National Institute of Allergy and Infectious Diseases (NIAID)
National Institutes of Health (NIH)

Protocol Face Sheet

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Protocol Title:	Characterization of skin immunity to *Aedes aegypti* saliva in dengue-endemic participants in Cambodia
Abbreviated Title:	PINCH
Institute Name:	NIAID
Accrual Period:	3 months
Proposed Dates:	01 May 2020 – 01 May 2022
Total Subjects to be Accrued:	42
Ionizing Radiation Use:	None
Is Tissue Being Collected for Research Purposes?	Yes (whole blood, sera, and skin punch biopsy)
Location of the Study	Kampong Speu Referral Hospital, Chbar Mon, Kampong Speu, Cambodia
Investigational New Drug/Device:	None

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STATEMENT OF COMPLIANCE

The trial will be carried out in accordance with International Council on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Good Clinical Practice (GCP) and the following:

- US Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46)

NIH-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection and ICH GCP Training.

The protocol, informed consent form (ICF), recruitment materials, and all participant materials will be submitted to the NIH Intramural Institutional Review Board (IRB) and Cambodian National Ethics Committee for Health Research (NECHR) for review and approval. Approval of both the protocol and the ICF must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the NIH IRB and NECHR before the changes are implemented to the study. In addition, all changes to the ICF will be approved by the NIH IRB and NECHR; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved ICF.

1 PROTOCOL SUMMARY

1.1 SYNOPSIS

Title:	Characterization of skin immunity to *Aedes aegypti* saliva in dengue-endemic participants in Cambodia
Study Description:	This is a single-center study characterizing the innate and adaptive immune response of endemic human skin to *Aedes aegypti* mosquito bites. Participants will undergo vector feeding and have a skin punch biopsy taken from the site of mosquito bites (exposed) and from unbitten skin (unexposed). Additionally, blood will be collected after feeding to evaluate systemic immune response. All protocol-specified data will be recorded and entered in a central data management system for the purposes of analysis of composite data from the study. Please see **Schema and Schedule of Activities (SoA), Sections 1.2 and 1.3**.
Study Intervention Description:	Controlled feeding by colony-reared *Aedes aegypti* mosquitoes.
Objectives:	The primary objectives of the study are to: 1. Compare the early and late innate immune response in the skin of *Aedes aegypti*–bitten (exposed) versus unbitten (unexposed) skin 2. Characterize the local skin adaptive immune response The secondary objective is to:

1. Analyze systemic immune response to vector salivary proteins in endemic participants with lifelong *Aedes aegypti* exposure after antigen encounter

The exploratory objectives are to:

1. Describe the development of vector saliva–specific tissue-resident memory responses
2. Validate development of reliable biomarkers of vector exposure

Endpoints:

The primary endpoints are:

1. Measurement of changes in the early and late innate immune response and cellular recruitment in bitten skin versus unbitten skin by:
    a. immunohistochemistry of target proteins at Day 0 timepoints
    b. immunophenotyping of innate immune cell subsets in dissociated skin sample at Day 0 timepoints
    c. determination of cytokine profile in dissociated skin sample supernatant at Day 0 timepoints
    d. differential complementary DNA (cDNA) expression prepared from skin RNA and analyzed via RNA sequencing (RNASeq) at Day 0 timepoints
2. Measurement of changes in the adaptive immune response and cellular recruitment bitten skin versus unbitten skin by:
    a. immunohistochemistry of target proteins at Day 2 timepoints
    b. phenotyping of adaptive immune cell subsets in dissociated skin sample at Day 2 timepoints
    c. determination of cytokine profile in dissociated skin sample supernatant at Day 2 timepoints
    d. differential cDNA expression prepared from skin RNA and analyzed via RNASeq at Day 2 timepoints

The secondary endpoint is:

1. Flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) collected Day 0 (baseline) and Days 2 and 14 after feeding for saliva-specific T cells

The exploratory endpoints are:

1. Comparison of T-cell receptor (TCR) sequencing of skin biopsies at Day 0 versus Day 2
2. Antibody determination in sera at Day 0 and Day 14

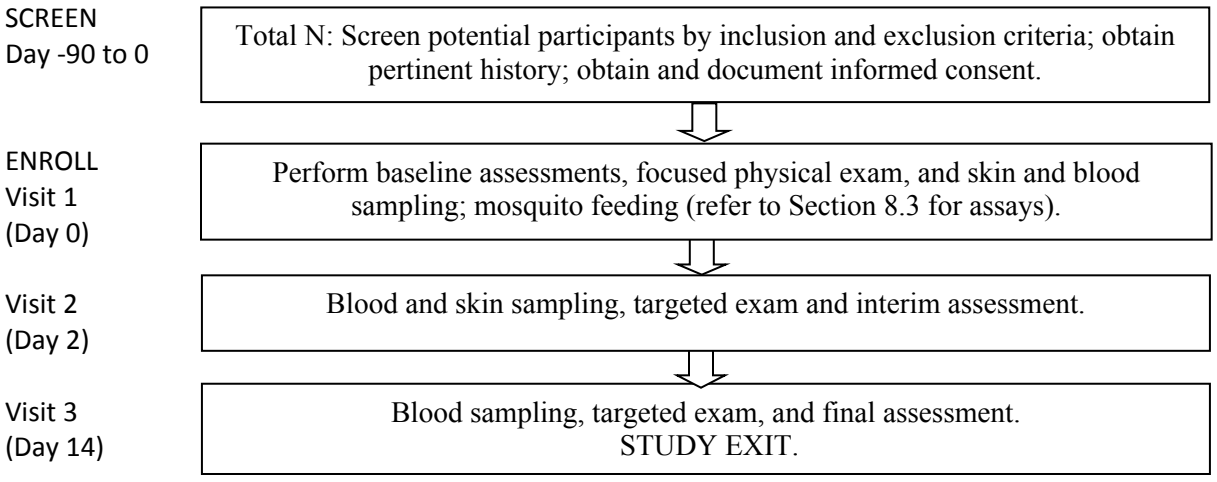
Study Population:	Healthy adults aged 18 to 45 years (at study entry)
Total Enrollment:	42 subjects
Description of Sites/Facilities Enrolling Participants:	Kampong Speu District Referral Hospital, Chbar Mon town, Cambodia
Study Duration:	24 months
Participant Duration:	2 weeks

1.2 PRECIS

Mosquito-borne viruses continue to cause significant global morbidity and mortality, particularly in Southeast Asia. When mosquitoes deliver the virus into the skin of humans while probing for a blood meal, they deposit also saliva, which contains a myriad of pharmacologically active compounds that modulate the host immune system. Little is known about skin immunity to mosquito saliva, particularly in endemic volunteers as most clinical studies are performed in naïve individuals who have never or rarely been exposed to a particular mosquito vector. People living in endemic areas have had long-term repeated exposure to these vectors and therefore have different immune response to mosquito saliva, which could interfere with mosquito-borne disease vaccine effectiveness. Characterization of skin immunity via various technical modalities will be important in order to identify critical aspects of the innate and adaptive immune responses after a vector bite.

Here, we will execute a paired study of exposed-unexposed skin to carefully examine the innate and adaptive immune responses in the skin and blood to exposure of the saliva of *Aedes aegypti*, the mosquito vector of dengue, Zika, and chikungunya viruses. We will enroll 42 participants to undergo vector feeding and give blood samples at baseline and 2 and 14 days later. Additionally, participants will give skin punch biopsy samples of bitten (exposed) and unbitten (unexposed) skin. For analysis, we will group 10-12 participants in each of 4 technical modality "cohorts" or "groups":
1) immunohistochemistry, 2) RNA sequencing, 3) flow cytometry, and 4) T-cell receptor sequencing. With the current rise of vector-borne diseases in the United States and around the world, we hope the results of this study contribute to future vaccine design and clinical development strategies for vector-borne diseases.

Schema 1: Study Flow Design



1.3 SCHEDULE OF ACTIVITIES (SOA)

Procedures	Screen (Day -90 to 0)	Enroll/Visit 1 (Day 0)	Visit 2 (Day 2) + 1 day	Visit 3 (Day 14) ±3 days
Informed Consent[1]	X			
Medical/Medication History	X	X		
Review of Eligibility Criteria	X	X		
Documentation of Usual Bite Response	X			
Clinician Assessment	X	X	X	X
Vital Signs[2]	X	X	X	X
Urine Pregnancy Test[3]	X	X		
Mosquito Feeding		X		
Skin Punch Biopsy		X[4]	X[5]	
Blood Draw[6]		X		X
SKIN BIOPSIES[7]				
Immunohistochemistry		X	X	
RNASeq		X	X	
Flow Cytometry		X	X	
TCR sequencing		X	X	
BLOOD SAMPLING				
Whole blood for PBMC isolation		X	X	X
Sera for antibody determination		X		X

Abbreviations: PBMC, peripheral blood mononuclear cell; RNASeq, RNA sequencing; TCR, T-cell receptor.

[1] Informed consent will be obtained prior to any study procedures.

[2] Vital signs include blood pressure, heart rate, respiratory rate, and temperature; height and weight are taken at Day 0 visit only.

[3] Females of childbearing potential only. Mosquito feeding will not be initiated until results are available.

[4] Two biopsies from bitten skin will be collected: one no earlier than 30 minutes and no later than 90 minutes after the feeding is completed, and one no earlier than 3.5 hours and no later than 4.5 hours after the feeding is completed. Two biopsies from unbitten skin on the opposite arm will be collected no earlier than 30 minutes before feeding and no later than 6 hours after the feeding is completed.

[5] One biopsy will be collected from bitten skin at least 40 hours after the feeding is completed.

[6] **Blood volume will not exceed 21 mL per visit;** each whole blood collection will be 16 mL in a heparin tube, and each serum collection will be 5 mL in a serum-separating tube (SST).

[7] Participants will be assigned to one of the following cohorts for skin sample technical evaluation modality: 10 participants to immunohistochemistry; 10 participants to RNASeq; 12 participants to flow cytometry; 10 participants to TCR sequencing.

2 INTRODUCTION

2.1 STUDY RATIONALE

Responsible for nearly one million deaths per year,[1] vector-borne diseases are on the rise.[2] Vector-borne disease occurs when a pathogen is transmitted by the bite of an infected blood-feeding arthropod such as a mosquito, fly, mite, flea, bug, or tick. Effective vaccines are needed to counter this global public health threat. At present, research efforts are mostly focused on the pathogen-host interactions without acknowledging the significant contribution of vector-derived products to disease development. Yet, as early as the 1940s, physicians observed humans' variable clinical responses to mosquito saliva and surmised that it may contain molecules with immunomodulatory capabilities.[3] However, not until the 1970s did scientists recognize the potential role of vector saliva in pathogen transmission.[4] Now, some attribute the complications of vector-borne disease vaccine development to the pleiotropic effects of vector saliva on both host and pathogen.[5] Pathogen-host interactions are complex in any disease setting but become more nuanced with the addition of the vector, a previously underappreciated disease determinant that is increasingly recognized as a vaccine target.[6–8]

For the few vaccines licensed for arthropod vector-borne disease and for the majority of the candidates in the pipeline, the focus is exclusively on the pathogen. However, vaccine development for these diseases may lie at the unique interface of the hematophagous insect vector, the pathogen, and the human host. The opportunity for vaccine development to disrupt disease transmission at "the bite site," where the host, pathogen, and vector initially intersect, is gaining traction.[7–9] Given the growing body of evidence of this concept in animal models, this protocol aims to achieve broader, translational understanding of cutaneous host-vector interactions via characterization of downstream immune cascades after exposure of endemic human skin to mosquito saliva. Specifically, we will carefully examine the early immune response to bites of *Aedes aegypti* mosquitoes in humans and explore how intense exposure in endemic areas may modulate future local skin responses, potentially affecting pathogen transmission and vaccine efficacy. We hypothesize that bites of arthropod vectors of disease egest specific vector-derived factors, including saliva,[10] that modulate the innate immune response in individuals and recall a saliva-specific adaptive immune response to the bite site, thereby altering the outcome of disease caused by pathogens transmitted by these vectors and potentially the immune response to vaccines in endemic populations. Results from this study will provide a better understanding of the role that vector-derived factors play in vector-borne infections in humans who live in endemic areas. Additionally, the findings will be essential in informing future vaccine design and clinical development for both well-established vector-borne diseases like dengue virus (DENV) as well as emerging threats requiring the rapid development of countermeasures like Zika virus (ZIKV) and chikungunya virus (CHIKV).[11,12]

2.2 BACKGROUND

Mosquito-borne disease is increasing worldwide, particularly in Southeast Asia.[1,12] Cambodia has multiple mosquito-borne diseases including DENV, CHIKV, ZIKV, *Plasmodium vivax* and *falciparum* malaria, and Japanese encephalitis virus (JEV).[13] With regard to *Aedes*-transmitted disease, DENV is the most common and is highly endemic with year-round transmission that peaks from July to November when rainfall is at its highest.[14,15] In 2019, Cambodia experienced its worst outbreak in decades with DENV incidence peaking at nearly 5,000 cases of symptomatic DENV per week (National Dengue Control

Program communication 2019).

Preliminary evidence from our ongoing pediatric cohort in Cambodia suggests intense exposure to *Aedes aegypti* mosquitoes particularly during the rainy season in July and August as evidenced by antibody reactivity against whole salivary gland homogenate of *Aedes aegypti* as measured by total immunoglobulin G enzyme-linked immunosorbent assay (ELISA). Others' retrospective studies of human sera have demonstrated the usefulness of anti-*Aedes* salivary gland extract antibodies as 1) a marker of *Aedes* exposure and 2) a general corollary of DENV transmission potential.[16–20]

2.2.1 *SCIENTIFIC* RATIONALE

When a mosquito inserts its proboscis and probes for blood, the mosquito ejects a salivary mix of vasodilators, anticoagulants, and other anti-hemostatic components into both the epidermis and the dermis.[7] In addition to components that facilitate a bloodmeal, mosquito saliva also contains proteins that modulate both innate and adaptive immune responses. Feeding by *Aedes* and *Culex* mosquitoes in C3H/HeJ mice downregulated T helper type 1 (Th1) cytokines like IFN-gamma and upregulated Th2 cytokines such as IL-4 and IL-10, cytokine shifts that were not seen when mosquitoes fed on flavivirus-resistant host mice.[21] Increased Th2 response is the cause of irritation and allergic reaction seen with mosquito bites.[9] While there is decreased T-cell recruitment to the site of injury after mosquito feeding, there is increased recruitment of neutrophils, eosinophils, and dendritic cells.[5] However, with repeated exposures to the vector's bites as one would expect in endemic areas, animals became desensitized to some components of the saliva and were able to mount a more effective proinflammatory Th1 response, presumably owing to the presence of anti-saliva antibodies.[23,25,26] Of the few human studies available, the majority of endemic participants in Mali had strong Th1-polarizing delayed type hypersensitivity reactions to sand fly saliva, suggesting a role for the adaptive immune response that will be investigated in this study with a 48-hour timepoint biopsy.[27]

Multiple arboviral studies in mice have demonstrated that the presence of saliva or salivary gland extract increases pathogenesis and dissemination of viruses. When a pathogen like DENV is present, mosquito saliva can facilitate the infectivity of an arbovirus by dampening the host's anti-viral Th1 immune response.[23,28–30] Further, codeposition of mosquito saliva with an offending pathogen allows the pathogen to enter the host and more easily replicate.[22,29,30,32,33] Mice inoculated intradermally with West Nile virus in the presence of *Culex tarsalis* saliva (via a mosquito feeding or co-inoculation) developed higher viral loads and faster onset of neuroinvasive disease compared to mice who were inoculated intradermally with virus alone.[34] LMVR investigators have shown that the immune profile and cellular recruitment at the injection/bite site of animals infected by needle compared with those infected by bites of infected sand flies are different.[35] Animals bitten by *Leishmania*-infected sand flies activated the host inflammasome and displayed an increased recruitment of neutrophils that was sustained for more than 18 hours, a completely different innate response than animals who received parasite injected by needle.[10] Pingen et al. also reported similar findings, showing that *Aedes aegypti* mosquito bites induced an inflammatory response in mice characterized by the presence of neutrophils, and this immune response augmented the severity of Semliki Forest and Bunyamwera virus infections.[32]

Given the majority of arboviral mouse models rely on type I interferon-deficiency, it is difficult to draw conclusions on the effects of saliva on the development of disease. A newer humanized mouse model was generated by transfer of human hematopoietic stem cells into NOD/SCID/IL1-gamma chain null (hu-NSG) mice.[36] The footpad skin of these hu-NSG mice exposed to *Aedes aegypti* mosquito bites showed changes in various T-cell populations as well as systemic effects in the blood (Table 1).[36]

Table 1. Changes in T-cell populations in bitten humanized NOD/SCID/IL1-gamma chain null mice

	6 Hours Post-bite	24 Hours Post-bite	7 Days Post-bite
Blood	↓ Regulatory T Cells ↑ Double-positive (DP) T Cells ↑ CD8+ T Cells ↑ Natural Killer T Cells	↑ Regulatory T Cells	↑ DP T Cells
Skin	No Significant Changes	↓ DP T Cells	↓ Regulatory T Cells ↑ DP T Cells
Spleen	↓ Total T Cells	↓ Regulatory T Cells	↑ CD8+ T Cells
Bone Marrow	↓ Total T Cells ↓ Regulatory T Cells	No Significant Changes	↑ DP T Cells

All these results strongly suggest that a mosquito bite alters the skin environment, and consequently, the establishment of the virus delivered by the mosquito vector. However, we know very little about saliva-mediated immune cascades in the skin, particularly in endemic populations. The majority of studies are animal-based, and the few retrospective studies available in endemic volunteers are focused on systemic antibody-mediated immunity to vector saliva.[19,37,38]

2.2.2 CONCLUSION

Our aim is to translate the aforementioned preliminary findings in animal models to better understand the immune response to *Aedes* mosquito saliva. Our preliminary cohort data confirm that this study area is endemic for biting *Aedes aegypti* mosquitoes and will provide a suitable adult population to study immune cascades in vector-experienced human skin. We hypothesize that there will be different innate and adaptive immune signatures in the bitten versus unbitten skin that can ultimately guide future rationale design of saliva-based, or vector-targeted, vaccines.

2.3 RISK/BENEFIT ASSESSMENT

2.3.1 KNOWN POTENTIAL RISKS

The risk of enrollment in the study is the effect of mosquito feeding, skin punch biopsy, and venipuncture.

Mosquito feeding may result in itchiness, pain, swelling, and redness at the site of bites. These reactions resolve after a short period of time and pose little risk to the participant. Applying cold packs and administering over-the-counter pain medications or antihistamines (topical or oral) if necessary can generally treat these reactions. Mosquito feeding or scratching after feeding may result in scarring such as hyperpigmentation. In rare cases a more severe irritation could occur, such as anaphylactic reaction or secondary infection at the site of the bite. Signs of infection include pain, redness, swelling, and drainage at the site. Oral antibiotics or anti-inflammatory medications can be administered if necessary.

Risks of skin punch biopsy include local pain, bleeding, redness, infection, a scar and possible keloid formation. Oral or topical antibiotics and oral analgesics will be used to manage pain and infection as

needed. Any non-routine complications resulting from the procedure will be addressed in consultation with available dermatological specialists in Phnom Penh. Injection of local anesthetic may cause a minimal burning discomfort or bruising at the site of the needle puncture.

Risks of blood draw include pain, bruising, bleeding, local discomfort, lightheadedness, dizziness or possibly fainting and rarely infection or blood clot. The amount of blood drawn is specified in the SoA table (Section 1.3). The amount of blood drawn will be within the limits allowed for adult participants by the local Cambodian authority NECHR, which is 21 mL in a single visit.

2.3.2 KNOWN POTENTIAL BENEFITS

There is no direct benefit to the participant. The information collected from this study will allow a better understanding of the skin immune response to vector bites to inform future vaccine development.

2.3.3 ASSESSMENT OF POTENTIAL RISKS AND BENEFITS

Although there is no direct benefit of participation in the study, study results could contribute to generalizable knowledge informing future vaccine development for diseases relevant to the study population and their communities. The study will only enroll healthy volunteers and will perform careful monitoring following study procedures to minimize risks and discomforts.

3 OBJECTIVES AND ENDPOINTS

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
Compare the early and late innate immune response in the skin of *Aedes aegypti*–bitten (exposed) versus unbitten (unexposed) skin	Measurement of changes in the early and late innate immune response and cellular recruitment in bitten skin versus unbitten skin by: a) immunohistochemistry of target proteins at Day 0 timepoints b) immunophenotyping of innate immune cell subsets in dissociated skin sample at Day 0 timepoints c) determination of cytokine profile in dissociated skin sample supernatant at Day 0 timepoints d) differential cDNA expression prepared from skin RNA and analyzed via RNASeq at Day 0 timepoints	Characterization of the responsible cell populations of the innate immune system in the epidermal-dermal microenvironment from exposure to mosquito saliva will highlight fundamental mechanisms of disease pathogenesis for mosquito-borne diseases.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Characterize the local skin adaptive immune response	Measurement of changes in the adaptive immune response and cellular recruitment in bitten skin versus unbitten skin by: a) immunohistochemistry of target proteins at Day 2 timepoints b) phenotyping of adaptive immune cell subsets in dissociated skin sample at Day 2 timepoints c) determination of cytokine profile in dissociated skin sample supernatant at Day 2 timepoints d) differential cDNA expression prepared from skin RNA and analyzed via RNASeq at Day 2 timepoints	Characterization of the cellular changes in the adaptive immune system in the epidermal-dermal microenvironment after exposure to mosquito saliva will also elucidate the underpinnings of mosquito saliva–mediated disease pathogenesis.
Secondary		
Analyze systemic immune response to vector salivary proteins in endemic participants with lifelong *Aedes aegypti* exposure after antigen encounter	Flow cytometry analysis of PBMCs collected Day 0 (baseline) and Days 2 and 14 after feeding for saliva-specific T-cells	Describing and understanding cellular immunity to *Aedes* saliva in heavily exposed individuals will simulate endemic conditions and will inform vaccine design in these target populations.
Tertiary/Exploratory		
Describe the development of vector saliva–specific tissue-resident memory responses	Comparison of TCR sequencing of skin biopsies at Day 0 versus Day 2	Understanding tissue-resident skin immune memory to *Aedes* saliva will highlight the responsible tissue-resident cell populations and possibly provide new targets for vaccine design of vector-borne disease.
Validate development of reliable biomarkers of vector exposure	Antibody determination in sera at Day 0 and Day 14	Assessing the anti-saliva antibody response in endemic volunteers in a quantitative fashion is critical to a biomonitoring strategy. Development and

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
		refinement of biomarkers are essential to targeted vector control initiatives in the future, particularly in resource-poor settings where limited supplies exist.

4 STUDY DESIGN

4.1 SITE DESCRIPTION

Located in Cambodia, the main town of Chbar Mon (estimated population approximately 55,000-60,000 over 10 km^2) is located 44 km from Phnom Penh city center. Chbar Mon is the largest operational district in Kampong Speu province and is the site of the Kampong Speu District Referral Hospital, a hospital of approximately 120 beds with 130 staff of medical doctors, nurses, and lab technicians. NIAID has a field house behind the hospital that serves as a temporary clinic in which to perform mosquito feedings, skin biopsies, and venipuncture.

In pupal surveys of *Aedes* mosquitoes in the region, 95.5% were *Aedes aegypti*, and the remainder were *Aedes albopictus*. This proportion was similar in both urban (94.7%) and rural (96.1%) areas.[14] In this part of the province, in Chbar Mon town, there is essentially no malaria transmission as *Anopheles* vectors are predominantly in the forested hills that make up the westernmost part of Kampong Speu.[39] However, *Culex* mosquitoes are fairly abundant throughout rural, peri-urban, and urban areas of Cambodia and are responsible for transmitting JEV. Our preliminary cohort data from Kampong Speu confirms *Aedes* exposure, and we have ongoing studies into cross-reactivity between *Culex* and *Anopheles* saliva in our cohort participants. Preliminary results via western blot with endemic human sera suggest little cross-reactivity between *Culex* and *Aedes* species. Mosquito trapping of adult specimens reveals abundant *Aedes aegypti, Aedes albopictus*, and *Culex* species, but much fewer anopheline vectors as would be expected. Our ongoing entomological surveys reveal that *Aedes aegypti* 1) has increased abundance compared to *Aedes albopictus*, 2) has 3-times increased odds of being found indoors compared to *Aedes Albopictus,* and 3) is equally distributed across the dense urban, transition, riverine, and rural areas in Chbar Mon.

4.2 OVERALL DESIGN

This is a single-center study of healthy skin immune response to bites of colony-reared *Aedes aegypti* mosquitoes not infected with any pathogen compared to unbitten (unexposed) skin. Healthy participants will undergo a single *Aedes aegypti* feeding on Day 0 and give blood and skin samples through 2 weeks following the feeding. A total of 5 skin punch biopsies will be performed: one from bitten skin at each of 3 different timepoints (30-90 minutes, 3.5-4.5 hours, and about 48 hours after feeding is completed), and two from unbitten skin collected on Day 0. For feasibility of skin sample analysis, each participant will be assigned to 1 of 4 technical modality cohorts:
1) immunohistochemistry, 2) RNASeq, 3) flow cytometry, and 4) TCR sequencing (see Section 9.1).

Additionally, systemic immune response will be evaluated on blood collected at baseline, and 2 and 14 days after feeding (see SoA, Section 1.3).

4.3 END OF STUDY DEFINITION

A participant is considered to have completed the study if he or she has completed all visits of the study as shown in the SoA, Section 1.3.

The end of the study is defined as completion of the last visit shown in the SoA (Visit 3).

If the participant has a complication, he or she will be followed until the PI deems that the complication or adverse event (AE) has resolved. In the unlikely event that an AE (such as a keloid) has not resolved by the end of the study at Day 14, the participant will be referred to the District Hospital for any necessary follow-up.

5 STUDY POPULATION

5.1 INCLUSION CRITERIA

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Provision of signed and dated ICF.
2. Stated willingness to comply with all study procedures and availability for the duration of the study.
3. Male or female, aged 18–45 years.
4. In good general health as evidenced by medical history.
5. Willing to allow biological samples to be stored for future research.
6. A female is eligible for this study if she meets 1 of the following:
    a. Of non-childbearing potential (i.e., women who have had a hysterectomy or tubal ligation or are postmenopausal, as defined by no menses in ≥1 year).
    b. Of childbearing potential but has negative urine pregnancy test on Day 0.
7. Agrees to not use scented lotions, deodorants, or topical creams on any part of the body on the feeding day.
8. Agrees to not take aspirin or any other nonsteroidal anti-inflammatory drug (e.g., ibuprofen) within 7 days of a biopsy.
9. Agrees to not use oral or topical antihistamines or steroid creams or ointments throughout the study without prior permission of PI.

5.2 EXCLUSION CRITERIA

1. Any underlying or current medical condition that, in the opinion of the investigator, would interfere with participation in the study.

2. History of severe allergic reaction (including to mosquito or other insect bites) with generalized urticaria, angioedema, anaphylaxis, anaphylactoid reaction, or any other reaction described by the participant and deemed severe by the PI.
3. Self-reported or known history of alcoholism or drug abuse within 6 months prior to enrollment.
4. Self-reported or known history of psychiatric or psychological issues that require treatment and are deemed by the PI to be a contraindication to protocol participation.
5. Any use of medications that affect blood clotting within 3 months or history of abnormal blood clotting.
6. History of significant scarring such as keloids after previous biopsies, lacerations, abrasions, surgeries, or other skin procedures (e.g., cosmetic piercings) that are deemed by the PI to be a contraindication to protocol participation.
7. Pregnant or breastfeeding.

5.3 SCREEN FAILURES

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently entered into the study. A minimal set of screen failure information is required to ensure transparent reporting of screen failure participants, to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any serious adverse event (SAE).

5.4 STRATEGIES FOR RECRUITMENT AND RETENTION

Advertising and recruitment in this province will mainly be via word-of-mouth using provincial health staff and the local village chiefs to organize informational meetings and subsequent dates for enrollment for those interested. Community engagement will begin 1-2 months prior to study start and will be organized in conjunction with the provincial health director of Kampong Speu province.

We anticipate having to screen no more than 80 to enroll goal number of 42 participants. We have no pre-set male:female ratios or age structure for the participant cohort. In Cambodia, it may be more likely for men to participate in healthy volunteer studies because they generally have more leisure time than women.

The anticipated length of individual study participation is approximately 2 weeks so participant retention should not be difficult. We will use multiple methods of contacting participants (e.g., phone number, Facebook Messenger username, and house visit if necessary). Retention compensation will include $5 US (to be paid in Cambodian Riel) for venipuncture blood draw, $10 for each biopsy, and $5 for mosquito feeding. The total amount for completion of all study procedures would be $70. These are standard compensation rates that have been approved by the local ethics boards in prior NIAID-led malaria studies over the last 12 years, although mosquito feedings and skin biopsies have not been performed and therefore a similar value was chosen.

Transportation for study visits will be provided to the referral hospital and NIAID Field Clinic as needed in a NIAID-CNM vehicle or be reimbursed (usually $1 maximum).

Lastly, the typical cost of an adult hospitalization is $10 in Kampong Speu District Referral Hospital. If any of the participants require hospitalization as a result of complications from the study (e.g., intravenous antibiotics for superimposed infection of bite site), they will have their hospitalizations and medications compensated.

5.4.1 JUSTIFICATION FOR EXCLUSION OF MINORS AND PREGNANT WOMEN

Exclusion of Pregnant Women: In this study, participants will undergo mosquito feeding. Because pregnant women are known to be immunosuppressed to some degree and the primary objective of the study is to examine immune response to vectors, they will be excluded so as not to compromise the scientific validity of the study.

Exclusion of Children: This is a study of the adult immune response to insect bites. Therefore, children will be excluded from the study.

6 PARTICIPANT DISCONTINUATION/WITHDRAWAL

6.1 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time upon request. An investigator may discontinue or withdraw a participant from the study if the subject meets study withdrawal criteria, as described in Section 8.4.13. If participants withdraw or are withdrawn prior to end of study, then they may be replaced as described in Section 8.4.14. The reason for participant discontinuation or withdrawal from the study will be recorded on the case report form (CRF).

6.2 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if he or she fails to return for a scheduled visit and he/she is unable to be contacted by the study site staff.

The following actions must be taken if a participant fails to return to the clinic for a required study visit:

- The site will attempt to contact the participant and reschedule the missed visit within the protocol window, counsel the participant on the importance of maintaining the assigned visit schedule, and ascertain if the participant wishes to and/or should continue in the study.
- Before a participant is deemed lost to follow-up, the investigator or designee will make every effort to regain contact with the participant (where possible, 3 telephone calls and local instant messaging methods).
- Should the participant continue to be unreachable, he or she will be considered to have withdrawn from the study with a primary reason of lost to follow-up. The PI may be able to replace the participant who is lost-to-follow-up if they have not followed up all procedures.

7 STUDY INTERVENTION

7.1 ARTHROPOD FEEDING

At the Malaria and Vector Research Laboratory (MVRL), an established state-of-the-art insectary for mosquitoes was built in 2014 to ACL2 (arthropod containment level 2) specifications.

The *Aedes aegypti* colony was first established in 2018, reared from larvae obtained in Chbar Mon, Kampong Speu province. The colonies have been raised in the CNM-MVRL insectaries since that time. The insectaries include temperature- and humidity-controlled rooms. We have experienced technicians that take care of the colonies on a daily basis. For our study, the mosquito pupae collected after emergence will be placed into clean new containers dedicated to mosquitoes for use in human studies, located in a separate room from the rest of the colony. The mosquitoes used in human studies never come into contact with human or any other blood prior to their use.

Colony-reared, clean female arthropods from *Aedes aegypti* mosquitoes will be bred in the CNM-MVRL insectary. Participants will be asked to not use scented lotions, deodorants, or topical creams on any part of the body starting 24 hours before the feeding day (or from their last shower as it is not common to shower every day).

Each feeding will be conducted as follows:

i. Assessment of the skin will be documented pre-feeding.
ii. 5 starved female *Aedes aegypti* mosquitoes will be aspirated or placed into a secured feeding device prior to feeding and brought to the Referral Hospital or NIAID Field Clinic (about 35 km away) from the CNM-MVRL insectary.
iii. The feeding site will be wiped clean with mild unscented soap and water, and the device will be placed on the participant's arm for up to 20 minutes. The insects will feed through a disposable mesh on the bottom of the feeding device. This device permits the evaluation of feeding on the human participant at the end of exposure, where the abdomens of blood-fed mosquitoes are clearly visible through the mesh.
    a. In the unlikely event of no feeding or poor feeding (only 0-2 insects fed or probed as noted by trained staff), the participant may undergo a repeat feed on the same arm with 5 fresh insects once.
    b. If a repeat feed is required, the post-feeding biopsies will be done based on the timing of the repeat feed.
    c. Post-feeding times will be calculated from the time the feeding device is removed.
iv. Post-feeding, redness, swelling, and number of visible bites will be assessed and documented immediately (+15 minutes) and 30 minutes (+15 minutes) after the feeding device is removed. This may also include taking photographs of the bites.
v. Once the mosquitoes have fed, they will be brought back to the lab for evaluation and then disposal.

Post-feeding, insect bites will be counted. In individuals that lack a robust response to saliva, each bite site will be distinguished by a red pinpoint on the skin, visible to the eye with careful inspection of the

skin. Other participants may be more reactive to component of saliva, such as vasodilators, and will exhibit clear redness and/or induration that denotes a clear site of bite.

After the feeding, participants may be offered standard treatment as needed to control reactions to vector bites, but we will otherwise try to avoid topical or oral antihistamine or steroid treatments. These treatments may include cold compresses, topical or oral antihistamines, or topical steroids. On days when biopsies will be performed, necessary treatment to control the reaction to the vector bite will be offered after the biopsy is completed.

To reduce confounding of new mosquito bites occurring between study visits, participants will be asked to wear long sleeves and pants and be offered available repellents that are commonly used in Cambodia (e.g., diethyltoluamide, bed nets, impregnated wall hangings).

7.2 EARLY TERMINATION VISIT

Participants who withdraw before the end of study will be encouraged to attend an early termination visit, where they will complete as many of the following procedures and evaluations as possible:

- Discussion to review reason for termination.
- Review of medical/medication history.
- Review of inclusion/exclusion criteria.
- Clinician exam/assessment.
- Vital signs.
- Blood draw for research labs if participant is willing.

7.3 UNEXPECTED OR INCIDENTAL MEDICAL CONDITIONS OF THE PARTICIPANT

If unexpected or incidental medical conditions are diagnosed during the medical evaluation in this protocol, the participant will be referred to an appropriate physician and/or hospital and encouraged to follow up for treatment of their condition. Standard-of-care treatment may be offered by the study team if necessary while the participant is being referred to appropriate outside medical care.

8 STUDY ASSESSMENTS AND PROCEDURES

8.1 CLINICAL EVALUATIONS

Prior to conducting any study-related procedures, informed consent in Khmer will be obtained from the participant via in-person interview with an investigator. If illiterate, a literate witness will read and sign the ICF on behalf of the participant, and the participant will provide a thumbprint. The participant will be queried regarding a history of significant medical disorders. A screening log will be kept to document eligibility of all participants.

Screening (-90 to Day 0)/Enrollment (Day 0):
The vector feeding will take place in the NIAID Field Clinic or the Kampong Speu District Referral Hospital. The following procedures will be performed:

- Informed consent.
- Review of medical/medication history.
    - Baseline demographics will be collected such as sex, age, mosquito-borne biting risk factors (e.g., number of domestic water containers at household), bed net use, past medical history, drug allergies, and any current or previous (within past month) use of over-the-counter, prescription, or traditional remedies. Other behaviors related to disease development may also be collected here.
    - Documentation of usual responses to mosquito or other insect bites as none (0), mild (1), moderate (2), or severe (3).
- Review of inclusion/exclusion criteria.
- Clinician targeted exam/assessment.
- Vital signs.
- Urine pregnancy test (for women of childbearing potential; confirmed negative before feeding).

After these screening and/or enrollment procedures, participants will undergo a blood draw (Section 1.3) and an *Aedes aegypti* mosquito feeding. Feeding and monitoring will be conducted as described in section 7.1.

After the feeding, participants will undergo the following procedures:

- Skin punch biopsy collections as follows:
    - 1 biopsy from bitten skin collected no earlier than 30 minutes and no later than 90 minutes after the vector feeding is completed.
    - 1 biopsy from bitten skin collected no earlier than 3.5 hours and no later than 4.5 hours after the vector feeding is completed.
- Any other clinical tests that are medically indicated or appropriate to ensure the safety of the individual participant as determined by the PI.

Additionally, at Day 0, we will collect 2 biopsies from unbitten skin on the opposite arm. The unbitten samples will be collected no earlier than 30 minutes before and no later than 6 hours after the vector feeding is completed.

Skin samples will be placed immediately and no later than 1 minute after excision into pre-labeled Eppendorf tubes containing either a fixative (10% buffered formalin), RNAlater, or other specified medium for immunohistochemical, transcriptomic, or cytometric analyses (depending on the participant's cohort for sample analysis).

Following these study procedures, participants will be allowed to leave as long as there are no safety concerns. They will be provided with medications if needed before they leave, and they will be asked to notify the study team if they have any reactions or side effects.

Day 2 (48-hour post-feeding visit +1 day):
All participants will return for a visit about 48 hours after the feeding. At this visit, they will be asked about interim medications, symptoms (e.g., fever), healing of previous biopsy sites and mosquito bites, and new mosquito bites, and the following procedures will be performed.

- Vital signs.
- Clinician assessment of bites and biopsy sites.
- 1 skin punch biopsy of bitten skin (at site of previously marked mosquito bite).
- Blood draw for research labs (ELISAs and cellular response assays, Section 1.3).

Day 14 (final study contact ±3 days):
All participants will return for a 14-day post-feeding visit. At this visit, they will be asked about interim medications, symptoms (e.g., fever), healing of previous biopsy sites and mosquito bites, and new mosquito bites, and the following procedures will be performed.

- Vital signs.
- Clinician assessment of bites and biopsy sites.
- Blood draw for research labs (ELISAs and cellular response assays) (Section 1.3)

Any participant who experiences complications due to feeding or biopsies will be examined in the clinic and followed until complications have resolved and/or referral to the necessary medical care has been made.

After the Day 14 visit, participation will be complete unless participants experience any AEs that need ongoing clinical monitoring.

8.2 STUDY PROCEDURES

8.2.1 VITAL SIGNS

Vital signs include blood pressure, heart rate, respiratory rate, and temperature. Height and weight will be taken at the Day 0 visit only. On feeding days, vital signs will be performed pre-feeding.

8.2.2 MOSQUITO FEEDING

The mosquito feeding procedure is described in Section 7.1.

8.2.3 SKIN PUNCH BIOPSIES

From each participant, five 3-mm skin biopsies will be taken: two at the bite site on Day 0, two at an unbitten site on the opposite arm (unexposed) on Day 0, and one at the bite site at 48 hours, using the following procedure:

1. The participant will be placed in a relaxed position with their arm out to expose the insect bites or unexposed site on the ventral side of the appropriate forearm.

2. A time-out will take place where the participant's name and medical record number and the site of the biopsy will be confirmed. The time and date of the time-out will be documented.
3. Non-sterile gloves will be worn and pads will be laid out to protect clothing as needed.
4. Biopsy location will be marked with small circle.
5. 2% lidocaine with epinephrine will be drawn up in a 3-mL syringe.
6. A 30-gauge needle will be used to inject lidocaine: It will be inserted into the skin just outside the circle drawn around the biopsy site; 1-1.5 cc of lidocaine will be injected slowly.
7. Anesthesia will be tested using sharp forceps. More lidocaine will be injected as needed to achieve proper local anesthesia.
8. Chlorhexidine or povidone iodide swabs will be used to disinfect the biopsy site by wiping concentrically from the center of the site to the outside of biopsy site.
9. A steri-drape may be applied around the biopsy site.
10. The biopsy will be performed by pulling skin taut with thumb and forefinger above and below the marked area. A punch biopsy tool will be used to apply pressure and turned back and forth 360 degrees. Forceps will be used to pull out the biopsy. Scissors may also be used if necessary to cut the specimen from the subcutaneous tissue.
11. Pressure will be held at the site using gauze, and then the site will be cleaned and dressed appropriately.
12. Steri-strips will be used to close biopsies, although sutures may rarely be placed if deemed necessary by the clinical team. If sutures are placed, the participant will return to clinic approximately 10 days later for suture removal.

Any necessary treatment to control bleeding, discomfort, or reduce scarring from the biopsy will also be offered. A member of the study team may contact or visit the participant after biopsies to monitor healing.

8.3 LABORATORY EVALUATIONS

Specific laboratory methods for each assay and instructions for handling and storage of samples will be maintained in a manual of procedures that includes all the standard operating procedures (SOPs) for the study.

8.3.1 SAFETY LABORATORY EVALUATIONS

For women of childbearing potential, a urine pregnancy test will be performed before the vector feeding procedure at the Kampong Speu District Referral Hospital laboratory or NIAID Field Clinic just behind hospital.

8.3.2 LABORATORY TESTING OF SAMPLES FOR STUDY ENDPOINTS

Skin samples collected from each participant (timepoints specified in Section 1.3) will be analyzed by one of the following technical modalities according to the participant's assigned cohort:

- Immunohistochemistry of target proteins (10 participants)

- Immunophenotyping of innate immune cell subsets in dissociated skin sample plus cytokine profile determination in dissociated skin sample supernatant (12 participants)
- Differential cDNA expression prepared from skin RNA and analyzed via RNASeq (10 participants)
- TCR sequencing prepared from skin RNA (10 participants)

Blood samples collected at each timepoint specified in Section 1.3 will be used for the following evaluations:

- Isolation of PBMCs to assess cellular response to salivary gland homogenate and/or salivary recombinant proteins
- Antibody determination in sera

Samples remaining after completion of these evaluations may be stored for future research use.

8.3.3 SPECIMEN COLLECTION, PREPARATION, HANDLING, AND SHIPPING

All human biological samples will be received, processed, aliquoted, and stored at the central laboratories of CNM-NIH and/or Institut Pasteur in Phnom Penh according to standard lab procedures.

Samples requiring transport from the clinical collection site to the designated central repository and, with host country permission, to the main NIH laboratory in the US will follow designated study sample shipping procedures, which will include shipment of samples with no participant identifiers. This will include all skin samples designated for sequencing.

8.4 SAFETY AND OTHER ASSESSMENTS

8.4.1 ADVERSE EVENT DEFINITIONS

AE: Any untoward medical occurrence in a human subject, including any abnormal sign (e.g., abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research.

SAE: Any AE that

- results in death;
- is life-threatening (places the subject at immediate risk of death from the event as it occurred);
- results in inpatient hospitalization or prolongation of existing hospitalization;
- results in a persistent or significant disability/incapacity
- results in a congenital anomaly/birth defect; or
- based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition (examples of such events include allergic bronchospasm requiring intensive treatment in the emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse).

***Unanticipated Problem Involving Risks to Subjects or Others (UP)*:** Any incident, experience, or outcome that meets all of the following criteria:

1. **unexpected** (in terms of nature, severity, or frequency) given
    a. the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and
    b. the characteristics of the subject population being studied; and
2. **related or possibly related** to participation in the research (possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
3. suggests that the research places subjects or others (which may include research staff, family members or other individuals not directly participating in the research) at a **greater risk of harm** (including physical, psychological, economic, or social harm) related to the research than was previously known or expected.

Protocol Deviation: Any change, divergence, or departure from the IRB-approved research protocol.

- *Major Deviations:* Deviations from the IRB-approved protocol that have or may have the potential to negatively impact the rights, welfare, or safety of the subject, or to substantially negatively impact the scientific integrity or validity of the study.
- *Minor Deviations:* Deviations that do not have the potential to negatively impact the rights, safety, or welfare of subjects or others, or the scientific integrity or validity of the study.

Non-compliance: Failure of investigator(s) to follow the applicable laws, regulations, or institutional policies governing the protection of human subjects in research, or the requirements or determinations of the IRB, whether intentional or not.

- *Serious non-compliance:* Non-compliance, whether intentional or not, that results in harm or otherwise materially compromises the rights, welfare and/or safety of the subject. Non-compliance that materially affects the scientific integrity or validity of the research may be considered serious non-compliance, even if it does not result in direct harm to research subjects.
- *Continuing non-compliance:* A pattern of recurring non-compliance that either has resulted, or, if continued, may result in harm to subjects or otherwise materially compromise the rights, welfare and/or safety of subjects, affect the scientific integrity of the study or validity of the results. The pattern may comprise repetition of the same non-compliant action(s), or different noncompliant events. Such non-compliance may be unintentional (e.g., due to lack of understanding, knowledge, or commitment), or intentional (e.g., due to deliberate choice to ignore or compromise the requirements of any applicable regulation, organizational policy, or determination of the IRB).

8.4.2 ADVERSE EVENT MANAGEMENT

Subjects entered onto this protocol will only be subject to skin biopsies, mosquito feedings, and limited blood sampling. There will be expected events as a result of these procedures.

Expected Events
The following are mild (Grade 1) to moderate (Grade 2) signs or symptoms that are induced by or associated with vector feeding or skin biopsy. If deemed related to vector feeding or skin biopsy by the PI, they will **not** be recorded as AEs in the Research Electronic Data Capture (REDCap) system per protocol, unless deemed by the PI to be abnormal or greater than Grade 2.

Feeding:

- Local swelling
- Pruritis
- Erythema
- Infection

Biopsy:

- Local swelling
- Erythema
- Bleeding at biopsy site
- Scar at biopsy site
- Infection

Information on AEs related to research procedures developing after the procedure will be collected by the investigators and entered into a computerized database (REDCap, Sections 10.1.6 and 10.1.7).

All AEs will be graded according to the following AE management guidelines, which are intended to ensure the safety of each subject while on the study. The AEs will be graded by severity (Table 2) and attributed to study procedures (unrelated, unlikely, possibly, probably, or definitely; Table 3).

Table 2. Grading of Adverse Events

1	Mild	Symptom barely noticeable to the subject; does not influence performance or functioning. Prescription drug not ordinarily needed for relief of symptom but may be given because of personality of subject.
2	Moderate	Symptom of a sufficient severity to make the subject uncomfortable; performance of daily activities influenced; subject is able to continue in study; treatment for symptom may be needed.
3	Severe	Symptom causes severe discomfort. May be of such severity that the subject cannot continue. Severity may cause cessation of study intervention; treatment for symptom may be given and/or subject hospitalized.
4	Life-threatening	Symptom places the subject at immediate risk of death from the reaction as it occurred; it does not include a reaction that, had it occurred in a more serious form, might have caused death.
5	Death	Symptom results in the death of the subject.

Table 3. Attribution of Adverse Events (AEs)

Relationship	Attribution	Description
Unrelated to intervention	Unrelated	The AE *is clearly **NOT** related* to the intervention
	Unlikely	The AE *is **doubtfully related*** to the intervention
Related to intervention	Possibly	The AE ***may be related*** to the intervention
	Probably	The AE ***is likely related*** to the intervention
	Definitely	The AE ***is clearly related*** to the intervention

8.4.3 REPORTING

The PI is responsible for reporting per NIH IRB and NECHR reporting requirements. Reportable events will be tracked and submitted to the NIH IRB and NECHR as outlined in NIH Human Research Protections Program Policy 801.

According to Policy 801, all UPs, major protocol deviations, all non-compliance, new information that might affect the willingness of a subject to enroll or remain in the study, and any suspension or termination of research activity will be reported within 7 calendar days of investigator awareness. Any death of a research subject that is possibly, probably, or definitely related to the research will be reported within 24 hours of investigator awareness.

The following events will be reported to the NIH IRB and NECHR in summary at the time of continuing review:

- AEs and SAEs that are not UPs, as a narrative summary indicating whether these events were within the expected range.
- Minor and major protocol deviations.
- UPs reported to the NIH IRB/NECHR.
- Non-compliance reported to the NIH IRB/NECHR that is not related to a protocol deviation.

The PI will report UPs, major protocol deviations, and deaths to the NIAID clinical director according to institutional timelines.

8.4.4 PREGNANCY

Although pregnancy itself is not an AE, events occurring during pregnancy, delivery, or in the neonate (e.g., congenital anomaly/birth defect) may be AEs or SAEs.

In the event of pregnancy, the following steps will be taken:

- Discontinue the study intervention and procedures but continue to follow-up for safety.
- Report to the NIH IRB and NECHR.

- Advise research participant to notify the obstetrician of study participation and study intervention exposure.

8.4.5 SPECIAL REPORTING SITUATIONS

Safety events of interest that may require expedited reporting and/or safety evaluation include but are not limited to:

- Excessive reactions to study intervention.
- Inadvertent or accidental exposure to a study intervention.
- Medication error involving a product (with or without participant exposure to the study intervention, e.g., name confusion).

Special reporting situations will be recorded. Any special reporting situation that meets the criteria of an SAE should be reported as described in sections 8.4.2 and 8.4.3.

8.4.6 TYPE AND DURATION OF THE FOLLOW-UP OF PARTICIPANTS AFTER AES

AEs that occur following signing the ICF will be followed until the final outcome is known or until the end of the study follow-up period. AEs that have not resolved by the end of the study follow-up period will be recorded as “not recovered/not resolved.” If a participant is lost to follow-up and AEs have not resolved, the outcome of these AEs will be recorded as “unknown.” Any participant who experiences complications due to feeding or biopsy will be followed until such complications have resolved or appropriate referral to the necessary medical care has been made. For SAEs, if it is not possible to obtain a final outcome (e.g., the participant is lost to follow-up), the reason a final outcome could not be obtained will be recorded by the investigator in REDCap.

8.4.7 PAUSING RULES FOR AN INDIVIDUAL SUBJECT

Pausing is the suspension of administration of study intervention to a single subject until a decision is made whether or not to resume administration of the study intervention.

The pausing criteria for a single subject in this study include any of the following:

- A subject experiences an SAE that is possibly, probably, or definitely related to a study intervention;
- A subject experiences two Grade 3 or greater AEs that are possibly, probably, or definitely related to a study intervention;
- Any safety issue that the investigator determines should pause administration of a study intervention to a single subject.

The PI will determine if study intervention administration to an individual participant should be paused. The study may also be paused for an entire group if a safety concern is identified.

8.4.8 REPORTING A PAUSE

If a pausing criterion is met, a description of the AE(s) or safety issue must be reported by the PI within 1 business day to the NIH IRB and NECHR.

8.4.9 RESUMPTION OF A PAUSED STUDY

The PI will determine whether or not it is safe to resume administration of the study intervention to the participant. The PI will notify the NIH IRB and NECHR of the decision on resumption of the study intervention.

A subject who does not resume study interventions will continue to be followed for safety.

8.4.10 HALTING RULES FOR THE PROTOCOL

Halting the study requires immediate discontinuation of study intervention administered for all participants and suspension of enrollment until a decision is made whether or not to continue enrollment and study intervention administration.

The halting rules are:

- 2 or more participants experience the same or similar SAEs that are possibly, probably, or definitely related to the study intervention;

OR

- 2 or more of the same or similar AEs in different participants that are grade 3 or above and are possibly, probably, or definitely related to the study intervention;

OR

- any safety issue that the PI determines should halt the study.

The PI will determine if the study should be halted.

8.4.11 REPORTING A STUDY HALT

If a halting rule is met, a description of the AE(s) or safety issue must be reported by the PI within 1 business day to the NIH IRB and NECHR.

8.4.12 RESUMPTION OF A HALTED STUDY

The PI will determine if it is safe to resume the study. The PI will notify the NIH IRB and NECHR of the decision on resumption of the study.

Participants who do not resume study intervention will continue to be followed for safety.

8.4.13 PREMATURE WITHDRAWAL OF A PARTICIPANT

An individual participant will be withdrawn for any of the following:

- An individual participant's decision. (The investigator should attempt to determine the reason for the participant's decision.)

- Non-compliance with study procedures to the extent that it is potentially harmful to the participant or to the integrity of the study data.
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation (e.g., moves outside of study area)
- The investigator determines that continued participation in the study would not be in the best interest of the participant.

8.4.14 REPLACEMENT OF WITHDRAWN PARTICIPANTS OR PARTICIPANTS WHO DISCONTINUE STUDY INTERVENTION

If participants withdraw or are withdrawn prior to end of study, then they may be replaced at the discretion of the PI if withdrawal or loss-to-follow up rates will impact study validity. Participants who withdraw at any time before all biopsies may be replaced. If a participant is removed prior to the feeding, none of their data will be used in the analysis or publication of the study. If a participant completes at a minimum the first feeding, data will be included in the analysis and publication. However, if they miss the second biopsy, they will not contribute to the 48-hour adaptive response studies.

9 STATISTICAL CONSIDERATIONS

9.1 STATISTICAL HYPOTHESES

This study is highly exploratory as very little is known about skin immunity to mosquito saliva in human skin. The simple characterization of this skin immunity will be important, as even with a small sample sizes we expect to be able to identify important aspects of the innate and adaptive immune response after a mosquito bite by identifying cell populations and examining the transcriptomic responses of differentially expressed genes.

The study hypothesis is that there will be differential phenotypic, transcriptomic, and immunohistochemical differences between the exposed (bitten) and unexposed (unbitten) skin with regard to innate immune markers at Day 0 after the mosquito feeding in an individual. We also expect to see differential phenotypic, transcriptomic, and immunohistochemical signals of adaptive immune markers at Day 2 as compared to unexposed (unbitten) skin.

To ensure sufficient sample amount to achieve study endpoints, skin samples from each participant will be assigned to a technical modality cohort. We are defining “cohort” as the 10-12 participants assigned to each technical evaluation modality. There are 4 cohorts for 4 types of evaluations:
1) immunohistochemistry, 2) RNASeq, 3) flow cytometry, and 4) TCR sequencing. To understand the real-world feasibility issues in Cambodia, we plan to first proceed with 10 participants in the immunohistochemistry cohort in Week 1 (since there are no temperature, time-sensitive storage, or processing requirements for formalin-preserved biopsies). Additionally in Week 1, we will enroll 2 participants in the flow cytometry cohort. These samples, which must be processed “fresh” in real-time due to time and temperature sensitivity, will be used for optimizing the flow cytometry cohort. The other 10 participants in the flow cytometry cohort will be sampled in Week 2.

9.2 SAMPLE SIZE JUSTIFICATION AND ANALYSIS PLAN

Given no prior saliva skin immunity studies published in humans, feasibility and sample sizes are adapted from literature concerning neoplastic or autoimmune disorders of the skin versus healthy comparator skin. With regard to flow cytometry of skin samples, 4-mm biopsies typically yield 2200 ±615 cells in healthy skin and 178,000 cells in lesional skin of psoriatic patients.[40] Previous studies of skin transcriptomes have estimated approximately 13,000 genes per biopsy of healthy individuals.[41]

Given the high cost of many of these analyses, several published human studies typically only have between 2 and 10 skin samples per group or total.[40,42,43] Currently, we do not know whether skin immune responses to bites of this vector will be uniform or how much variability will be observed. Therefore, it is very difficult to estimate an exact samples size. As this study is very exploratory in nature and has a relatively small number of individuals in each cohort, it is likely that the results will be very informative of future studies as it will offer more insight into the diversity or lack thereof in the skin responses. The cohort sizes of 10 (+2 additional participants in the flow cytometry cohort for optimization) will allow of us to have adequate numbers to perform these detailed and expensive immunohistochemical and transcriptomic techniques that could lead to future larger studies with more focused testing once the differential genes and important areas of immune response are identified.

As we expect transcriptomic analyses to yield at least 13,000 genes, this large number of genes will be examined to find a smaller list of differentially expressed genes within cohorts. For transcriptomic analysis, non-normalized raw counts will be used with an analysis program (e.g., the EdgeR package) to perform differential gene expression analysis after quality control (QC) of samples. Our anticipated analysis program will utilize model-based scale normalization, estimate dispersions, and apply a negative binomial model for RNA transcriptomic data analysis to find differentially expressed genes.

Single comparisons will be made within cohorts of bitten skin at a particular timepoint to the unbitten skin biopsy. Because our study will analyze paired samples (bitten skin and unbitten skin biopsies from the same individual), we can employ two different approaches. First, we can apply general linear modelling (GLM) using a GLM likelihood ratio test for comparing bitten versus unbitten skin. Also, we can employ group-wise comparisons where negative binomial fitting is followed by exact test. False discovery rate (FDR) adjustment will be used for multiple testing correction. An FDR threshold of 0.1 for statistical significance is typically applied. Because this is exploratory, an FDR or a q-value equivalent of 0.05 will be applied. Genes with larger differential expression will be defined with a 4-fold change threshold between experimental conditions.

For flow cytometric analysis, data will be collected using the BDCanto software (BD Biosciences). Data will be analyzed using FlowJo (v10.2; FlowJo, LLC). Differences will be assessed by Mann Whitney-U test or Wilcoxon matched-pairs signed rank test. A p value below 0.05 is considered significant for a single comparison, but flow cytometry will likely need to be adjusted for multiple comparisons as we anticipate using a minimum of 8 markers.

10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

10.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

10.1.1 INFORMED CONSENT PROCESS

10.1.1.1 CONSENT AND OTHER INFORMATIONAL DOCUMENTS PROVIDED TO PARTICIPANTS

ICFs describing in detail the study procedures and risks are given to the participants, and written documentation of informed consent is required prior to starting research procedures and evaluations. The ICFs in English (to be translated into Khmer for submission for Cambodian ethics review and for use in study) are submitted with this protocol.

10.1.1.2 CONSENT PROCEDURES AND DOCUMENTATION

Informed consent is a process where information is presented to enable persons to voluntarily decide whether or not to participate as a research subject. It is an ongoing conversation between the human research subject and the researchers that begins before consent is given and continues until the end of the subject's involvement in the research. Discussions about the research will provide essential information about the study in Khmer and include at the very least the purpose, duration, procedures, alternatives, and risks and benefits. Potential participants will be given the opportunity to ask questions and have them answered. Those who are literate will sign and date the ICF (written in Khmer) prior to undergoing any research procedures. In this case, a witness will not sign and date the ICF. If the potential participant is illiterate, a thumbprint will be obtained and the date will be documented by the investigator. In this case, an impartial witness who is literate and not involved with the study will then sign and date the ICF.

Participants may withdraw consent at any time throughout the course of the study. A signed copy of the ICF will be given to them for their records. The investigator will document the signing of the ICF in the participant’s medical record. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

10.1.2 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended (paused) or prematurely terminated (halted) as described in Sections 8.4.7 and 8.4.10. If the PI prematurely terminates or suspends the study, then the PI will promptly inform study participants and the NIH IRB and NECHR in writing, and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule. The study may resume once concerns about safety, protocol compliance, and data quality are addressed.

10.1.3 CONFIDENTIALITY AND PRIVACY

Participant confidentiality and privacy is strictly held in trust by the participating investigators and their staff. This confidentiality is extended to cover testing of biological samples in addition to the clinical

information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

All research activities will be conducted in as private a setting as possible.

The study monitor, other authorized representatives of the sponsor, representatives of the NIH IRB, NECHR, or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site staff will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB and ethics committee, institutional policies, or sponsor requirements.

Study participant research data, which is captured on tablets for purposes of statistical analysis and scientific reporting, will be transmitted to and stored on a central server either in Phnom Penh and/or LMVR at NIH main campus. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used by research staff will be secured and password protected. At the end of the study, the study database will be de-identified and archived at LMVR in Rockville, MD.

10.1.4 FUTURE USE OF STORED SPECIMENS AND DATA

Intended Use: Samples, specimens, and data collected under this protocol may be used to study the following:

- Development of new diagnostic tests to enable more sensitive and specific tests for vector-borne diseases.
- Determination of immune response to vector saliva, vector-borne diseases, or other infectious diseases or immunologic phenomena.
- Determination of incidence and pathogenesis of co-circulating infections (e.g., DENV, JEV, CHIKV, ZIKV, influenza), as it contributes to this community.
- Use of samples for developing vaccines and therapeutic interventions to prevent or treat vector-borne infections or other infectious diseases.
- Isolation or sequencing of viruses (e.g., DENV, CHIKV, ZIKV), parasites, or bacteria.

Storage: All of the stored study research samples are labeled by a code that only the investigators can link to the subject. Samples are stored at the Malaria Vector and Research Laboratory at the National Center for Parasitology, Entomology, and Malaria Control and the Institut Pasteur Cambodge, which are both secure facilities with limited access. During the high-volume biopsy collection, samples may be temporarily stored in the Kampong Speu District Referral Hospital main laboratory, which is also a secure facility with limited access. In the future, it will be necessary to ship a limited number of samples to NIH for analyses that cannot be performed in Cambodia, and these would be kept in a secure facility

at the LMVR in Rockville, MD. Data will be kept in password-protected computers. Only investigators or their designees will have access to the samples and data.

Tracking: Samples and data acquired under this protocol will be tracked using either Microsoft Excel (for input into the Biological Specimen Inventory) or REDCap.

Disposition at the Completion of the Protocol:

- In the future, other investigators (both at NIH and outside) may wish to study these samples and/or data for research purposes. If the planned research falls within the category of "human subjects research," appropriate NIH IRB and NECHR review and approval will be obtained. This includes the NIH or CNM researchers sending out coded and linked samples or data and getting results that they can link back to their subjects.
- At the time of protocol termination, samples will be either destroyed or, after NIH IRB and NECHR approval, transferred to another existing protocol. Data will be archived by the study team in compliance with requirements for retention of research records; alternatively, after NIH IRB and NECHR approval, the data may be either destroyed or transferred to another repository.

Loss or Destruction:

- Any loss or unanticipated destruction of samples (for example, due to freezer malfunction) or data (for example, misplacing a printout of data with identifiers) that meets the definition of a reportable event will be reported to the NIH IRB and the NECHR according to NIH Policy 801.
- Additionally, subjects may decide at any point not to have their samples stored. In this case, the PI will destroy all known remaining samples and report what was done to both the subject and to the NIH IRB and NECHR. This decision will not affect the individual's participation in this protocol or any other protocols at NIH.
- However, withdrawal of consent with regard to bio-sample storage may not be possible after the study is completed. If a subject had previously consented to future use and some of their blood has already been used for research purposes, then the information from that research may still be used even after consent is revoked.

10.1.5 CLINICAL AND SAFETY MONITORING

Clinical site monitoring is conducted to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with ICH GCP, and with applicable regulatory requirement(s).

According to ICH-GCP 5.18 clinical protocols are required to be adequately monitored. This study monitoring will be conducted according to the "NIAID Intramural Clinical Monitoring Guidelines." Monitors under contract to NIAID/Office of Clinical Research Policy and Regulatory Operations (OCRPRO) will visit the research site to monitor aspects of the study in accordance with the appropriate regulations and the approved protocol. The objectives of a monitoring visit will be: (1) to verify the existence of signed ICFs and documentation of the informed consent process for each monitored subject; (2) to verify the prompt and accurate recording of all monitored data points, and prompt reporting of all SAEs; (3) to compare abstracted information with individual subjects' records and source documents (subjects' charts, laboratory analyses and test results, physicians' progress notes, nurses'

notes, and any other relevant original subject information); and (4) to help ensure investigators are in compliance with the protocol. The monitors also will inspect the clinical site regulatory files to ensure that regulatory requirements (Office for Human Research Protections), and applicable guidelines (ICH GCP) are being followed. During the monitoring visits, the investigator (and/or designee) and other study personnel will be available to discuss the study progress and monitoring visit.

During monitoring visits, the PI (and/or designee) and other study personnel will be available to discuss the study progress and monitoring visit. The PI will be provided copies of monitoring follow-up letters within 20 days of a visit.

The investigator (and/or designee) will make study documents (e.g., ICFs) and pertinent hospital or clinical records readily available for inspection by the NIH IRB, NECHR, the site monitors, and the NIAID staff for confirmation of the study data.

A specific protocol monitoring plan will be discussed with the PI and study staff prior to enrollment. The plan will outline the frequency of monitoring visits based on such factors as study enrollment, data collection status, and regulatory obligations.

The data gathered during this study will be monitored by the PI for safety and compliance with protocol-specified requirements.

10.1.6 QUALITY ASSURANCE AND QUALITY CONTROL

QC procedures will be implemented beginning with the data entry system and data QC checks that will be run on the database will be generated. Any missing data or data anomalies will be communicated to the PI for clarification/resolution.

REDCap will serve as the core data collection component of the data management system. REDCap, which has been deployed in approximately 100 countries in support of 244,000 studies, is already in use at the site in an ongoing cohort study. It has an extensive range of features, the most important of which are the following:

- the ability to operate from a web browser on a server or laptop and from Android and Apple tablets in disconnected mode and to synchronize data between any of these devices;
- the ability to interface with and receive data from external data management systems and adjudicate data imports prior to incorporation into the database;
- a lightweight application that requires minimal processing power or memory;
- the ability to define and run data quality rules over the duration of the study that can be run on both a real-time and ad hoc basis;
- support for updates through multiple mechanisms, including programming directly in the system at the site and transmission of updates from the Data Coordinating Center;
- support for multiple languages, both in the user interface and data collection instruments; and
- real-time reports, data summaries, and data exports, including frequencies for outlier detection.

Precision and accuracy of actual data collected will be checked by internal procedures at random (5%). Data editing and error resolution will be performed monthly.

Following written SOPs, the monitors will verify that the clinical trial is conducted, data are generated, and biological specimens are collected, documented (recorded), and reported in compliance with the protocol, ICH GCP, and applicable regulatory requirements

The study site will provide direct access to source data/documents, reports for the purpose of monitoring and auditing, and inspection by local and regulatory authorities.

10.1.7 DATA HANDLING AND RECORD KEEPING

10.1.7.1 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

Upon enrollment in the protocol, all subjects will be assigned a unique identifying combination code of four to seven numbers depending on assigned protocol number (e.g. 001-000) as it is a single-site study. The identifier will serve to link this identifying code with subject name. CRFs, databases, and samples will be coded with unique identifying numbers and will not contain subject names. Study personnel involved in data acquisition, entry, and analysis will therefore not have access to names, assuring the privacy and confidentiality of subjects. Standard CRFs containing clinical and laboratory data will be completed on tablets using the aforementioned REDCap that will be locked with a code and uploaded to password-protected servers. Relevant subject data will be entered into a hybrid system (REDCap plus Datafax as needed which is already established in the NIAID sites in Cambodia). Electronically captured data will be checked by study investigators and verified by a data manager. All electronic data will be stored on a secure server.

Access to subject charts and databases for data analysis will be restricted to the investigators listed on this protocol and those working under their direct supervision. Access to ICFs, which link identifying numbers to names, will be restricted to those investigators authorized to obtain consent. The OCRPRO and their authorized representatives may also have access to the subjects' study records, including ICFs and source documents. CRFs, ICFs, and source documents will eventually be transported from the study site to our offices in Phnom Penh, where they will be retained under lock and key by the investigators listed on this protocol and made available for clinical monitoring.

10.1.7.2 STUDY RECORDS RETENTION

The PI will retain all study records for at least 7 years in compliance with institutional, IRB and ethics committee, state, and federal medical records retention requirements, whichever is longest. The PI will keep all stored records confidential to the extent required by federal, state, and local law. Data captured electronically via tablets will be backed up nightly to the site's central server and transmitted on a weekly basis. All data will be archived at the end of the study and retained for a period of time consistent with IRB and ethics committee requirements.

Should the PI wish to assign the study records to another party and/or move them to another location, the PI will provide written notification of such intent to OCRPRO/NIAID with the name of the person who will accept responsibility for the transferred records and/or their new location. NIAID will be notified in writing and written OCRPRO/NIAID permission shall be obtained by the site prior to destruction or relocation of research records.

10.1.8 PROTOCOL DEVIATIONS

As a result of a protocol deviation (as defined in Section 8.4.1), corrective actions are to be developed by the site and implemented promptly.

These practices are consistent with ICH GCP:

- 4.5 Compliance with Protocol, sections 4.5.1, 4.5.2, and 4.5.3.
- 5.1 Quality Assurance and Quality Control, section 5.1.1.
- 5.20 Noncompliance, sections 5.20.1, and 5.20.2.

Protocol deviations must be sent to the NIH IRB and NECHR per their policies (Section 8.4.3). The site investigator is responsible for knowing and adhering to the reviewing IRB and ethics committee requirements.

10.1.9 PUBLICATION AND DATA SHARING POLICY

This study will be conducted in accordance with the following publication and data sharing policies and regulations:

NIH Public Access Policy, which ensures that the public has access to the published results of NIH funded research. It requires scientists to submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication.

This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH-Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial will be registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer-reviewed journals. Data from this study may be requested from other researchers 5 years after the completion of the primary endpoint by contacting Jessica Manning (NIAID).

Human data generated in this study will be shared for future research as follows:

- De-identified data in an NIH-funded or approved public repository.
- De-identified data in another public repository.
- De-identified data with approved outside collaborators under appropriate agreements.

10.2 ABBREVIATIONS

ACL2	Arthropod Containment Level 2
AE	Adverse Event
cDNA	Complementary Deoxyribonucleic Acid
CFR	Code of Federal Regulations
CHIKV	Chikungunya Virus
CNM	National Malaria Center
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
DENV	Dengue Virus
DP	Double-positive (T Cell)
ELISA	Enzyme-linked Immunosorbent Assay
FDR	False Discovery Rate
GCP	Good Clinical Practice
GLM	General Linear Modelling
hu-NSG	Humanized NOD/SCID/IL1-Gamma Chain Null Mice
ICF	Informed Consent Form
ICH	International Council on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IRB	Institutional Review Board
JEV	Japanese Encephalitis Virus
LMVR	Laboratory of Malaria and Vector Research
MVRL	Malaria and Vector Research Laboratory
NECHR	National Ethics Committee for Health Research
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
OCRPRO	Office of Clinical Research Policy and Regulatory Operations
PBMC	Peripheral Blood Mononuclear Cell
PI	Principal Investigator
QC	Quality Control
REDCap	Research Electronic Data Capture
RNASeq	Ribonucleic Acid Sequencing
SAE	Serious Adverse Event
SoA	Schedule of Activities
SOP	Standard Operating Procedure
SST	Serum-separating Tube
TCR	T-cell Receptor
Th	T Helper
UP	Unanticipated Problem Involving Risks to Subjects or Others
US	United States
ZIKV	Zika Virus

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