]. Mosquito saliva is composed of hundreds of biologically active molecules that play essential roles in the physiology of blood feeding[18]. Many saliva components have immunogenic properties and some of these antigens elicit detectable levels of antibody responses in the blood following biting exposure[19]. Assessment of antibody responses directed against mosquito salivary antigens as a surrogate measure of human exposure to mosquito bites has been proposed[20]. Individuals repeatedly bitten by mosquitoes develop a long-lasting broad and variable sero-reactivity to mosquito salivary antigens of the biting species[21]. Serum can cross-react with salivary antigens of other mosquito species to which an individual has never been exposed to[22]. As a result, it can be difficult to identify antigenic peptides that elicit transient antibody responses with adequate sensitivity and specificity. Only two *An. gambiae* (gSG6-P1 and cE5) and two *Ae. sp.* (Nterm-34kDa and D7) peptides have been used for assessing exposure in large-scale epidemiological surveys and trials of vector-control[23,24]. However, critical parameters including sensitivity, specificity and half-life of the antibody responses have not been assessed precisely: only one prospective study strived to characterize the immune responses of humans (n = 1 subject exposed to *Culex quinquefasciatus* bites) and rabbits (n = 1 subject exposed to *Ae. aegypti* bites) to mosquito salivary antigens in a challenge model of controlled exposure[19]. We therefore propose to conduct a world-first clinical trial of controlled exposure to bites of uninfected laboratory-reared *Anopheles* sp. and *Aedes* sp. mosquitoes to identify and validate biomarkers of exposure to dengue and malaria vector bites.

Objectives

The primary objective of this study is to identify and validate biomarkers of exposure to bites of *An. dirus*, *An. maculatus*, *An. minimus*, *Ae. aegypti* and *Ae. albopictus*. The secondary objectives are to characterize the dose-response relationship between the number of mosquito bites and antibody titers and to compare the performance of capillary blood spotted on filter paper and serum from venous blood for measuring antibody responses to mosquito salivary antigens.

Trial design

This study is an exploratory, factorial randomized controlled trial of controlled exposure to mosquito bites with 10 arms corresponding to different species (*An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* and *Ae. albopictus*) and numbers of bites (35 or 305 bites in total over 6 weeks). Participants will be assigned randomly to one of the 10 study arms with a 1:1 ratio using a block randomization schedule. Those with incomplete follow-up will be replaced until complete follow-up of 15 participants per arm is achieved. Serum from venous blood

and capillary blood spotted on filter paper will be collected weekly from each study participant before, during and after the challenges. In addition to those described in the published literature, candidate peptides will be identified with antigen prediction algorithms using mosquito salivary proteins sequences data and immunoproteomic assays carried out using protein extracts of dissected mosquito salivary glands and participants samples[25]. Antibody titers will be determined with ELISA and electrochemiluminescence immunoassays[26,27].

Methods

Participants, intervention and outcomes

Study setting. The study will be conducted at the research center of the Shoklo Malaria Research Unit in Mae Sot, Thailand. Critical to the trial design, Mae sot is a small town in Thailand where *Anopheles* mosquitoes have disappeared after decades of development and urbanization[28]. Therefore, people who live in Mae Sot are not exposed to *Anopheles* bites if they do not travel in rural areas located outside the city. Unlike *Anopheles* mosquitoes, *Aedes* are ubiquitous and it will not be possible to avoid participant exposure to *Aedes* bites not related to the study challenges. This is a limitation to the evaluation of antibody responses to *Aedes* salivary proteins, which will be treated as a secondary outcome and analyzed descriptively.

Eligibility criteria. Volunteer eligibility to the study will be assessed using the criteria presented in Table 1.

Table 1. Eligibility criteria.

Inclusion criteria
Generally healthy male or female aged 18 to 60 years old as assessed by a medical doctor
Thai, Burmese or Karen ethnicity
Living in Mae Sot city for the last 12 months
Able to tolerate direct mosquito exposure
Exclusion criteria
History of travel in a rural area (*i.e.*, where participant may be exposed *Anopheles* bites) in the last 12 months, or plan to do so during the study
Medication or condition deemed to interfere with the outcome measure or increase the risk of an adverse reaction to the study procedures (hypersensitivity to mosquito bites, atopy, systemic mastocytosis, immunodeficiencies, Epstein-Barr virus-associated lymphoproliferative disease, and longue-course oral treatment with a steroidal anti-inflammatory drug)
Moderate and severe anaemia (haemoglobin concentration less than 110 g/L of blood)
Pregnancy
Breastfeeding

Intervention. Participants will be exposed to bites of laboratory-adapted colonies of *An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* or *Ae. albopictus* weekly for six weeks (seven challenges per participant in total). Participants in the low-exposure arms will be challenged on each occasion with five mosquito bites (35 bites in total), those in the high-exposure arms will be challenged once with five bites and then six times with 50 bites (305 bites in total). The numbers of bites were chosen based on previous entomological investigations conducted in this area[8] and published reports of human challenge with mosquito bites[29–31]. In order to assess participant skin reactions to mosquito bites, three bites will be administered on one participant arm using single mosquitoes in 50 mL tubes topped with netting material. The remaining mosquitoes will be put into plastic cups of 10 cm in diameter topped with netting material and offered to feed on participant counter arm, calf, thigh or back skin. The mosquitoes will be left undisturbed and allowed to feed for 30 min. The number of bites actually received by the participant will be assessed by counting the number of engorged mosquitoes at the end of the exposure time. If not all mosquitoes engorge, the participant will be exposed to additional mosquitoes using the same procedure in order to reach the target number of bites. All challenges will be performed with 5- to 7-day-old starved nulliparous female imagoes (*i.e.*, that have never blood fed before) of laboratory-adapted mosquito colonies reared in insectaries. Participants with hypersensitivity to mosquito bites will be withdrawn from the study. In order to increase adherence to the study protocol, the study coordinator will call participants on the day before a scheduled visit and remind them about the appointment. Participants who miss a visit will be given the opportunity to come for a retake visit until the day of the next scheduled visit. All participants will be provided antipruritic medication (chlorphenoxamine cream and cetirizine pills) to relieve itching from mosquito bites. Participants will be informed during the screening visit about the importance of avoiding being bitten by *Anopheles* mosquitoes during the study and how to do so. They will be provided with an insecticide-impregnated mosquito bed net (PermaNet 2.0®, Vestergaard) and skin repellent (N,N-diethyl-meta-toluamide, D.E.E.T., 20%). They will be asked not to travel to rural areas and the travel history will be recorded at every visit.

Outcomes. The primary outcome of this study will be the participant antibody responses to *Anopheles* salivary proteins. The secondary outcome of this study will be the participant antibody responses to *Aedes* salivary proteins. The measurement variables will be the amino-acid sequence of candidate peptides, and the titers of antibodies directed against these peptides determined in participants blood samples (serum from venous blood and capillary blood spotted on filter paper) collected at one-week intervals before, during and after the challenges. Antibody titers, assessed by measuring the optical density of immunoassay reactions, will be analyzed as a continuous outcome and also as a binary outcome (seropositive/seronegative compared to unexposed sera).

The size and type of participant skin reactions to mosquito bites will be assessed after every challenge. Reactions greater than 30 mm in diameter, ecchymosis, vesicle, blister, bullae, Skeeter syndrome and systemic symptoms (generalized urticaria, angioedema and anaphylaxis) will require withdrawing the participant from the study.

Participant timeline. The participant timeline is presented in Table 2. Volunteers interested in participating in the study will be appointed for enrollment and the eligibility of those who consent will be assessed. Eligible participants will be appointed to attend weekly visits for 16 weeks and the complete follow-up will be 112 days. Participants will be challenged with mosquito bites seven times between day 14 and day 56. Immediate skin reactions will be recorded 20 to 30 minutes after every challenge and delayed skin reactions will be recorded 24 to 36 hours after the first and second challenges. The level of antibody titers against mosquito salivary antigens will be measured in participant serum from venous blood and capillary blood spotted on filter paper collected at one-week intervals for the entire follow-up.

Sample size. There is no data to calculate the sample size *a priori* because the characteristics of immune responses to candidate peptides is not known at this stage. A sample size of 15 participants with complete follow-up per study arm was deemed appropriate for this study given the number of repeated assessments and expected variation in the continuous individual antibody responses. Participants with incomplete follow-up will be replaced to ensure there are 15 participants per study arm with complete follow-up.

Recruitment. Information on the study will be spread through word of mouth by the study team to people who live in Mae

Table 2. Participant timeline.

	Study period																			
	Enrollment	**Allocation**	**Post-allocation**																	**Closeout**
Visit	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Time point (in days)	-30 to 0	0	7	14	15	21	22	28	35	42	49	56	63	70	77	84	91	98	105	112
Enrollment:																				
Eligibility screen	x																			
Informed consent	x																			
Allocation		x																		
Intervention:																				
Challenge				x		x		x	x	x	x	x								
Assesments:																				
Antibody titers																				
Skin reactions				x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination	x	x	x	x		x		x	x	x	x	x								
Complete blood count	x																			
Pregnancy and lactation	x																			
Vital signs	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x
Medical history	x																			
Travel history	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x
Concomitant medication	x	x	x	x		x		x	x	x	x	x	x	x	x	x	x	x	x	x
Adverse events	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Sot city and people interested in participating will be invited to contact the study team. In order to reach the planned sample size, volunteers interested to take part in the study will be asked to spread information to their network of acquaintances.

Assignment of intervention

Allocation sequence generation. A block randomization schedule will be generated using the *block.random* function of the R package *psych* version 1.8.12[32] with variables species (*An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* and *Ae. albopictus*) and dose (35 or 305 bites in total), yielding an ordered list of 15 blocks with 10 participants per block randomly assigned to one of the 10 study arms.

Allocation concealment mechanism. An allocation sequence will be implemented using individual, sealed and sequentially numbered envelopes. Following screening and eligibility assessment, participants will be assigned to a study arm during visit two using the randomization schedule.

Implementation. An allocation sequence will be generated by a study investigator. At the beginning of the study, the study coordinator will prepare a set of case report forms (CRFs) with preprinted subject identification codes and attach the sealed envelope containing intervention allocation to the CRF. Study nurses will then assign a subject identification code to participants by chronological order of enrollment in the study and the envelope will be opened during visit two.

Blinding. Allocation of intervention will be masked to outcome assessors (laboratory personnel who will process the serum samples) and data analysts. In order to do so, allocation of intervention in study datasets that contain this information will be masked until the results of the final analysis are made available to study investigators.

Data collection, management and analysis

Data collection methods. A screening panel will be constituted and the antibody titers against the candidate markers included in the panel will be determined with ELISA and electrochemiluminescence immunoassays[26,27]. Both measurements are being taken because different investigating centers implement different technologies, and because high-throughput assays will be ultimately needed to carry out large-scale vector serosurveillance studies. Moreover, electrochemiluminescence assays can be multiplexed and the analytical performances of this technology are sometime claimed to be superior to that of ELISA[33,34]. The specific titer of several immunoglobulin isotypes and subclasses (including IgM, IgG1-4, IgA and IgE) will be measured separately. The experimental conditions in the assays (quantity of peptide, serum dilution, incubation times etc...) are not yet known since they will require peptide-specific and laboratory-specific optimization.

The final composition of the screening panel is not known at this stage. In addition to biomarkers of exposure reported in the literature, candidate markers will be identified with antigen prediction algorithms using mosquito salivary proteins sequence data and with immunoproteomic assays using protein extract of dissected mosquito salivary glands and participants samples collected during visits four, 18 and 20[25,35]. In order to decipher antibody responses, tests will be carried out with salivary gland protein extracts, recombinant proteins and recombinant peptides (including orthologs in different mosquito species). However, the ultimate goal of this study is to identify short species-specific and genus-specific recombinant peptides that can be used for large-scale vector serosurveillance.

Antibody titers, assessed by measuring the optical density of immunoassay reactions, will be analyzed as a continuous outcome measure and used to define a binary responder status. Optical density measurements in test samples will be normalized over a blank reaction carried out with reaction buffer instead of the test sample in order to subtract noise from the signal. The seropositivity threshold of the assay will be set using reference serum specimens of individuals not exposed to bites of anopheline or aedine mosquitoes and defined as three standard deviations above the mean optical density measured in this unexposed population. Reactions with an optical density above this cut-off will be considered seropositive (*i.e.*, presence of antibodies recognizing the test peptide in participant serum sample). Participants with incomplete follow-up will be replaced to achieve a sample size of 15 participants per arm with complete follow-up, however, their data will still be included in the final analysis.

Data management. Data will be managed by a dedicated data management team composed of a data manager and data entry clerks independent from the study team. The data management team will design the CRF, build the study database, capture the data and perform quality checks. All study data with the exception of the results of the complete blood count (CBC) test will be recorded on the CRF and entered in the study database. The results of the CBC test, outputted by the machine and stored electronically in a separate laboratory database, will be linked to the study database using the subject identification code (printed results will also be attached to the CRF). The study database will be built using MACRO software and stored on a secured server. Double-entry of data critical to the analysis (age and sex of participants, allocation of intervention, visit dates and actual number of bites administered during each challenge) will be carried out. Validation rules allowing range checks for data values will be incorporated into the database during its design.

Statistical methods. The analysis of antibody responses to *Anopheles* salivary proteins will be carried out as follow. The change in individual antibody titers (as a continuous outcome and binary response) over time will be analyzed using generalized linear mixed-effects models. Optical density measurements (*i.e.*, antibody titers) will be analyzed using a Gaussian distribution family and identity link function (*i.e.*, a linear mixed-effects model) and binary responder status will be analyzed using a Binomial distribution family and a logit link function (*i.e.*, a logistic mixed-effects model). Age and sex of participant, mosquito species, number of bites,

follow-up period (baseline, exposure and post-exposure) and antibody type will be included as covariates in the mixed-effects models; a random effect (*i.e.*, intercept) will be included for participant to allow random variations of immune responses in individuals. In order to investigate if changes in antibody titers vary according to mosquito species or level of exposure, interaction terms will be fitted between time, species and number of bites, respectively. Estimates from the linear mixed-effects models will be used to calculate the half-lives of each antibody measure. Results obtained with capillary blood and serum specimens will be compared using the Bland and Altman method, to assess the reliability of assessing exposure with dried blood spots and point-of-care tests.

The analysis of antibody responses to *Aedes* salivary proteins will be descriptive and limited to before-after comparisons among exposed participants because participant exposure to *Aedes* bites not related to the study challenges could not be avoided.

Monitoring

Data monitoring. Monitoring of trial and safety data will be carried out by an internal data monitor and a safety review board independent of the study team and sponsor, following data and safety monitoring plans established before study initiation. Data monitoring will include checks of the investigator site file, consent forms, randomization, CRFs and study logbooks, adverse and serious adverse events, sample inventory and data entry. Checks will be carried out for each randomization block upon block closeout. Detected issues will be formally reported to the principal investigator and addressed by the study team following pre-specified timelines. The safety monitoring board will be composed of a medical doctor experienced in working with the border population, a biostatistician and a dermatologist. The safety monitoring board will meet within 7 days after the occurrence of any serious adverse event and issue a formal report of its recommendations to the trial executive committee and principal investigator, potentially leading to early stopping of the trial if deemed necessary to guarantee participant safety.

Harms. The main risk associated with this trial are hypersensitivity reactions to mosquito bites, skin infections and accidental transmission of mosquito-borne pathogens. Adverse events will be documented according to the standard definitions of the Common Terminology Criteria for Adverse Events guidelines[36]. Pre-specified adverse events include blood and lymphatic system disorders (leukocytosis 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 (papulopustular rash, rash pustular, sepsis, skin infection), injury 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). Adverse events will be diagnosed and graded by a medical doctor of the study team; study nurses will be trained to detect adverse events for the visits that do not include a physical examination by a medical doctor. Adverse events will be managed by the study team, who will refer participants to a secondary or tertiary care center if deemed necessary. Adverse events will be reported to the study sponsor and ethics committees following applicable regulation.

Auditing. An internal and external trial audit may be conducted at any time upon request by the sponsor or a third party.

Ethics and dissemination

Trial registration. This study is registered with clinicaltrials.gov (NCT04478370 on July 20th, 2020)[37]. The herein article reports the protocol version 2.0 dated June 14th, 2020.

Research ethics approval. The protocol, informed consent form, participant information sheet and consent form will be submitted and approved by the Oxford Tropical Research Ethics Committee, the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, the Alfred Hospital Ethics Committee, Burnet Institute and the Tak Community Advisory Board, a community-based committee assembling members of the communities in which the study will be performed[38].

Protocol amendments. Any modifications to the protocol which may impact on the conduct of the study, potential benefit of the participant or may affect participant safety, including changes of study objectives, study design, participant population, sample sizes, study procedures, or significant administrative aspects will require a formal amendment to the protocol. Such amendment will be agreed upon by the study group, approved by the Ethics Committee prior to implementation and notified to the health authorities in accordance with local regulations.

Consent. During the enrollment visit, study nurses will lead a group discussion with potential participants interested in taking part in the study and meet privately with individual volunteers. During both group and individual discussions, the participant information sheet and informed consent form will be presented to attendants in appropriate language (Thai, Karen or Burmese), detailing 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. Participants will be allowed as much time as they wish to consider the information, and the opportunity to question study team members or other independent parties to decide whether they will participate. Written informed consent will be obtained by asking the participant to sign and date the informed consent form. Illiterate participants 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.

Confidentiality. The CRF will be pseudonymized using the subject identification code, date of enrolment, initials, sex and

date of birth. Samples will be identified with a specimen number, subject identification code, initials and date. Other personal data (name, telephone number and address) will be recorded and stored separately in a password-protected electronic file stored on a secure server, allowing linkage of study data with participant's details by the study coordinator and site investigator using the subject identification code.

Access to data. Direct access to data will be granted to authorized representatives from the sponsor, Burnet Institute, A*STAR Infectious Diseases Labs, Walter Reed Army Institute of Research, Shoklo Malaria Research Unit, Mahidol-Oxford Research Unit, and any host institution, ethics committee and regulatory authorities for monitoring, audits and inspections of the study to ensure compliance with regulations.

Ancillary and post-trial care. The project is covered under the sponsorship of the University of Oxford. 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.

Dissemination policy. The scientific integrity of the project requires that the data from this study be analyzed study-wide and reported as such. All presentations and publications are expected to protect the integrity of the major objectives of the study; data that break the blind will not be presented prior to the release of mainline results. Each paper, abstract or presentation will be submitted to the principal investigator and other contributors for review of its appropriateness and scientific merit prior to submission. Every attempt will be made to reduce to an absolute minimum the interval between the completion of data collection and the release of the study results. The study results will be released to the participating physicians, referring physicians, patients and the general medical community as they become available during and after the study.

Study status

The study started on January 21st, 2021, and the last visit was on September 14th, 2022. Laboratory processing of the study samples is on-going.

Data availability

No data are associated with this article.

Reporting guidelines

Zenodo: SPIRIT checklist for 'Characterizing antibody responses to mosquito salivary antigens of the Southeast Asian vectors of malaria and dengue with a human challenge model of controlled exposure: a protocol'. https://doi.org/10.5281/zenodo.7703965.

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Open Peer Review

Current Peer Review Status: ✔ ? ?

Version 2

Reviewer Report 12 July 2023

https://doi.org/10.21956/wellcomeopenres.21758.r62568

✔ **Julien Pompon**
MIVEGEC, IRD, CNRS, Universite de Montpellier, Montpellier, Occitanie, France

The authors correctly addressed all my comments. I Approve the paper for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Flaviviral transmission, molecular entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 22 May 2023

https://doi.org/10.21956/wellcomeopenres.21120.r56423

? **Paolo Gabrieli**
[1] Department of Biosciences, Universita degli Studi di Milano, Milan, Lombardy, Italy
[2] Department of Biosciences, Universita degli Studi di Milano, Milan, Lombardy, Italy

The authors in this manuscript present the protocol to study the antibody response of humans to mosquito bites. The rationale is clear and adequate, but it lacks some details.

1. The authors put major attention on malaria. Because the chosen site lacks *Anopheles*

mosquito, they can use this population as naive or not exposed to *Anopheles* bites. They include an exclusion criteria about excluding people who traveled in the rural area in the last 12 months. Based on the data in literature[1] the antibodies levels seem to last for more than 12 months in some subjects. I would opt for a screening of the sera of volunteers before the enrolment as exclusion criteria, to minimise, as possible, subjects with detectable levels of antibodies.

2. Even if the absence is true for *Anopheles* mosquitoes, what is the situation for *Aedes* mosquitoes? I imagine that there are in the chosen country. How can the authors deal with that?

3. In the exclusion criteria, I would suggest to add also past history of malaria/dengue/Chikungunya or other mosquito-borne pathogens. Or history of undiagnosed fever with cutaneous rash.

4. Generally, the ELISA test is not clear to me. The authors talk about "candidate peptides". Do they test the response to whole salivary gland extracts first? Do they want to use synthetic peptides of immunogenic proteins? This is central in the study, because the authors claim to identify possible marker of exposure with this study. However, synthetic peptides do not always work[1] and immune response is really dependant on the single individual. How many markers do they plan to test for each species? How can decide if the marker is species specific or genus specific?

5. The authors talk about antibody response. Which class of antibodies they are going to test? Total IgG, IgG1, etc?

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Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?

No

Are the datasets clearly presented in a useable and accessible format?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mosquito saliva markers

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Jun 2023

Victor Chaumeau

We thank the reviewer for his feedback on the manuscript. A point-by-point answer to reviewer's comments is given below:

1. The authors put major attention on malaria. Because the chosen site lacks *Anopheles* mosquito, they can use this population as naive or not exposed to *Anopheles* bites. They include an exclusion criteria about excluding people who traveled in the rural area in the last 12 months. Based on the data in literature, the antibodies levels seem to last for more than 12 months in some subjects. I would opt for a screening of the sera of volunteers before the enrollment as exclusion criteria, to minimize, as possible, subjects with detectable levels of antibodies.

Response: To our knowledge, the decay rate of antibodies directed against mosquito salivary proteins is not known in detail and likely varies with the nature of the immunogenic peptide and previous exposure. Natural antibody responses to mosquito salivary proteins are indeed broad, variable, long-lasting and sometime cross-react with salivary proteins of multiple mosquito species. The aim of this study is to identify biomarkers of exposure that can be used to reflect changes in transmission dynamics in people who live in endemic areas. Therefore, we will try to identify immunogenic peptides that elicit short, sensitive and specific antibody responses. Baseline reactivity to salivary proteins that generate long-lasting antibody responses is, in our opinion, not a relevant exclusion criteria for this study.

2. Even if the absence is true for *Anopheles* mosquitoes, what is the situation for *Aedes* mosquitoes? I imagine that there are in the chosen country. How can the authors deal with that?

Response: Avoiding participant exposure to mosquito bites not related to the biting challenges is indeed key to the design of this study, which is focused on malaria. Unlike *Anopheles* mosquitoes, *Aedes* mosquitoes are ubiquitous and therefore it will not be possible to avoid participant exposure to *Aedes* bites. This is a limitation to the evaluation of antibody responses to *Aedes* salivary proteins which will be treated as a secondary outcome. The evaluation of antibody responses to *Aedes* salivary proteins will be descriptive and limited to before/after comparisons. This point has been clarified in the revised version of the manuscript.

Revision to the paragraph Study setting: "Unlike *Anopheles* mosquitoes, *Aedes* are ubiquitous and it will not be possible to avoid participant exposure to *Aedes* bites not related to the study challenges. This is a limitation to the evaluation of antibody responses to *Aedes* salivary proteins, which will be treated as a secondary outcome and analyzed descriptively."

Revision to the paragraph Statistical methods: "The analysis of antibody responses to *Aedes* salivary proteins will be descriptive and limited to before-after comparisons among exposed participants because participant exposure to *Aedes* bites not related to the study challenges could not be avoided."

3. In the exclusion criteria, I would suggest to add also past history of malaria/dengue/Chikungunya or other mosquito-borne pathogens. Or history of undiagnosed fever with cutaneous rash.

Response: It is not clear to us why the reviewer suggests to exclude people with previous mosquito-borne infection from the study. To our knowledge, the effect of previous mosquito-borne infection on baseline serum reactivity to mosquito salivary proteins has not been described precisely. Excluding people with previous history of mosquito-borne infection would have been unreliable since many people have only limited access to health care and many infections are asymptomatic. Moreover, it would have created difficulties in recruiting participants in this population where dengue and malaria are endemic, and where outbreaks of Chikungunya have occurred in recent years.

4. Generally, the ELISA test is not clear to me. The authors talk about "candidate peptides". Do they test the response to whole salivary gland extracts first? Do they want to use synthetic peptides of immunogenic proteins? This is central in the study, because the authors claim to identify possible marker of exposure with this study. However, synthetic peptides do not always work and immune response is really dependent on the single individual. How many markers do they plan to test for each species? How can decide if the marker is species specific or genus specific?

Response: In order to decipher antibody responses, whole gland extract, saliva, recombinant proteins and recombinant peptides will be tested in ELISA and electrochemiluminescence screens. There are several advantages and limitations associated with the use of either whole gland extract and recombinant peptides. In general, salivary gland protein extracts are impractical to use in large-scale epidemiological studies. Therefore, the ultimate goal of this study is to identify short species-specific and genus-specific recombinant peptides. Detailed information on the screening panel is not known at this stage. The number and nature of salivary proteins tested will depend on the published literature, the output of in silico B-cell epitope predictions and immunoproteomic experiments. The comparison of antibody responses in participants of the different study arms will be used to assess cross-reactivity of the antibody responses to different mosquito species or genus (see Statistical Methods: "In order to investigate if changes in antibody titers vary according to mosquito species or level of exposure, interaction terms will be fitted between time, species and number of bites, respectively." The reviewer's point has been clarified in the revised version of the manuscript.

Revision to the paragraph Data collection methods: In order to decipher antibody responses, tests will be carried out with salivary gland protein extracts, recombinant proteins and recombinant peptides (including orthologs in different mosquito species). However, the ultimate goal of this study is to identify short species-specific and genus-

specific recombinant peptides that can be used for large-scale vector serosurveillance.

5. The authors talk about antibody response. Which class of antibodies they are going to test? Total IgG, IgG1, etc?

Response: We are going to investigate different classes and subtypes of immunoglobulins including IgM, IgG1, IgG2, IgG3, IgG4 and IgA. This has now been clarified in the revised version of the manuscript.

Revision to the paragraph Data collection methods: The specific titer of several immunoglobulin isotypes and subclasses (including IgM, IgG1-4, IgA and IgE) will be measured separately.

Competing Interests: The authors had no competing interest to disclose.

Reviewer Report 12 May 2023

https://doi.org/10.21956/wellcomeopenres.21120.r56424

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Cassandra Modahl
[1] Centre for Snakebite Research and Interventions, Liverpool Institute of Tropical Medicine, Liverpool, UK
[2] Centre for Snakebite Research and Interventions, Liverpool Institute of Tropical Medicine, Liverpool, UK

The clinical trial protocol “Characterizing antibody responses to mosquito salivary antigens of the Southeast Asian vectors of malaria and dengue with a human challenge model of controlled exposure” reports on a study being conducted involving the controlled exposure of individuals to mosquito bites and the quantification of antibody responses to mosquito salivary proteins over time and in relation to the number of bites. I have the following suggestions for improvement:

Abstract:
What is the high-throughput cutting-edge immuno-assay? In the Data collection, management and analysis it details a high-throughput ELISA and mesoscale screening assay (using electrochemiluminescence I assume from the cited paper). Is there a reason why both of these measurements are being taken, and which one is being referenced in the abstract?

Intervention:
Are these bite numbers realistic? How many bites maximum is an average individual exposed to? Bites are also being assessed by if a mosquito has successfully blood-fed, but there could also be exposure to mosquito salivary proteins from unsuccessful blood-feeding attempts.

Why such a focus on avoiding *Anopheles* bites when *Aedes* responses are also being evaluated?

Recruitment:
If recruitment is only being done by word of mouth, will this create any bias? Will there be any considerations to making sure there are equal numbers of recruited males and females, and ages?

Data collection, management, and analysis:
Could more detail be provided regarding the antigens? For the biomarkers of exposure previously reported in literature, will recombinant forms of these antigens be used? For the antigens identified using prediction algorithms, is it is just the antigen epitopes that will be synthesized and used to coat the ELISA plates? Will these be specific for each species?

In the case of the salivary gland extracts, there will likely be a lot of non-salivary proteins present in the mixture too as there are housekeeping proteins present in salivary gland tissues that are not secreted into the saliva. These housekeeping proteins will likely share sequence conservation across the tested mosquito species, making it difficult to evaluate vector species-specific antibody responses.

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Partly

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Proteomics, immunology, neglected tropical diseases, bioinformatics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Jun 2023

Victor Chaumeau

We thank the reviewer for her feedback on the manuscript. A point-by-point answer to reviewer's comments is given below: What is the high-throughput cutting-edge immunoassay? In the Data collection, management and analysis it details a high-throughput ELISA and mesoscale screening assay (using electrochemiluminescence I

assume from the cited paper). Is there a reason why both of these measurements are being taken, and which one is being referenced in the abstract?

Response: The wording high-throughput cutting-edge immunoassay has been revised to ELISA and electrochemiluminescence immunoassays, and an explanation of the reason why both of these measurements are being taken is now provided.

Revision to the paragraph Data collection methods – "A screening panel will be constituted and the antibody titers against the candidate markers included in the panel will be determined with ELISA and electrochemiluminescence immunoassays [26, 27]. Both measurements are being taken because different investigating centers implement different technologies, and because high-throughput assays will be ultimately needed to carry out large-scale vector serosurveillance studies. Moreover, electrochemiluminescence assays can be multiplexed and the analytical performances of this technology are sometime claimed to be superior to that of ELISA [33, 34]. Are these bite numbers realistic? How many bites maximum is an average individual exposed to? Bites are also being assessed by if a mosquito has successfully blood-fed, but there could also be exposure to mosquito salivary proteins from unsuccessful blood-feeding attempts.

Response: Yes, the numbers of bites used in this study are realistic. These numbers are based on previous entomological investigations conducted in this area and published reports of human challenge with mosquito bites. Additional details and references to the published literature were added in the revised version of the manuscript (see our response to comment 4 from reviewer Julien Pompom). We acknowledge the reviewer's point on the potential bias of assessing the number of bites from blood-feed mosquitoes. The methodology was chosen because it will not be possible to directly observe and record the actual number of bites received by participants during the challenges. We assumed that the bias will be distributed at random across participants and challenges. Moreover, the number of bites in the high exposure group is one order of magnitude bigger than in the low exposure group. This difference was deemed large enough to ensure that the number of bites in the low exposure group will be actually smaller than in the high exposure group, despite the random bias associated with counting blood-fed mosquitoes as a proxy of the number of bites. Why such a focus on avoiding *Anopheles* bites when *Aedes* responses are also being evaluated?

Response: Avoiding participant exposure to mosquito bites not related to the biting challenges is indeed key to the design of this study, which is focused on malaria. Unlike *Anopheles* mosquitoes, *Aedes* mosquitoes are ubiquitous and therefore it will not be possible to avoid participant exposure to *Aedes* bites. This is a limitation to the evaluation of antibody responses to *Aedes* salivary proteins which will be treated as a secondary outcome. The evaluation of antibody responses to *Aedes* salivary proteins will be descriptive and limited to before/after comparisons. This point has been clarified in the revised version of the manuscript.

Revision to the paragraph Study setting: "Unlike *Anopheles* mosquitoes, *Aedes* are ubiquitous and it will not be possible to avoid participant exposure to *Aedes* bites not related to the study challenges. This is a limitation to the evaluation of antibody responses to *Aedes*

salivary proteins, which will be treated as a secondary outcome and analyzed descriptively."

Revision to the paragraph Statistical methods: "The analysis of antibody responses to *Aedes* salivary proteins will be descriptive and limited to before-after comparisons among exposed participants because participant exposure to *Aedes* bites not related to the study challenges could not be avoided."

If recruitment is only being done by word of mouth, will this create any bias? Will there be any considerations to making sure there are equal numbers of recruited males and females, and ages?

Response: No, we don't think that recruiting participants by word of mouth will be a significant source of bias in this study. Age and sex will not be taken into account in the randomization procedure because the sample size is too small and it will create difficulties in enrolling participants, but they will be included as fixed effect covariates in the models to adjust for their potential effects on outcome measures.

Could more detail be provided regarding the antigens? For the biomarkers of exposure previously reported in literature, will recombinant forms of these antigens be used? For the antigens identified using prediction algorithms, is it is just the antigen epitopes that will be synthesized and used to coat the ELISA plates? Will these be specific for each species?

Response: Detailed information on the antigens is not known at this stage. In order to decipher antibody responses, tests will be carried out with salivary gland protein extracts, recombinant proteins and recombinant peptides (including orthologs in different mosquito species). However, the ultimate goal of this study is to identify short species-specific and genus-specific recombinant peptides that can be used for large-scale vector serosurveillance. More details were added in the revised version of the manuscript to address the reviewer's point.

Revision to the paragraph Data collection methods: In order to decipher antibody responses, tests will be carried out with salivary gland protein extracts, recombinant proteins and recombinant peptides (including orthologs in different mosquito species). However, the ultimate goal of this study is to identify short species-specific and genus-specific recombinant peptides that can be used for large-scale vector serosurveillance.

In the case of the salivary gland extracts, there will likely be a lot of non-salivary proteins present in the mixture too as there are housekeeping proteins present in salivary gland tissues that are not secreted into the saliva. These housekeeping proteins will likely share sequence conservation across the tested mosquito species, making it difficult to evaluate vector species-specific antibody responses.

Response: We acknowledge the reviewer's point about the limitations of using salivary gland protein extracts to assess exposure to mosquito bites and mosquito saliva may be used as an alternative. There are other limitations which make salivary gland and saliva extracts impractical to use for large-scale studies. Therefore, the ultimate goal of this study is to identify short species-specific and genus-specific recombinant peptides.

Competing Interests: The authors had no competing interest to disclose

Reviewer Report 17 April 2023

https://doi.org/10.21956/wellcomeopenres.21120.r55743

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Julien Pompon
[1] MIVEGEC, IRD, CNRS, Universite de Montpellier, Montpellier, Occitanie, France
[2] MIVEGEC, IRD, CNRS, Universite de Montpellier, Montpellier, Occitanie, France

The authors present a protocol to identify the immunogenic antigens in mosquito saliva. The objectives are sound and the protocol is clearly written and of interest to the communities studying viral transmission by mosquitoes. However, there are a few points that need to be addressed before indexing:

In Introduction:

1. There is no mention of the two vaccines against DENV that have been licenced. Please mention them and explain why the uptake among patients is limited.

In Assignment of intervention:

1. There is a confusion between 15 study arms with 10 participants in each. It is the opposite...

General points:

1. The participants will be selected from an urban area where they should not have been bitten by *Anopheles*. The absence of *Anopheles* bite based on geographic restriction is not very strong and there should be another way to control that the participants did not experience *Anopheles* bites. The authors could test the response to *Anopheles* salivary proteins before the challenge, for instance.

2. Why 35 and 305? The authors should explain why they selected this number of bites. For instance, compare these bites with the average number of bites in this region of the world and elsewhere.

3. Recruitment will be done through word of mouth. It seems, but maybe I am wrong, that they overestimate the recruitment power of word of mouth. What about advertising in social media? They would also recruit a more diverse crowd of participants.

4. It is not clear in the study if the authors will measure the antibodies to salivary proteins from all mosquito species or just against *Anopheles* salivary proteins; please clarify in the text.

5. More details should be given about the methods to quantify the antibody response, among

others the number of salivary proteins tested, their quantity, volume of serum...

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Flaviviral transmission, molecular entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 23 Jun 2023

Victor Chaumeau

We thank the reviewer for his feedback on the manuscript. A point-by-point answer to reviewer's comments is given below:

1. There is no mention of the two vaccines against DENV that have been licensed. Please mention them and explain why the uptake among patients is limited.

Response: Mention of the two vaccines against DENV that have been licensed and explanation of the reasons why the uptake among patients is limited have now been added to the revised version of the manuscript.

Revision to the paragraph Background and rationale – "Two vaccines against dengue viruses were licensed but the uptake among patient is limited: one is indicated in children with previous infection and the other is licensed only in Europe."

1. There is a confusion between 15 study arms with 10 participants in each. It is the opposite...

Response: We have reviewed the manuscript and cannot find any text specifically stating "15 study arms". We confirm that the sentence "A block randomization schedule will be generated using the block.random function of the R package psych version 1.8.12 [32] with variables species (*An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* and *Ae. albopictus*) and dose (35 or 305 bites in total), yielding an ordered list of 15 blocks with 10 participants per block randomly assigned to one of the 10 study arms." is correct.

1. The participants will be selected from an urban area where they should not have been bitten by *Anopheles*. The absence of *Anopheles* bite based on geographic

restriction is not very strong and there should be another way to control that the participants did not experience *Anopheles* bites. The authors could test the response to *Anopheles* salivary proteins before the challenge, for instance.

Response: Three blood samples will be collected from each participant at baseline (before the mosquito challenges). In the analysis, baseline reactivity will be included to take into account previous exposure by including a random intercept for participant to allow the immune response at baseline to vary between individuals (see paragraph Statistical Methods).

1. Why 35 and 305? The authors should explain why they selected this number of bites. For instance, compare these bites with the average number of bites in this region of the world and elsewhere.

Response: The numbers of bites used in this study were chosen based on previous entomological investigations conducted in this area and published reports of human challenge with mosquito bites. Justification of the number of bites and references to the published literature were added in the revised version of the manuscript.

Revision to the paragraph Intervention – "The numbers of bites were chosen based on previous entomological investigations conducted in this area [8] and published reports of human challenge with mosquito bites [29, 30, 31]."

1. Recruitment will be done through word of mouth. It seems, but maybe I am wrong, that they overestimate the recruitment power of word of mouth. What about advertising in social media? They would also recruit a more diverse crowd of participants.

Response: Based on our experience in working with the population on the Thailand-Myanmar border, social media were deemed inappropriate for advertising the study because they reach mostly adolescent and young adults, and exclude illiterate people and people who don't use social media (an important proportion of the study population). Word of mouth has been used to enroll study participants in many of our studies and was not associated with difficulties in recruiting.

1. It is not clear in the study if the authors will measure the antibodies to salivary proteins from all mosquito species or just against *Anopheles* salivary proteins; please clarify in the text.

Response: We will measure antibody responses to salivary proteins from all mosquito species. This has been clarified in the revised version of the manuscript.

Revision to the paragraph Outcomes – "The primary outcome of this study will be the participant antibody responses to Anopheles salivary proteins. The secondary outcome of this study will be the participant antibody responses to *Aedes* salivary proteins."

1. More details should be given about the methods to quantify the antibody response, among others the number of salivary proteins tested, their quantity, volume of serum

Response: Detailed information about the methods to quantify antibody responses is not known at this stage. The number of salivary proteins tested will depend on the published literature, the output of *in silico* B-cell epitope predictions and immunoproteomic experiments. The experimental conditions in the assays (quantity of peptide, serum dilution, incubation times etc.) will require peptide-specific and laboratory-specific optimization. This was clarified in the revised version of the manuscript.

Revision to the paragraph Data collection methods – “A screening panel will be constituted and the antibody titers against the candidate markers included in the panel will be determined with ELISA and electrochemiluminescence immunoassays [26, 27]. Both measurements are being taken because different investigating centers implement different technologies, and because high-throughput assays will be ultimately needed to carry out large-scale vector serosurveillance studies. Moreover, electrochemiluminescence assays can be multiplexed and the analytical performances of this technology are sometime claimed to be superior to that of ELISA [33, 34]. The specific titer of several immunoglobulin isotypes and subclasses (including IgM, IgG1-4, IgA and IgE) will be measured separately. The experimental conditions in the assays (quantity of peptide, serum dilution, incubation times etc…) are not yet known since they will require peptide-specific and laboratory-specific optimization. The final composition of the screening panel is not known at this stage. In addition to biomarkers of exposure re ported in the literature, candidate markers will be identified with antigen prediction algorithms using mosquito salivary proteins sequence data and with immunoproteomic assays using protein extract of dissected mosquito salivary glands and participants samples collected during visits four, 18 and 20 [35, 25].”

Competing Interests: The authors had no competing interest to disclose.

Author Response 23 Jun 2023

Victor Chaumeau

We thank the reviewer for his feedback on the manuscript. A point-by-point answer to reviewer’s comments is given below:

1. There is no mention of the two vaccines against DENV that have been licensed. Please mention them and explain why the uptake among patients is limited.

Response: Mention of the two vaccines against DENV that have been licensed and explanation of the reasons why the uptake among patients is limited have now been added to the revised version of the manuscript.

Revision to the paragraph Background and rationale – “Two vaccines against dengue viruses were licensed but the uptake among patient is limited: one is indicated in children with previous infection and the other is licensed only in Europe.”

2. There is a confusion between 15 study arms with 10 participants in each. It is the opposite…

Response: We have reviewed the manuscript and cannot find any text specifically stating “15 study arms”. We confirm that the sentence “A block randomization schedule will be generated using the block.random function of the R package psych version 1.8.12 [32] with variables species (*An. minimus*, *An. maculatus*, *An. dirus*, *Ae. aegypti* and *Ae. albopictus*) and dose (35 or 305 bites in total), yielding an ordered list of 15 blocks with 10 participants per block randomly assigned to one of the 10 study arms.” is correct.

3. The participants will be selected from an urban area where they should not have been bitten by *Anopheles*. The absence of *Anopheles* bite based on geographic restriction is not very strong and there should be another way to control that the participants did not experience *Anopheles* bites. The authors could test the response to *Anopheles* salivary proteins before the challenge, for instance.

Response: Three blood samples will be collected from each participant at baseline (before the mosquito challenges). In the analysis, baseline reactivity will be included to take into account previous exposure by including a random intercept for participant to allow the immune response at baseline to vary between individuals (see paragraph Statistical Methods).

4. Why 35 and 305? The authors should explain why they selected this number of bites. For instance, compare these bites with the average number of bites in this region of the world and elsewhere.

Response: The numbers of bites used in this study were chosen based on previous entomological investigations conducted in this area and published reports of human challenge with mosquito bites. Justification of the number of bites and references to the published literature were added in the revised version of the manuscript.

Revision to the paragraph Intervention – "The numbers of bites were chosen based on previous entomological investigations conducted in this area [8] and published reports of human challenge with mosquito bites [29, 30, 31]."

5. Recruitment will be done through word of mouth. It seems, but maybe I am wrong, that they overestimate the recruitment power of word of mouth. What about advertising in social media? They would also recruit a more diverse crowd of participants.

Response: Based on our experience in working with the population on the Thailand-Myanmar border, social media were deemed inappropriate for advertising the study because they reach mostly adolescent and young adults, and exclude illiterate people and people who don't use social media (an important proportion of the study population). Word of mouth has been used to enroll study participants in many of our studies and was not associated with difficulties in recruiting.

6. It is not clear in the study if the authors will measure the antibodies to salivary proteins from all mosquito species or just against *Anopheles* salivary proteins; please clarify in the text.

Response: We will measure antibody responses to salivary proteins from all mosquito species. This has been clarified in the revised version of the manuscript.

Revision to the paragraph Outcomes – "The primary outcome of this study will be the participant antibody responses to Anopheles salivary proteins. The secondary outcome of this study will be the participant antibody responses to Aedes salivary proteins."

7. More details should be given about the methods to quantify the antibody response, among others the number of salivary proteins tested, their quantity, volume of serum

Response: Detailed information about the methods to quantify antibody responses is not known at this stage. The number of salivary proteins tested will depend on the published literature, the output of *in silico* B-cell epitope predictions and immunoproteomic experiments. The experimental conditions in the assays (quantity of peptide, serum dilution, incubation times etc.) will require peptide-specific and laboratory-specific optimization. This was clarified in the revised version of the manuscript.

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Competing Interests: The authors had no competing interest to disclose.