# Understanding dengue transmission in remote settings through innovative and integrated diagnostic and surveillance systems

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# Declaration of originality

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## COVID-19 Impact Statement

Studies were disrupted during 2020 and 2021 due to the worldwide coronavirus disease 2019 (COVID-19) pandemic and associated social distancing and international travel restrictions. Activities in the UK, Thailand and Timor-Leste were affected, particularly those relating to the implementation and evaluation of technology within surveillance systems. Despite these challenges, and because I worked with a committed, adaptable and brave team, I was able to complete the majority of my planned laboratory and epidemiological research.

# Abstract

**Introduction:** Dengue is a major cause of acute febrile illness (AFI), health-seeking, morbidity and mortality across tropical nations. Current surveillance systems are incomplete and slow because they rely on sub-optimal, laboratory-based diagnostic tests and a lack of effective information transfer and analysis. Rural and remote settings are particularly affected.

**Hypothesis:** Innovation in molecular and serological diagnostic techniques and their use within surveillance systems can enhance understanding of dengue transmission in remote settings.

**Methods:** A pilot observational study described the presentation of dengue and use of available molecular diagnostics in a remote setting. A qualitative, focus group study described challenges and requirements for dengue diagnostics. The role of an emerging diagnostic solution, loop-mediated isothermal amplification (LAMP), was investigated in a systematic literature review. Its application was studied in-silico and in-vitro and assays were optimised for use in a portable diagnostic workflow. Dengue transmission in remote settings was further investigated through serological surveillance studies estimating force-of-infection (FOI) and using satellite-derived environmental data in predictive mapping.

**Results:** The observational study demonstrated the potential improved diagnosis and surveillance of dengue with molecular tests. The qualitative study defined a need for faster, more accurate, remote-connected, serotype-specific tests which can be operated outside laboratories. The literature review identified LAMP assays with high analytical and diagnostic performance. In-silico and in-vitro experiments optimised these with a 25-minute incubation duration and translated them into a lyophilised, colorimetric format. Serological surveillance studies measured high dengue FOI in Timor-Leste which is spatially heterogenous and closely associated with environmental factors, demonstrating the need for enhanced surveillance systems.

**Conclusion:** Significant advances were made in the molecular and serological diagnosis of dengue which may enhance surveillance in remote areas. Spatial variation in transmission was characterised across a previously unstudied region.

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# Abbreviations

| | |
|---|---|
| AFI | Acute febrile illness |
| ASSURED | Affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free and deliverable to end-users criteria |
| BIP | Backward inner primer |
| BLP | Backward loop primer |
| CAMO | Centre for Antimicrobial Optimisation |
| cDNA | Complementary deoxyribonucleic acid |
| CHC | Community health centre |
| CI | Confidence interval (or credibility interval) |
| CLIA | Clinical Laboratory Improvement Amendments |
| COVID-19 | Coronavirus disease 2019 |
| CT | Cycle threshold |
| DBS | Dried blood spot |
| DHFRU | Dengue Haemorrhagic Fever Research Unit |
| DNA | Deoxyribonucleic acid |
| DSS | Dried serum spot |
| EA | Enumeration area |
| ED | Emergency Department |
| ELISA | Enzyme-linked immunosorbent assay |
| EVI | Enhanced vegetation index |
| FFU | Focus forming units |
| FIP | Forward inner primer |
| FLP | Forward loop primer |
| FOI | Force-of-infection |
| FPE | Filter paper elute |
| GPS | Global Positioning System |
| HGC | Human genome control |
| HNGV | Hospital Nacional Guido Valadares |
| IgG | immunoglobulin G |
| IgM | immunoglobulin M |
| INS | Institute Nacional da Saúde |
| IQR | Interquartile range |

| | |
|---|---|
| ISFET | Ion-sensitive field-effect transistor |
| IV | Index value |
| LAMP | Loop-mediated isothermal amplification |
| LB | Backward outer primer |
| LF | Forward outer primer |
| LF | Lymphatic filariasis |
| LFT | Lateral flow test |
| LLOD | Lower limit of detection |
| LLOQ | Lower limit-of-quantification |
| LNS | Laboratorio Nacional da Saúde |
| LyoLAMP | Lyophilised colorimetric loop-mediated isothermal amplification strips |
| MIR | Middle infrared reflectance |
| NA | Nucleic acid |
| NPV | Negative predictive value |
| NS1 | Non-structural protein 1 |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PFU | Plaque forming units |
| POC | Point-of-care |
| PPV | Positive predictive value |
| PRNT | Plaque reduction neutralisation testing |
| R | R programming language / R Studio |
| RCF | Relative centrifugal force |
| RDT | Rapid diagnostic test |
| RH | Relative humidity |
| RNA | Ribonucleic acid |
| ROC | Receiver operator characteristic |
| RT-LAMP | Reverse-transcriptase loop-mediated isothermal amplification |
| RT-PCR | Reverse-transcriptase polymerase chain reaction |
| RT-qPCR | Reverse-transcriptase quantitative (real-time) polymerase chain reaction |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| SD | Standard deviation |
| SOP | Standard operating procedure |
| SST | Serum separation tube |

| | |
|---|---|
| TE | Tris EDTA |
| Tmax | Maximum daily temperature |
| Tmin | Minim daily temperature |
| WB | Whole blood |
| WHO | World Health Organisation |
| WHODAS-12 | World Health Organisation Disability Assessment Schedule 12-item questionnaire |

# CHAPTER 1

## 1.0 Introduction to the use of diagnostics in dengue surveillance

### 1.1 Dengue as an emerging cause of acute febrile illness in remote tropical regions

Dengue is a mosquito-borne tropical disease. Due to international travel and climate change, global incidence has increased rapidly during the last 50 years. Half the world's population is now at risk and over 400 million infections occur annually.(1–4) Contemporary observational studies consistently identify dengue as a major cause of health-seeking behaviour, morbidity and mortality among adults and children across Asia, Africa and Latin America.(5–7) Although dengue has historically been considered an urban disease associated with dense human population and the built environment, increasing evidence suggests high transmission in many rural settings, including remote areas where healthcare and other infrastructure is lacking.(8)

### 1.2 Dengue viruses

Dengue can be caused by any of four closely related viruses (dengue viruses serotypes 1-4) encoded by a single positive strand of ribonucleic acid (RNA). This genome encodes ten proteins including three 'structural proteins' (see figure 1) and seven 'non-structural proteins'. Structural proteins include the capsid protein ('C-protein', which forms the nucleocapsid at the core of the virus particle, containing the genome), as well as envelope and membrane proteins (E- and M-proteins, respectively, which embed the viral envelope and form a protective layer which controls entry into host cells. Non-structural proteins 1, 2A, 2B, 3, 4A, 4B, and 5 (NS-1-5) play roles in viral replication and assembly once inside a host cell, and in pathogenesis.

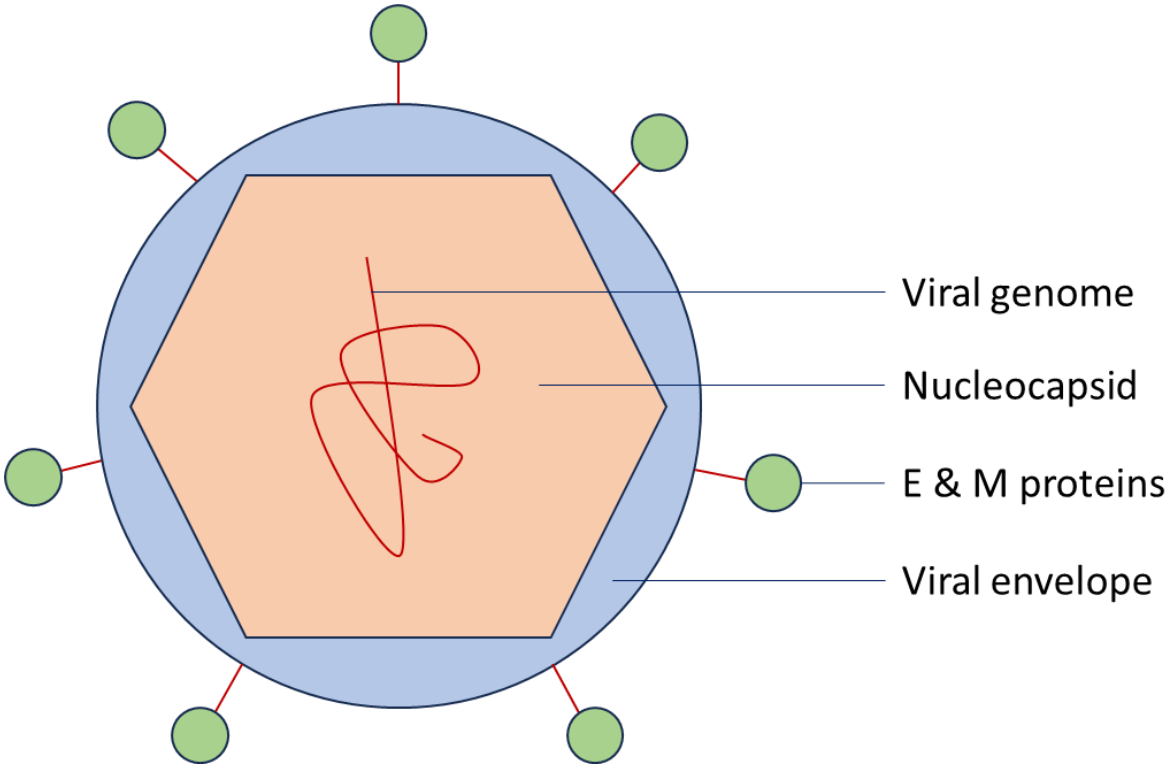



**Figure 1: Schematic of dengue virus particle showing the viral genome, nucleocapsid, viral envelope and outer layer formed by envelope (E) and membrane (M) proteins.**

**Legend:** This figure has been adapted from Kuhn et al, 2014.(9)

## 1.3 Transmission of dengue

Dengue viruses are transmitted by *Aedes* species mosquitos and maintained in a cycle involving humans and mosquitos (see Figure 2). *Aedes aegypti* is the primary vector, with *Aedes albopictus* also contributing to transmission in some areas. Female mosquitos are infected when they take a blood meal from an human during the viraemic (febrile) phase of illness. Virus particles first infect midgut cells, then disseminate throughout mosquito tissues including the salivary glands. From this time, mosquitos can infect humans when feeding.(10)

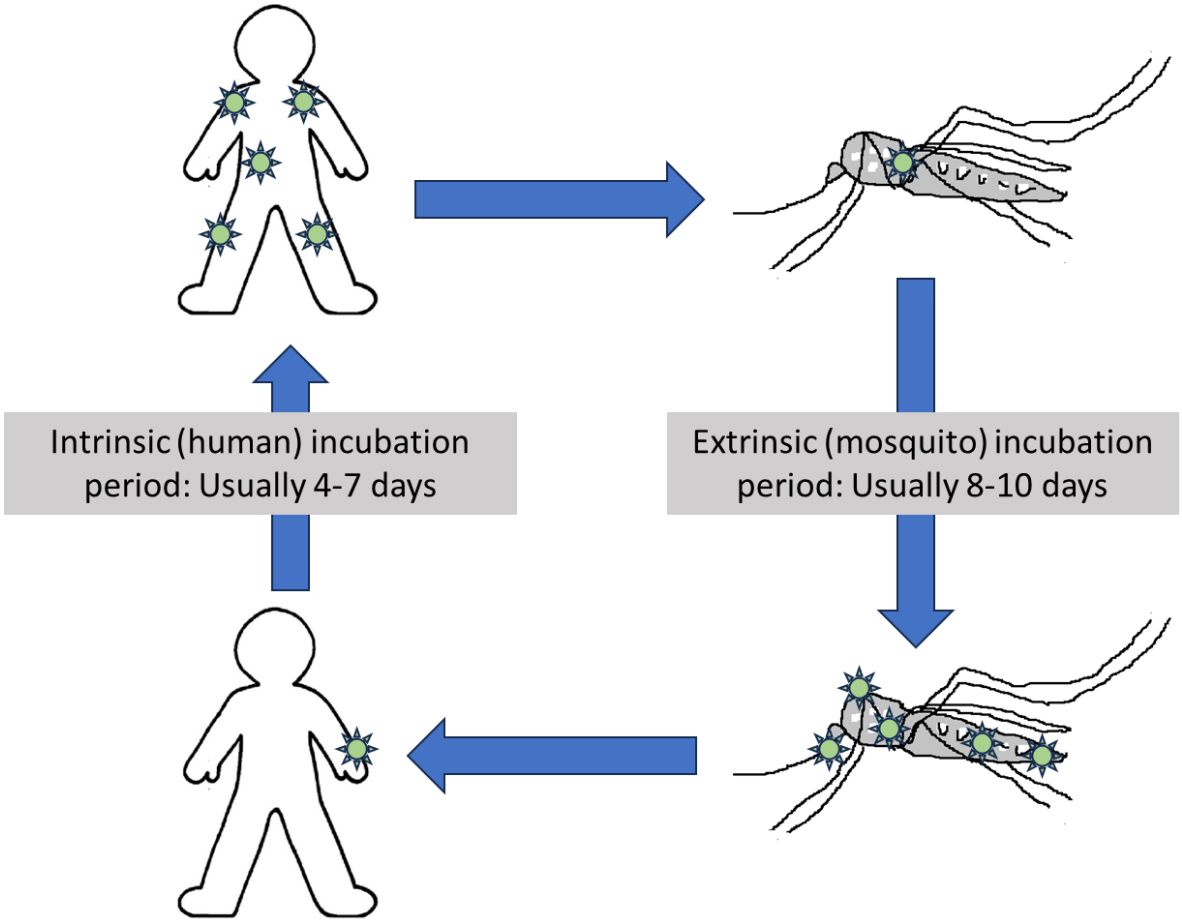



**Figure 2: The human-mosquito cycle which maintains dengue viruses**

**Legend:** This figure has been adapted from Guzman et al, 2006.(10)

## 1.4 Clinical manifestations of dengue and the management of cases

Dengue causes a diverse clinical syndrome ranging from asymptomatic or mild, self-limiting acute febrile illness (AFI) to dengue haemorrhagic fever, dengue shock and death.(10). Of the estimated 400 million cases of dengue infection which occur each year globally, approximately 25% (100 million) are 'clinically apparent'. Of these, 5% (500,000) lead to severe dengue, of which 2.5% (12,500) can be fatal.(3,11)

After a usual incubation period lasting 3-7 days, clinically apparent disease occurs with symptoms in three phases. The 'febrile phase' is characterised by high temperature, headache, vomiting, myalgia

and a sometimes a macular rash. This usually lasts 3-7 days after which most patients recover. However, a small proportion of patients progress to the 'critical phase', lasting 2-3 days, in which systemic vascular leakage leads to various serious complications including shock, pulmonary oedema and pleural effusion. Haemorrhage may also occur due to thrombocytopenia and coagulation abnormalities. 'Secondary dengue infections', when an individual is infected for a second (or subsequent) time by a different serotype to their earlier 'primary infection', are most likely to progress to the critical phase. This is thought to be due to an immunological phenomenon called 'antibody-dependant enhancement' (ADE), in which non-neutralising antibodies enhance uptake of virus particles into phagocytes where a T-helper-2-type immune response is activated, accelerating viral replication and causing excessive cytokine production.(12) In the 'recovery phase', vascular permeability returns to normal and there is improvement in the patient's symptoms.(13)

Despite numerous trials of antiviral and immunomodulatory agents, there are currently specific effective virus- or host-targeted treatments for dengue. Clinical management of cases is therefore supportive, with key interventions being early recognition of severe cases, careful fluid management and blood transfusion where there is severe bleeding which compromises cardiovascular function. Observational studies have shown that this supportive treatment can reduce the mortality of severe dengue to <1%.

## 1.5 Detecting and diagnosing infection

### 1.5.1 Syndromic diagnosis

Diagnoses of dengue can be suspected based on clinical features and may be supported by routinely available laboratory data including raised blood haematocrit and serum liver enzymes.(14) However, in many cases, clinical features do not definitively distinguish dengue from other potential causes of AFI, including bacterial infections and malaria which require disparate treatment. Suspected dengue should therefore be confirmed with diagnostic tests wherever possible.(15)

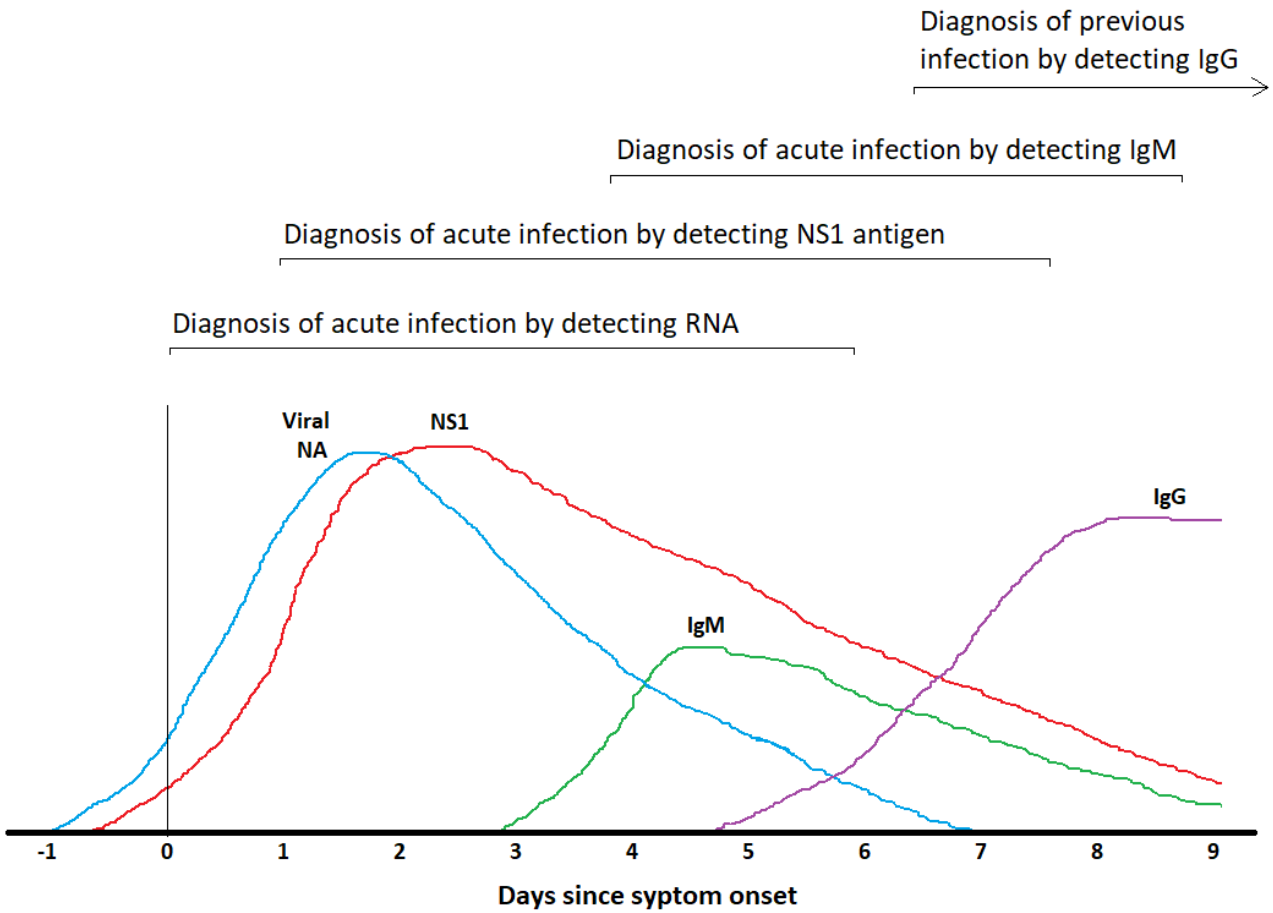
### 1.5.2 Molecular techniques

Dengue virus serotypes 1-4 are encoded by a single positive strand of ribonucleic acid (RNA) which is present in clinical samples from the onset of symptoms in both primary and secondary infection and can be detected using molecular diagnostic techniques. These include reverse-transcriptase polymerase chain reaction (RT-PCR) assays which use a RT enzyme to synthesise complementary deoxyribonucleic acid (cDNA) from the target RNA sequence, and a DNA polymerase enzyme to amplify the cDNA. PCR primers can be designed to either bind regions of the dengue virus genome which are conserved across serotypes (resulting in a 'generic' assay), or regions which are specific to one serotype (resulting in a 'serotype-specific' assay).(16) Assays can quantify RNA if performed in 'real-time' format (RT-qPCR), and can be multiplexed with assays for other pathogens.(17,18) RT-qPCR is the modern gold-standard in diagnosis and serotyping of dengue infections.(15) However, it requires significant laboratory infrastructure, a reliable power supply, skilled operators and systems for quality and safety. Deploying RT-qPCR assays in rural and remote settings has proven impractical.(19)

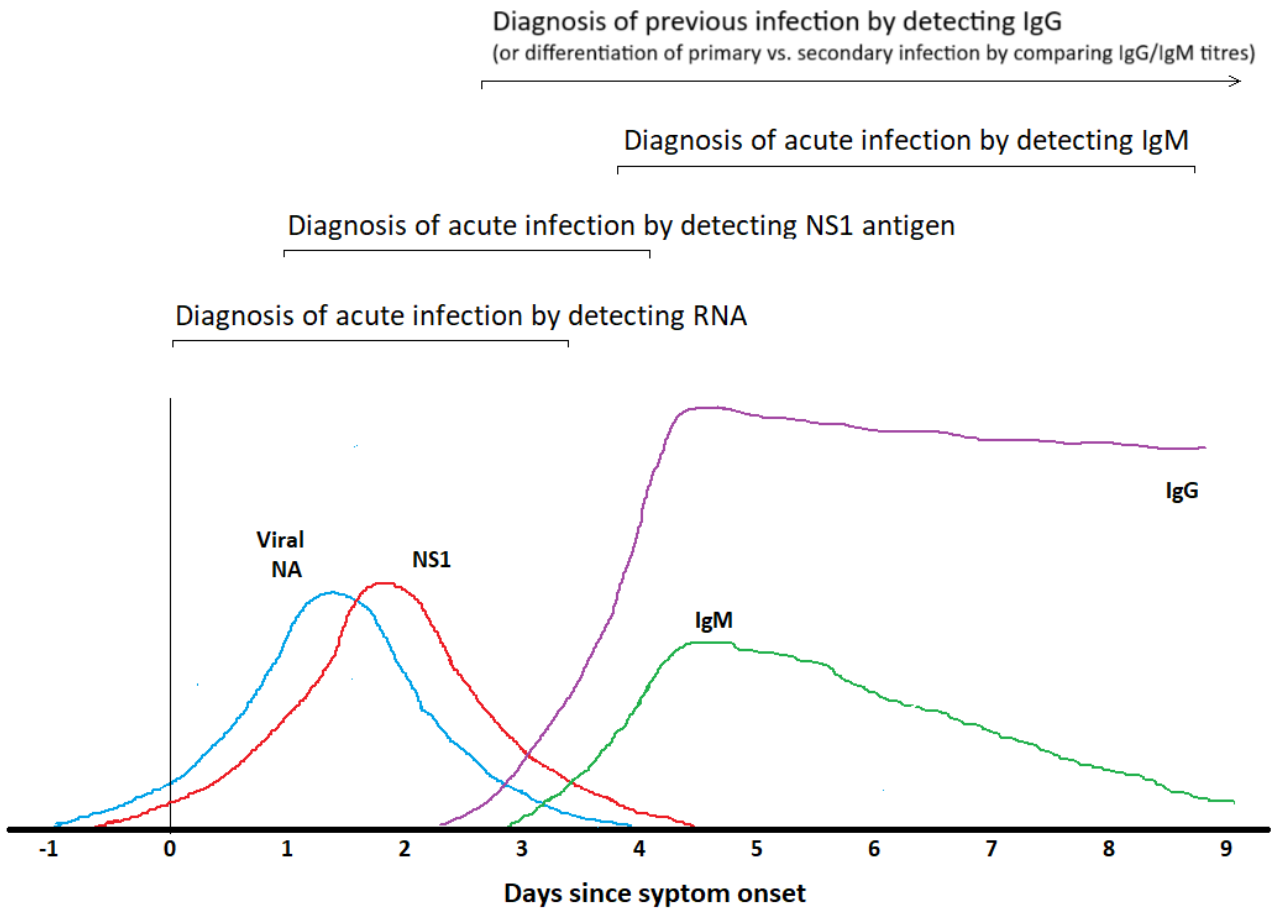
Innovations in molecular diagnostics include the discovery of loop-mediated isothermal amplification (LAMP) which is analogous to PCR, but uses a different DNA polymerase enzyme which allows DNA synthesis to occur at a constant temperature.(20) Because LAMP assays do not require thermocycling equipment, they may be more suited to miniaturisation and/or deployment outside the laboratory setting than PCR assays, including for the diagnosis of dengue and other diseases relevant to rural and remote settings. (21,22)

### 1.5.3 Serological techniques

Dengue infection induces strong host humoral immune responses including the production of antibodies against various viral proteins. In primary infection, specific immunoglobulin M (IgM) is typically detectable in clinical samples 3-5 days after symptom-onset. This response is short-lived and therefore its observation indicates acute or recent infection. Immunoglobulin G (IgG) is detectable 7-9 days after symptom onset and is long-lasting. Its observation in the absence of IgM indicates previous but not recent infection. In secondary infection there is a lesser IgM response but an earlier, anamnestic IgG response compared to primary infection and thus comparison of IgG and IgM titres can help distinguish primary versus secondary infection. Dengue infection also results in the continuous production of the antigen non-structural protein 1 (NS1) by infected host cells, which is a protein essential for viral replication with a significant role in pathogenesis.(23) It is detectable in blood shortly after the onset of symptoms. Serological techniques which detect host- and virus-derived proteins are therefore commonly used in the diagnosis of dengue. Enzyme-linked immunosorbent assays (ELISAs) and hemagglutination inhibition assays require laboratory equipment and expertise, whereas rapid diagnostic tests (RDTs) based on lateral flow technology can be operated by non-specialist users at the point-of-care (POC). Figure 1 A and B are schematics showing the dynamic concentrations of different molecular and serological diagnostic targets during primary and secondary infection, respectively.


Diagnosis of previous infection by detecting IgG
Diagnosis of acute infection by detecting IgM
Diagnosis of acute infection by detecting NS1 antigen
Diagnosis of acute infection by detecting RNA
Viral NA
NS1
IgG
IgM
-1
0
1
2
3
4
5
6
7
8
9
Days since syptom onset

A




B

**Figure 3: Schematic showing dynamic concentrations of potential targets for molecular and serological diagnosis of dengue.**

**Legend:** Primary infection (A) is compared to secondary infection (B) which typically cases shorter-lived viraemia and NS1 proteinemia, lesser IgM response and an earlier anamnestic IgG response. This figure is adapted from Muller DA et al, 2017.(15)

## 1.6 Surveillance of dengue

Outbreaks of dengue are typically seasonal and occur with rain. The number of cases and proportion causing severe disease can be highly variable between years. This can be particularly explained by shifts in the predominant circulating serotype: When a greater proportion of the population are at-risk of a new serotype (and many have 'sero-discordant' immunity from the previous serotype), outbreaks with more severe cases may occur.(24) Effective surveillance of dengue is crucial to provide outbreak warning, guide empirical therapy for AFI, deploy disease-control interventions and allocate public health resources more generally. (25,26)

### 1.6.1 Passive and active surveillance systems

The commonest approach to understand and monitor dengue transmission is through 'passive surveillance'. This involves cases being identified within a healthcare system via the routine assessment of patients, and their notification to a central surveillance authority. It relies on availability and utilisation of accurate diagnostic tests and effective, timely communication of results, ideally alongside clinically-derived metadata. In some countries an additional network of 'sentinel-sites' augments passive surveillance by standardising inclusion criteria, data collection and testing of patients. This is with the intention of estimating trends in the wider population. Samples may undergo additional testing, for example to determine the infecting dengue virus serotype.(27,28)

'Active surveillance' programmes involve assigned personnel purposively contacting healthcare providers or the population to collect data. This includes serological surveillance studies, where blood samples from the general population are tested for the presence of anti-dengue virus antibodies, most commonly to detect the proportion who have had previous infection ('seroprevalence'), rather than those who are actively or recently infected. Estimating seroprevalence at different age strata in a population is a key method to understand and compare transmission ('force-of-infection', FOI) in different geographical areas: In those with higher

transmission, individuals become seropositive earlier in their lifetime compared with those with lower transmission.(29) Active surveillance systems for dengue typically result in a more detailed, representative understanding of transmission than passive surveillance, but are resource-intensive and time-consuming.

**Table 1: Passive vs. active dengue surveillance**

| **Features** | **Passive surveillance** | **Active surveillance** |
|---|---|---|
| **Case identification** | Cases identified passively when they present to a health facility. | Cases identified actively among the general public or specific populations of interest (e.g. contacts of cases, in 'active case finding'). |
| **Data collection** | Collection of clinically-derived meta-data and laboratory results (which may be augmented at 'sentinel sites'). | Bespoke collection of data for surveillance purposes +/- collection of samples for testing |
| **Diagnostic tests** | Where used, diagnostic testing is for acute infection (including molecular methods for detection of viral RNA) | Diagnostic tests for previous infection may be used (i.e. serological techniques for detection of host IgG antibodies) |
| **Responsible personnel** | Cases usually reported by individuals working within the health facility | Cases usually reported by individuals working within the surveillance authority or in a research study. |
| **Strengths/ limitations** | Inexpensive but may suffer from underreporting, delayed reporting, and incomplete data | Resource-intensive and time-consuming but usually results in more detailed and representative data |

**Legend:** This table is adapted from Runge-Ranzinger et al, 2008. (30)

### 1.6.2 Traditional dengue disease control methods

Traditional methods for prevention of dengue cases in populations rely on the control of *Aedes aegypti* (and to a lesser extend *Aedes albopictus)* mosquitos, or the interruption of human-mosquito contact. Methods are targeted at disrupting vector breeding sites and habitats which are in the vicinity of households and other settings which humans occupy. The WHO promote a strategy of 'integrated vector management', which includes 'advocacy, social mobilisation and legalisation', 'collaboration within health and other sectors', 'evidence-based decision making' and 'capacity building', with the primary intention to eliminate containers containing water where mosquitoes may breed. This can be done by preventing mosquito-access, emptying and cleaning containers, or the use of insecticides.(14) In some systems, these activities are intensified in regions where risk of transmission is identified to be high or where there are high numbers of cases, in attempt to attenuate outbreaks.

### 1.6.3 Emerging dengue disease control methods

The live attenuated tetravalent CYD-TDV vaccine (Dengvaxia) is licensed in several dengue-endemic countries for individuals 9–45 years of age based on data from phase three trials which showed efficacy against symptomatic virologically-confirmed dengue.(31) However, in 2017, long-term safety data showed a 1.75-fold increased risk of hospitalisation due to dengue and severe dengue among seronegative participants who received vaccination (compared with those who were seronegative but unvaccinated).(32) The WHO therefore recommends vaccination after individuals are screened for prior infection in most settings. A lack of rapid diagnostic tests (RDTs) which can accurately determine individual dengue serostatus has meant this vaccine has scarcely been used.(33–35) In September 2023 data from phase three trials of the live attenuated quadrivalent TAK-003 vaccine (QDENGA) were presented and have since been shared with the WHO. These are reported to show efficacy against all four dengue virus serotypes in baseline seropositive children, and efficacy against dengue virus serotypes 1 and 2 in baseline seronegative children. However, the trial was

underpowered to assess efficacy in baseline seronegative participants against dengue virus serotypes 3 and 4, and cannot rule out the potential risk of enhanced disease due to serotypes 3 and 4. Despite this, WHO now recommends the vaccine be considered for introduction in settings with high dengue disease burden and high transmission intensity. Post-authorisation studies which include virological confirmation of dengue virus infections including serotyping are required.(36)

*Wolbachia pipientis* is a common obligate intracellular bacteria which can infect *Aedes aegypti* and manipulates reproductive outcomes between wild-type and wolbachia-infected mosquitoes such that only infected progeny are viable. It confers resistance to disseminated dengue infection in mosquitos. In 2021 a cluster-randomised controlled trial assessed the impact of releasing wolbachia-infected mosquitos and showed significantly reduced incidence of virologically-confirmed dengue cases and hospitalised cases of dengue in treated areas compared with control areas. This approach is increasingly being used in high-transmission, high population-density areas in multiple countries. Follow-up so far shows reduction in dengue incidence is sustained, but questions remain regarding the relative effectiveness against different dengue virus serotypes. Ongoing surveillance in treated regions including virological confirmation of serotypes and/or assessment of population immunity to different serotypes is required to understand this.(37)

### 1.6.4 Statement of research gap: A need for improved diagnostics in dengue surveillance

Detailed understanding of spatial and temporal patterns of dengue transmission are required to direct disease control interventions including the deployment of vaccines and wolbachia-infected mosquitos. This can only be achieved through the use of appropriate diagnostic tests within surveillance systems. However, a wealth of scientific writing, including a 2021 Lancet Commission on diagnostics and accompanying multicentre observational study on the global availability of testing

have highlighted a major gap in diagnostic availability particularly affecting rural and remote areas. This exists within the broader issue of global inequity and lack of access to healthcare. It includes diagnostic tests for infectious disease.(38,39) Lack of access to diagnostics results in poorly targeted treatments for patients with AFI. It also affects all downstream systems of passive and active surveillance where case-definitions incorporate results from testing. Surveillance systems can therefore be incomplete (i.e. lacking data on cases who are not diagnosed, or where reporting does not occur) or delayed (i.e. where information is not generated, transferred, analysed or interpreted in time to support any response). An international collaboration on dengue surveillance identified common weaknesses which include centralised laboratory services which batch-test and provide delayed results, a lack of serotype-specific diagnostic tests, and limited 'local data usage' with missed opportunities for rapid, local disease control responses.(40) The World Health Organization (WHO) Special Program for Research and Training in Tropical Diseases recommend that, to address disease control needs, diagnostics should be affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free and deliverable to end-users ('ASSURED criteria').(41) There is increasing recognition that modern diagnostics should be remote-connected, such that case-reporting can occur at the point-of-use.(26)

**Table 2: Definitions of rural and remote settings**

| **Rural setting** | **Remote setting** |
|---|---|
| Areas outside of urban or suburban areas, with low population density. | Areas which are isolated from urban or suburban areas, with very low population density. These may be difficult to access and lack healthcare and other infrastructure. |

**Legend:** This table was adapted from the World Health Organization global policy recommendations for increasing access to health workers in remote and rural areas through improved retention, 2010. (42)

## 1.7 Hypothesis

In this thesis I investigated the following hypothesis:

*Innovation in molecular and serological diagnostic techniques and their integration with active and passive surveillance systems can enhance understanding of dengue transmission in remote settings.*

### 1.7.1 Aims and objectives

My aim was to investigate whether molecular and serological diagnostic techniques to detect dengue infection (both those in-development and novel applications of existing technology) could strengthen dengue surveillance systems.

I collected data in Thailand, where systems of passive dengue surveillance have existed for many years but where there is significant interest among surveillance officials and academics in how new diagnostics may improve the diagnosis and reporting of dengue cases in rural and remote locations. This setting was ideal to guide the development of new diagnostics.

I also undertook studies in Timor-Leste, a country with a highly dispersed rural population, widespread annual dengue outbreaks and significant current shortfalls in dengue surveillance. This represented the ideal setting to explore how diagnostics can be implemented within surveillance systems, in particular within active serological surveillance, to significantly improve understanding of dengue transmission.

To achieve my aim, I set the following objectives

1. To understand existing systems of dengue surveillance, identify specific challenges and define user-requirements for new diagnostic techniques.

2. To review the use of LAMP for the molecular diagnosis of dengue, and assess its potential for deployment remote settings.
3. To develop a new molecular diagnostic for dengue which fulfils user-requirements.
4. To investigate innovations in serological diagnostics including LFTs and the analysis of DBSs, and assess their potential to overcome challenges to active disease surveillance in remote settings.
5. To study the transmission of dengue in a remote setting through the deployment of serological techniques in a population-representative survey and undertake predictive mapping to identify areas with high FOI.

## 1.8 Thesis overview

Figure 2 is an overview of my thesis.

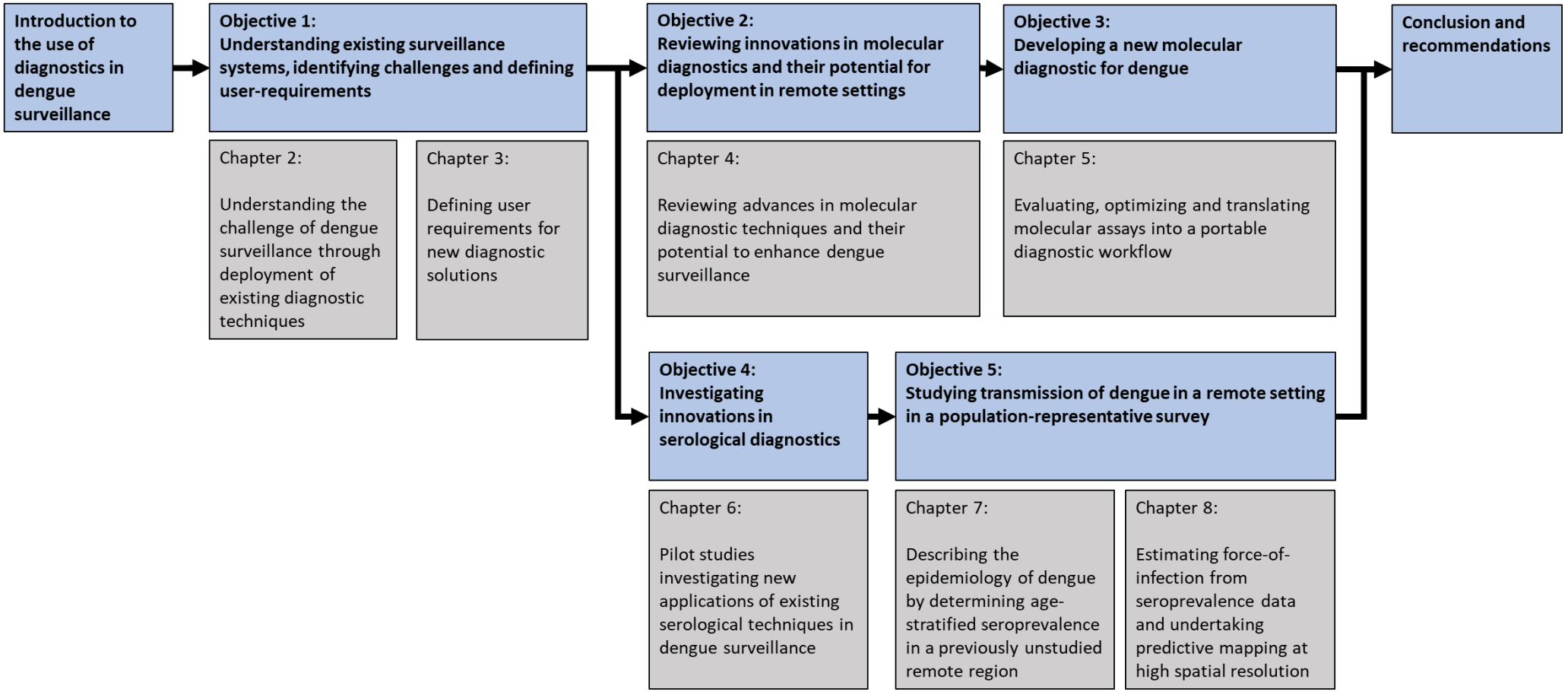



**Figure 4: Thesis overview**

# CHAPTER 2

## 2.0 Understanding the challenge of dengue surveillance through deployment of existing diagnostic techniques

## 2.1 Chapter introduction

Dengue infection causes a clinical syndrome in adults and children which is often indistinguishable from other causes of AFI. In many rural and remote areas there is a lack of access to accurate diagnostic testing. This affects the clinical management of patients (who may be given inappropriate empiric treatments) and effective passive disease surveillance (which relies on accurate diagnosis and reporting of cases). RDTs are increasingly used, but these are not serotype-specific and can be insensitive, particularly in cases of secondary infection. Deployment of RT-qPCR assays in such settings may bring improvements but is likely to be challenging due to laboratory infrastructure and workforce requirements.(15).

## 2.2 Chapter objectives

The aims of this chapter were to (a) observe patients presenting with AFI to a single centre in a remote setting, (b) describe their patient pathways, clinical diagnoses, management and outcomes, and (c) assess how the deployment of existing molecular diagnostic technology affected passive dengue surveillance. This would allow me to understand challenges of dengue surveillance and identify potential areas for innovation.

## 2.3 Methods

### 2.3.1 Study design

Pilot prospective observational cohort study enrolling patients with AFI.

### 2.3.2 Setting

Timor-Leste is a half-island nation located between Australia and Indonesia with a population of 1.3 million people. Much of the population live in rural areas and experience significant barriers to accessing healthcare.(43) While dengue RDTs are available at six referral hospitals, there is a lack of access to diagnostic testing for AFI outside these facilities. Suspected and confirmed cases of dengue are reported through the passive surveillance system, with seasonal outbreaks occurring in the wet season peak (December-January). However, the annual number of notifications is low and there is anecdotal evidence of underdiagnosis and underreporting.(44) Molecular diagnostic techniques for dengue have not been available in Timor-Leste and hence the dengue virus serotypes currently circulating were not known before the occurrence of the work presented in this chapter.(45)

Participant recruitment for this study took place at the Emergency Department (ED) at Hospital Nacional Guido Valadares (HNGV). This hospital provides care to adults and children in Dili, the capital city of Timor-Leste who are either self-presenting or referred from district referral hospitals and community health centres (CHCs). Assay implementation and diagnostic testing occurred at Laboratorio Nacional da Saúde (LNS) which is a clinical and surveillance laboratory adjacent to HNGV.

### 2.3.3 Participant assessment, recruitment, sampling and follow-up

All patients presenting to HNGV Emergency Department between 22/03/2023 and 23/06/2023 were assessed for potential inclusion during their initial patient triage by a study nurse.

Inclusion criteria were:

- Age >1 year
- Tympanic or axillary temperature ≥37.5 degrees Celsius at presentation
- Blood culture being taken as part of routine care

- Provides informed written consent (or parental consent +/- verbal assent from individuals under 16 years or next-of-kin declaration for those who lack capacity).

Initially, individuals who had been an 'inpatient' at any other health facility during the previous 28 days were excluded (defined as those staying for more than 8 hours, or overnight). This was to prioritise patients who had AFI and exclude patients who had chronic symptomatology and/or likely 'hospital-acquired infections'. However, an early interim analysis revealed high exclusion rate, with most excluded patients having spent 1-3 nights at another health facility. The inclusion criteria were therefore changed during week six, after which those who had been an inpatient for four or more nights were excluded.

Baseline data including demographic, clinical and treatment-related details were collected by ED nurses or clinicians by interviewing the participant and their family and by reviewing their medical notes. This occurred after the participant's initial clinical assessment by a clinician, and included provisional clinical diagnoses made based on history-taking, clinical examination and vital signs alone. Where dengue was included as a clinical diagnosis, warning signs or severe clinical features (as per WHO criteria) were noted if they were identified by the consulting clinician, but participants were not systematically assessed for disease severity by research staff.(14) Data were collected onto a paper questionnaire and later transcribed into electronic format on a mobile application with REDCap application installed. Adult participants then provided 4mL of whole blood and child participants provided 1mL of whole blood by phlebotomy into an EDTA sample collection tube. This was transported within eight hours to the laboratory where it was refrigerated at four degrees Celsius until it was analysed.

Follow-up data were collected by research nurses interviewing participants on days 1-10 and day 30 following their recruitment. This was done face-to-face if the participant was an inpatient at HNGV or by telephone otherwise. If a participant was not contactable (and when follow-up was scheduled for a weekend) the interview was marked as unsuccessful and the research nurse moved to the next

scheduled interview (usually the following day). If the 30-day interview was 'unsuccessful' then up to two further attempts were made, on days 31 and 32. Each follow-up was focused on ascertaining whether the participant was still experiencing fever (or fever symptoms), whether they were being treated with antimicrobial medications, and whether they had experienced any adverse outcomes since the last follow-up. For adult participants, disability was assessed at the 30-day interview only, using the WHODAS 12-item functional questionnaire. Follow-up data were input directly into electronic format on a mobile application with REDCap application installed. Data collection tools are shown in Appendix 2.

### 2.3.4 Ethics

Participants 16 years of age or over provided informed written consent. Those under 16 years provided verbal assent, with their parent or guardian providing written consent. For those who were assessed by the clinical team and/or research nurse to lack capacity to make decisions about participation in this study, the next-of-kin were asked to provide a declaration that they support their relative's participation. Participant information sheets and consent forms are shown in Appendix 2. I was awarded ethical approval to conduct this study from the Institute Nacional da Saúde (INS) Research Ethics Committee, Timor-Leste (Reference 157-MS-INS/GDE/I/2023) and the Northern Territory Research Ethics Committee, Australia (Reference 2023-4505)

### 2.3.5 Sample analysis

Blood samples underwent RNA extraction using the AusDiagnostics MT-Prep Extraction System and were then tested using the AusDiagnostics Mosquito Panel which is a multiplexed qPCR assay which detects dengue viruses 1-4, chikungunya virus, West Nile virus, Barma Forest virus, zika virus, Japanese encephalitis virus, Murray Valley encephalitis virus, Ross River virus and malaria.(46) This

assay was chosen because scientists at LNS had significant experience using a similar AusDiagnostics assay for detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Blood culture was performed using internationally recognised techniques, according to a local standard operating procedure.

### 2.3.6 Reporting of individual results

Research nurses reported all positive results from AusDiagnostics Mosquito Panel or blood culture testing to the clinician looking after the participant at the time of result reporting, and to the participant. Positive dengue results were also notified to the Communicable Disease Control Department, Ministry of Health, Timor-Leste along with relevant associated meta-data.

### 2.3.7 Data analysis

I undertook a descriptive analysis on 24/07/2023, including all participants who would have completed follow-up (i.e. those recruited between 22/03/2023 and 23/06/2023. The primary outcome was the number of participants who were diagnosed with dengue viruses 1-4 as a proportion of all recruited participants with AFI. Secondary outcomes included the number of participants who were diagnosed with any other pathogen by AusDiagnostics Mosquito Panel testing or by blood culture. I also reported meta-data and clinical outcomes including age, gender, duration of fever at presentation, clinical diagnosis at presentation, severity of any presumptive dengue diagnoses, need for re-admission, functional outcome at day 30 as measured by the World Health Organisation Disability Assessment Schedule 12-item questionnaire (WHODAS-12), mortality, and causes of death. Duration of antimicrobial therapy in whole days was calculated from available data at each follow-up. Where a participant went from receiving antimicrobials to not receiving antimicrobials (or vice versa) during a period when they missed one or more day of follow-up, the

change was assumed to have taken place on the middle day (rounded up). Duration of inpatient hospital admission was calculated in a similar way.

## 2.4 Results

### 2.4.1 Recruitment

Out of 93 individuals who were screened as eligible to participate between 22/03/2023 and 23/06/2023, 90 agreed to participate. Forty-eight (53.3%) were male. Fifty-two (57.8%) participants were adults whose median (interquartile range, IQR) age was 33 (25-52) years. 44/52 (84.6%) adults had capacity to make decisions about study recruitment and gave consent themselves, while 8/52 (15.4%) did not, with their next-of-kin providing a declaration instead. Adults' median (IQR) duration of fever symptom was 2 (1-4) days. Thirty-eight (42.2%) participants were children whose median (IQR) age was 3 (1-6.75) years. Childrens' median (IQR) duration of fever was 2 (1-3) days. All participants reported clinical features which localised infection to a possible anatomical site, including seventy-eight (86.7%) with one or more gastro-intestinal feature and 33 (36.7%) with one or more respiratory feature. Thirty-seven (41.1%) participants had received antimicrobials before recruitment and 19 (21.1%) had been an inpatient for 1-3 days at another healthcare facility before recruitment. There was a presumptive diagnosis of dengue in 30 (33.3%) cases, of which 8/30 (26.7%) either had clinical 'warning signs' or were 'severe'. Figure 3 shows weekly participant recruitment during the study and Figure 6 shows the flow of individuals through recruitment and participation.

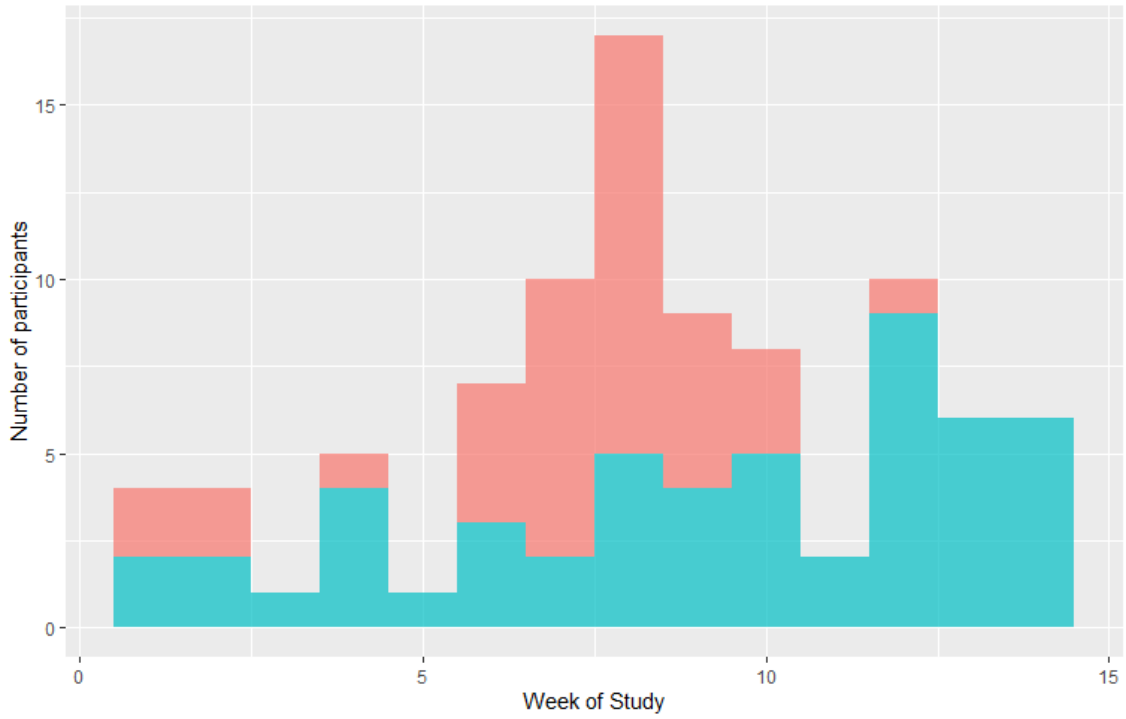



**Figure 5: Weekly participant recruitment during prospective observational cohort study of patients with acute febrile illness**

**Legend:** Bars are stacked with adults shown in blue and children shown in red. Inclusion criteria were modified such that those who had been an inpatient at another healthcare facility for 1-3 days could participate during week six.

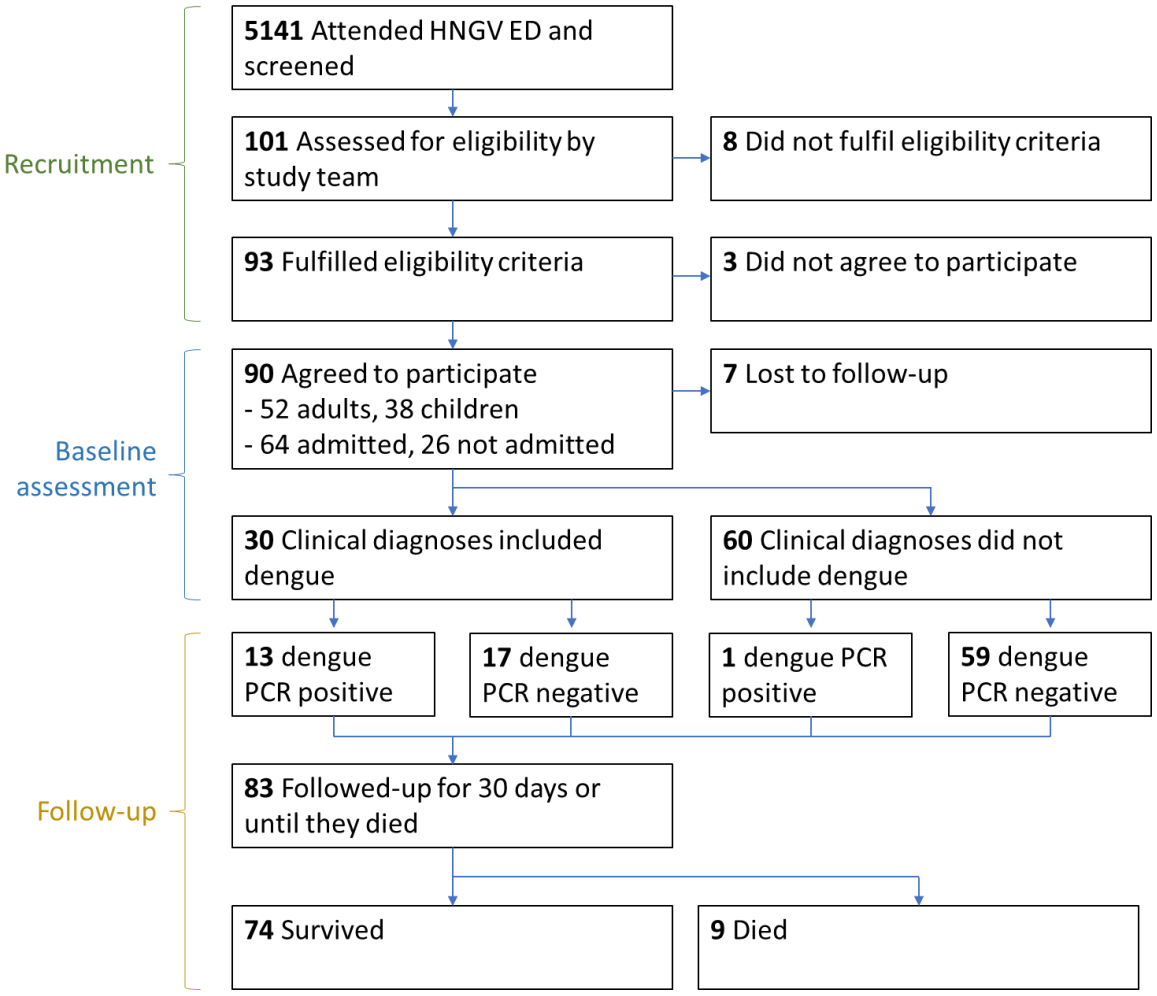



**Figure 6: Flow of individuals through recruitment and study participation**

### 2.4.2 Dengue virus serotypes and aetiology of AFI

PCR testing was positive for dengue in 14 (15.6%) participants. This included 10 participants with serotype 3 infection and one participant each with serotypes 1, 2 and 4 infection. One participant tested positive for both serotypes 1 and 3. One patient with a clinical diagnosis of acute appendicitis (and not dengue) was PCR positive for dengue. In 17/30 (56.7%) cases, PCR testing was negative, despite a clinical diagnosis of dengue having been made. Blood culture was positive in 4 (4.4%) cases with *Klebsiella pneumoniae* in two participants, *Staphylococcus aureus* in 1 participant, and *Pseudomonas stutzeri* (not considered to be a clinically significant result, rather blood culture contamination) in 1 participant. Table 1 summarises results from diagnostic testing.

**Table 3: Summary of results from RT-qPCR and blood culture testing**

| | Adults | Children | Total |
|---|---|---|---|
| Positive RT-qPCR (AusDiagnostics Mosquito Panel) result: | | | |
| - Dengue 1 detected | 1 | 0 | 1 |
| - Dengue 2 detected | 1 | 0 | 1 |
| - Dengue 3 detected | 2 | 8 | 10 |
| - Dengue 4 detected | 0 | 1 | 1 |
| - Dengue 1 & 3 detected | 0 | 1 | 1 |
| No target detected | 48 | 28 | 76 |
| Significant blood culture result: | | | |
| - *Klebsiella pneumoniae* isolated | 1 | 1 | 2 |
| - *Staphylococcus aureus* isolated | 1 | 0 | 1 |
| No significant blood culture result: | | | |
| - *Pseudomonas stutzeri* isolated | 0 | 1 | 1 |
| - No growth | 49 | 28 | 77 |
| - Blood culture not successfully undertaken | 1 | 8 | 9 |
| Total | 52 | 38 | 90 |

### 2.4.3 Participant follow-up and outcomes

Of the 90 included participants, 83 (92.2%) were followed-up for the full 30 days, or until they died. The median (IQR) follow-up duration for the 7 (7.8%) individuals who were lost-to-follow-up was 9 (8-10) days. Median (IQR) antimicrobial treatment duration of all 50 treated participants was 7 (5-9) days. The most commonly used antimicrobials were ceftriaxone (received by 70 % of participants), ampicillin/amoxicillin (42%), metronidazole (40%), cloxacillin (22%), and meropenem (4%). Median (IQR) duration of initial hospital admission was 5 (1-8) days and there were no re-admissions to

HNGV or any other healthcare facilities. At 30 days, median (IQR) WHODAS-12 outcome (only collected for adults) was 60 (58-60). Nine (10 .0%) participants died within 30 days of recruitment. The cause of death was 'hypoxic encephalopathy', 'meningoencephalitis', 'bronchopneumonia and cardiomyopathy', 'severe hypokalaemia', 'tuberculosis', 'severe dengue with haemorrhagic complications' and 'aplastic anaemia' for one participant each. The cause of death was 'unknown, reported by the family' for two participants. Table 4 summarises demographic, clinical, treatment-related, and outcome data, and compares cases where dengue virus 1-4 was detected to those where it was not. In univariable analyses, participants where dengue was detected by PCR were significantly more likely to be children ($p = 0.02$), and had shorter antimicrobial treatment durations ($p < 0.001$) compared to those where it was not. Figure 4 summarises the patient pathway for each participant.

| | Dengue PCR positive | Dengue PCR negative | | All participants |
|---|---|---|---|---|
| | | Clinical dx inc den | Clinical dx not inc den | |
| Demographic information | | | | |
| - Male gender (%) | 9 (64.2) | 8 (47.1) | 25 (42.4) | 42 (46.7) |
| - Median (IQR) age in years | 9.5(7.2-17.2) | 4 (1-24) | 29 (6.5-49) | 20.5 (4.2-36) |
| - Child (<16 years) (%) | 10 (62.5) | 5 (29.5) | 19 (15.3) | 38 (42.2) |
| - Inpatient for 1-3d before recruitment (%) | 1 (7.1) | 1 (5.9) | 17 (28.8) | 19 (21.1) |
| Clinical features at presentation | | | | |
| - Median (IQR) duration of fever in days | 3 (2-3) | 2 (2-3) | 2 (1-4) | 2 (1-3.7) |
| - Fever for > 3 days | 3 (21.4) | 3 (17.6) | 19 (15.3) | 23 (25.6) |
| - Clinical features localising potential infection (%) | 14 (100.0) | 17 (100.0) | 59 (100.0) | 90 (100.0) |
| - Gastro-intestinal tract (%) | 14 (100.0) | 15 (88.2) | 49 (83.1) | 78 (86.7) |
| - Respiratory tract (%) | 2 (14.3) | 17 (100.0) | 59 (100.0) | 78 (86.7) |
| - Other (%) | 3 (21.4) | 4 (23.6) | 7 (11.9) | 14 (15.6) |
| - Antimicrobials received before recruitment (%) | 3 (21.4) | 2 (11.8) | 32 (54.2) | 37 (41.1) |
| Clinical diagnosis at presentation | | | | |
| - Dengue (%) | 13 (92.9) | 17 (100.0) | 0 (0.0) | 30 (33.3) |
| - Warning signs/severe dengue (%) | 6 (24.9) | 2 (11.8) | 0 (0.0) | 8 (8.8) |
| - Other (not including dengue) (%) | 1 (7.1) | 0 (0.0) | 59 (100.0) | 60 (66.6) |
| Outcomes | | | | |
| - Dengue PCR positive (%) | 14 (100.0) | 0 (0.0) | 0 (0.0) | 14 (15.6) |
| - Blood culture with significant organism (%) | 0 (0.0) | 1 (5.9) | 2 (3.4) | 3 (3.3) |
| - Completed 30d follow-up (or died during follow-up) (%) | 14 (100.0) | 2 (11.8) | 5 (8.5) | 83 (92.2) |
| - Antimicrobial treatment given (%) | 6 (42.8) | 4 (23.6) | 40 (67.8) | 50 (55.5) |
| - Median (IQR) antimicrobial duration in days | 3 (2.2-3.7) | 9 (3-11) | 7 (5-10) | 7 (5-9) |
| - Admitted to hospital (%) | 13 (92.8) | 14 (82.4) | 37 (62.7) | 64 (71.1) |
| - Median (IQR) admission duration in days | 5 (4-7) | 7 (3-9) | 6 (3-12) | 6 (4-9) |
| - Need for readmission after discharge (%) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| - Median (IQR) WHODAS functional score at 30-days (/60) | 60 (60-60) | 60 (60-60) | 60 (58-60) | 60 (58-60) |
| - 30-day mortality (%) | 0 (0.0) | 2 (11.8) | 7 | 9 (10.0) |
| Total | 14 | 17 | 59 | 90 |

**Table 4: Summary of demographic data, clinical features and clinical outcomes**

**Abbreviations:** dx = diagnosis, inc = including, den = dengue, IQR = interquartile range, PCR = polymerase chain reaction, WHODAS = World Health Organisation Disability Assessment Schedule. *Only calculated for adults.

**Legend:** Cases where dengue viruses 1-4 were detected are compared to those where it was not. Fishers exact and Mann-Whitney U tests were used were used to compare categorical and ordinal variables, respectively.

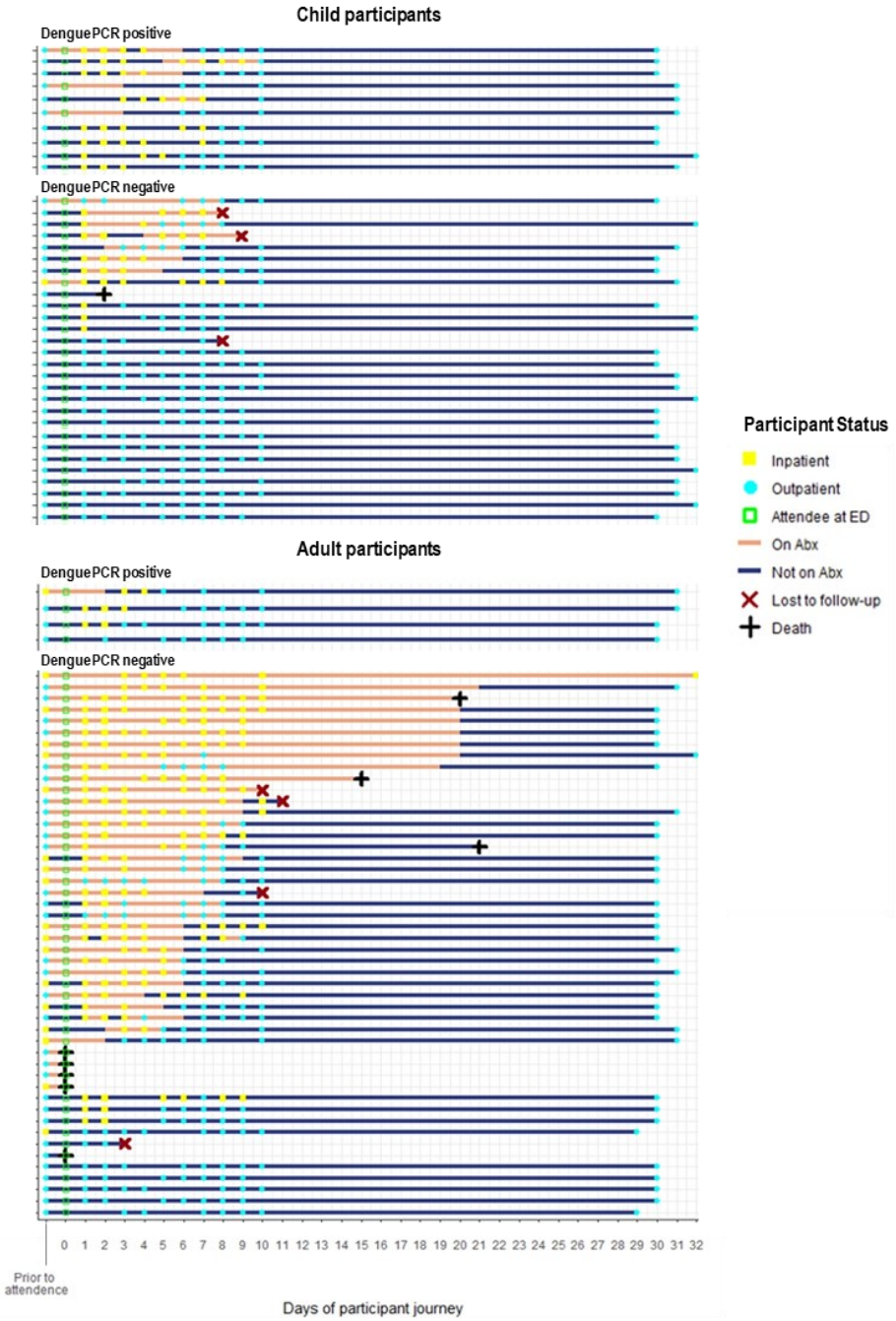



**Figure 7: Summary of participants' patient pathways after attending hospital with acute febrile illness**

**Legend:** Each of 90 participants are shown (y-axis) with details of their patient pathway during the study (x-axis). Duration on (orange line) or off (dark blue line) antibiotic treatment and successful inpatient (yellow dot) and outpatient (light blue dot) follow-up interviews, are show. Participants have been stratified based on whether they were children (aged <16 years) or adults (16+ years) and whether their sample was PCR-positive for any dengue virus (serotypes 1-4). Within these strata they have been ordered based on their duration of antimicrobial therapy.

### 2.4.4 Case-notification and surveillance

Fourteen cases of dengue were reported to the Communicable Disease Control Department, Ministry of Health along with relevant meta-data and the infecting dengue serotypes. Of these, 13 were suspected to have dengue infection on admission, but one was only diagnosed with dengue through participation and molecular testing in this study. There were 19 participants who had received a clinical diagnosis of dengue but were PCR negative. These may have been reported falsely had they not participated in the study.

## 2.5 Discussion

During this chapter I undertook a pilot observational study of patients presenting with AFI to a single site. An existing molecular diagnostic assay was successfully deployed and used to test participants for dengue viruses 1-4 and other arthropod-borne infections. This was the first prospective observational study enrolling participants with severe, community-acquired AFI in Timor-Leste, describing the patient pathway of 90 individuals presenting to the national referral hospital in Dili, including their clinical features, aetiology, antimicrobial treatment and outcomes.

Dengue was identified as a significant cause of AFI in this cohort. A high rate of admission, broad-spectrum antimicrobial use and mortality were observed. There was significant discrepancy between clinical diagnoses at admission and PCR results, highlighting the potential for such testing to improve individual treatment and reduce inappropriate use of antimicrobials. However, it is possible that the positive detection rate of PCR in cases of dengue and/or other arthropod-borne infections would have been higher had participants presented earlier in their patient pathway (i.e. during the viraemic phase of illness). Additional serological testing of participants, including comparison of convalescent with acute antibody titres to assess for seroconversion, would have been useful in understanding the role of molecular diagnostics when deployed in this situation.

Serotype-specific results were reported to the local surveillance authority for the first time and represent the only contemporary genomic data on currently circulating dengue virus serotypes in Timor-Leste. The potential role of molecular testing to enhance understanding of dengue transmission is therefore demonstrated.

Limitations of this study include its small sample size and occurrence at only one site in Timor-Leste during a three-month period. Findings are therefore limited in their generalisability. Larger, multicentre studies conducted throughout the year would yield more representative data on the clinical features, antimicrobial treatment and outcomes from AFI in Timor-Leste. Additionally, while the majority of participants completed follow-up (i.e. were contactable at 30 days post recruitment), many missed follow-up interviews between day 0 and day 10. In part this was because interviews were not attempted on weekends. When this occurred, some antimicrobial stop or start dates and hospital discharge dates were estimated to have occurred at the midpoint between successful follow-ups. Future studies could include follow-up on weekends and potentially home visits for discharged participants who are not contactable by telephone. However, multi-site surveillance over larger geographical areas would better support outbreak detection or prediction, and guide disease control interventions.

Two main challenges relating to the deployment and use of molecular diagnostic techniques were experienced during this study. First, only laboratory-based assays were available. This meant that the study was undertaken at a single site which is adjacent to a diagnostic laboratory with appropriate existing equipment and expertise. Replicating methods elsewhere would require significant sample transportation logistics and/or laboratory capacity building at smaller laboratory sites. Second, because a traditional RT-qPCR assay was used, samples were necessarily batch-tested once per week. This resulted in delays in reporting results, both to clinical teams and to the surveillance authority. Third, effective pathways for timely data transfer from the point of diagnosis

to the surveillance authority were not established before this study occurred. This likely contributed to further delays in potential usage of surveillance data.

## 2.6 Conclusion and key messages

A pilot observational study of patients with AFI in Timor-Leste identified a high rate of dengue infection and reported serotype-specific results to the local surveillance authority for the first time. The potential for molecular diagnostic techniques to enhance passive dengue surveillance was highlighted, alongside significant challenges relating to the deployment of assays in remote settings.

# CHAPTER 3

## 3.0 Defining user requirements for new diagnostic solutions

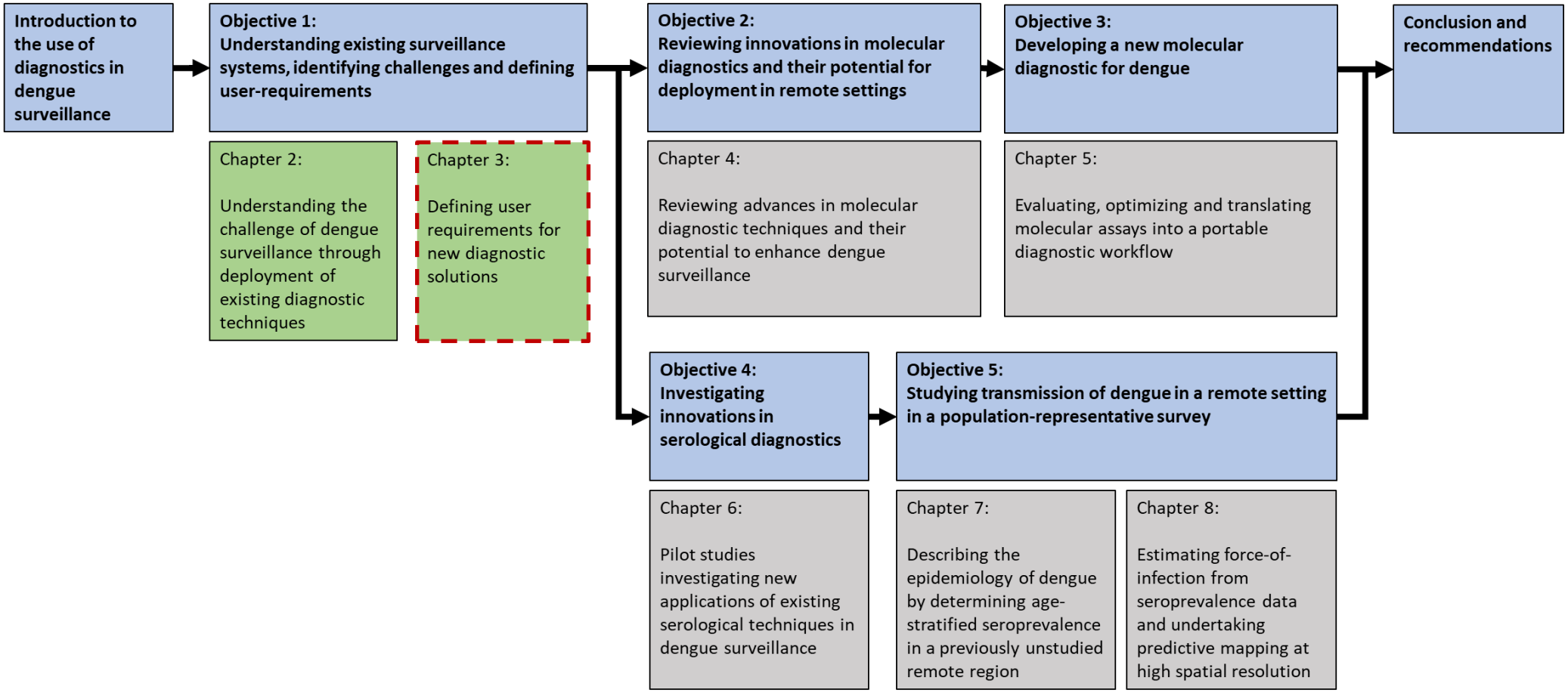



**Figure 8: Thesis overview**

## 3.1 Chapter introduction

In chapter 2 I undertook a pilot observational study in which existing molecular diagnostic techniques were used to test patients with AFI. A high rate of dengue infection was identified and serotype-specific results were reported to the local surveillance authority for the first time. However, significant challenges relating to the deployment of assays and potential scalability of this approach were experienced. Describing and understanding these challenges is likely to be crucial in developing appropriate solutions.

In the context of dengue surveillance, 'users' of technology may include individuals who are involved in the operation and interpretation of diagnostic devices, and/or the use of data they generate to make decisions about management of patients, surveillance or disease control.(47) The professional occupation of individuals undertaking each of these activities is likely to vary between healthcare settings and surveillance systems, but may include doctors, nurses, allied health professionals, laboratory scientists, public health practitioners, surveillance officials, and academics. Patients and the general public are also important users. Previous studies evaluating the implementation of RDTs for infectious diseases have identified potential barriers to their use. These include user training requirements, practical challenges in operating devices, difficulties interpreting and recording results, distrust of results, and a lack of direct impact on clinical decision making.(48–52) Outside the context of infectious disease diagnosis and surveillance, there is frequent non-adoption of health technology, including in rural and remote areas.(47,53,54)

It is crucial that technology is developed in collaboration with intended users and evaluated in the appropriate setting. Engagement throughout the design process is likely to result in optimised solutions and a maximised chance of technology adoption. (55–57). The Consolidated Framework for Implementation Research (CFIR) provides a set of domains which can be used to systematically assess barriers and facilitators to implementing health intervention. These include the intervention itself (and how it may be adapted), the setting, the processes, and individuals involved, (58,59)

## 3.2 Chapter objectives

The aims of this chapter were to (a) understand in detail the use of diagnostics in an existing surveillance system for dengue, (b) identify specific challenges, and (c) define user-requirements for new techniques.

## 3.3 Methods

### 3.3.1 Study design

To fulfil the aims of this chapter I designed a qualitative, focus group study of prospective diagnostic technology users who work in the Thai dengue surveillance system. I chose this setting because, while systems of passive dengue surveillance have existed for many years in Thailand, anecdotal evidence suggested they may perform sub-optimally and collaborators there have significant interest in diagnostics and their application to surveillance.

### 3.3.2 Setting

I conducted this study during July 2022 at four institutions in Thailand:

1. The Division of Vector Borne Diseases, Department of Disease Control at the Ministry of Public Health is the national authority responsible for surveillance of dengue and strategies for dengue control in Thailand.
2. The Hospital for Tropical Diseases, located in Bangkok, is a tertiary care hospital specialised in tropical diseases including dengue.

3. Khon Kaen hospital is a public hospital located in Khon Kaen province in the northeastern part of Thailand. The hospital provides inpatient and outpatient care for patients from rural areas who are either self-presenting or referred from community hospitals.
4. The Dengue Haemorrhagic Fever Research Unit (DHFRU) at Mahidol University, Bangkok is an academic centre with a multidisciplinary dengue research portfolio spanning pathology, diagnostics, clinical studies and epidemiology.

### 3.3.3 Participant recruitment

I took a purposive sample to ensure inclusion of participants with a range of experience across the dengue surveillance system in Thailand. This included public health practitioners, surveillance officials, doctors, nurses, laboratory scientists and dengue researchers. These individuals were identified by Dr Saranath Lawpoolsri and Dr Sanhapon Ketklao, who are researchers at Mahidol University, Bangkok with whom I collaborated during this chapter.

### 3.3.4 Ethics

Potential participants were approached by Dr Lawpoolsri during their usual working day and received verbal and written information about the purpose of the proposed study and its procedures (see participant information sheet and consent form, Appendix 3). I was awarded ethical approval to conduct this study from Mahidol University Faculty of Tropical Medicine Research Ethics Committee (Reference MUTM-2022-031-01)

### 3.3.5 Data collection

I travelled to the study sites in Thailand in July 2022 to collect data for this study. Together with Dr Lawpoolsri, I conducted a series of four focus group discussions, each of which included between

four and seven participants. We conducted these either in English or Thai language, depending on the preference of the participants when asked at the beginning of the session. Two occurred in person, and two by teleconference. I facilitated discussion using a topic guide which I developed in advance based on an up-to-date literature review and expert opinion on innovation in dengue diagnosis and surveillance from colleagues at Imperial College, London, Mahidol University, Bangkok, and Menzies School of Health Research, Dili (see Table 5). I reviewed and revised this topic guide iteratively during and between sessions, to ensure that emerging themes could be identified, explored further and/or triangulated within and between groups of participants. I audio-recorded focus groups and wrote notes including the details of any potentially important non-verbal communication. I organised for sections in Thai to be translated to English language and then transcribed recordings into word processing files.

**Table 5: Focus group topic guide**

| |
|---|
| **A. Contextual understanding and needs assessment.**<br>- How is dengue surveillance done, at your workplace (and more broadly)?<br>- Where do patients present to with symptoms of dengue and how do they get diagnosed?<br>- If tests are not always done, why do you think this is?<br>- Where / how should cases of dengue get reported, to surveillance?<br>- If positive results are not always reported, why do you think this is?<br>- How are surveillance data used? |
| **B. Requirements for new diagnostic devices: The assay.**<br>- Where does diagnostic testing usually occur, and what laboratory equipment is available there (if any)?<br>- Who typically operates diagnostic devices, and what sample preparation / analysis skills are required (if any)?<br>- What do you think would be the preferred sample type and sample volume, that would go into any new diagnostic device?<br>- What do you think would be the preferred (and maximum) time from sample to result (i.e. test duration), of any new diagnostic device?<br>- What do you think the preferred (and minimum) sensitivity and specificity, of any new diagnostic device?<br>- Is knowing the dengue serotype important?<br>- Is knowing the quantity of dengue (level of 'viraemia') in a patient's sample important? |
| **C. Requirements for new diagnostic devices: Remote connectivity and reporting.**<br>- How are results from diagnostic tests generally reported, and where are they stored?<br>- If a new diagnostic device could be remote-connected, where should results be reported to?<br>- Which information about cases would be most useful to report alongside test results, to enhance dengue surveillance?<br>- Would it be useful if a new diagnostic device could receive and display real-time information about local dengue incidence to the user (as well as transmitting data for case-reporting)? |

### 3.3.6 Data analysis

Together with Dr Ketklao, I conducted a thematic analysis. We each printed transcripts from all focus groups, annotated them and assigned codes independently. We then openly discussed and aggregated them into themes and subthemes. 'Current practices and challenges' and 'requirements for new diagnostics in surveillance' were overarching themes which we agreed *a priori*, as they were central to the aim of the study and the topic guide was developed specifically to explore these ideas. Within these, data were mapped to CFIR domains using a deductive approach.

## 3.4 Results

Identified themes mapped onto CFIR domains are summarised in Figures 9 and 10.

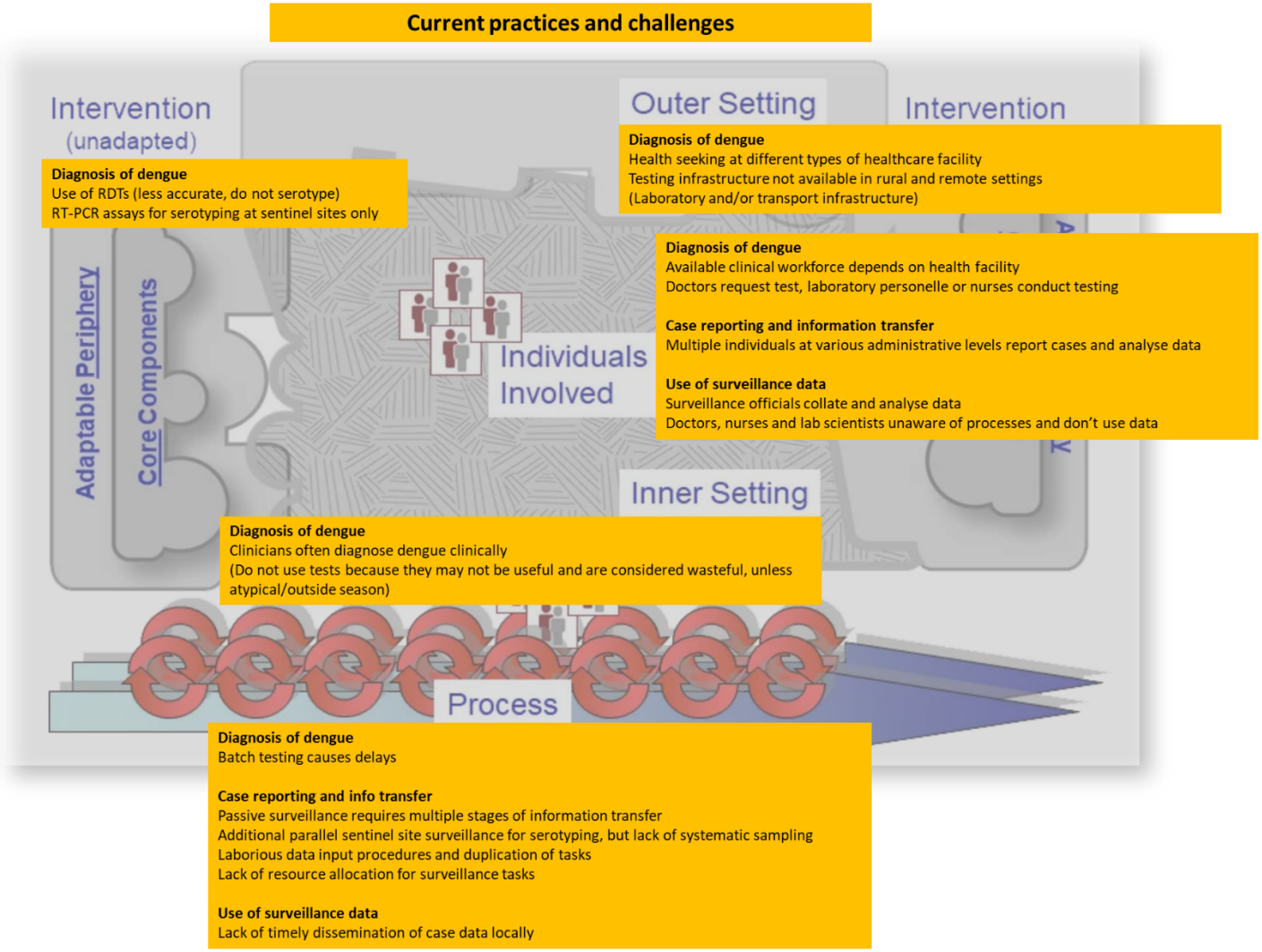



**Figure 9: Identified themes within 'current practices and challenges relating to dengue diagnosis and surveillance' mapped to the Consolidated Framework for Implementation Research (CFIR) domains**

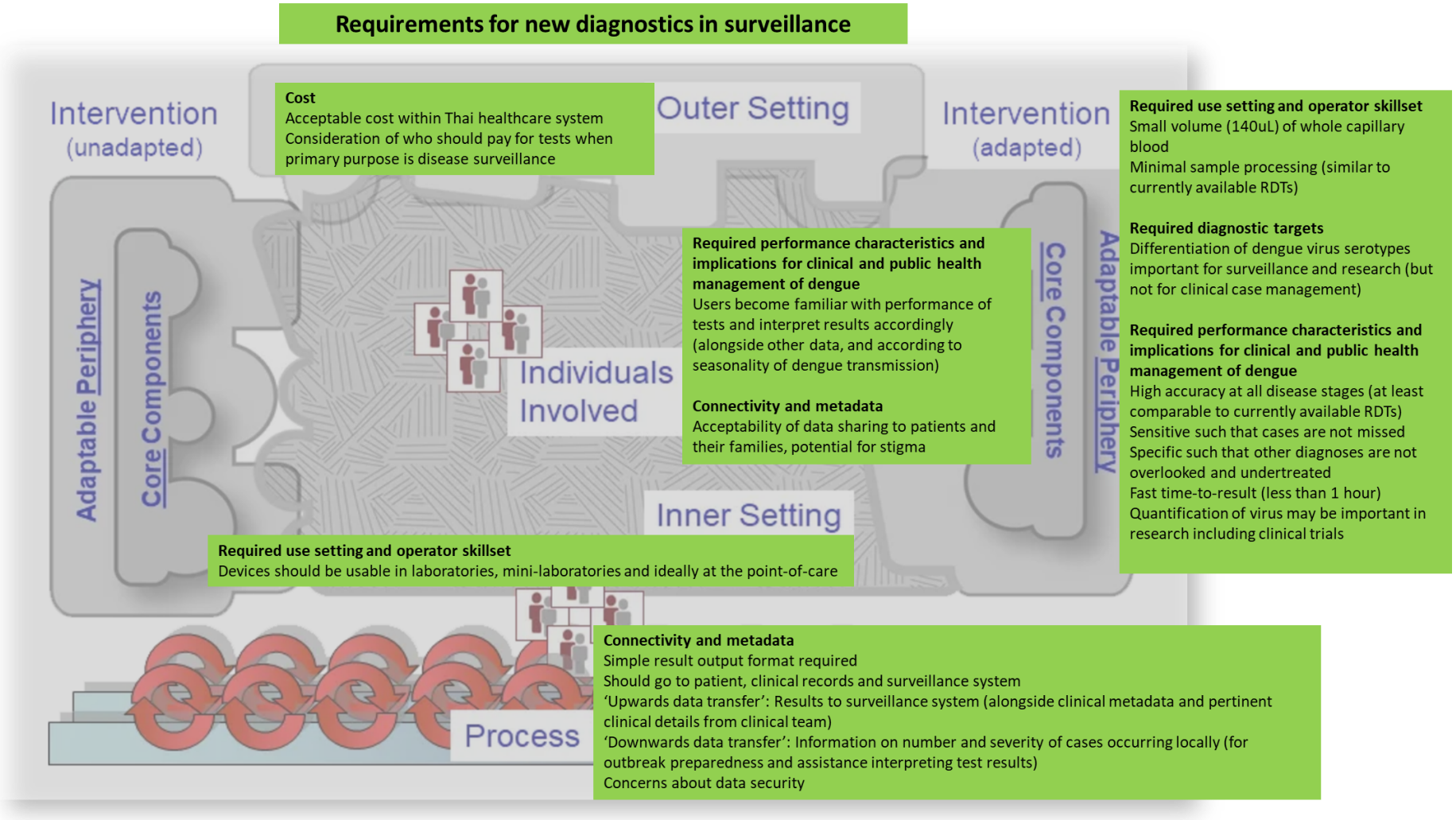



**Figure 10: Identified themes within 'requirements for new diagnostics' mapped to the Consolidated Framework for Implementation Research (CFIR) domains**

### 3.4.1 Current practices and challenges in diagnosing dengue

Participants described how individuals with dengue may present to different types of healthcare facility in Thailand, including primary health centres, district hospitals, regional hospitals, referral hospitals, pharmacies or private clinics, with each type typically having different clinical workforce and diagnostic test availability. There is a lack of access to diagnostic testing in healthcare settings in remote areas.

*"It depends on the level of [healthcare facility], if located in a very remote area, they cannot do a blood test [at the facility]."*

Senior doctors described frequently diagnosing dengue based on clinical features and many said they often didn't use a diagnostic test.

*"I think the senior doctors like me are very used to following the clinical, but I think the new generation of doctors are more likely to use the [RDT]."*

Cited reasons for not testing included participants having a high degree of confidence in their ability to make a clinical diagnosis, potential for tests to be inaccurate (particularly in the early phases of disease) and use of tests being a waste of resources. Some preferred to only use tests in atypical cases and/or during periods when incidence is low (i.e. outside dengue season).

When dengue tests are carried out, RDTs are most frequently used. These are usually operated at laboratory or 'mini laboratory' by a laboratory scientist but may be operated at the point-of-care by a nurse or another member of the clinical team. Participants were aware of other types of tests being available in Thailand, including RT-qPCR. However, RT-qPCR tests are rarely used because samples need to be transported to specialist laboratories and results may be delayed and therefore not useful in clinical management.

*"If you go to a [primary health centre] or small hospital they cannot do [PCR], they have to send it somewhere else"*

### 3.4.2 Current practices and challenges in case reporting and information transfer

Participants described a system of passive disease surveillance requiring multiple stages of information transfer. After a diagnosis is made by a doctor, this will usually be communicated to a nurse who will then inform the department or individual at their healthcare facility who has responsibility for disease reporting. This communication may occur by telephone or face-to-face conversation, but more often occurs using instant messages between personal mobile telephones. This informal communication was perceived to speed up the process of case investigation and notification.

*"In the past we reported using [a specific system] but it is not so successful because there is a lot of information to type and it requires a lot of time to do this. So we change to use the Line application, by taking pictures of the information required"*

Participants reported that cases should then be reported via a standardised form which is sent electronically, first to the District Surveillance Information Centre, then to the Provincial Epidemiological Unit, then to the Provincial Epidemiological Unit, then to the Ministry of Public Health (Figure 6).

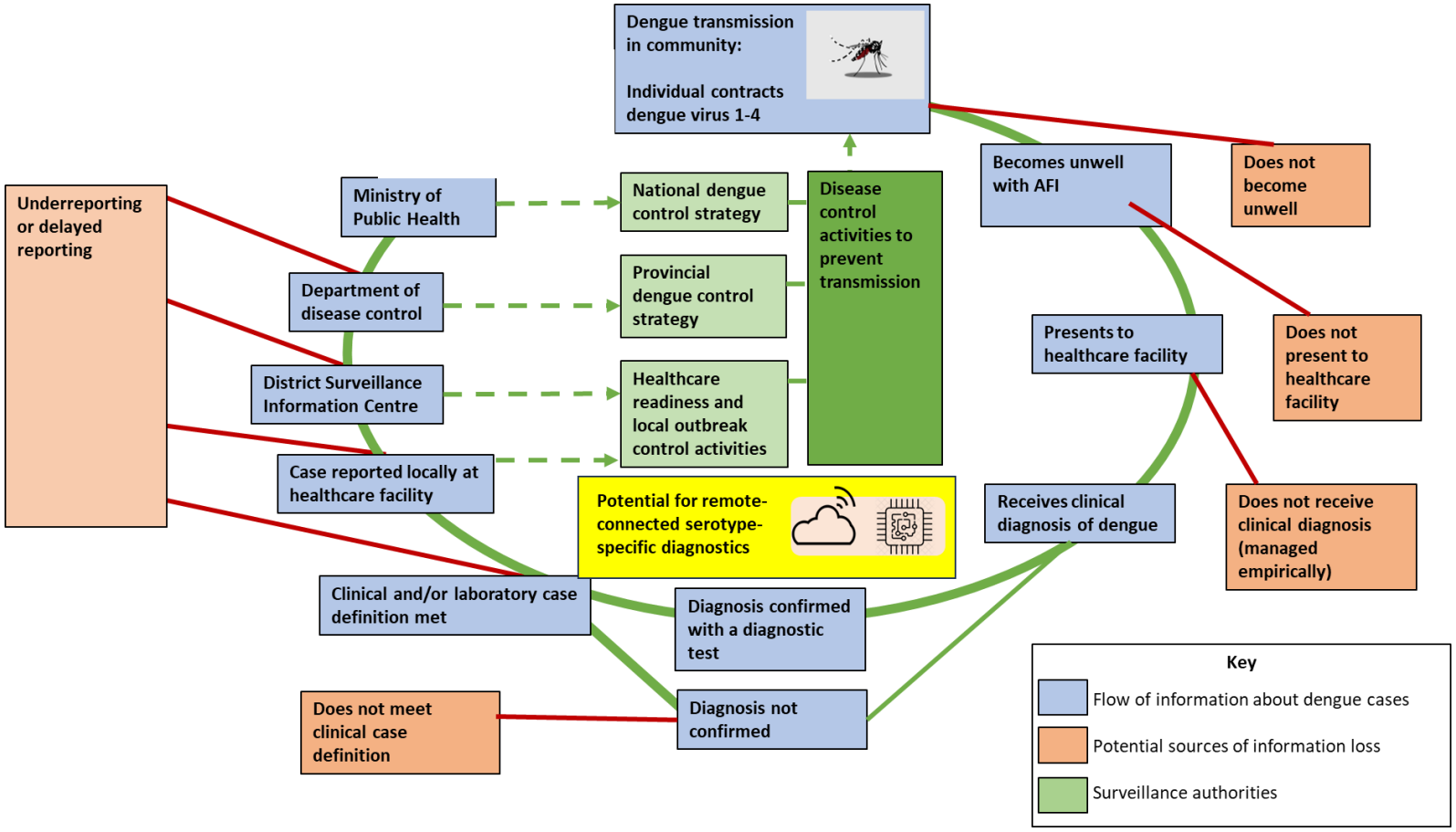



**Figure 11: Schematic diagram showing the pathway from individuals contracting dengue to their data usage in disease control.**

**Legend:** Flow of information about dengue cases and potential sources of information-loss (and hence causes of incomplete or delayed surveillance) are shown. Better availability of diagnostic testing (including in rural and remote healthcare and community settings) and remote-connected diagnostic devices for automated case-reporting may improve passive surveillance in this setting.

However, they reported that information transfer could be incomplete and/or delayed at each stage, and that delayed reporting could take up to 2-4 weeks.

*"Oh I'm really sad … to tell you, not only do we have an underdiagnosis situation, but we have an underreporting situation also."*

Particular barriers to case reporting at the hospital and district level included laborious data input procedures, frequent duplication of tasks and lack of time- and resource- allocation for these activities.

*"One of the reasons they don't report is they have to sit down and key in the result."*

Some participants also described an additional (parallel) system of sentinel site surveillance of dengue, with samples undergoing additional serotype-specific testing at a central location. However, only low numbers of cases are generally included and these are not recruited in any systematic manor. Furthermore, there can be a delay in obtaining serotype data because samples are batch-tested.

*"In order to get the benefits from these data, we should have a systematic sampling and have enough data. But now, some provinces send the data, some not. So the data we receive is not a good representative of the population."*

### 3.4.3 Current practices and challenges in the use of surveillance data

When participants were asked about the benefits of collecting surveillance data, responses varied according to professional occupation. Doctors, nurses and laboratory scientists were largely unaware of any process for data collation or analysis and said they did not benefit from any local or regional reporting of dengue cases. They had not received any information or warning about outbreaks as a result of the official surveillance system. However, some had occasionally received unofficial

communications from colleagues in nearby healthcare facilities (for example if a high number of cases had been experienced recently).

*"No one tells us, we just know when a large number of patients is coming!"*

Public health practitioners and surveillance officials explained how national and regional data is collated into reports but agreed that information could be disseminated more rapidly and used more efficiently at a local level.

### 3.4.4 User-requirements relating to use-setting and operator skillset

Participants stated that new devices for the diagnosis of dengue should be usable in a wide range of settings, including at the point-of-care (inpatient and outpatient) and in laboratories and 'mini laboratories' (non-clinical areas attached to smaller healthcare facilities where simple testing can occur). As such, there was a preference for analysing a small volume (up to 4 drops, ~140 µL) of capillary blood, which would be obtainable by finger-prick techniques and transferred directly into the device (as opposed to analysing larger volumes of venous blood or serum collected by venepuncture).

*"If we use it in outpatients where there are many patients, obtaining blood from the fingertip would be suitable"*

*"In terms of the amount … 3-4 drops will be fine, because [parents] don't want you to obtain large amounts of blood from their child."*

There was a strong desire for devices which require minimal or no sample processing prior to analysis (i.e. do not require centrifugation, pipetting, mixing or addition of reagents). These responses were frequently explained by reference to currently available RDTs, which are simple to use and can be operated by individuals without specific training in laboratory methods.

*"Nurses are not using pipette. If that's needed, it needs to be in the lab."*

*"We have to try to mimic the [RDTs]"*

### 3.4.5 User-requirements for diagnostic targets

Participants emphasised that dengue is very common across Thailand and is a significant public health concern. Many stated that new diagnostic devices should have the ability to differentiate dengue virus serotypes. Public health practitioners, surveillance officials and several dengue researchers had particularly strong desires for this, noting that it has not been achieved by currently available RDTs.

*"If we can get the serotype in real-time of course it will make our control measures more effective."*

Doctors and nurses could also understand this potential surveillance benefit but stated that serotype determination is of little consequence for individual patient management.

### 3.4.6 User-requirements for assay performance

Most participants cited 'accuracy' as an important characteristic for a new diagnostic device. They recognised that existing dengue tests were sometimes insensitive and could affect clinical management. Insensitive tests which give falsely negative results may lead to diagnosis of dengue being missed, with further tests and treatments for other causes (for example bacterial infections) being initiated or continued unnecessarily.

*"If the doctors see that the test is negative, [they] might diagnose something else and treat something else, like bacterial infection… they might prescribe some kind of [non-steroidal anti-inflammatory medication] or something that might harm the patient."*

They suggested that new devices should have at least the same sensitivity as currently available RDTs, and desired new tests which had high sensitivity regardless of the stage of disease.

*"If the test will be more useful than [RDTs] it should have the same sensitivity along the course of the disease."*

Participants also recognised that falsely positive results could have adverse impacts. They explained that non-specific tests which give falsely positive dengue results may lead to alternative diagnoses being missed, with potential discontinuation of important specific treatments (for example antibiotics), which was considered unacceptable.

*"If it has false positive it may lead to mistreatment of other diseases"*

*"This means it's not dengue but something else. Yes definitely, this delays the treatment. Yes it's going to be a problem."*

They caveated this by suggesting that users would become familiar with the performance of any new test, and would interpret results accordingly. They also described how clinical and epidemiological context are considered, when interpreting dengue test results.

*"We use it along with [routine laboratory data]. If [the test] is negative, but the case is likely to be dengue, we still have [routine laboratory data] to follow-up the patient"*

*"If the local prevalence of the infection is high, then the test-negative will not ensure that the patient has no dengue infection. But if the patient is in a without dengue area, we will have high confidence that this patient does not have dengue infection. It will depend on the prevalence at the time and in the local area ."*

Many participants also cited 'fast result' as an important characteristic for a new diagnostic device. This was particularly important for nurses and laboratory scientists who are frequent operators of RDTs. More than one suggested that the target sample-to-result time should definitely be less than one hour, and ideally less than 15-20 minutes.

*"If it can be reported fast, it will be good in terms of management and better turnaround time. I think less than 1 hour, but practically I think 15 minutes, like [RDTs]"*

The 'ability to quantify virus' was not considered an important characteristic, either for clinical or surveillance purposes.

### 3.4.7 User-requirements for connectivity and metadata

Participants recommended that diagnostic devices should have a simple way of displaying results to users and/or patients which has low chance of misinterpretation. They also stated that results should be recorded permanently on a patient's record. This could be achieved by integrating devices with electronic patient records and/or laboratory information systems (if being used in larger healthcare facilities or hospitals), or by allowing results to be printed (likely more feasible in smaller facilities).

*"I think it should give an official report so we can store the result in medical record or other systems"*

There was agreement among all participants that integrating diagnostic devices with surveillance systems could be helpful, and that receiving serotype data in this way would support surveillance efforts. Many suggested that it would reduce requirements for informal communication, paper records, data input and duplication of work at several levels of the surveillance system, hence improving case reporting. Public health practitioners and surveillance officials detailed which metadata should be reported routinely alongside the test results (table 2). They also felt that optional reporting of pertinent clinical details could be useful (for example details of particularly severe or atypical cases which may warrant further investigation).

**Table 6: Metadata which could be reported automatically from diagnostic devices to the surveillance authority**

**Upwards data transfer (device to surveillance system)**

**A. Test-related data**

- Date of test (date)
- Geo-location of test (latitude, longitude)
- Dengue test result (positive/negative)
- Serotype result (dengue virus 1 / 2 / 3 / 4)

**B. Identifiers**

- Name (free text)
- National identification number (number)
- Home address (free text)
- Patient's (or parent/guardian's) telephone number (number)

**C. Clinical details**

- Duration of symptoms in days (number)
- Severity of case at time of testing if dengue suspected clinically (non-severe/dengue with warning signs/severe dengue/patient died)
- Additional information for communication to surveillance authority. For example, details of particularly severe or atypical cases, or those where multiple family members are unwell, which may warrant further investigation (free text)

**Downwards data transfer (surveillance system to device)**

D. All test-related data (see A, above) from other devices.

- Note: This information could be output to the clinical user. For example, the number and proportion of recent positive tests in the surrounding area could be displayed, to aid interpretation of the current result. Graphs or maps showing temporal or geographical trends could also be displayed.

**Legend:** 'Upwards data transfer' (from diagnostic devices to the surveillance authority) and 'downwards data transfer' (from surveillance to the diagnostic device) should be combined.

As well as performing automated case notification ('upwards data transfer'), participants suggested that a new diagnostic device could also receive and display epidemiological data to the user ('downwards data transfer'). They expressed their desires for up-to-date information about the numbers and severity of dengue cases in their area and agreed that devices which provide early warning of dengue outbreaks would be useful.

*"If we know the information about the outbreak of dengue cases in the surrounding area, we will be more aware of the possibility of more severe cases coming to the hospital"*

Some explained how this knowledge could be used to assist in the interpretation of the dengue test itself.

*"When patients present with fever during the outbreak season the clinician usually ask where they come from. If we know that they come from an outbreak area, it increases the possibility that the case may be dengue "*

*"Knowing the map and the number of cases in the hospitals nearby would be helpful to let doctors be aware. If there are patients with fever coming it is more likely to be dengue. And also help to prepare beds."*

However, some participants had concerns relating to data security, particularly if devices could receive, store or display potentially sensitive information about other cases in the region (for example their location).

*"My concern is that it would interfere with the [Personal Data Protection Act]… When patient walk into the hospital, so they consent for the treatment and give the information and consent for us to use the information. So that's the way we can use it. But for point-of-care tests, which is possibly used by whoever in the field… they might need consent first, to enter the data"*

*"Someone can think about stigmatisation. OK so this family has dengue and someone can think that they are spreading dengue to the village, or something like that ."*

### 3.4.8 User-requirements relating to cost

Participants emphasised the importance of cost when considering the potential introduction of new diagnostic devices in Thailand. Usually, diagnostic testing is paid for by government insurance coverage, private insurance, or personal funds. Many participants considered a conceptual difference between testing which is undertaken for individual patient benefit (i.e. for diagnostic purposes) and that which is undertaken for potential collective population benefit (i.e. for surveillance), and felt that using personal funds to pay for the latter would be unfair.

## 3.5 Discussion

The participants I collected data from in this chapter desired new tests for acute dengue which are rapid, accurate and can be operated easily by non-expert users outside laboratory settings. These features largely reflect a broader existing literature on user requirements for diagnostics, including the ASSURED criteria.(41) They also supported integration of diagnostic technologies with surveillance systems because this may increase quantity and speed of case notification. . These requirements align with The World Health Organization (WHO) Special Program for Research and Training in Tropical Diseases 'ASSURED' criteria for diagnostics, and subsequent publications supporting real-time connectivity and ease of specimen collection ('REASSURED' characteristics).(41,60,61)

Tests which can differentiate dengue serotypes may be very important for surveillance and assessment of outbreak risk but would be less likely to benefit individual patients. As well as 'upwards data transfer' (such that cases are notified by devices to the surveillance authority), it was felt that 'downwards data transfer' (transfer of information from the surveillance system to each diagnostic device) would also be very useful. This could provide healthcare professionals with up-to-date information about local dengue cases, assisting them in the interpretation of individual test results as well as giving them early-warning of outbreaks. Cautions relating to this overall approach

included those relating to data security and the potential cost of new devices when compared to currently available diagnostic tests.

Various previous publications have described requirements for tests for acute dengue, including the Global Health Innovation Technology Fund 2013 target product profile.(62–64) However, there is no contemporary, agreed TPP, and surveillance requirements have been largely overlooked (with a focus on case management instead). Findings from this study may form a 'target product profile' (TPP) for dengue diagnostics which support real-time surveillance activities.

Previous studies have explored healthcare workers' and/or community members' perceptions of new diagnostic devices for tropical infections, particularly those intended to be used at the point-of-care. Diggle et al conducted interviews and focus groups in Northern Kenya about malaria RDTs, finding significant knowledge gaps, misconceptions and evidence of low uptake. Reasons included perceptions that testing was unnecessary, distrust of results, fear that devices might also test for other, potentially stigmatised conditions, and cost. However, RDTs were noted for their ease of use, portability and non-reliance on electricity.(50) Rasti et al conducted focus group discussions with healthcare workers in Southwestern Uganda exploring their use and perceptions of point-of-care tests. Findings supported the notion that such assays can bring diagnostic testing to under-resourced areas and improve clinical decision making. However, participants also described limitations, including the potential for inaccurate results and the importance of careful interpretation and corroboration using other clinical information.(51) Boadu et al interviewed healthcare workers in central Ghana, specifically to identify influencers of malaria RDT implementation in primary care facilities. Key factors included those relating to healthcare delivery constraints, provider perceptions and social dynamics of care delivery.(52) More recently, a scoping review of the use of mobile phones in the prevention and control of arboviral infections identified six studies where mobile phone technology formed part of a diagnostic solution and 25 studies where mobile telephones were used in surveillance (but not diagnosis). Cited benefits of using mobile telephone technology in

surveillance were a ‘reduction in error of transcribed data’, ‘rapid data transfer to a central server’, and ‘good completeness in terms of more dengue case reporting’, which are highly relatable to the present study’s findings.(65)

Previous studies have examined

Data collected in this chapter are the first specifically relating to user requirements for diagnostic devices which would optimise dengue surveillance. They are collected from a wide range of diagnostic technology users, including those who make decisions to test, those with hands-on experience of operating tests, and those who are involved in downstream analysis and/or usage of derived data (both in terms of individual patient management, surveillance and disease control). Broad inclusion appears to have been important because user requirements sometimes varied between occupational groups. Innovation in technology should account for this and may need to balance priorities of different users.

Limitations of these data include their collection from participants who only have experience of dengue diagnosis and surveillance in one country, which could mean that findings are geographically specific and are not fully transferrable to other settings. However, many of the practices and challenges described appear similar to those experienced in other Southeast Asian nations(27) and more widely.(40) An additional limitation is the exclusion of patients or members of the general public, who are important users of diagnostic technology. Results from the present study highlight a need to engage this group, particularly around the importance of data security. Additionally, requirements for diagnostic devices may be different if they are used by members of the public in the community to ‘self-test’. This potential application was not explored in the present study. However, studies of self-testing interventions using RDTs for COVID-19, malaria and human immunodeficiency virus have highlighted the importance of appropriate user interface, instructions for use, sampling and testing steps, and method of result interpretation.(66–68)

## 3.6 Conclusion and key messages

Systems of passive dengue surveillance are limited by a lack of access to diagnostic testing in rural and remote settings and a lack of effective, timely case-notification. New diagnostic technologies should be accurate, serotype-specific, easy to use outside laboratory settings and remote-connected such that results can be communicated to surveillance authorities from the point of testing Epidemiological information could also be communicated to users in real-time. These design features are most likely to result in user uptake and would optimally support systems of outbreak risk-assessment and responsive, targeted disease-control activities.

Findings from this chapter directly influenced my efforts to innovate and implement a new diagnostic workflow for dengue, which are presented in chapters 4-6.

# CHAPTER 4

## 4.0 Reviewing advances in molecular diagnostic techniques and their potential to enhance dengue surveillance

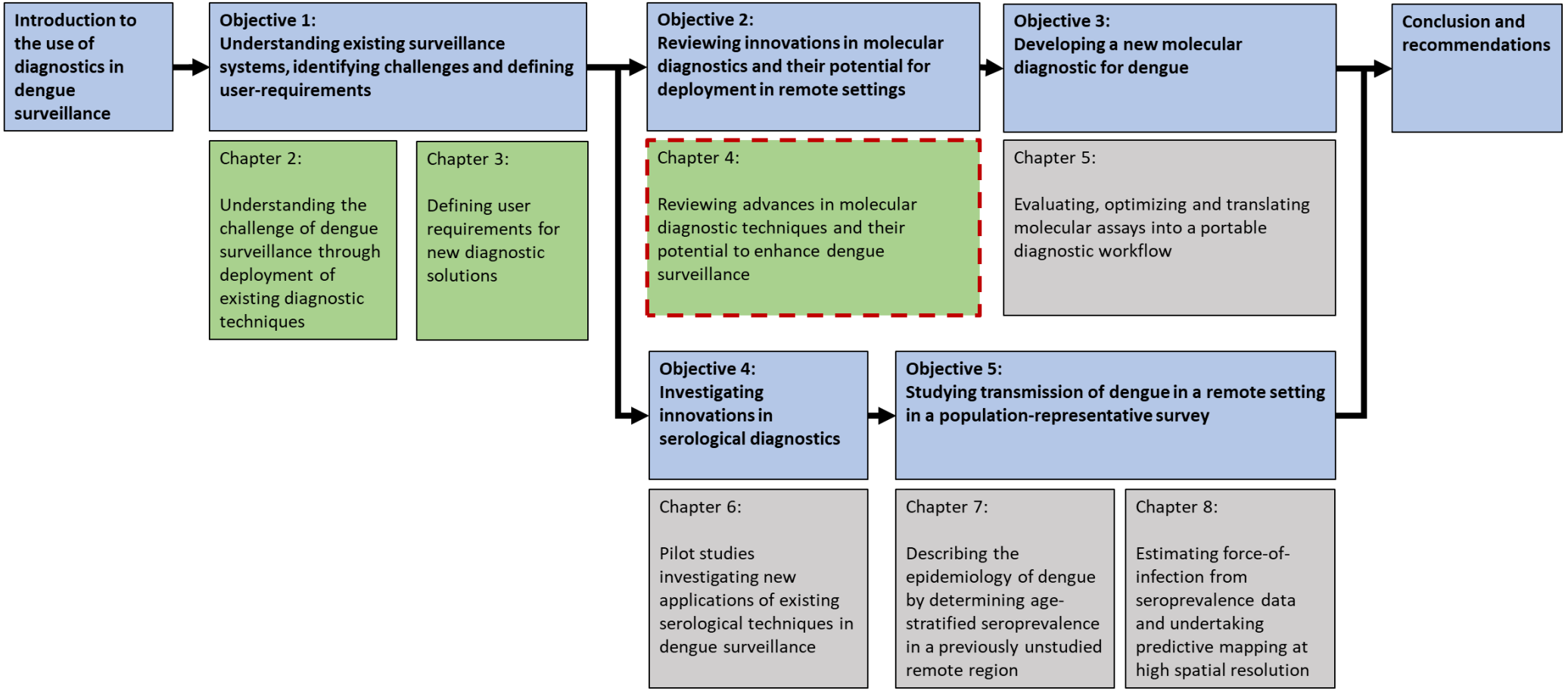



**Figure 12: Thesis overview**

## 4.1 Chapter Introduction

Molecular diagnostic techniques are methods used to help identify a disease by studying molecules in a clinical sample. In the broadest sense this may include assays which detect a wide range of endogenous and exogenous biological molecules.(69) However, in the field of infectious disease diagnostics, the term 'molecular' typically refers to the detection and/or characterisation of specific sequences of nucleic acids (DNA or RNA), but not proteins (for example antibodies or antigens). These are usually pathogen-derived, with the intention of determining the aetiology of an infection and/or other genomic features which may guide treatment. In this context, molecular diagnostics are synonymous with 'nucleic acid amplification tests'. RT-qPCR is the most commonly used molecular technique. Assays have high sensitivity and specificity and are the modern gold-standard in diagnosis and serotyping of infections. However, their use in remote and rural settings has been limited due to laboratory infrastructure requirements.(19) Currently available assays do not fulfil the user requirements I identified in chapter 2.

Molecular assays typically include four key components: First, the sample undergoes 'nucleic acid extraction', to isolate and purify DNA or RNA, removing undesired components which may inhibit or otherwise affect the efficiency of the amplification reaction. Second 'nucleic acid amplification' occurs, such that sequences of interest are replicated (and others are not). This requires 'nucleic acid primers', which are purposively designed to complement and bind to the target sequence. If the target is RNA (for example the dengue virus genome) it must be 'reverse transcribed' into cDNA before amplification can occur. Third, the amplified product must be 'detected'. When assays are designed such that nucleic acid is amplified and detected simultaneously, this is called 'real-time detection'. In these assays, signal strength is monitored over time and is related to the quantity of product. Together with sample collection, transport and storage (occurring before the assay is used), and result verification and reporting (occurring after the assay is used), they form a 'diagnostic workflow'. Results from chapter 2 highlight the need for diagnostic workflows to include result

reporting directly to relevant surveillance authorities as well as to clinical team members. There may also be a role for reporting to patients themselves. Figure 8 is a schematic, outlining these processes.

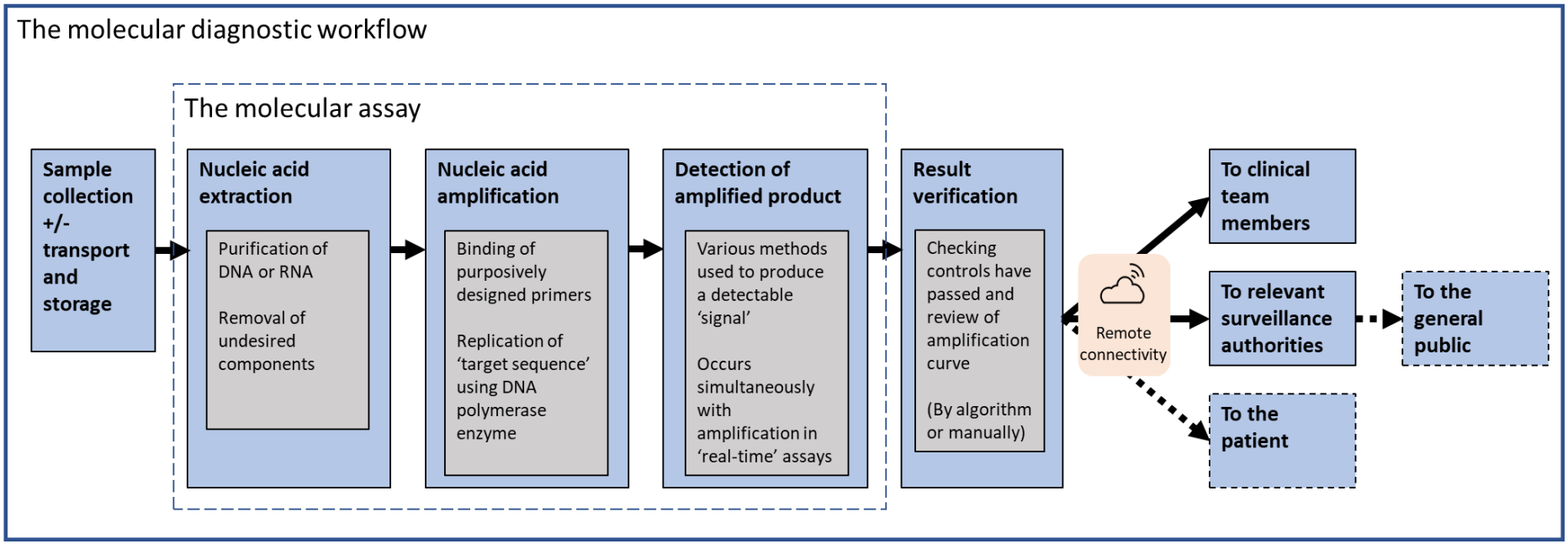
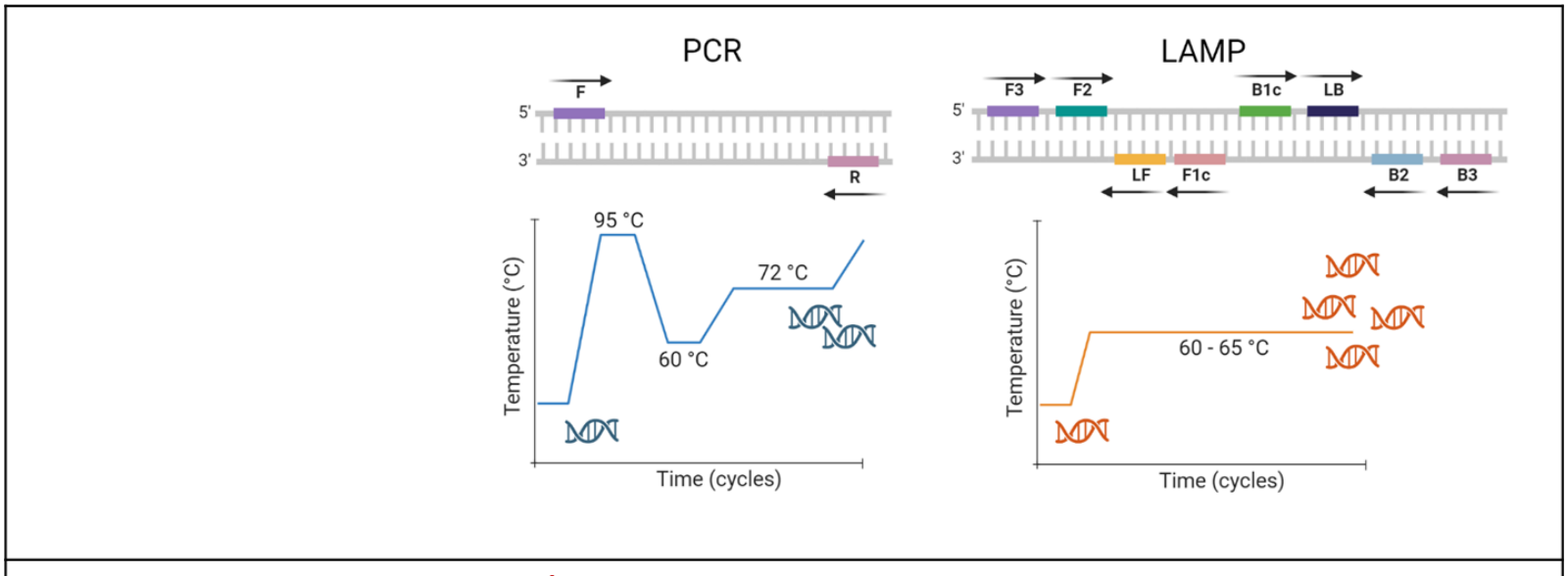



**Figure 13: Components of a molecular assay within a diagnostic workflow**

### 4.1.1 Loop-mediated isothermal amplification as a potential solution

Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification method first described in 2000 by Notomi et al.(20) When coupled with a reverse-transcriptase step using either Avian Myeloblastosis Virus or RTx (New England Biolabs) reverse transcriptase, which are two different reverse transcriptase enzymes suited to RT-LAMP because they are active at 63 degrees Celsius, it can be used to detect RNA (RT-LAMP). Samples can be analysed directly, but usually undergo a nucleic acid extraction step. LAMP uses a *Bacillus stearothermophilus* (*Bst*) DNA polymerase enzyme which possesses high autocycling strand displacement activity, allowing DNA synthesis to occur at a constant temperature (typically between 60-65 degrees Celsius). Multiple primers are used to create continuous loop structures during amplification. Primers may be designed to produce either generic or serotype-specific dengue assays. LAMP primers include forward and backward outer primers (F3 and B3, respectively), forward and backward inner primers (FIP and BIP), and forward and backward loop primers (FLP and BLP, which are not essential but can improve efficiency of the reaction). Amplified nucleic acids can be detected and/or quantified using various methods including visual inspection, turbidometry, gel electrophoresis, real-time monitoring using intercalating dyes and hybridisation with fluorescent probes, or non-fluorescence methods such electrochemical sensors.(70–73) Because LAMP assays do not require thermocycling equipment, they have been considered potentially more suited to miniaturisation and/or deployment outside the laboratory setting than PCR assays, including for dengue and other neglected tropical diseases.(21,22) Figure 9 shows how RT-LAMP compares to RT-qPCR in terms of primer-binding, incubation requirement and other characteristics.




| | | |
|---|---|---|
| Reaction time | **Slow** | **Fast** |
| Hardware requirements | **High** | **Low** |
| Yield of nucleic acid | + | ++ |
| Time/effort required to design high-performing assays | + | ++ |
| Potential for miniaturization and use in portable diagnostic devices | + | ++ |

**Figure 14 Comparison between polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP).**

**Legend:** Primer binding regions and thermal cycling conditions are shown at the top. Key differences between the methods are highlighted in the text with relative benefits shown in green and relative limitations shown in red.

### 4.1.2 Evaluating RT-LAMP assay performance

Studies which evaluate RT-LAMP assays may include measurement of various analytical performance characteristics (i.e. those which are inherent to the assay) and/or diagnostic (clinical) performance characteristics (i.e. those which become relevant when the assay is used to detect a condition or disease).(62,74,75)

Analytical performance characteristics include lower limit of detection (LLOD, also known as 'analytical sensitivity' which is defined as the lowest concentration of a given substance that can be detected) and the linear range of quantification (also known as the 'reportable range', which for quantitative assays is the span of test result values over which the accuracy of the measurement can be verified). Additionally, analytical specificity (which is the ability of an assay to detect only the intended target and the absence of 'cross-reaction' with potentially interfering nucleic acids or specimen-related conditions) can be determined by interference studies which test 'no template controls' and/or samples containing potentially interfering substances or non-targeted biomarkers. If/when amplification does occur, various techniques can be used to verify authenticity of the DNA product, and hence analytical specificity of the assay. These include assessment of amplicon specificity using a specific restriction enzyme and agarose gel electrophoresis, amplification and melting curve studies, and nucleic acid sequencing techniques.(76,77) Assuring analytical specificity is particularly important in assay design because several phenomena including the formation of amplifiable primer-dimers and hairpin structures can lead to false-positivity.(78–80)

Diagnostic performance characteristics include diagnostic sensitivity (which is the ability of a test to correctly classify an individual as having a condition or disease, i.e. the number of true positive results as a fraction of the total number individuals with the condition or disease) and diagnostic specificity (which is the ability of a test to correctly classify an individual as not having a condition or disease, i.e. the number of true negative results as a fraction of the total number of individuals without the condition or disease). An important consideration for diagnostic accuracy studies is the

choice of reference standard, which is used to compare the performance of the 'index test' against. This may be an alternative 'gold-standard' assay or may be based on validated clinical diagnostic criteria.(74)

## 4.2 Chapter objectives

The aims of this chapter were to describe the technical features and performance characteristics of previously developed serotype-specific dengue RT-LAMP assays and to assess whether they have potential for use in new molecular diagnostic devices or workflows which could be deployed in remote areas.

## 4.3 Methods

### 4.3.1 Study design

To fulfil the aims of this chapter I designed a scoping literature review with reference to the Preferred Reporting Items for Systematic reviews and Meta-Analyses extension for Scoping Reviews guideline.(81)

### 4.3.2 Search strategy

I conducted a search in Medline on 4$^{th}$ May 2022. I developed a search strategy using a combination of medical subject headings (MeSH) and keywords (Table 7). I imported results into Covidence software and deduplicated them.(82) Together with Dr Dumrong Mairiang, who is a researcher at Mahidol University, Bangkok, I reviewed citations at a title/abstract level for potential inclusion, and then at full text level for inclusion. We resolved discrepancies through discussion until consensus was reached. We also examined citation lists from all studies which were examined at full text level, as

well as those from any review articles, to identify any studies which may have been missed by the original search.

**Table 7: Search terms used in this chapter**

| | |
|---|---|
| 1 | exp Dengue/ or exp Dengue Virus/ |
| 2 | dengue.mp. |
| 3 | loop mediated.mp. |
| 4 | isothermal amplification.mp. |
| 5 | LAMP.mp. |
| 6 | 1 or 2 |
| 7 | 3 or 4 or 5 |
| 8 | 6 and 7 |

### 4.3.3 Inclusion criteria

I included studies if they were listed before 4th May 2022 (no prior time limit set) and fulfilled either of the following criteria

1) Described one or more newly developed RT-LAMP assay which was designed to detect a single dengue virus serotype ('original assays').

2) Described the further evaluation, adaption or implementation of one these assays.

I excluded studies if they did not detail the primer sets which were used (either within the publication, supplementary material or by reference) or if they were not written in English language. I also excluded studies if they described only generic dengue assays, because data from chapter 2 indicated a strong desire for serotype-specific diagnostic techniques. All types of laboratory or clinical study design were eligible.

### 4.3.4 Data collection and analysis

I collected data from included studies according to a pre-determined data collection form. I designed this based on the required performance characteristics which are needed before implementation of laboratory-developed tests, as detailed in the Revised Clinical Laboratory Improvement Amendments (CLIA) of 2003(83) (analytical performance characteristics) and the updated Standards for Reporting Diagnostic Accuracy statement of 2015 (diagnostic performance characteristics).(84) Where LLOD was given in plaque forming units (PFU) or RNA copies per µL, I converted this to PFU or RNA copies per reaction (by multiplying by the reported reaction volume), in order to make some comparison between the analytical sensitivity of assays. I also collected data on the technical features of each assay including its extraction method, reaction mixture ingredients, primer sequences, incubation temperature and detection method.

## 4.4 Results

My database search retrieved 87 unique articles, of which 46 were excluded based on titles and abstracts. Two articles requiring full text review were also identified through review of citations. I therefore assessed 43 full texts for eligibility. Twenty-two met inclusion criteria and 21 did not. The commonest reason for exclusion at full text review was 'assay was generic (i.e. not a serotype-specific assay)', which applied to 12 articles. Of the 22 studies included, 8 described original assays and 14 described the further evaluation, adaption, or implementation of a previously developed assay. A workflow showing the assessment of articles and their inclusion is shown in Figure 10.

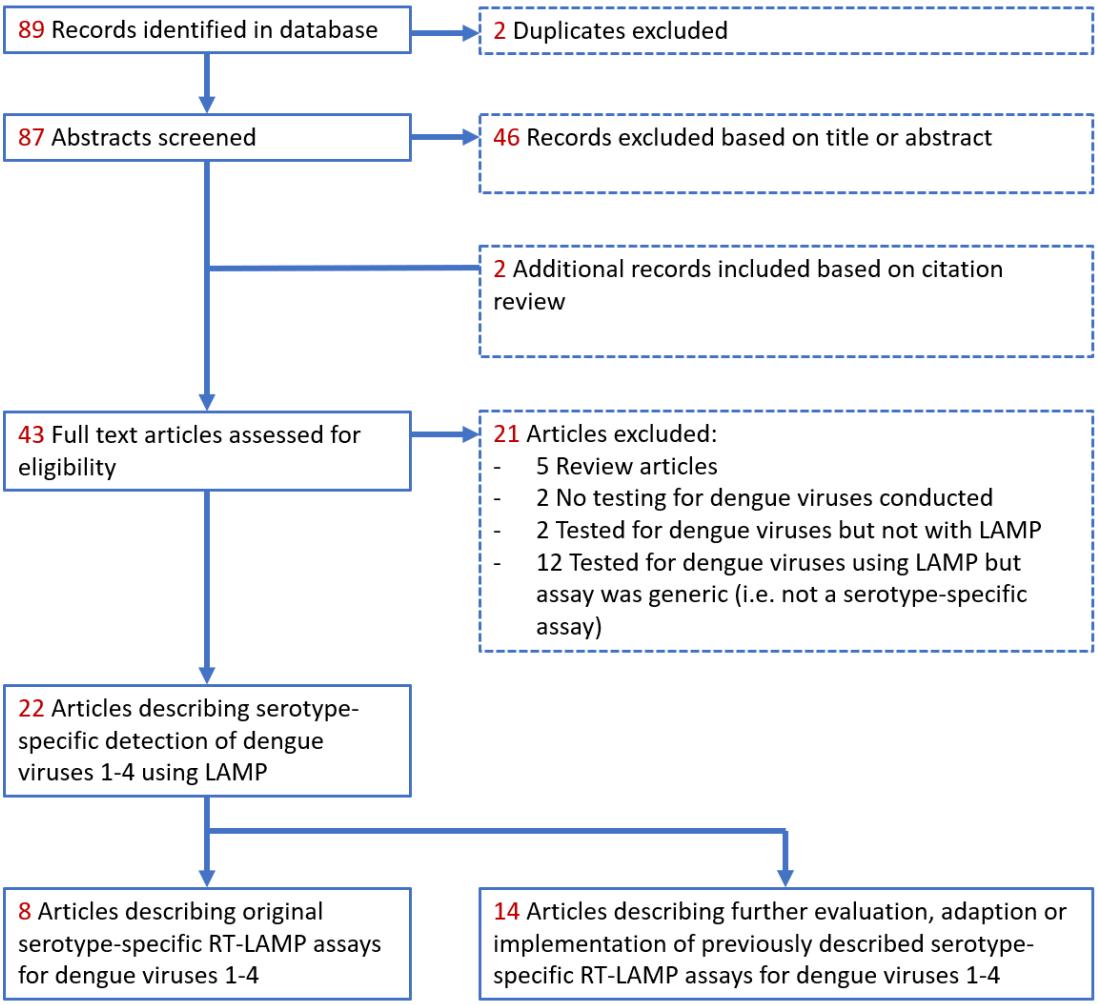



**Figure 15: Review workflow showing the assessment of articles and their inclusion in this chapter**

### 4.4.1 Technical features of original RT-LAMP assays

Eight sets of original RT-LAMP assays were developed by Parida et al (2005), Neeraja et al (2015), Hu et al (2015), Lau et al (2015), Yaren et al (2017), Kim et al (2018), Lopez-Jimena et al (2018) and Shoushtari et al (2021). (85–92) They were designed by obtaining sequences for dengue virus serotypes 1-4 from GenBank/NCBI database (7/8) or other sources (1/8). Various methods and software packages were used to identify potential template regions where sequences were conserved within sequences from the same serotype (but distinct from other serotypes and organisms) and assess possible secondary structures of primers. This included DNASIS software (Hitachi, Japan, 2/8 studies), Primer-Explorer V5 (Eiken Chemical Co. LTD, Tokyo, Japan, 2/8), LAMP

designer (Primer biosoft, America, 1/8), Primer-Explorer V3 (Eiken Chemical Co. LTD, Tokyo, Japan, 1/8), OligArch v2 (FfAME, Alachua, FL, 1/8), PrimerCompare v1 (FfAME, Alachua, FL, 1/8), and various R (R programming language / R Studio) packages (1/8). Figure 11 shows a schematic of the dengue genome and the position of primer-binding for each serotype-specific RT-LAMP assay.

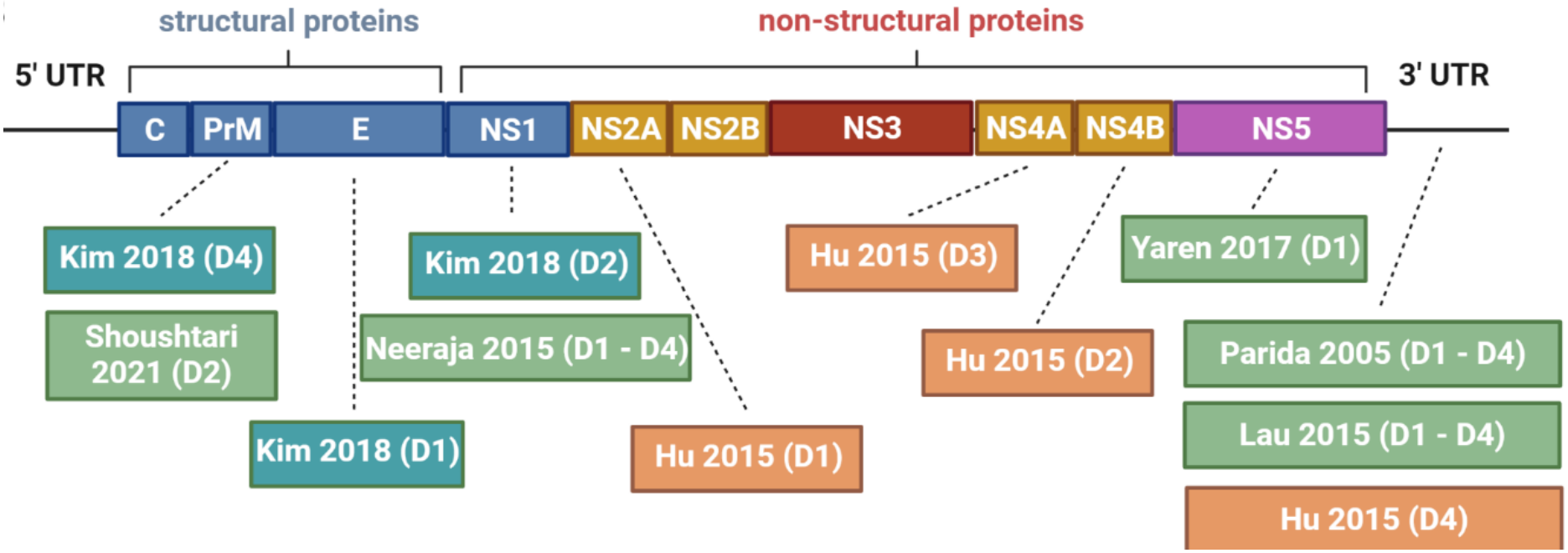



**Figure 16: Schematic of dengue genome showing the position of primer-binding in published serotype-specific RT-LAMP assays**

**Legend:** Primer binding positions of seven sets of published assays are shown. The eighth set of assays (Lopez-Jimena et al, 2018) could not be shown because primer sequences were not published.

Out of the eight original assays, seven performed nucleic acid extraction using commercially available kits (most commonly QIAamp viral RNA mini kit, QIAGEN, Hilden Germany). All assays used commercially available preparations of *Bst* DNA polymerase and a reverse transcriptase enzyme (Avian Myeloblastosis Virus or RTx reverse transcriptase). Some assays used commercially available LAMP reaction mixes, while others used bespoke mixes which included deoxynucleoside triphosphates, betaine, Tween 20, $(NH4)_2SO4$, $MgSO_4$ (or $MgCl_2$), KCl and Tris–HCl.

### 4.4.2 Analytical performance characteristics

When determined in 7/8 studies, LLOD of assays was between $2.5x10^{-3}$ and $1.22x10^{0}$ plaque-forming unit (PFU)/reaction (for studies which quantified target in PFU) and between $2.2x10^{1}$ and $8.25x10^{2}$ copies/reaction (for studies which quantified target in RNA copies). However, some studies did not give detailed description of the method for quantification of viral particles and/or copies of template RNA used in LLOD experiments. Furthermore, it was sometimes not clear whether the cited concentrations referred to those of original samples, the elution buffer after nucleic acid extraction (i.e. the extract) or the final reaction mix. In this review, concentrations have been converted to 'per-reaction values', as best possible from the information available in manuscripts. When determined in 3/8 studies, the linear range of quantification went as low as $2.5x10^{0}$ PFU/reaction (for the study which quantified target in PFU) and as low as $1.98x10^{2}$ copies/reaction (for studies which quantified target in RNA copies), but similar difficulties interpreting quantification methods and cited concentrations were encountered.

Analytical specificity was usually assessed by testing viral particles of similar viruses (including discordant dengue virus serotypes and other flaviviruses), or their synthetic RNA or DNA templates. Virtually no incidents of non-specific amplification were reported across all studies. However, the total number of reactions conducted during these interference studies was often not reported, or was fewer than ten. Authenticity of the amplified product was evaluated using digestion with a

specific restriction enzyme and agarose gel electrophoresis in 3/8 studies, nucleic acid sequencing in 3/8 studies, melting curve studies in 1/8 study, and was not done in 1/8 study. However, sometimes these data were not presented, and it was rarely clear whether authentication was undertaken for all experiments (i.e. every sample which was determined positive), or only a subset.

### 4.4.3 Diagnostic performance characteristics of published assays

Assessment of diagnostic performance occurred in 6/8 studies. Most often panel of 'positive samples' were used, which had been tested in parallel using alternative methods (5/6 studies), or had been characterised previously and assigned 'dengue positive' based on clinical criteria (1/6 studies). A panel of 'negative samples' from healthy individuals were also used in most studies (5/6). RT-qPCR was most often used as a reference standard (in 5/6 studies), but some studies did not clearly detail which test and/or clinical case definition was being used as a reference standard. In 1/6 study RT-LAMP testing resulted in significantly higher positivity than RT-qPCR, which was interpreted as superior diagnostic sensitivity but may also have been due to low diagnostic specificity.

### 4.4.4 Adaption of assays and potential for use in remote areas

Fourteen studies described the further evaluation, adaption, or implementation of a previously developed assay. None of these studies present a molecular workflow which has been deployed and thoroughly evaluated outside the laboratory setting. However, the following technological advances were presented:

Yamagishi et al (2017) adapted the Parida et al assay, analysing samples directly (i.e. without nucleic acid extraction) and loading amplified products into a portable MinION sequencer to determine the serotype. Sequencing was deemed necessary because erroneous LAMP signals were observed from

negative control samples, and the workflow was ultimately used under 'field conditions' at a small clinic in Indonesia.(93)

Ganguli et al (2017) printed and dried Hu et al primers onto microfluidic channels in bespoke sample-processing and amplification chips. Amplification occurred on the chip while it is housed within a 3d-printed light-proof cradle and a smart phone was used to perform real time detection of fluorescence in each channel.(94)

Minero et al (2017) developed and applied two different detection methods using optomagnetic spectroscopy to the Lau et al assays. First, the interaction between biotinylated FIP or LF primers (which incorporate into amplicons during the LAMP reaction) and streptavidin-coated magnetic nanoparticles (included in the reaction mixture) was observed in real-time. Second, a method was devised to try and discriminate between 'true positive' and 'spurious' LAMP amplicons using a 3'-biotiylated 'loop-validating' DNA probe. In this study the authors highlight the common problem of spurious amplicons in LAMP assays and the importance of having a readout method which is not prone to detection of these. (95)

Priye et al (2017) described a 'quenching of unincorporated amplification signal reporters' technique which was used to multiplex the Lau et al dengue virus 1 and 2 assays. The BIP primer was labelled with cyanine-5 and a short complimentary quenching probe was included, resulting in fluorescence upon cooling if specific amplification had occurred. The whole workflow was transferred into a 'smart phone-operated LAMP box' which included a heating module, an assay reaction housing module and an optical-detection/image-analysis module and gave a qualitative result for each target.(96)

Hin et al (2021) used The Lopez-Jimena assays in an automated device performing sample lysis, nucleic acid extraction, and up to 12 parallel LAMP reactions which are detected in real-time using fluorescence (the 'FeverDisk'). Analytical performance characteristics for the dengue virus assays in this format were not determined but specimens from some participants were tested positive.(97)

Kumar et al (2022) coated Prida et al primers with either biotin or digoxigenin, precipitated the amplified product using polyethylene glycol, and induced clumping with streptavidin- or anti-digoxigenin-coated magnetic particles. This produced a ‘magnetic’ assay which could be interpreted visually and multiplexed with another assay.(98)

Table 8 summarises the studies included in this review.

**Table 8: Summary of studies describing original RT-LAMP assays for dengue virus serotypes 1-4**

| Publication (year) | Assay details | | | | | Analytical performance | | | Diagnostic performance | | | | Subsequent publications (year) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Extraction method | Reaction mixture | Incubation | Detection | Target (gene) | Lower limit-of-detection | Specificity: other organisms | Specificity: examination of amplified product | Specimens | Reference standard | Sensitivity | Specificity | |
| Parida et al (2005) | QIAamp viral RNA mini kit (QIAGEN, Hilden Germany) | Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Japan) | 63.0 degrees Celsius for 60 mins (but determined positive at 30 mins) | Visual inspection (+/- addition of SYBR Green I) and real-time monitoring of turbidity and agarose gel analysis* | DENV1 (3' UTR)<br>DENV2 (3' UTR)<br>DENV3 (3' UTR)<br>DENV4 (3' UTR) | 1 PFU/ml (=2.5x10^-2 PFU/reaction)<br>0.1 PFU/ml (=2.5x10^-3 PFU/reaction)<br>0.1 PFU/ml (=2.5x10^-3 PFU/reaction)<br>0.1 PFU/ml (=2.5x10^-3 PFU/reaction) | JEV, WNV or SLEV templates - no amplification | Restriction enzyme digestion + agarose gel electrophoresis - product sizes in good agreement with predicted. Further confirmation with sequencing - nucleotide sequences matched target | 83 serum samples:<br>- 25 confirmed dengue cases<br>- 38 suspected dengue cases<br>- 20 healthy individuals | Considered positive if:<br>Conventional RT-PCR (+)<br>OR nested RT-PCR (+)<br>OR virus isolation (+) | 31/31 (100.0%) | 20/20 (100.0%) | Li et al (2011)<br>Chagan-Yasutan et al (2013)<br>Lo et al (2013)<br>Yamagishi et al (2017)<br>Kumar et al (2022)<br>Gaber et al (2022) |
| Neeraja et al (2015) | QIAamp viral RNA mini kit (QIAGEN, Hilden Germany) | Isothermal Master Mix ISO-001 (Optigene, U.K.) | 63.0 degrees Celsius for 35 minutes | Visual inspection (+/- addition of SYBR Green I) and agarose gel analysis* | DENV1 (NS1)<br>DENV2 (NS1)<br>DENV3 (NS1)<br>DENV4 (NS1) | N/A | Other flaviviruses (sic) including JEV, WNV, HCV and CHIKV - no amplification | Restriction enzyme digestion + agarose gel electrophoresis - product sizes in agreement with predicted. Confirmation using sequencing - results not in manuscript | 300 serum or plasma samples:<br>- 250 dengue cases<br>- 50 healthy individuals | Considered positive if:<br>RT-qPCR (+) | 140/140 (100.0%)** | 152/160 (95.0%)** | Dave et al (2022) |
| Hu et al (2015) | QIAamp viral RNA mini kit (QIAGEN, Hilden Germany) | Bespoke mix | 63.0 degrees Celsius for 45 minutes | Visual inspection (+/- addition of SYBR Green I) and real-time monitoring of flourescence (SYBR Green I) and agarose gel analysis* | DENV1 (NS2A)<br>DENV2 (NS4B)<br>DENV3 (NS4A)<br>DENV4 (3'UTR) | 1x10^1 copies/uL (=2.5x10^2 copies/reaction)<br>1x10^1 copies/uL (=2.5x10^2 copies/reaction)<br>1x10^1 copies/uL (=2.5x10^2 copies/reaction)<br>1x10^1 copies/uL (=2.5x10^2 copies/reaction) | JEV, YFV, HSV and Epstein-Barr virus x10 times - no amplification | Restriction enzyme digestion + agarose gel electrophoresis - images shown in manuscript. Further confirmation using sequencing - 'specificity of amplification confirmed' | 210 serum samples:<br>- 190 confirmed dengue cases<br>- 20 healthy individuals | Considered positive if:<br>'confirmed to be infected by dengue by clinical diagnosis' | 50/50 (100.0%)<br>59/60 (98.3%)<br>40/40 (100%)<br>39/40 (97.5%) | 20/20 (100.0%)<br>20/20 (100.0%)<br>20/20 (100%)<br>20/20 (100%) | Ganguli et al (2017) |

| Publication (year) | Assay details | | | | | Analytical performance | | | Diagnostic performance | | | | Subsequent publications (year) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Extraction method | Reaction mixture | Incubation | Detection | Target (gene) | Lower limit-of-detection | Specificity: other organisms | Specificity: examination of amplified product | Specimens | Reference standard | Sensitivity | Specificity | |
| Lau et al (2015) | QIAamp viral RNA mini kit (QIAGEN, Hilden Germany) | Loopamp RNA amplification kit (Eiken Chemical Co. Ltd., Japan) | 65.0 degrees Celsius for 30 min (DENV1-3 assays) or 45 min (DENV4 assay) | Visual inspection (+ HNB dye, Sigma, USA) and real-time monitoring of turbidity* | DENV1 (3' NCR) DENV2 (3' NCR) DENV3 (3' NCR) DENV4 (3' NCR) | The detection limit of RT-LAMP for 3'-NCR was as low as ten copies (=2.5x10^2 copies/reaction) | JEV, CHIKV and Sindbis virus - no amplification | Agarose gel electrophoresis - typical DNA ladder observed (though analysis of amplicon size using restriction enzyme not reported) | 213 serum samples - 189 suspected dengue cases - 24 healthy individuals | Considered positive if 2 or more of the following were true: RT-qPCR (+), ELISA (+), RT-LAMP (+). | 115/115 (100%) | 98/98 (100%) | Minero et al (2017) Priye et al (2017) Meagher et al (2018) Sigera et al (2019) |
| Yaren et al (2017) | Unclear | Bespoke mix | 65.0 degrees Celsius for 60-90 minutes | Real-time detection of fluorescence (TAMARA-labelled LB or LF probe) then modification to include 'target specific strand-displaceable probe' (fluorescence detected by cell phone camera). | DENV1 (NS5) | 1.22 PFU per assay (=1.22x10^0 PFU/reaction) | ZIKV and CHIKV RNA - no amplification | Agarose gel electrophoresis - typical DNA ladder observed (though analysis of amplicon size using restriction enzyme not reported) | N/A | N/A | N/A | N/A | Yaren et al (2018) |
| Kim et al (2018) | QIAamp viral RNA mini kit (QIAGEN, Hilden Germany) | Bespoke mix | 69.7 (DENV1 assay), 65.0 (DENV2 assay) or 66.5 (DENV4 assay) degrees Celsius for 40 minutes | Visual inspection (UV light illumination) | DENV1 (E) DENV2 (NS1) DENV4 (PrM) | 33 copies / uL (=8.25x10^2 copies/reaction) 3.55 copies / uL (=8.88x10^1 copies/reaction) 9.06 copies / uL (2.27x10^2 copies/reaction) | DENV3, norovirus, rotavirus and bovine viral diarrhea - no amplification | Agarose gel electrophoresis - typical DNA ladder observed (though analysis of amplicon size using restriction enzyme not reported) | N/A | N/A | N/A | N/A | |

| Publication (year) | Assay details | | | | | Analytical performance | | | Diagnostic performance | | | | Subsequent publications (year) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Extraction method | Reaction mixture | Incubation | Detection | Target (gene) | Lower limit-of-detection | Specificity: other organisms | Specificity: examination of amplified product | Specimens | Reference standard | Sensitivity | Specificity | |
| Lopez-Jimena et al (2018) | Various commercially available extraction methods depending on source of samples/viruses | Bespoke mix | 64.0 degrees Celsius for 45 min (DENV1 assay), 90 min (DENV2 assay), 75 min (DENV3 assay) or 50 min (DENV4 assay) | Real-time detection of fluorescence | DENV1 (various)<br>DENV2 (various)<br>DENV3 (various)<br>DENV4 (various) | 22 RNA molecules per reaction (=2.2x10^1 copies/reaction)<br>542 RNA molecules per reaction (=5.42x10^2 copies/reaction)<br>92 RNA molecules per reaction (9.2x10^1 copies/reaction)<br>197 RNA molecules per reaction (=1.97x10^2 copies/reaction) | ZIKV, YFV, WNV, Ntaya virus, *S. typhi*, *S. paratyphi*, *S. pneumoniae* and *P. falciparum* - no amplification | Melting curve analysis - single peak temperatures indicated specific amplification | 78 samples:<br>- 42 imported blood/serum samples<br>- 36 imported RNA extracts | Considered positive if: RT-qPCR (+) | Initially sensitivity = 17/24 (70.8%), then false-negative samples re-extracted re-run and sensitivity = 23/24 (95.8%)** | 7/7 (100%)** | Hin et al (2021) |
| Shoushtari et al (2021) | QIAamp viral RNA mini kit (QIAGEN, Hilden Germany) | Bespoke mix | 65.0 degrees Celsius for 60 minutes | Agarose gel analysis | DENV2 (C-PrM) | 100 RNA copies per reaction (=1x10^2 copies/reaction) | DENV1, DENV3, DENV4, WNV, YFV, ZIKV RNA (and serum from hepatitis C patient) - no amplification | N/A | 31 serum samples<br>- 20 dengue cases<br>- 11 healthy individuals | Considered positive if: RT-qPCR (+) | 15/15 (100%) | Results for 11 healthy sera not presented | |

**Abbreviations:** DENV = dengue virus, PFU = plaque forming units, JEV = Japanese encephalitis virus, WNV = West Nile virus, SLE = St Louis encephalitis virus, HCV = hepatitis C virus, CHIKV = chikungunya virus, HSV = herpes simplex virus, ZIKV = Zika virus, RT-PCR = reverse-transcriptase polymerase chain reaction, ELISA = enzyme-linked immunosorbent assay, RT-LAMP = reverse transcriptase loop-mediated isothermal amplification

* Studies frequently described more than one method for detecting amplified products of RT-LAMP. However, it was sometimes not clear how discrepant results were handled in analysis of assay analytical and diagnostic performance

** Multiple alternative analyses are reported in the manuscript

## 4.5 Discussion

During this review I identified eight studies which described original serotype-specific dengue RT-LAMP assays. All assays underwent evaluation of analytical performance with some also undergoing evaluation of diagnostic performance. However, studies were heterogenous in their design and reporting, and some omitted key experimental details. This made it difficult for me to objectively assess and compare assays, and would likely affect my attempts to replicate assays and verify findings. That said, I acknowledge that reports of assay development published in academic literature are often a preliminary step, occurring before more rigorous efforts are made to achieve validation and accreditation. As such, authors might not be expected to fulfil requirements such as those set by CLIA for implementation of laboratory-developed tests (which I used as a template for data collection in this study). Nevertheless, standardised assay evaluation and more detailed reporting of performance would have been beneficial in fully understanding the performance and potential of each of these assays.

When detailed, I found the method of nucleic acid extraction, ingredients of the reaction mix (apart from primers) and enzymes to be broadly similar across original assays. However, incubation temperature, incubation duration and method for detecting the amplified product varied considerably. Analytical sensitivity (LLOD) and linear ranges were comparable to those which are achievable with many RT-qPCR assays.(99) Analytical specificity was also reportedly good, with virtually no incidents of non-specific amplification being reported. However, I found that the numbers of experiments conducted using 'no-template controls' was generally low, and subsequent studies which used the same primer sets cited non-specificity as a particular reason for modifying incubation settings, primers and/or the detection method. Some studies interpreted higher positivity by RT-LAMP (index test) when compared to RT-qPCR (reference test) as evidence of superior RT-LAMP sensitivity, when in fact this could have been due to lower RT-LAMP specificity. Non-specific reactions are a feature of some nucleic acid amplification assays, including LAMP.(78–80) Therefore, assays must be designed carefully and evaluated thoroughly to ensure analytical specificity.

When evaluated, diagnostic performance characteristics were also reportedly good. However, I found that several studies did not state the gold-standard assay or clinical diagnostic criteria (or composite thereof) that was being used as a comparator (reference test). In studies which did give these details, RT-qPCR was most frequently used. However, if novel RT-LAMP based assays are developed which are truly portable and can be used at the POC (including sample preparation, amplification, and detection steps), then comparing their diagnostic performance to existing RDTs, which can be used in similar settings, may also be appropriate.

I found that assays had gone on to be used in 14 subsequent studies. These included studies where samples were tested directly (i.e. without any nucleic acid extraction prior to amplification). Assays which do not need sample preparation would be of huge benefit when considering their potential deployment into remote settings. However, the performance of so called 'direct LAMP' and superiority of LAMP assays over PCR assays in this regard is contentious.(100) They also included studies where reagents were lyophilised or 'printed' into microfluidic channels and those which used novel detection methods including the use of smart phone cameras, electrochemical sensing, and sequencing (MinION). These innovations overcome various challenges relating to RT-LAMP-based dengue diagnosis. However, no portable diagnostic workflow has so far been deployed and thoroughly evaluated outside the laboratory setting.

The restriction of my search strategy to include only serotype-specific assays for detecting dengue viruses 1-4 is a limitation, and I acknowledged that other relevant technological advances are likely to have been made and demonstrated in other applications of LAMP-based diagnostics. Additionally, it is possible that other data on dengue RT-LAMP assays which have not been published or included in Medline could have been missed by my search strategy. Nevertheless, conducting this literature review gave me an in-depth and up-to-date understanding of LAMP-based diagnostic techniques and their application to dengue diagnosis and surveillance.

## 4.6 Conclusion and key messages

Eight sets of serotype-specific dengue RT-LAMP assays have been developed previously. Available data suggest high analytical and diagnostic performance characteristics. However, study reporting is not sufficiently standardised to compare assays' analytical or diagnostic performance. While the RT-LAMP amplification technique does not, in and of itself, require significant laboratory equipment, none have so far been deployed or thoroughly evaluated in remote areas, and none have been used in programmes of passive surveillance. This is likely due to the ongoing requirement for sample extraction and signal detection which occurs separately using laboratory equipment in most published workflows. Development of equipment-free methods for these parts of the diagnostic workflow, and their integration with RT-LAMP assays, is therefore a priority.

Findings from this chapter shaped my research which objectively evaluated, optimised and translated RT-LAMP assays towards their use in a portable molecular diagnostic workflow (chapter 4), and implement a new system of dengue serotyping and surveillance in a remote setting (chapter 5).

# CHAPTER 5

## 5.0 Evaluating, optimizing and translating molecular assays into a portable diagnostic workflow

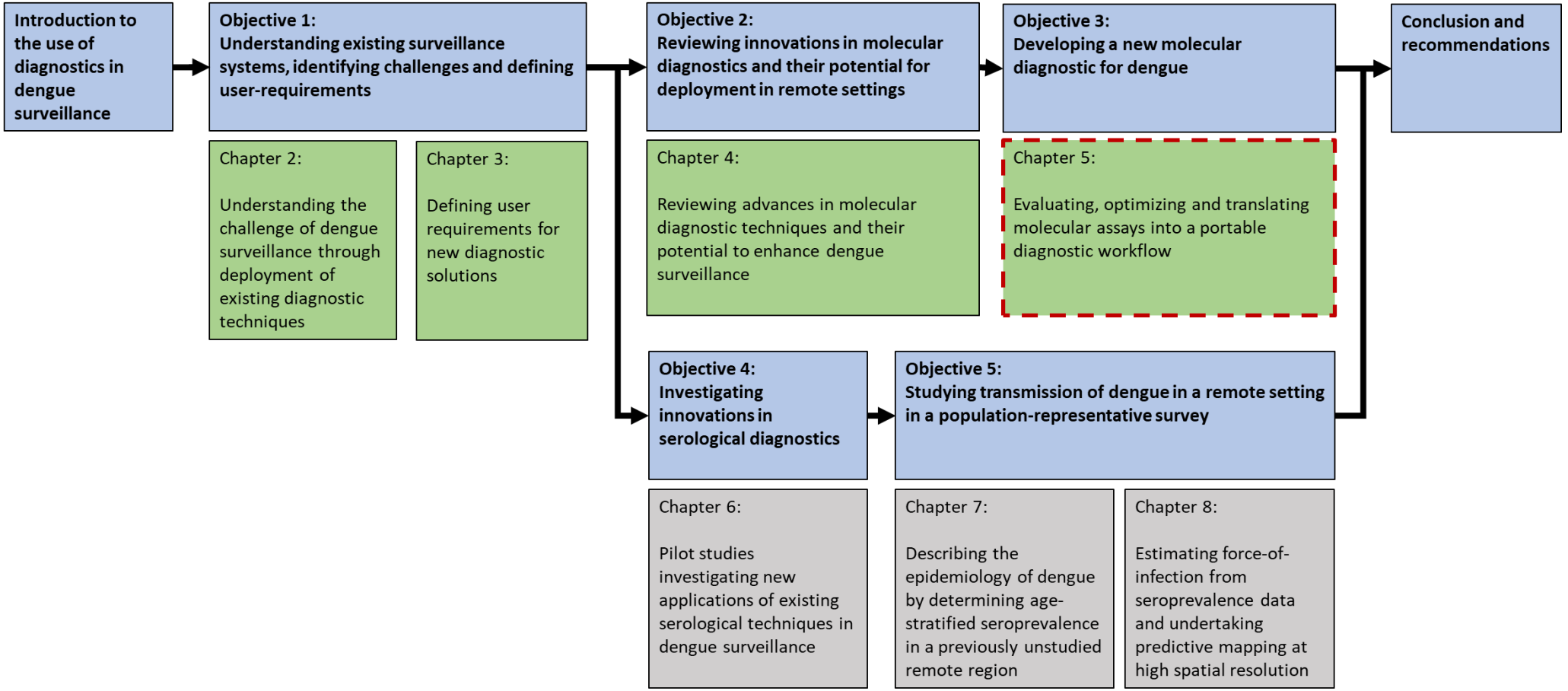



**Figure 17: Thesis overview**

## 5.1 Chapter introduction

In chapter four I identified eight sets of previously published serotype-specific dengue RT-LAMP assays. These reportedly had good analytical and (in some cases) diagnostic performance. However, reporting was not sufficiently detailed or standardised to allow comparison between assays, and most included nucleic acid extraction and/or detection stages which required laboratory equipment. Despite some advances presented in 14 subsequent studies, no workflow has so far been deployed and thoroughly evaluated in a remote setting, and none had been used in a programme of passive dengue surveillance. This would require all stages of the diagnostic workflow to be reproducible outside the laboratory setting by users without specialist training in laboratory methods. It would also require any reagents and consumables to be easily transportable and storable, ideally without the need for refrigeration. Simple, low cost equipment which can be used on a desk or in a 'mini-lab' with access to a standard power supply would be acceptable. Hereafter, methods which fulfil these requirements, which were identified by users in chapter 2, will be termed 'portable'.

## 5.2 Chapter objectives

The aims of this chapter were to (a) perform an objective in-silico and in-vitro evaluation of published serotype-specific RT-LAMP assays, (b) optimise performance of the most promising assay for each dengue virus serotype, and (c) translate them into a new diagnostic workflow through integration with portable nucleic acid extraction and signal detection techniques.

## 5.3 Methods

### 5.3.1 Study design

To fulfil the aims of this project I designed a series of in-silico and in-vitro laboratory experiments employing a variety of molecular methods which I undertook at the Centre for Antimicrobial Optimisation (CAMO) at Imperial College, London. I undertook these in four stages:

1. In-silico evaluation

2. In-vitro evaluation and comparison

3. Optimisation

4. Translation

### 5.3.2 In-silico evaluation of assays

I performed an in-silico evaluation of five sets of serotype-specific dengue RT-LAMP assays published by Parida et al (2005)**,** Neeraja et al (2015), Hu et al (2015), Lau et al (2015) and Kim et al (2018). Three other sets of assays were excluded from this analysis (and all downstream experiments presented hereafter) because the exact primer nucleic acid sequences used were not reported (Lopez-Jimena et al, 2018), assays for only one serotype were published (Yaren et al, 2017, Shoushtari et al, 2021), or there was less evidence of their high-performance identified during my literature review in chapter 3 (all three).

For these experiments I used Geneious Prime, which is the preferred bioinformatics software platform used by other researchers at CAMO.(101) I imported primer sequences from each publication and performed a nucleic acid alignment using the reference strains for each dengue virus serotype which were used in the original publications as templates. These were available on Genbank and are shown in Table 9.

**Table 9 Reference strains of dengue viruses 1-4 and gene sequences used during in-silico evaluation of serotype-specific RT-LAMP assays**

| Target | Reference strain accession number | Gene | Assay |
|---|---|---|---|
| Dengue virus 1 | U88535.1 | 3'UTR | Parida (2005) |
| | MG957435.1 | NS1 | Neeraja (2015) |
| | MK506262.1 | NS2A | Hu (2015) |
| | OK562744.1 | 3'UTR | Lau (2015) |
| | FJ687475.1 | E | Kim (2018) |
| Dengue virus 2 | AF038403 | 3'UTR | Parida (2005), Lau (2015) |
| | | NS4B | Hu (2015) |
| | AF162448.1 | NS1 | Neeraja (2015) |
| | KP406804 | NS1 | Kim (2018) |
| Dengue virus 3 | M93130.1 | 3'UTR | Parida (2005), Lau (2015) |
| | | NS4A | Hu (2015) |
| | EU371057 | NS1 | Neeraja (2015) |
| Dengue virus 4 | AF289029.1 | 3'UTR | Parida (2005), Hu (2015), Lau (2015) |
| | KC620380.1 | NS1 | Neeraja (2015) |

I used the *align* function in Geneious Prime to assess primer binding and identify any features which may adversely affect analytical sensitivity (primer melting temperature outside optimal range, primer guanine or cytosine content outside optimal range, and distance between primers when aligned to target nucleic acid sequence) or specificity (potential for primers to form secondary structures or off-target binding). Parameter targets were defined as those used in the PrimerExplorer package, which is the preferred package for LAMP primer design used by other researchers at CAMO.(102)

### 5.3.3 In-vitro evaluation and comparison of assays

Having identified and characterised assays' potential averse features in-silico, I proceeded to assess their analytical performance in-vitro. As the primary aim was to verify published data relating to the

LAMP reaction itself, and to compare analytical performance of each assay, I performed these experiments under laboratory conditions (i.e. without any modification which would make the assays portable). I used a standardised LAMP reaction mixture and incubation settings for all reactions, which has previously been optimised by other researchers in our group. Table 10 shows my LAMP mixture, which is comparable to that used in original publications .

**Table 10: Loop-mediated isothermal amplification (LAMP) reaction mixture used in in-vitro evaluation and assay optimisation experiments**

| Reagent | Stock concentration | Final concentration | Volume per reaction (µL) |
|---|---|---|---|
| Nuclease-free water | - | - | 2.82 |
| 10X isothermal buffer (without Tris) | 10X | 1X | 1 |
| MgSO4 | 100mM | 5mM | 0.6 |
| dNTPs | 25mM | 1.4mM | 0.56 |
| BSA (glycerol-free) | 20mg/mL | 1.2mg/ml | 0.5 |
| Betaine | 5M | 0.8M | 1.6 |
| 10X primer mix | 10X | 1X | 1 |
| NaOH | 0.2M | $4.6x10^{-3}$ | 0.23 |
| Bst 2.0 DNA Polymerase (warmstart) | 120U/µL | 0.5U/ µL | 0.04 |
| SYTO 9 | 20uM | 0.5uM | 0.25 |
| Target RNA | * | * | 1 |
| Total reaction volume | - | - | 10 |

**Abbreviations:** MgSO4 = magnesium sulphate, dNTPs = Deoxyribonucleotide triphosphates, BSA = bovine serum albumin, NaOH = sodium hydroxide

* Concentration of target RNA (or sample RNA extract) varied depending on the specific experiment or reaction being conducted

For these experiments I used synthetic double stranded DNA as the nucleic acid template. I chose relevant sequences from the aforementioned dengue virus serotypes 1-4 reference genomes, which I procured from Integrated DNA Technologies in the form of a lyophilised pellet 'gblock'. I reconstituted these using Tris EDTA (TE) Buffer according to a departmental protocol, such that my

primary stock solution would be approximately 10 ng per µL. I did this on a cleaned benchtop using aseptic technique and personal protective equipment including gloves and a mask, to ensure there was no contamination. I quantified my primary stock solutions using a Nanodrop device, which uses spectrophotometry to determine the concentration of DNA in ng/µL, from a drop of fluid.(103) I performed each measurement three times, and took the means as true values. I then used the Thermofisher DNA Copy Number and Dilution Calculator to calculate dilution protocols based on each DNA sequence length, which would give me secondary stock solutions containing $1x10^8$ copies of template DNA per µL.(104) Using these, I performed serial dilutions, resulting in series of eight 'test solutions' from $1x10^8$ copies/µL to $1x10^1$ copies/µL for each target sequence. Because 1µL of each test solution was included in each reaction, these concentrations are equivalent to the quantities of target DNA included in each reaction (i.e. the copies per reaction).

I analysed test solutions in triplicate, with each relevant assay. A negative control was included on each run, consisting of TE Buffer only. Figure 13 shows the LAMP plate setup I used in these experiments.

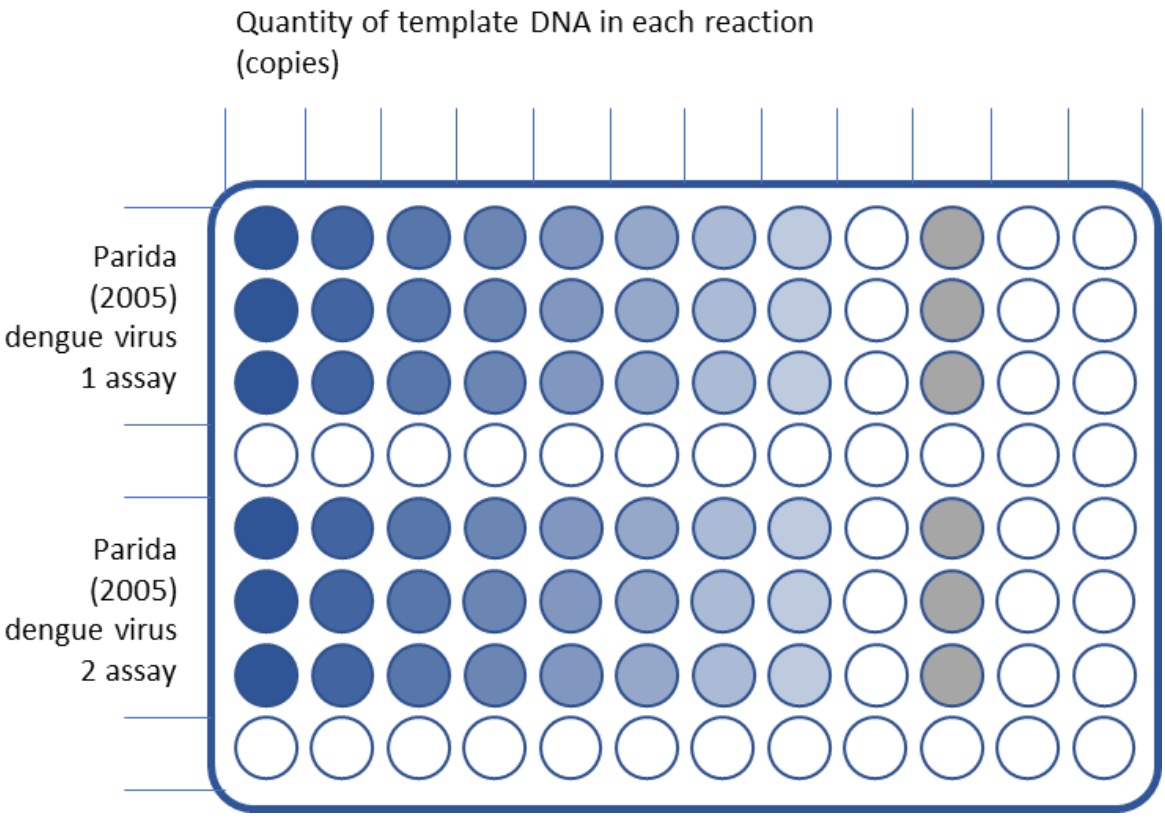



**Figure 18: Loop-mediated isothermal amplification (LAMP) plate setup used in in-vitro evaluation experiments**

**Legend:** Concentration of template DNA per reaction ($1x10^8$ – $1x10^1$) is denoted by the shade of each plate well. Negative control reactions are grey. Parida (2005) dengue virus 1 and 2 assays are used as an example, with the same pattern being replicated in subsequent plates for other assays.

Incubation was for 40 minutes at a constant 63 degrees Celsius. SYTO 9 was used to produce fluorescence in the presence of amplified DNA, which was monitored in real-time using a Qiagen QIAquant PCR machine. After each run I downloaded files from the PCR machine and visually inspected amplification curves. I assigned each reaction as either 'dengue DNA detected' or 'dengue DNA not detected' based on whether an exponential amplification curve crossing the automatically determined fluorescence threshold, and noted the 'time-to-positivity'. If any negative control reactions were assigned 'dengue DNA detected', or if any of the highest concentration test solution reactions were assigned 'dengue DNA not detected', I repeated the run.

I determined the LLOD of each assay to be the lowest concentration of template DNA at which all three reactions were positive. I also plotted the time-to-positivity against the log10 template DNA concentration to assess linearity in the assay, and calculated coefficients of variation to indicate intra-run variability. These analytical performance metrics were used to compare assays and decide which should proceed into further experiments.

### 5.3.4 Optimisation

Having identified the best-performing dengue RT-LAMP assay for each serotype, I further characterised and optimised their analytical performance in a series of experiments using two strains of each dengue virus serotype: First, I used 'laboratory strains' of dengue viruses 1-4, which were commercially available and commonly used in research. Second, I used 'Thai strains', which had been recently been isolated from samples in a clinical study conducted at DHFRU, Mahidol University, Bangkok. The purpose was to ensure that any newly developed diagnostic workflow would perform optimally in patients infected with viruses currently circulating in Southeast Asia, as well as those previously identified. I arranged for concentrations of solutions containing these viruses to be quantified in focus forming units (FFU/µL) at DHFRU. These were then spiked into three types of media at five different concentrations ($2x10^5$ - $2x10^1$ FFU/ml): Blood, serum and a commercially available virus transport medium. This was to mimic 'real' samples, and to assess whether assay performance would vary according to sample type. This can occur in molecular assays because of 'reaction inhibitors' which can be present in blood and other biological samples, despite effective nucleic acid extraction.(105) Nucleic acids were then extracted using a commercially available TRIzol extraction kit and protocol, and the resulting 'nucleic acid extracts' ($8x10^3$ – $8x10^{-1}$ FFU/reaction) were transported to CAMO for my analysis (see Material Transfer Agreement, Appendix 4).(106)

I ran an 'analytical sensitivity experiment' for each assay, in which I analysed each extract in triplicate (equalling a total of 90 reactions). Figure 14 shows the RT-LAMP plate setup I used in these experiments.

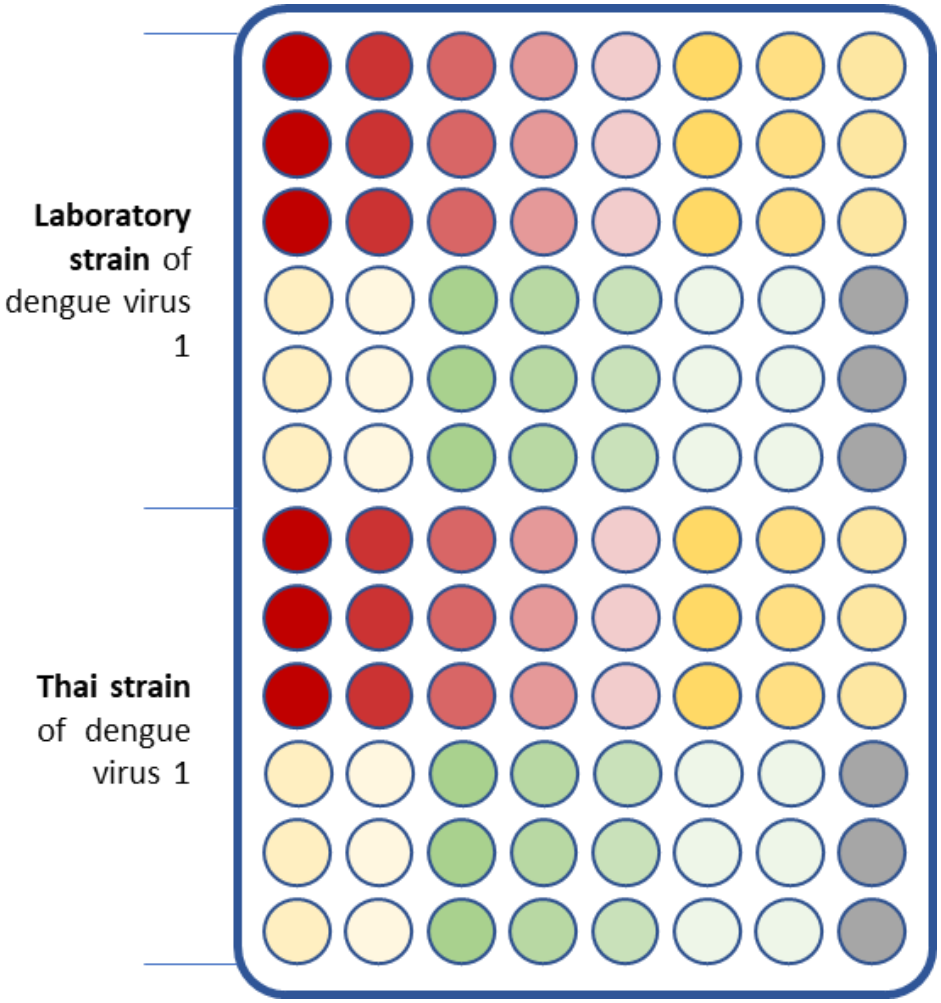



**Figure 19: Reverse transcriptase-Loop-mediated isothermal amplification (RT-LAMP) reaction plate setup used in analytical sensitivity experiments**

**Legend:** Two different strains of dengue virus particles were spiked into blood (red), serum (yellow) and a commercial transport medium (green), then underwent RNA extraction. They were tested at different concentrations in triplicate. Concentration of virus particles in FFU/reaction ($8x10^{3}$ – $8x10^{-1}$) is denoted by the shade of each plate well. Negative control reactions are grey. The setup for the Hu (2015) dengue virus 1 assay is used as an example, with the same pattern being replicated in subsequent plates for other assays.

I also ran two 'analytical specificity experiments' for each assay, in which I analysed a series of 'no-template controls': In the first experiment, these consisted of TE Buffer only (18 reactions per assay) and sero-discordant dengue viruses (72 reactions per assay). This was to assess whether assays had analytical specificity, even in the presence of RNA from similar viruses (and hence could be used to distinguish dengue virus serotypes). In the second experiment, these consisted of extracted RNA from a UK study enrolling adult patients with sepsis (84 reactions per assay, see authorisation from the Imperial College Tissue Bank to use these extracts in Appendix 4). This was to assess analytical specificity in patients who have relevant alternative diagnoses, in whom a false-positive dengue result could have significant adverse consequences. Both of these experiments were designed to ensure assays fulfilled specific user requirements when used in patients with and without dengue infection, identified in chapter 2. Figure 15 shows the LAMP plate setup I used in these experiments.

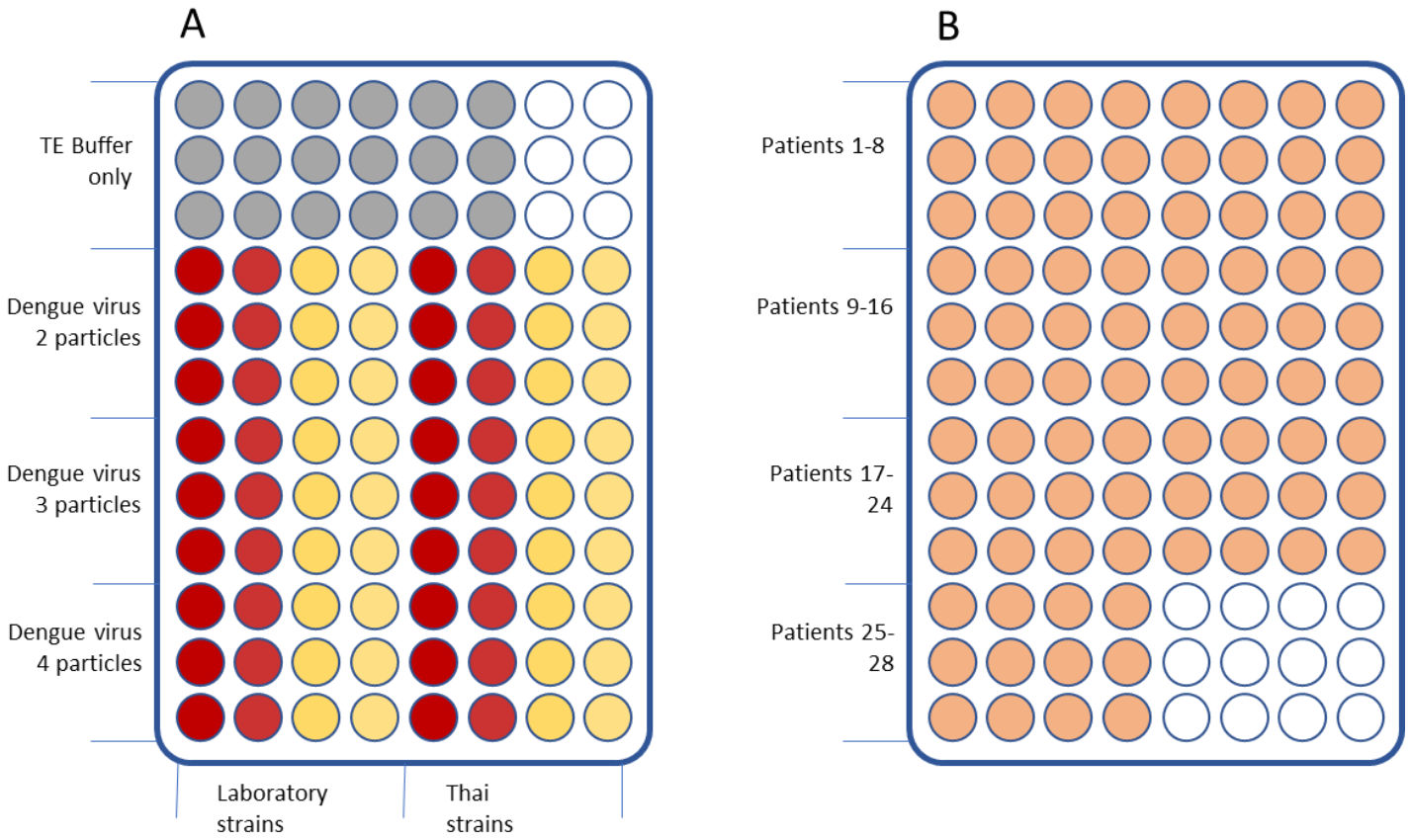



**Figure 20: Reverse transcriptase-Loop-mediated isothermal amplification (RT-LAMP) reaction plate setup used in analytical specificity experiments**

**Legend:** (A) TE Buffer (grey) and sero-discordant dengue particles spiked into blood (red) and serum (yellow) which had undergone RNA extraction were tested in triplicate. The concentration of virus particles per reaction ($8x10^3$-$8x10^2$) is denoted by the shade of each well. (B) Extracted RNA from 28 participants in a UK study enrolling adult patients with sepsis was tested in triplicate. The setup for the Hu (2015) dengue virus 1 assay is shown as an example, with the same pattern being replicated in subsequent plates for other assays.

I used the same LAMP reaction mix as in previous experiments, with the addition of a RTx reverse transcriptase (to reverse transcribe dengue virus RNA into cDNA) and RNAse inhibitor (to prevent destruction of target RNA by endogenous RNAse enzymes which may be present in samples). Incubation was for 60 minutes at a constant 63 degrees Celsius. I chose a longer incubation duration based on some earlier in-vitro evaluation experiments in which I noticed amplification occurring late in the 40-minute incubation in some assays, which I assumed to be non-specific but which needed further characterisation. Similar to previous experiments, I conducted these optimisation experiments in a Qiagen QIAquant PCR machine with SYTO9, used the same criteria to assigned samples as 'dengue RNA detected' or 'dengue RNA not detected', and noted time-to-positivity for each where dengue RNA was detected.

For each assay I plotted a histogram of all 'dengue RNA detected' results, with time-to-positivity on the x-axis. I stratified the results according to whether they occurred in a 'sensitivity experiment' (i.e. where the detection of dengue RNA was expected and hence the result was considered to be 'true positive') or in a 'specificity experiment' (i.e. where only 'no-template control' material was being tested and hence the result was considered to be 'false positive'). This was to visually assessed the difference in time-to-positivity between true and false positive results. I then went on to undertake a receiver operator characteristic (ROC) analysis in R using the pROC package, to determine specific incubation durations which would optimise assay performance. Data from chapter 2 highlighted a particular need to maximise assay specificity, to avoid the erroneous diagnosis of dengue and withholding of treatment for other conditions. Therefore, I prioritised specificity over sensitivity and set the target for this at 95%. In all potential diagnostic workflows assays will be used concurrently (i.e. with the same incubation settings). Therefore, I needed to combine data from all assays and determine the optimal overall incubation duration.

### 5.3.5 Translation: Integration of nucleic acid extraction methods

Having identified and optimised a RT-LAMP assay for each dengue virus serotype, I proceeded to conduct experiments to integrate these with portable methods for nucleic acid extraction and signal detection, with the intention of producing a diagnostic workflow which could be deployed remote areas.

For nucleic acid extraction, my original plan had been to collaborate with researchers at the Centre for Bio- Inspired Technology (CBIT), Imperial College, London who are currently developing a 'nucleic acid extraction module' which uses microfluidic technology to mix and manipulate reagents in a series of steps. However, this project has been delayed and a working prototype has not been made available to me thus-far. My second plan was to use an electricity-free magnetic bead-based RNA extraction technique which has also been developed at CBIT and evaluated for respiratory samples with good results (not yet published). The quantity of dengue RNA present in the respiratory tract during acute infection is low, and testing capillary blood obtained by finger-prick technique was a specific user-requirement identified in chapter 2 .(107,108) Therefore, this extraction method required adaptation and optimisation for small volumes of blood. However, a working prototype has not been made available for me thus-far. My third plan was developed after thoroughly reviewing the literature and discussing the challenge of nucleic extraction from blood with several collaborators at CAMO and DHFRU. I noted that other groups had used the filter paper inside RDTs as a source of dengue RNA for further molecular characterisation of infections. Various workflows have been presented, with some not requiring specific nucleic acid extraction, presumably because RNA is exposed by the drying and subsequent elution of blood.(109–111) In the detection of other pathogens, some authors have suggested that LAMP assays are more suited to analysing un-extracted samples than PCR assays ('direct LAMP').(100,112) Together with Dr Kenny Malpartida-Cardenas, who is a molecular scientist and researcher at CAMO, Imperial College, London, and Mr Adisak Songgjaeng, I conducted a series of experiments to validate an 'electricity-free filter paper nucleic acid extraction technique' for dried blood spots containing dengue viruses.

I used dengue viruses 1-4 which had been recently been isolated from samples in a clinical study conducted at DHFRU, Mahidol University, Bangkok. I spiked these into whole blood to give five different concentrations ($2x10^4$-$2x10^0$ FFU/µL). These were determined such that results would be comparable to earlier assay optimisation experiments in terms of the starting concentration of dengue virus particles (see Table 11). I pipetted 50µL of each onto Whatman 903 filter paper and dried them at ambient temperature for 4 hours to produce a 'dried blood spot'. This volume was chosen and was not varied because it is roughly equivalent to one drop, the most desirable sample volume identified by users in chapter 2. I then punched two 3mm circular discs from each dried blood spots using a hole-punch which was sterilised each time. I eluted the circular discs according to a previously published protocol, which was designed to serve as an RNA extraction method.(112) I tested 4µL of the resulting elute in serotype-specific RT-PCR assays. I noted the cycle threshold (CT) value for each where dengue RNA was detected. To determine the quality of the electricity-free filter paper nucleic acid extraction technique, I compared results to those obtained when testing the same spiked blood samples after they had been extracted using a commercially available blood RNA extraction method. I also compared results to those obtained when the filter paper elute was extracted using this extraction method.(106)

**Table 11: Expected concentrations of dengue virus 1 particles during evaluation of 'electricity-free filter paper nucleic acid extraction technique'**

| Sample concentration (FFU/µL) | A: Electricity-free filter paper nucleic acid extraction technique' (index method) | | | B TRIzol extraction (reference method) | |
|---|---|---|---|---|---|
| | Copies per filter paper disc (µL) (x50) | Conc. of filter paper elute (copies/ µL) (/80) | Expected particles per reaction after 4µL elute added to RT-PCR reaction (x4) | Conc. of extract after TRIzol extraction method (FFU/µL) (x4) | Expected particles per reaction after 4µL extract added to RT-PCR reaction (x4) |
| $2x10^{1}$ | $1x10^{3}$ | $1.25x10^{1}$ | $5x10^{1}$ | $8x10^{1}$ | $3.2x10^{2}$ |
| $2x10^{0}$ | $1x10^{2}$ | $1.25x10^{0}$ | $5x10^{0}$ | $8x10^{0}$ | $3.2x10^{1}$ |
| $2x10^{-1}$ | $1x10^{1}$ | $1.25x10^{-1}$ | $5x10^{-1}$ | $8x10^{-1}$ | $3.2x10^{0}$ |
| $2x10^{-2}$ | $1x10^{0}$ | $1.25x10^{-2}$ | $5x10^{-2}$ | $8x10^{-2}$ | $3.2x10^{-1}$ |
| $2x10^{-3}$ | $1x10^{-1}$ | $1.25x10^{-3}$ | $5x10^{-3}$ | $8x10^{-3}$ | $3.2x10^{-2}$ |

**Abbreviations:** FFU = Focus forming units

**Legend:** A commercial TRIzol extraction was used as the reference method.

### 5.3.6 Translation: Integration of signal detection methods

For signal detection, I first collaborated with researchers at CBIT, who are developing electrochemical detection methods for molecular assays. Because the LAMP produces hydrogen ions, it can be detected as a pH change using an ion-sensitive field-effect transistor (ISFET). This is potentially a more portable technique than optical detection of fluorescence, which is bulky, expensive and requires a greater supply of power. Therefore, together with Dr Nicholas Moser, a researcher in the Electrical Engineering Department at Imperial College, London, I conducted a series of experiments demonstrating this technique using my serotype-specific dengue LAMP assays. I used aforementioned synthetic double stranded DNA as the nucleic acid template, adding these to a pre-made LAMP reaction mix and applying the mixture to an ISFET chip in a 3D printed reaction chamber using a pipette. An adjacent heat block, housed within the same prototype device, was used to

incubate the reaction at 63 degrees Celsius for 30 minutes. The electrochemical signal was monitored, processed, log10-transformed, and plotted against time. It was visually assessed and determined as 'dengue DNA detected' if the signal crossed a pre-determined threshold.

I also investigated a second option for signal detection: I arranged for the reagents in my serotype-specific dengue assays to be lyophilised and integrated with colorimetric dyes, creating 'dengue lyophilised colorimetric loop-mediated isothermal amplification strips ('LyoLAMP strips'). This process has been developed and optimised for other RT-LAMP assays by collaborators at CAMO. Dengue LyoLAMP strip setup is shown in Figure 16.

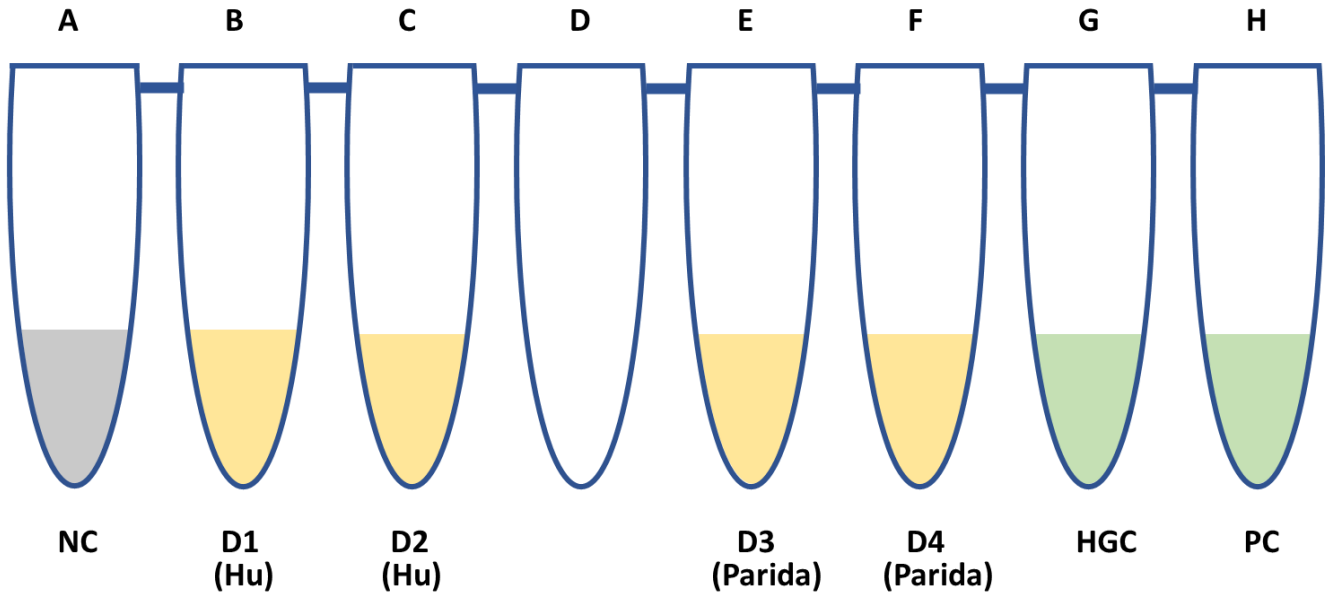



**Figure 21: Lyophilized colorimetric loop-mediated isothermal amplification ('LyoLAMP') strip setup**

**Legend:** Tube A is a negative control (NC), which contains all reaction ingredients apart from enzymes. Tubes B, C, E and F include primers for dengue viruses 1 and 2 (Hu, 2015), and 3 and 4 (Parida 2005), respectively. Tube G contains primers for a human gene target (b-Actin), and acts as a 'sample adequacy control'. Tube H contains primers and template DNA for a well-performing LAMP assay and is the positive control.

I designed a series of experiments to evaluate the performance of dengue LyoLAMP strips, using the aforementioned 'laboratory strains' and 'Thai strains' of each of dengue virus 1-4, which had been spiked into blood and serum and undergone RNA extraction. I diluted these to give three different concentrations ($2x10^{1}$-$2x10^{-1}$ FFU/ µL), such that quantities of dengue particles per LyoLAMP reaction were directly comparable to those in earlier standard RT-LAMP reactions. An RT-qPCR assay was used as the reference standard.(99) Table 12 shows the concentrations of dengue virus 1-4 particles which were used in this experiment. Reference testing was conducted in Thailand by Mr Adisak Songgjaeng, who is a molecular scientist and researcher at DHRFU, Bangkok, Thailand. After inoculating each LyoLAMP strip, it was incubated at 63 degrees Celsius in a small heat-block incubator for 20 minutes and then interpreted visually, with reactions being assigned 'dengue RNA detected' if the solution had turned from pink to yellow. This duration was informed by my earlier, 'assay optimisation' experiments. If any false-negative results were observed (i.e. where dengue RNA was not detected despite a sero-concordant dengue virus being tested) the strip underwent two further 3-minute incubations, with visual interpretation after each one. This was in case RT-LAMP reactions occurred slower in the LyoLAMP format compared to the standard RT-LAMP format used in earlier assay optimisation experiments, and hence to investigate whether a slightly longer LyoLAMP incubation should be used. If any negative control reactions were assigned 'dengue RNA detected', or if either the sample adequacy control or positive control was assigned 'dengue DNA not detected', I repeated the run.

**Table 12: Expected concentrations of dengue virus particles during lyophilized colorimetric loop-mediated isothermal amplification (LyoLAMP) evaluation**

| Conc. of spiked sample (FFU/µL) | Conc. of extract after TRIzol extraction method (FFU/µL) (x4) | A: LyoLAMP testing (index method) | | B: Standard LAMP testing (used in earlier experiments | C: RT-qPCR reference method |
|---|---|---|---|---|---|
| | | Conc. after 20-fold dilution (FFU/ µL) (/20) | Extracted particles in each LyoLAMP reaction after 20 µL of diluted extract added (x20) | Expected particles in each standard LAMP reaction after 1 µL extract added (FFU) (x1) | Expected in each RT-qPCR reaction after 5 µL extract added (FFU) (x5) |
| $2x10^{1}$ | $8x10^{1}$ | $4x10^{0}$ | $8x10^{1}$ | $8x10^{1}$ | $4x10^{2}$ |
| $2x10^{0}$ | $8x10^{0}$ | $4x10^{-1}$ | $8x10^{0}$ | $8x10^{0}$ | $4x10^{1}$ |
| $2x10^{-1}$ | $8x10^{-1}$ | $4x10^{-2}$ | $8x10^{-1}$ | $8x10^{-1}$ | $4x10^{0}$ |

**Abbreviations:** FFU = Focus forming units

**Legend:** An RT-qPCR assay was used as the reference method

## 5.4 Results

### 5.4.1 In-silico evaluation of assays

I successfully aligned primer sequences from five previously published sets of serotype-specific dengue RT-LAMP assays with dengue reference genomes. Figure 17 is a screenshot showing an example of a set of LAMP assay primers aligned against a reference genome. Tables 13 and 14 show the in-silico characteristics of each assay and highlights potential adverse features and how they may affect performance.

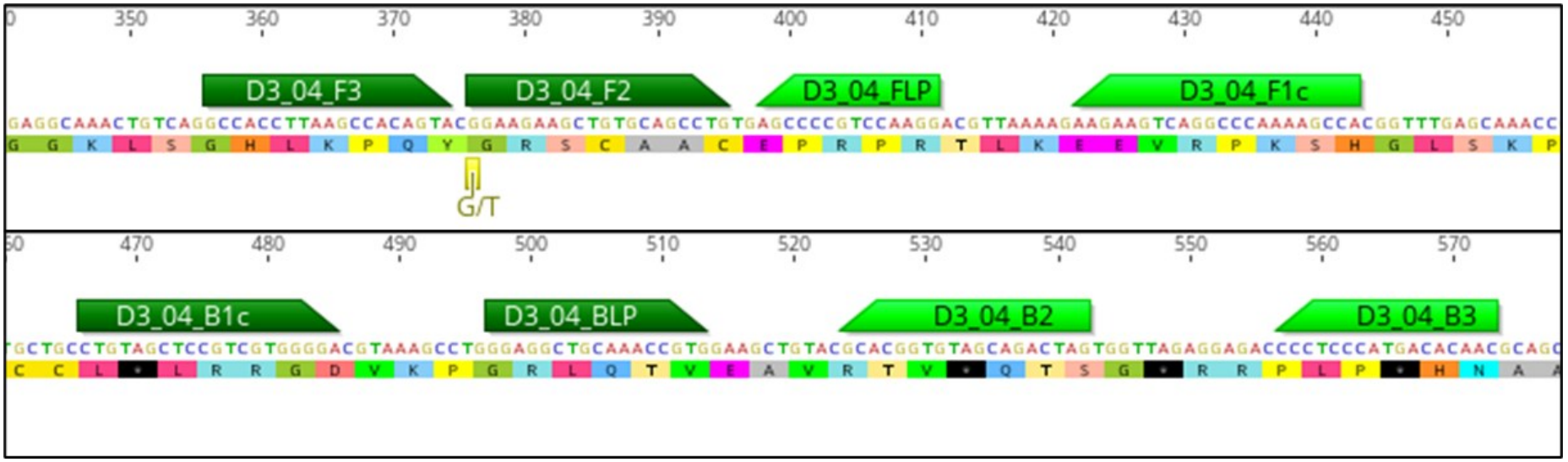



**Figure 22: Screenshot from Geneous Prime software showing alignment of a published set of serotype-specific dengue LAMP primers**

**Legend:** Reference dengue virus genomes were used as templates. The Lau (2015) dengue virus 3 assay is shown as an example, binding to the 3'UTR region of the OK562744.1 reference genome. A G/T base pair mismatch at the F2 primer binding site is highlighted in yellow.

**Table 13: Summary of data from in-silico evaluation of serotype-specific dengue RT-LAMP assays relating to analytical sensitivity**

| | | gblock ref sequence | Tm (degrees Celsius, optimal = 64-66 for F1c, B1c, LF and LB & 59-61 for F2, B2, F3 & B3) | | | | | | | | Primer GC content (%, optimal = 50-60) | | | | | | | | Base pair mismatches (n) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | F1c | B1c | F2 | B2 | F3 | B3 | LF | LB | F1c | B1c | F2 | B2 | F3 | B3 | LF | LB | F1c | B1c | F2 | B2 | F3 | B3 | LF | LB |
| Parida (2005) | D1 | U88535.1 | 64.1 | 64.7 | 57.6 | 59.5 | 21.5 | 50.3 | 49.9 | 52.5 | 61.9 | 61.9 | 61.1 | 52.6 | 52.6 | 55 | 50 | 55.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | D2 | AF038403 | 63.7 | 62.7 | 58.1 | 59.2 | 57.4 | 56.1 | 52.1 | 54.1 | 60 | 61.9 | 52.4 | 60 | 55.6 | 55.6 | 58.8 | 52.9 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | D3 | M93130.1 | 62.7 | 63.4 | 60.9 | 57.7 | 57.1 | 54.2 | 53.7 | 58.6 | 50 | 65.5 | 55 | 57.9 | 52.6 | 58.8 | 71.4 | 64.7 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | D4 | AF289029.1 | 62.5 | 47.3 | 62.4 | 68.6 | 52.3 | 54.3 | 59.8 | 53.1 | 50 | 60 | 50 | 61.1 | 50 | 55.6 | 70.6 | 47.1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| Neeraja (2015) | D1 | MG957435.1 | 58.2 | 60.1 | 55.5 | 55.4 | 54.9 | 55.9 | 59.4 | 58.5 | 40 | 52.4 | 47.4 | 55.6 | 47.4 | 57.9 | 47.6 | 45.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | D2 | AF162448.1 | 58.2 | 58.6 | 54.6 | 53.4 | 55.9 | 53.9 | 59.9 | 58.7 | 47.6 | 43.5 | 50 | 52.9 | 55.6 | 50 | 47.6 | 43.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | D3 | EU371057 | 58.8 | 59.1 | 57.1 | 57.4 | 55.1 | 53.2 | 59.8 | 58.8 | 45.8 | 47.8 | 47.4 | 50 | 50 | 44.4 | 50 | 57.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | D4 | KC620380.1 | 67.2 | 69 | 61.8 | 61 | 59.5 | 54.2 | 67.9 | 71.7 | 52.6 | 60 | 52.9 | 42.1 | 42.9 | 31.8 | 57.9 | 56.5 | 1 | 0 | 0 | 2 | 0 | 1 | 1 | 0 |
| Hu (2015) | D1 | MK506262.1 | 57.6 | 59.2 | 51.3 | 55.9 | 55.5 | 55.7 | 57.3 | 57.5 | 50 | 55 | 40.9 | 45.5 | 40.9 | 40.9 | 47.6 | 45 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | D2 | AF038403 | 58.9 | 55.1 | 55.4 | 59.5 | 55.7 | 55.6 | 57.8 | 58.2 | 61.1 | 47.4 | 50 | 45.5 | 52.6 | 50 | 47.6 | 45.5 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | D3 | M93130.1 | 61.7 | 59.8 | 52.7 | 55.6 | 56.6 | 57.2 | 57.7 | 57.3 | 63.2 | 63.2 | 47.1 | 47.6 | 55.6 | 55.6 | 55.6 | 55.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | D4 | AF289029.1 | 52.3 | 61.1 | 55.4 | N/A | 55.4 | 55.1 | 57.1 | 56.9 | 52.9 | 57.1 | 52.6 | N/A | 52.6 | 55.6 | 55.6 | 52.6 | 0 | 0 | 0 | N/A | 1 | 0 | 0 | 1 |
| Lau (2015) | D1 | OK562744.1 | 62.2 | 61.8 | 57.5 | 57.9 | 57.7 | 57.8 | 64.2 | 52 | 65 | 61.9 | 52.6 | 55 | 57.9 | 52.6 | 70.6 | 47.1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | D2 | AF038403 | 61.3 | 61.8 | 60.8 | 57.7 | 58.4 | 58 | 50.5 | 52 | 63.2 | 61.9 | 65 | 55 | 55.6 | 55.6 | 41.2 | 47.1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | D3 | M93130.1 | 71.2 | 59.4 | 66.8 | 59.3 | 58.2 | 57.7 | 55.4 | 52.9 | 59.1 | 60 | 58.8 | 57.1 | 61.1 | 55 | 52.9 | 52.9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | D4 | AF289029.1 | 60.4 | 59.4 | 61.4 | 58.8 | 57.7 | 54 | 55.9 | 50.2 | 63.2 | 60 | 65 | 52.4 | 52.6 | 42.1 | 52.9 | 41.2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| Kim (2018) | D1 | FJ687475.1 | 63.4 | 62.9 | 55.7 | 57.5 | 58.1 | 59 | N/A | N/A | 50 | 57.1 | 52.6 | 52.6 | 50 | 50 | N/A | N/A | 0 | 0 | 0 | 0 | 1 | 0 | N/A | N/A |
| | D2 | KP406804 | 59.5 | 57.3 | 56.2 | 53 | 55.2 | 54.5 | N/A | N/A | 40 | 39.1 | 52.6 | 47.4 | 47.4 | 40 | N/A | N/A | 0 | 0 | 0 | 0 | 0 | 0 | N/A | N/A |
| | D4 | KP406806.1 | 64.8 | 65.3 | 58 | 52.8 | 58.7 | 57.8 | N/A | N/A | 56.5 | 58.3 | 55.6 | 52.9 | 50 | 50 | N/A | N/A | 0 | 0 | 1 | 0 | 0 | 0 | N/A | N/A |

**Legend:** Primers were assessed in terms of their melting temperature (Tm), GC content, and base pair mismatches . Adverse features are highlighted orange.

**Table 14: Summary of data from in-silico evaluation of serotype-specific dengue RT-LAMP assays relating to analytical specificity**

| | | gblock ref sequence | Distance (optimal = 120-160 bases for F2-B2, 40-60 bases for F2-F1c, 0-60 bases for F2-F3 | | | | | Potential to form secondary structures | | | | | | | | | | | | | | | Off target binding |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Hairpin Tm | | | | | | | | Self-dimer Tm | | | | | | | | |
| | | | F2 -> B2 | F2 -> F1c | B2 -> B1c | F2 -> F3 | B2 -> B3 | F1c | B1c | F2 | B2 | F3 | B3 | LF | LB | F1c | B1c | F2 | B2 | F3 | B3 | LF | LB | |
| Parida (2005) | D1 | U88535.1 | 142 | 40 | 74 | 1 | 17 | | 48 | 32 | | | 50 | | | | | 15 | | 22 | 12 | | | LF |
| | D2 | AF038403 | 172 | 42 | 52 | 2 | 1 | 47 | 35 | 33 | 44 | 43 | | | | 22 | | 5.9 | 12 | 11 | | | | |
| | D3 | M93130.1 | 167 | 46 | 57 | 1 | 14 | 51 | 43 | 55 | | | | | | 4.5 | | 19 | | | | | | |
| | D4 | AF289029.1 | 185 | 47 | 55 | 1 | 8 | 57 | | 44 | 32 | | | 61 | | 6.4 | | 5.2 | | 5 | | 9 | | B1c |
| Neeraja (2015) | D1 | MG957435.1 | 153 | 46 | 60 | 0 | 0 | | | 36 | | | | | 46 | | | | 5.9 | | 6.5 | | | |
| | D2 | AF162448.1 | 148 | 41 | 57 | 7 | 8 | 45 | 34 | | | | | 46 | 36 | 1.1 | 13 | 30 | | | | | | |
| | D3 | EU371057 | 150 | 41 | 60 | 0 | -2 | 43 | | 43 | 34 | 48 | | 43 | | 5.2 | | 11 | | 12 | | | | |
| | D4 | KC620380.1 | 139 | 42 | 55 | 1 | 2 | 46.1 | 53.7 | 44.8 | | | | 46.7 | | 20.1 | | 30.1 | | | 1 | 13.8 | 14.2 | LF and LB |
| Hu (2015) | D1 | MK506262.1 | 166 | 47 | 60 | 0 | 7 | 58 | | 51 | | | 59 | | | | 8.2 | 14 | | | | | | |
| | D2 | AF038403 | 142 | 43 | 52 | 54 | 2 | 46 | 36 | | | | | 47 | 34 | 19 | | | | | | 22 | | |
| | D3 | M93130.1 | 131 | 40 | 52 | 3 | 10 | 45 | | | | 59 | | 44 | 38 | | | | | 32 | | 13 | 9.8 | |
| | D4 | AF289029.1 | N/A | 51 | N/A | 45 | N/A | 61 | | 35 | N/A | 42 | 35 | 63 | | | | 1.9 | N/A | | | | 25 | |
| Lau (2015) | D1 | OK562744.1 | 142 | 43 | 50 | 8 | 9 | 44 | 37 | | 50 | | | | 56 | 9.6 | | | 9.3 | | | 22 | 9.7 | |
| | D2 | AF038403 | 155 | 43 | 48 | 3 | 15 | | 37 | 58 | 44 | 63 | | | 56 | 22 | | | 12 | 18 | | 9.7 | | |
| | D3 | M93130.1 | 152 | 47 | 50 | 9 | 1 | 35 | 37 | 53 | 42 | 39 | | | 49 | 28 | 3.9 | | 5.9 | 10 | | | 3.5 | F2 |
| | D4 | AF289029.1 | 159 | 54 | 47 | 0 | 7 | 34 | 37 | 58 | 43 | | | | | | | | 4.7 | | | | | B1c and F2 |
| Kim (2018) | D1 | FJ687475.1 | 129 | 39 | 46 | 1 | 9 | 45 | | 31 | | | 34 | | | | 8.7 | | | | 12 | | | |
| | D2 | KP406804 | 146 | 41 | 55 | 4 | 8 | 42 | 55 | 43 | | 51 | | | | 6.9 | 5.6 | | | 14 | | | | |
| | D4 | KP406806.1 | 149 | 71 | 87 | 1 | 3 | 46 | 63 | 47 | | | | | | 20 | | | 9.9 | 5 | | | | |

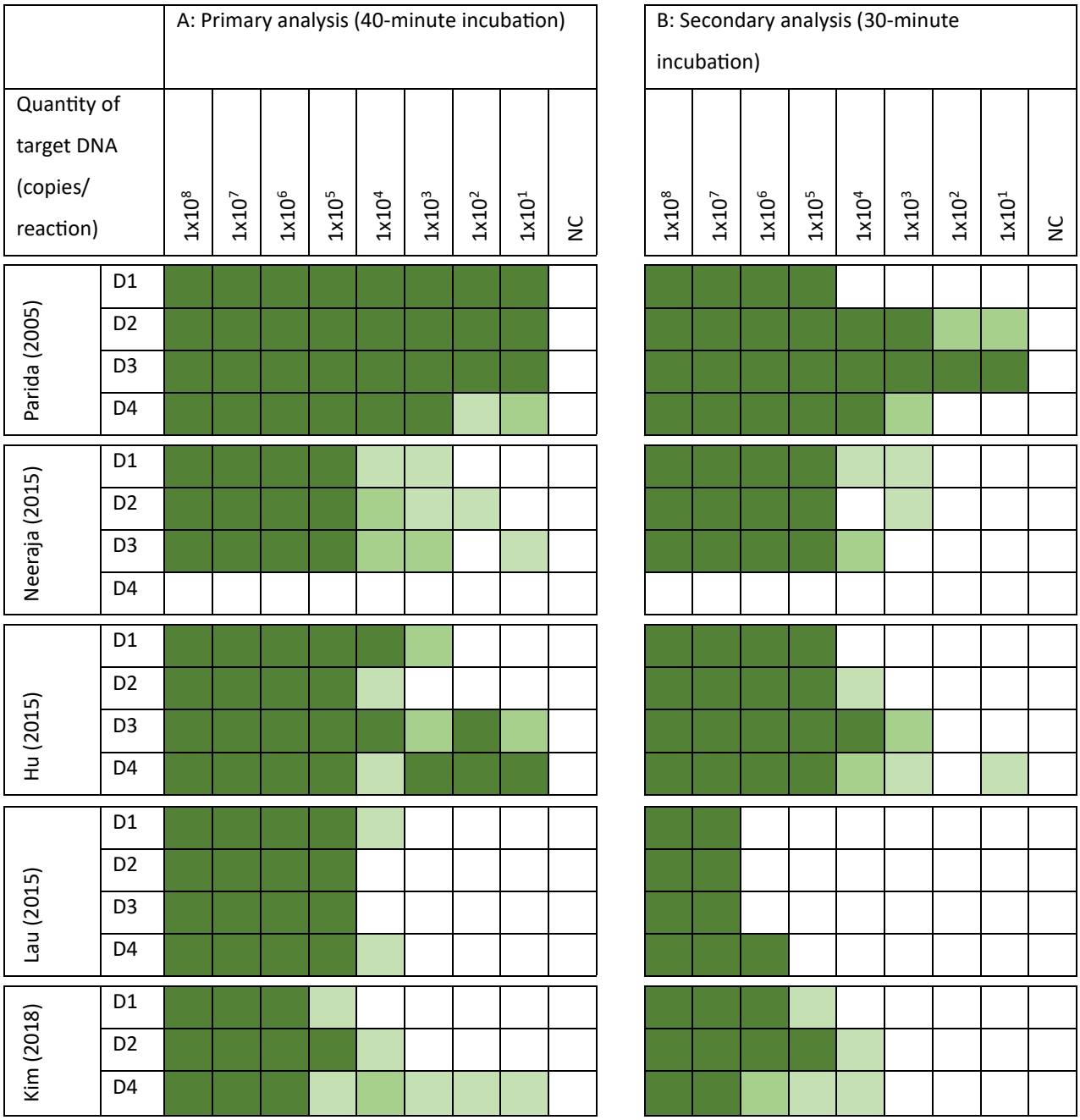
**Legend:** Primers were assessed in terms of their relative distances, potential to form secondary structures (hairpin structures and self-dimers) and off-target binding. Adverse features are highlighted orange.

### 5.4.2 In-vitro evaluation of assays

I performed in-vitro evaluation of five previously published sets of serotype-specific dengue RT-LAMP assays. In all but one case I was able to amplify and detect synthetic double stranded dengue DNA from the appropriate serotype. In one case this was not possible because the procured gblock did not contain any nucleic acid (verified using a Nanodrop device, which gave no signal). I observed significant variation in assays' LLODs. I also noticed that, in many reactions containing low concentrations of template DNA ($1x10^3$ – $1x10^1$ copies per reaction), amplification occurred very late during the 40-minute incubation (i.e. at 30-40 minutes). I also needed to repeat 3 runs because the negative control was positive. I considered it possible that this observation was due to laboratory contamination and therefore re-ordered reagents and undertook experiments in a different part of the laboratory, but the observation remained. I then considered it possible that this observation was due to intrinsic assay non-specificity ('non-specific reactions'). This can occur in molecular assays, including LAMP, due to interaction between primers. (78–80) Visual inspection of shape and peak temperature of the melting curve was not significantly different in reactions which occurred early, when compared to those which occurred late or in negative control reactions. Without being able to determine definitively which of these late reactions were non-specific (if any) I undertook a secondary analysis, in which I considered reactions to be positive if the signal crossed threshold before 30 minutes.

Table 15 summarises these findings. Positive detection rates for each assay when analysing a range of different concentrations of target DNA (ranging from $1x10^8$ – $1x10^1$ copies per reaction) are shown. The number of reactions which were positive is indicated by the shade of green (ranging from 0/3 – 3/3. In the primary analysis (A) reactions were assigned positive if amplification occurred any time during the 40 minute incubation, while in the secondary analysis (B) they were only assigned positive if it occurred during the first 30 minutes of the incubation.

**Table 15: Summary of data from in-vitro evaluation of previously published serotype-specific dengue RT-LAMP assays**




**Legend:** Reactions were assigned 'dengue DNA detected' if signal crossed a pre-determined threshold during the whole 40-minute incubation (primary analysis, A) or during the first 30 minutes of incubation (secondary analysis, B). Each quantity of template DNA was tested in triplicate, with the positive detection rate (0/3 – 3/3 reactions) being denoted by the matrix shading (white – dark green).

I also demonstrated that there was a linear relationship between log10 concentraton of template DNA and time-to-positivity when relatively high concentrations of template DNA was tested ($1x10^8$ – $1x10^5$ copies per reaction). Coefficients of variation at this range were between 0.6% and 3.8%. However, this relationship was generally not observed when lower concentrations were tested ($1x10^4$ – $1x10^1$). This suggests that the assays' lower limit-of-quantification (LLOQ) is higher than the LLOD, or it may be due to some values in this range being the result of 'non-specific reactivity'. Figure 17 shows an analysis of linearity for four assays, as examples. In chapter 2, users indicated they had little desire to know the quantity of dengue virus in patients' blood. Therefore, I decided not to assess the linear (quantitative) range of assays in experients hereafter, instead I prioritised the consideration of assay results to be qualitative i.e. either 'detected' or 'not detected'.

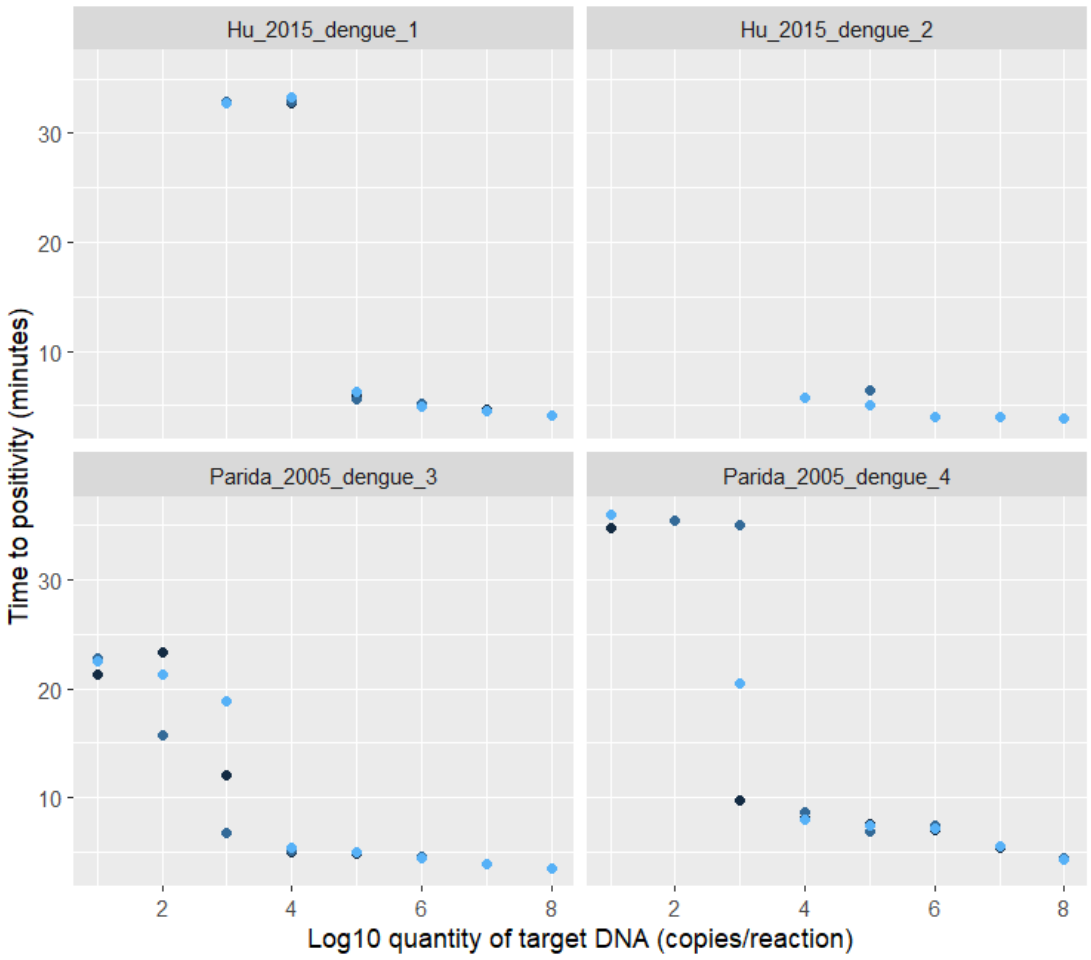



**Figure 23: Analysis of reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP) assay linearity**

**Legend:** Between $1x10^8$ and $1x10^5$ copies per reaction, there is a linear relationship between Log10 quantity of target DNA and time-to-positivity in most assays. Hu (2015) dengue virus 1 and 2 and Parida (2005) dengue virus 3 and 4 assays are used as an example. Graphs for the other assays were constructed in a similar way.

When interpreted together with data from my in-silico evaluation experiments, these findings indicated that the Hu (2015) assays for dengue viruses 1-2 and the Parida (2005) assays for dengue viruses 3-4 had among the lowest LLODs (i.e. had relatively high analytical sensitivity), with less risk of non-specific reactivity. However, I was sufficiently concerned to undertake further experiments in order to further characterise late reactions, and optimise assays in an attempt to improve specificity.

### 5.4.3 Optimisation

Results from analytical sensitivity experiments are depicted in Figure 19. With the longer 60-minute incubation used in these experiments, I observed a significant number of false-positive reactions during 'analytical specificity experiments' (i.e. sero-discordant dengue viruses, TE buffer, or RNA from UK sepsis patients were assigned 'dengue detected'). The total number of false-positive reactions was 229/696, with more occurring in the Hu (2015) dengue virus 1 assay (135/174) than in the Hu (2015) dengue virus 2 and Parida (2005) dengue virus 3 and 4 assays (42/174, 27/174 and 25/174, respectively). Most non-specific reactions occurred late during incubation, whereas true-positive reactions generally occurred earlier. Results from ROC analyses are shown in Figure 20. Each plot shows the trade-off between the sensitivity and specificity of each assay (and if all assays were to be combined) as the incubation duration and hence criteria for positivity is varied. I found that a 25-minute incubation would result in combined assay sensitivity and specificity of 76.9% and 98.7%, respectively. This was the incubation duration I used in subsequent experiments. Individual and combined assay sensitivity and specificity estimates at various incubation durations are shown in Table 16.

It is important to note that these estimates are based on testing 90 nucleic acid extracts (between $18x10^{3}$ – $8x10^{-1}$ FFU/reaction) per assay, which were RT-qPCR positive and as reference positives. I understand that it is unlikely 'real' patients presenting with acute dengue in any particular setting will do so with evenly distributed blood virus concentrations through the same range. Therefore, estimates are hypothetical, for the purpose of assay optimisation, and these assays must be evaluated within a diagnostic workflow using a representative sample of 'real' patient samples.

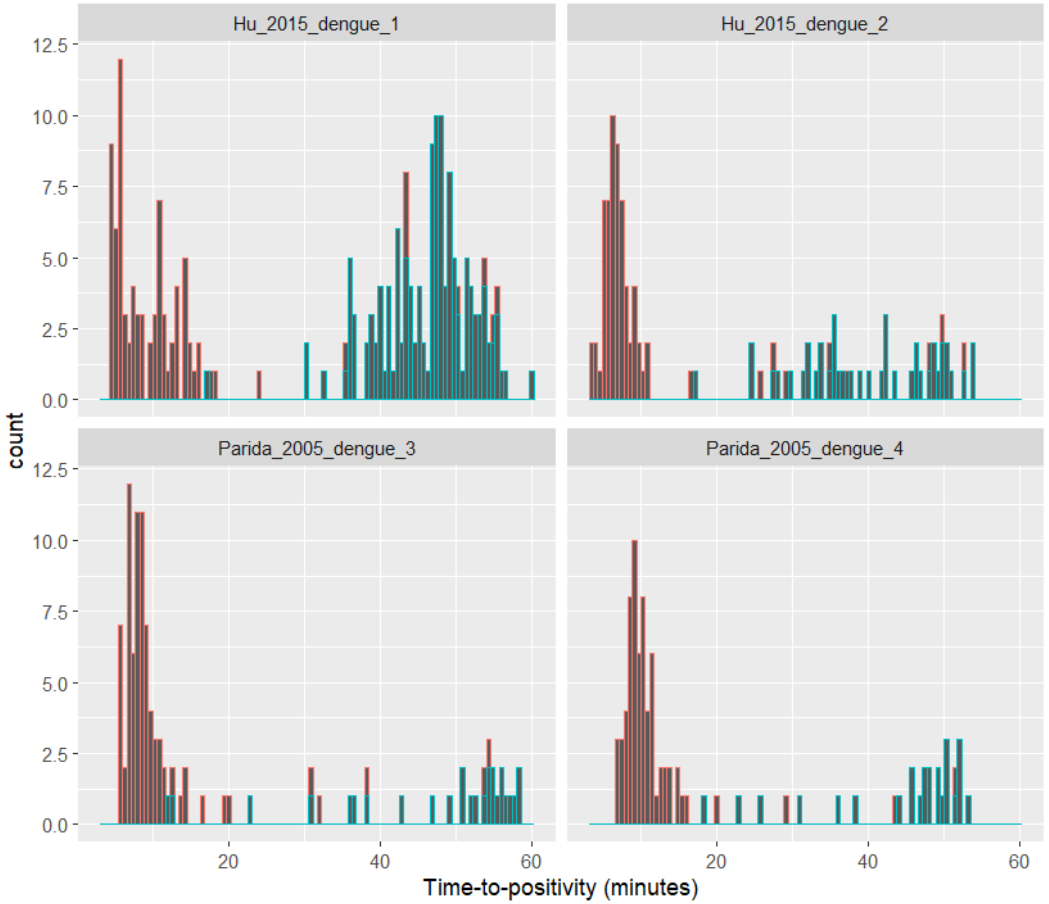



**Figure 24: Histograms showing reaction time-to-positivity during analytical sensitivity and specificity experiments**

**Legend:** Results from analytical sensitivity experiments (considered to be true-positive) are shown in red, while results from analytical specificity experiments (considered false-positive results) are shown in blue). Most true-positive reactions occurred early during incubation, whereas false-positive reactions occurred late.

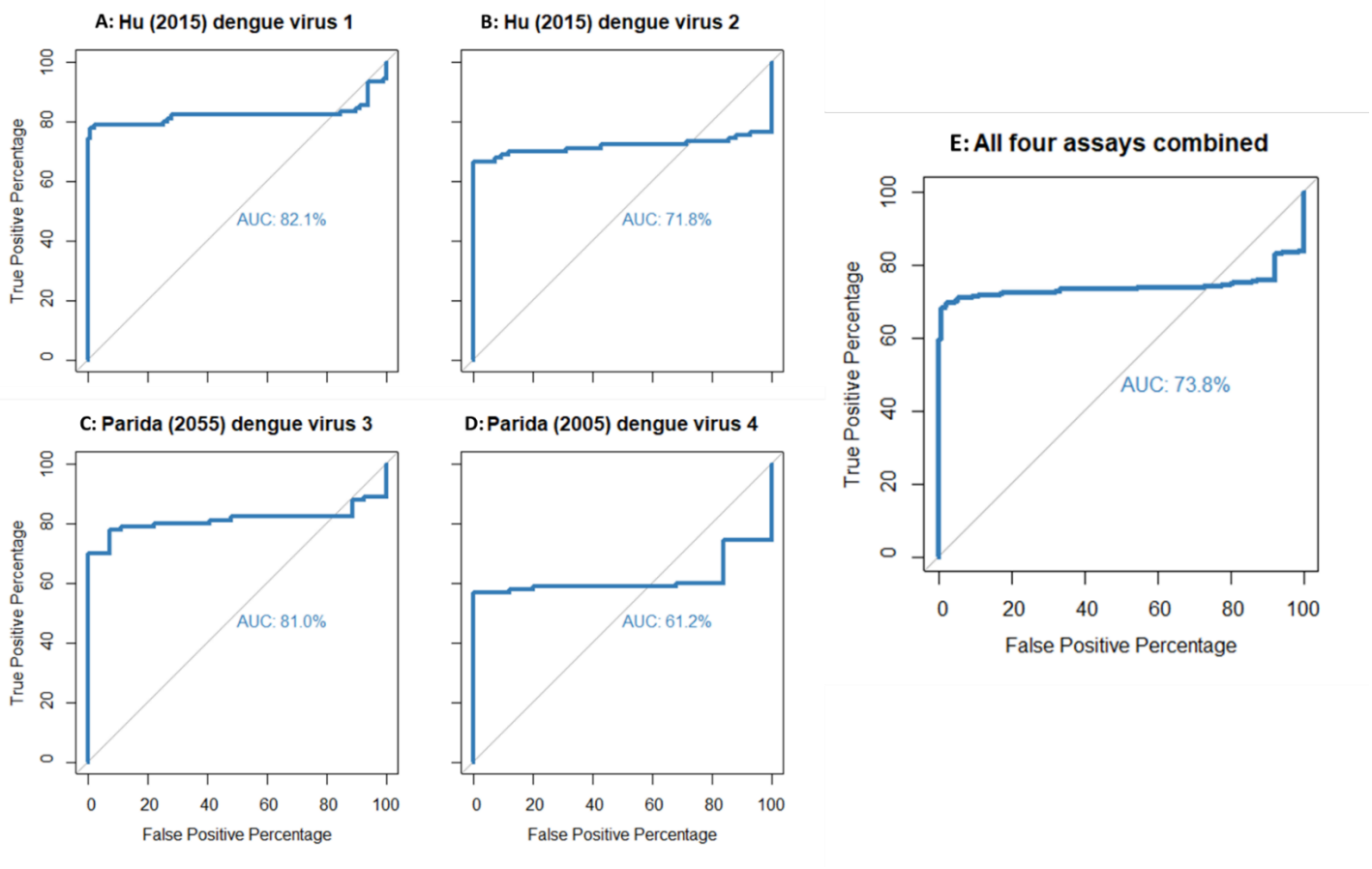



**Figure 25: Receiver operator characteristic (ROC) curves for dengue reverse-transcriptase loop-mediated isothermal amplification (RT-LAMP) assays.**

**Legend:** Assays were each used to test 90 nucleic acid extracts ($8x10^{3}$ – $8x10^{-1}$ FFU/reaction) which were RT-qPCR positive (reference positives) and 174 non-template controls (reference negatives). ROC curves are shown for individual assays (A-D) and when data from all assays are combined (E). These plots are a visual representation of trade-off between the sensitivity and specificity of each assay as the incubation duration (and hence criteria for positivity) is varied

**Table 16: Individual and combined assay sensitivity and specificity estimates at various incubation durations**

| | 20-minute incubation | | 25-minute incubation | | 30-minute incubation | |
|---|---|---|---|---|---|---|
| | Sens | Spec | Sens | Spec | Sens | Spec |
| Hu (2015) dengue virus 1 | 84.4% | 99.4% | 85.6% | 99.4% | 85.5% | 99.4% |
| Hu (2015) dengue virus 2 | 67.8% | 99.4% | 67.8% | 98.3% | 71.1% | 96.6% |
| Parida (2005) dengue virus 3 | 83.3% | 98.9% | 83.3% | 98.3% | 83.3% | 98.3% |
| Parida (2005) dengue virus 4 | 70.0% | 99.4% | 71.1% | 98.9% | 72.2% | 98.3% |
| All four assays combined | 76.4% | 99.3% | 76.9% | 98.7% | 78.1% | 98.1% |

**Legend:** Receiver operator characteristic (ROC) analyses were undertaken to examine the trade-off between assay sensitivity and specificity when varying incubation duration. 25-minute incubation duration was chosen for subsequent experiments.

### 5.4.4 Translation: Integration of nucleic acid extraction methods

Results from experiments evaluating the 'electricity-free filter paper nucleic acid extraction technique' are shown in Table 17 and Figure 21. When it was used to extract blood spiked with high concentration of virus particles ($2x10^1$ FFU/µL), these were detected by RT-qPCR. However when it was used to extract lower concentrations ($2x10^0$ - $2x10^{-3}$ FFU/µL), these were not detected. Positive detection rate was significantly better after a commercially available extraction technique was used, both on the original whole blood and the filter paper elute.

**Table 17: Comparison of 'electricity-free filter paper nucleic extraction' with a commercially available (TRIzol) blood RNA extraction.**

| Dengue virus 1 blood conc. (FFU/µL) | Electricity-free filter paper nucleic acid extraction technique | | | TRIzol extraction of whole blood | | TRIzol extraction of filter paper elute | | |
|---|---|---|---|---|---|---|---|---|
| | CT Replicate 1 | CT Replicate 2 | CT replicate 3 | CT Replicate 1 | CT Replicate 2 | CT Replicate 1 | CT Replicate 2 | CT Replicate 3 |
| $2x10^{1}$ | 44.43 | 41.84 | 42.30 | 19.35 | 19.48 | 20.1 | 20.69 | 20.23 |
| $2x10^{0}$ | ND | ND | ND | 22.30 | 22.69 | 23.63 | 24.51 | 23.91 |
| $2x10^{-1}$ | ND | ND | ND | 26.55 | 26.33 | 27.20 | 27.52 | 27.47 |
| $2x10^{-2}$ | ND | ND | ND | 29.32 | 29.45 | 30.93 | 30.50 | 30.66 |
| $2x10^{-3}$ | ND | ND | ND | 33.24 | 33.20 | 34.38 | 34.26 | 34.45 |

**Legend:** Dengue virus 1 was spiked into blood at various concentrations (2x10 – $2x10^{-3}$ FFU/µL) and then extracted using each technique. Cycle threshold (CT) values of RT-qPCR reactions performed following this are shown.

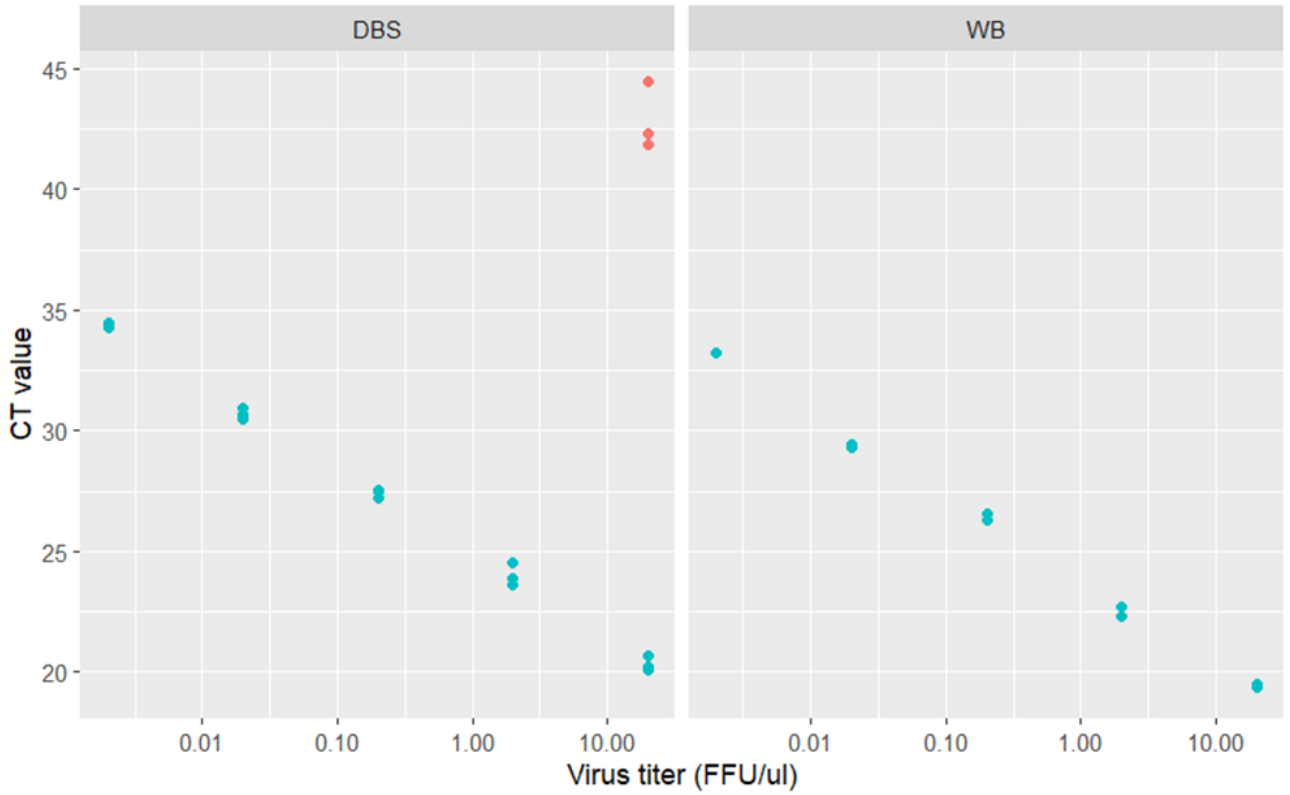



**Figure 26: Comparison of 'electricity-free filter paper nucleic extraction' with a commercially available (TRIzol) blood RNA extraction**

**Legend:** Cycle threshold (CT) values of RT-qPCR reactions are shown. When 'electricity-free filter paper nucleic acid extraction' (red) was used, only the highest concentration of dengue 1 virus was detectable. When TRIzol extraction (blue) was used on both whole blood (WB) and the filter paper elute (FPE) from the electricity-free filter paper nucleic acid extraction, all concentrations were detectable.

### 5.4.5 Translation: Integration of signal detection methods

I successfully demonstrated that amplification of DNA occurring in LAMP assays for dengue viruses 1 and 2 can be detected electrochemically. Results from these experiments are shown in Figure 22. Voltage at the chip surface is detected and plotted over time. Where a LAMP reaction has occurred (wells 3 and 4), there is an obvious inflection point in the curve (akin to that which is observed during real-time PCR amplification. Where a LAMP reaction has not occurred (wells 1 and 2), there is somechange in voltage (attributable to 'sensor drift') but no infection point. While these results were positive, significant challenges relating to the reliable application of reagents, stable heat-transfer, and signal processing mean that this method is not yet deployable in a portable diagnostic workflow. I therefore did not investigate it further as part of this PhD research. However, my findings will lead to further work within our group to leverage this electrochemical detection method within diagnostic devices.

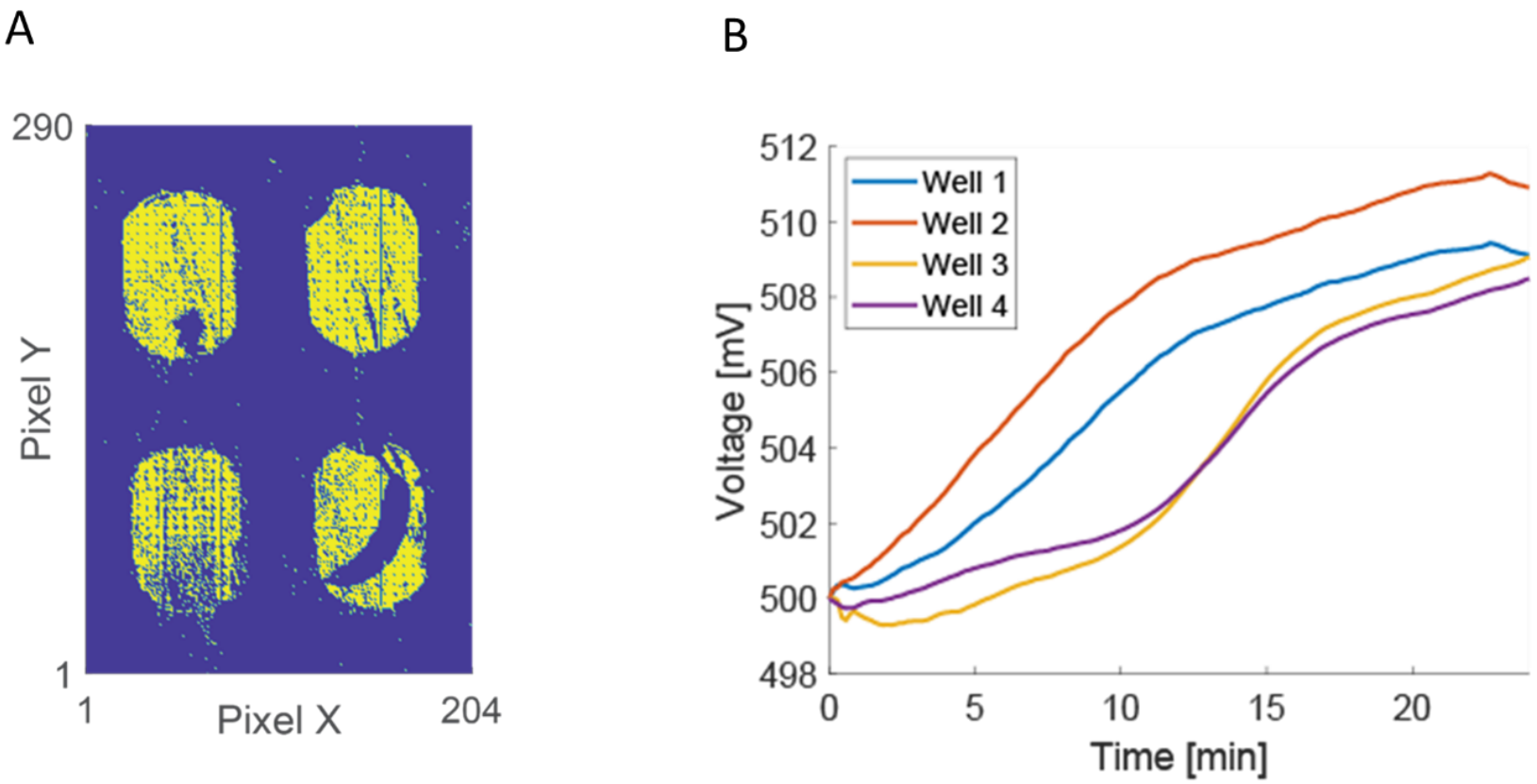



**Figure 27: Electrochemical detection of dengue viruses 1 and 2.**

**Legend:** (A) Visual representation of a 290x204 pixel ion-sensitive field-effect transistor (ISFET). In four distinct areas a voltage change has been detected (yellow). These correspond to four wells, in which LAMP reactions have occurred. (B) Graph showing cumulative voltage change over time in each well. Amplification of dengue virus 1 has occurred in wells 1 and 2 and amplification of dengue virus 2 has occurred in wells 3 and 4.

I successfully detected dengue viruses spiked into blood and serum and then extracted, using LyoLAMP strips. An photograph of a LyoLAMP strip upon interpretation is shown in Figure 23.




**Figure 28: Photograph showing lyophelized colorimetric loop-mediated isothermal amplification ('LyoLAMP') strip upon interpretation.**

**Legend:** A colour change from pink to yellow has occurred in the dengue virus 2 (D2) tube, human genome control (HGC) tube and positive control (PC) tube, but not in the negative control (NC) tube or D1, D3, or D4 tubes. This strip was therefore interpreted as 'dengue virus 2 detected'.

Results from my experiments evaluating the analytical performance of LyoLAMP strips are presented in Table 18. Positive detection rate was high with concentrations of virus particles between 80 and 0.8 FFU/reaction, similar to standard RT-LAMP testing (i.e. with detection using SYBR I using a Qiagen QIAquant PCR machine). However, I did not assess performance at lower concentrations. This was because the supply of LyoLAMP strips was limited and I considered it most appropriate to save the remainder for use with any forthcoming portable nucleic acid extraction methods, such that the whole workflow could be evaluated. This work will occur after my PhD.

**Table 18: Positive detection rate of dengue virus RNA using lyophilized colorimetric loop-mediated isothermal amplification ('LyoLAMP') strips compared to standard LAMP and reverse-transcriptase polymerase chain reaction (RT-qPCR).**

| Target | Conc. (FFU/ reaction) | LyoLAMP results | | | Standard LAMP results | RT-qPCR results |
|---|---|---|---|---|---|---|
| | | 25-minute incubation | 28-minute incubation* | 31-minute incubation* | | |
| Dengue virus 1 | $8x10^{1}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 17/18 (94%) | 18/18 (100%) |
| | $8x10^{0}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 17/18 (94%) | 18/18 (100%) |
| | $8x10^{-1}$ | 3/4 (75%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| Dengue virus 2 | $8x10^{1}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| | $8x10^{0}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| | $8x10^{-1}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 17/18 (94%) | 18/18 (100%) |
| Dengue virus 3 | $8x10^{1}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| | $8x10^{0}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| | $8x10^{-1}$ | 3/4 (75%) | 3/4 (75%) | 4/4 (100%) | 15/18 (83%) | 18/18 (100%) |
| Dengue virus 4 | $8x10^{1}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| | $8x10^{0}$ | 4/4 (100%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| | $8x10^{-1}$ | 0/4 (0%) | 4/4 (100%) | 4/4 (100%) | 18/18 (100%) | 18/18 (100%) |
| All serotypes combined | $8x10^{1}$ | 16/16 (100%) | 16/16 (100%) | 16/16 (100%) | 53/54 (98%) | 54/54 (100%) |
| | $8x10^{0}$ | 16/16 (100%) | 16/16 (100%) | 16/16 (100%) | 52/54 (96%) | 54/54 (100%) |
| | $8x10^{-1}$ | 10/16 (63%) | 15/16 (94%) | 16/16 (100%) | 50/54 (93%) | 54/54 (100%) |

*Strips where false-negative results were observed at 25-minutes underwent two further 3-minute incubations.

## 5.5 Discussion

In this chapter I conducted a series of experiments to evaluate and optimise serotype-specific dengue RT-LAMP assay and made significant advances towards translating them into a portable diagnostic workflow. I found that the Hu (2015) dengue virus 1 and 2 and Parida (2005) dengue virus 3 and 4 assays had better in-silico and in-vitro analytical performance, relative to other previously published assays. However, I observed that these assays exhibited significant non-specific reactivity, likely due to primer-primer or other non-specific interaction. This is a feature of many nucleic acid amplification techniques, in particular LAMP. (78–80) My earlier systematic review (presented in chapter 4) showed that this feature of LAMP is rarely investigated, characterised or reported in existing literature.(113) It is important because diagnostic devices using LAMP assays affected by this issue may produce sporadic false-positive results which could adversely affect clinical decision-making or surveillance efforts. I undertook a large number of experiments using various non-template control material to understand exactly when non-specific reactions occurred, and whether varying incubation duration could improve assays to an acceptable performance. To my knowledge, this was the first time ROC analyses have been applied to data from LAMP reactions to optimise assays. I found that an incubation duration of 25-minutes gave optimal combined assay sensitivity and specificity when analysing extracted dengue viruses at a range of concentrations. These methods will be relevant to other investigators developing and optimising LAMP assays for diagnostic and surveillance purposes. Detailed examination and reporting of analytical specificity using significant reactions containing non-template control material should become a standard in LAMP assay validation literature.

I demonstrated that these assays could be translated into a lyophilised, colorimetric format which would allow portable amplification and signal detection (LyoLAMP strips). These can be used to accurately detect and differentiate dengue viruses from blood samples, after they have undergone RNA extraction. They fulfil user-requirements for assay performance and diagnostic targets identified in chapter 2.

Despite these significant advances, further work is required to develop a diagnostic workflow for dengue in which all stages are portable. This is because a high-performing, portable technique for extracting RNA from blood was not ready for integration with LyoLAMP strips within the time available. My evaluation of a previously published 'electricity-free filter paper nucleic acid extraction technique' suggested that recovery of dengue virus 1 RNA from blood was not sufficient for this application. Further research into the integration of nucleic acid extraction techniques and remote connectivity with LyoLAMP strips are ongoing within our group, but will not be included in this thesis.

## 5.6 Conclusion and key messages

A new method which can be used to accurately detect and differentiate dengue viruses from extracted blood samples has been demonstrated (LyoLAMP strips). Efforts to integrate this with a high-performing, portable RNA extraction method and remote-connected mobile application are ongoing.

# CHAPTER 6

## 6.0 Pilot studies investigating new applications of existing serological techniques in dengue surveillance

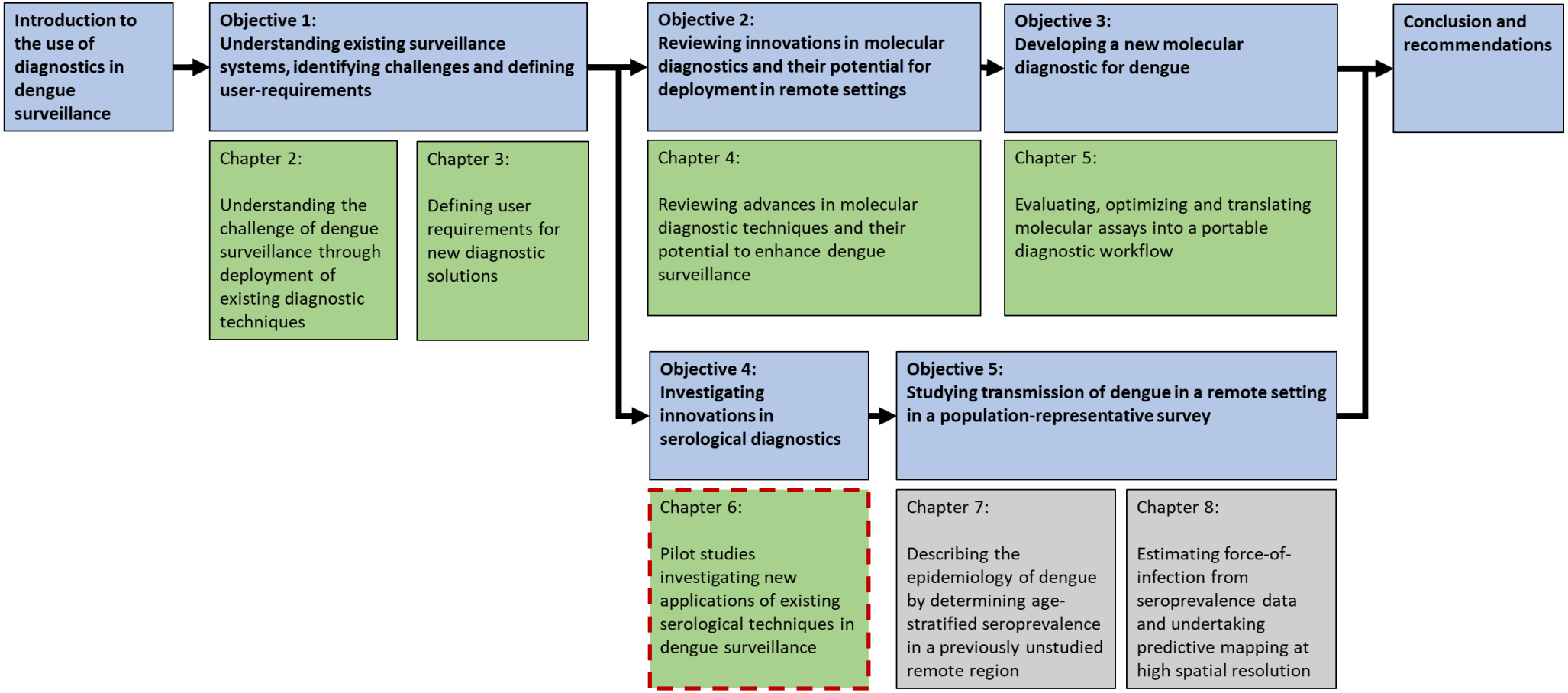



**Figure 29: Thesis overview**

## 6.1 Chapter introduction

In chapters 2-5, I investigated how innovation in molecular diagnostics may be used to improve the diagnosis of acute dengue infection and enhance case-detection in systems of passive dengue surveillance. I made significant advances towards developing a portable, serotype-specific molecular diagnostic workflow for this purpose. Despite this, implementation and thorough evaluation in a remote setting has not been possible within the time available for my PhD. Therefore, I considered methods of active disease surveillance which could be advanced more quickly with the aim of studying and understanding dengue transmission in remote regions. These would ultimately complement passive disease surveillance if used in the same geographical area.

In serological surveillance studies, samples are taken from the general population and tested for the presence of antibodies against dengue. Because the specific IgG response to dengue virus infection is long-lasting, these studies are used to determine the proportion of individuals who have had previous dengue infection ('seroprevalence').(114,115) This is in contrast to most passive surveillance systems, which rely on individuals presenting to healthcare facilities when they are unwell and undergoing testing for active infection. Serological surveillance studies for dengue can result in a detailed, representative understanding of transmission. However, various challenges relating to sample collection, storage and analysis make them challenging to undertake in remote settings.

### 6.1.1 Challenges to serological surveillance in remote settings relating to participant recruitment

If results from serological surveillance studies are intended to be generalisable, participants in the study should be representative of the wider population from which they are sampled. This is usually achieved using a random selection process which uses the whole population as a 'sampling frame'. Clustered sample designs can be used to facilitate study logistics (i.e. to reduce the total number of communities or households which must be visited). However, these must be accounted for in

statistical analysis, and sample collection remains time-consuming and expensive to undertake in remote areas.

### 6.1.2 Challenges to serological surveillance in remote settings relating to sample analysis

In traditional serological surveillance whole blood is collected from participants by phlebotomy and undergoes centrifugation to separate serum (or plasma) from the cellular component. Samples then require refrigerated storage and transport until they are analysed at a laboratory. This workflow is challenging to undertake in remote regions, where equipment and/or a reliable power supply may be lacking. Furthermore, participants may not wish to undergo phlebotomy. Previous serological surveillance studies of dengue have reported low participation rates among young children.(116,117) They have also transported and batch-tested samples at reference laboratories because appropriate serological assays for dengue are not in routine use in many remote regions where studies take place.(115) This may lead to significant delays in reporting.

### 6.1.3 Potential solutions through new applications of existing methods

Various groups of individuals in remote areas may undergo blood sampling for reasons unrelated to dengue. This may include pregnant women receiving antenatal care, blood donors and patients attending hospitals for the management of acute or chronic illness. It may also include participants enrolled in studies where the primary aim is not to investigate the epidemiology of dengue. These groups may be significantly easier to access and recruit into surveillance programmes than randomly selected members of the general population and have previously been used as the sampling frame for studies investigating seroprevalence of dengue.(118–120) Integration of surveillance efforts in this manner may significantly reduce cost of recruiting participants and obtaining samples for dengue serological surveillance (but may affect generalisability of findings to the general population).

Dried blood spot (DBS) samples can be collected by applying whole capillary blood to filter paper. This can be collected by finger-prick techniques which are easier to perform and may be more

acceptable to participants in surveillance systems than phlebotomy. Once dried, they can be transported and stored without refrigeration, but must undergo elution before they can be analysed. DBSs have been proposed as an alternative to serum for use in serological surveillance studies of tropical infection in remote areas.(121,122) However, potential limitations include variable sample volume due to incomplete saturation of the filter paper and variable performance of serological assays with different filter paper varieties or the presence of inhibitors derived from the cellular component of the sample.(122) A small number of studies have used DBSs to investigate dengue but many did not thoroughly validate serological assays for this sample type.(123)

RDTs which use lateral flow technology (lateral flow tests, LFTs) have been designed and optimised for the diagnosis of dengue in patients with AFI. Many of these detect anti-dengue IgG (in addition to IgM and/or NS1) and could therefore be used in programmes of serological surveillance. This would remove the need for laboratory analysis of samples. However, the blood concentration of anti-dengue IgG in previous dengue infection is typically lower than in acute or recent infection (see Figure 1), and hence the diagnostic accuracy of LFTs for this new purpose needs to be established. Some LFTs and other RDTs have undergone such evaluation with the view to them being used in pre-vaccination screening (see Table 19).(124–131) However, all studies, apart from one, have so far been conducted in controlled laboratory conditions and none have assessed diagnostic performance in a system of surveillance.

**Table 19: Summary of previous studies assessing performance of rapid diagnostic tests in the diagnosis of previous dengue infection**

| Publication | Participants | Reference testing | Overall seropositivity among participants by reference test | Sample type used for index testing | Setting of index testing | Index test(s) | Use of lateral flow reader | Performance of index test | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Sens. | Spec. |
| Bonaparte M et al. (Oct 2019) | 804 individuals who were participants in multiple clinical trials of CYD-TDV or employees of Sanofi Pasteur in the USA. | Serostatus for each sample defined using a 'combination of epidemiological, clinical (when available) and assay result information'. Sensitivity (positive) and specificity (negative) panels created. | 33.6% | Serum | Laboratory: Global Clinical Immunology Laboratory, Sanofi Pasteur, Swiftwater, USA. | RDT Dengue IgA/IgG (Bio-Rad, Hercules, USA) (IgG component read) | No | 69.6% | 99.4% |
| | | | | | | OnSite Dengue IgG/IgM (CTK Biotech, San Diego, USA) (IgG component read) | No | 67.0% | 98.9% |
| | | | | | | SD Bioline Dengue IgG/IgM (Abbott, Chicago, USA) (IgG component read) | No | 53.7% | 99.6% |
| | | | | | | Dengue IgG/IgM Rapid Test (GenBody/ Bahiafarma, Cheonan, Korea) (IgG component read) | No | 39.6% | 99.1% |
| Bonaparte M et al. (Mar 2020) | 709 individuals from both dengue endemic and non-endemic regions who were participants in CYD-TDV (Dengvaxia) trials. | Seronegative status defined as $PRNT_{50}$-negative (non-endemic) and according to algorithm including data from: $PRNT_{50}$, $PRNT_{90}$, anti-dengue NS1 IgG ELISA (endemic). Seropositive status defined by Dengvaxia | 53.5% | Serum | Laboratory: A 'Puerto Rican laboratory certified under the Clinical Laboratory Improvement Amendments statute for | Tell Me Fast Dengue IgG/IgM Combo Test Device (Biocan Diagnostics, Vancouver, Canada) (IgG component read) | No | 61.0% | 99.1% |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | phase 3 trial. Sensitivity (positive) and specificity (negative) panels created. | | | diagnosis of acute dengue infection'. | | | | |
| DiazGrandos C et al. (Nov 2020) | 3962 children previously included in phase 3 CYD-TDV studies including those aged 2-14 years in Asia-Pacific region and those aged 9-16 years in Latin American region. | Assigned seropositive/ negative according to reference algorithm including data from:<br>1. $PRNT_{50}$<br>2. $PRNT_{90}$<br>3. Anti-dengue NS1 IgG ELISA | 69.0% | Serum | Laboratory: Central Virology Laboratory (Chaim Sheba Medical Center, Ramat Gan, Israel | SD Bioline Dengue IgG/IgM (Abbott, Chicago, USA) (IgG component read) | No | 71.1% | 96.0% |
| | | | | | Laboratory: Sanofi Pasteur's Global Clinical Immunology Laboratory, Swiftwater, USA | Tell Me Fast Dengue IgG/IgM Combo Test Device (Biocan Diagnostics, Vancouver, Canada) (IgG component read) | No | 52.5% | 99.0% |
| | | | | | | OnSite Dengue IgG/IgM Combo Rapid Test CE (CTK Biotech, San Diego, USA) (IgG component read) | No | 47.6% | 99.5% |
| Chong ZL et al. (Feb 2021) | 484 individuals in communities covered by District Health Office dengue vector control activities. Mean (SD) age in years = 32.0 (18.4) | Two or more positive tests required from:<br>1. Panbio indirect dengue IgG ELISA (Abbott, Chicago, USA)<br>2. In-house HI assay<br>3. In-house FRNT | 79.1% | Capillary blood | Field: Two communities in Petaling District, Selangor State, Malaysia | ViroTrack Dengue Serostate (BluSense Diagnostics, Copenhagen, Denmark) (Detects IgG) | N/A | 91.1% | 91.1% |
| Daag JV et al. (Jun 2021) | 1000 Healthy children aged 9-14 years in a cohort study in Bogo and Balamban in the Phillipines | mFRNT | 91.4% | Serum | Laboratory: University of the Philippines Manila National Institutes of Health | Ichroma II (Boditech Med Incorporated, Gang-won-do, Republic of Korea) (Detects IgG) | N/A | 91.8% | 90.7% |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Echegaray F et al. (July 2021) | 24 individuals aged 18-93 years who had confirmed dengue virus or zika virus infection between 1-30 years prior | FRNT | 66.7% | Blood | Laboratory: Unclear exact locations in USA and Ecuador | SD BIOLINE Dengue IgG/IgM RDT (Bitrodiagnostico, Quito, Ecuador) (IgG component read) | No | 0.0% | 100.0% |
| | 69 participants in the EcoDess cohort in Borbón, Ecuador (aged 2-60 years). | Dengue PanBio IgG ELISA, using cutoff values established by DENV1-4 PRNT titers:<br>- DENV-naïve (<0.2 units)<br>- DENV-primary (0.2-0.9)<br>- DENV-secondary (>0.9) | 63.8% | Serum | | | | 40.9% | 100.0% |
| | 52 children aged 2-17 years from cohort studies in Managua, Nicaragua, who had confirmed dengue infections 6-12 months prior. | Initial infection confirmed by RT-PCR, virus isolation, or a serological algorithm. Serostatus confirmed by testing paired annual samples using the 'dengue inhibition ELISA' and/or 'reporter-virus based neutralization assay'. | 63.5% | Serum/ plasma | Laboratory: Unclear exact locations in Nicaragua and/or Ecuador | Excivion Dengue RDT (Excivion Ltd, Cambridge, UK) (Detects IgG) | No | 78.8% | 78.9% |
| | 115 children aged 8 in the ECUAVIDA birth cohort in Ecuador | Abcam Human Anti-Dengue virus IgG ELISA Kit. | 65.2% | Serum | | | | 63.5%* | >=90%* |
| Limothai et al. (Sep 2021) | Healthy children aged 9-22 years in Ratchaburi province, Thailand. | PRNT | 81.7% | Serum | Laboratory: Central Laboratory, Chulalongkorn hospital, Thailand | SD Bioline Dengue Duo NS1 Ag & IgG/IgM (SD Bioline, Korea) (IgG component read) | No | 35.1% | 100.0% |

**Abbreviations:** Sens. = sensitivity, spec. = specificity, $PRNT_{50}$ = plaque reduction neutralisation test with 50% cut-off, $PRNT_{90}$ = plaque reduction neutralisation test with 90% cut-off, ELISA = enzyme-linked immunosorbent assay, NS1 – non-structural protein 1, HI = hemagglutination inhibition, FRNT = focus reduction neutralisation test, mFRNT = micro focus reduction neutralization test,

## 6.2 Chapter objectives

The aim of this chapter was to investigate how new applications of existing methods for participant recruitment and sample analysis may enhance serological surveillance of dengue.

## 6.3 Methods

### 6.3.1 Study design

To fulfil the aims of this chapter I undertook two pilot studies:

1. A study which integrated participants recruitment and analysis of dried blood spot (DBS) samples with another epidemiological survey occurring across a remote areas ('dried blood spot study').

2. A field evaluation and diagnostic accuracy study of available dengue LFTs when repurposed for the diagnosis of previous dengue infection ('lateral flow test study')

### 6.3.2 Dried blood spot study setting

Participant recruitment took place in remote community locations in seven of the 13 municipalities in Timor-Leste. Sample analysis took place at LNS in Dili, Timor-Leste.

### 6.3.3 Dried blood spot study participant recruitment

I designed this study to take place alongside another epidemiological survey which was being conducted to assess the impact of mass drug administration on lymphatic filariasis (LF) prevalence. In that survey, individuals were being visited by field teams in villages and tested for the presence LF antigen and antibodies using RDTs. I arranged for a subset of the same individuals to participate in my dried blood spot study, with the first 10-15 individuals in 40 villages being approached.

### 6.3.4 Dried blood spot study collection, transportation and analysis

Capillary blood was obtained by finger-prick technique from each participant and used to create DBS samples on Whatman 903 filter paper. These were dried at ambient temperature out of direct

sunlight for four hours, then put individually into zip-lock bags alongside desiccant sachets. They were transported and stored at ambient temperature until serological analysis.

DBS samples were eluted using a published method which has been used for in studies of dengue IgG.(132) Samples which were not fully saturated with blood on visual inspection were rejected. The Panbio Dengue IgG Capture ELISA assay was used to analyse eluted DBSs according to the manufacturer's instructions.

### 6.3.5 Dried blood spot assay validation

Unfortunately it was not possible to obtain known reference-positive and -negative serum samples to LNS for verification of this assay for DBS samples. This was due to a lack of sample availability at collaborating reference laboratories as well as difficulties maintaining cold-chain transport to Timor-Leste during the COVID-19 pandemic. Therefore, I verified the assay in the following manner: I created dried serum spots (DSSs) from 20 deidentified sera with unknown dengue serostatus as well as each of the control materials provided with the assay. I eluted these and tested the resulting elutes alongside the original serum samples, comparing optical density (OD) values in a coefficient of variation analysis

### 6.3.6 Dried blood spot study data analysis

I determined the proportion of individuals in the study whose sample was assigned 'dengue IgG detected' ('seroprevalence') and compared this among individuals of different demographic groups using Fisher's exact and Mann–Whitney U tests for categorical and ordinal data, respectively. I chose to categorise age into four groups (1-15 years, 16-30 years, 31-45 years, 46+ years). This was a pragmatic decision which meant that age groups were of even interval (15 years each) and which would result in approximately even numbers of participants in each group. It also meant that a seroprevalence estimate for children (considered <16 in Timor-Leste) could be estimated.

### 6.3.7 Dried blood spot study ethics

Potential participants received verbal and written information about the purpose of the proposed study and its procedures (see participant information sheet and consent form, Appendix 5) and provided informed written consent. I was awarded ethical approval to conduct this study from the INS Research Ethics Committee, Timor-Leste (Reference 340/MSINS/GDE/III/2019,) and the University of New South Wales Human Research Ethics Committee, Australia (Reference HC190056)

### 6.3.8 Lateral flow test study setting

Participant recruitment and lateral flow testing took place in remote community locations in Dili Municipality, Timor-Leste. Reference serological testing took place at LNS in Dili, Timor-Leste

### 6.3.9 Lateral flow test study participant recruitment

I designed this study to take place alongside the initial phases of another epidemiological survey which I conducted. In that survey, individuals were eligible if they lived in households which were randomly selected from 2015 National Census-enumerated households in a three-stage cluster sample (see chapter 7). Communities were visited and individuals recruited at their households, with Dili municipality being visited first. I arranged for the first 400 participants in the survey to additionally participate in my lateral flow test study.

### 6.3.10 Lateral flow test study sample collection and analysis

Research nurses collected whole blood using either standard phlebotomy or by finger-prick technique, depending on participant preference.

Participants were tested for the presence of dengue antibodies using two different commercially available dengue LFTs (index tests). I chose the Bioline Dengue IgG/IgM, Abbott LFT ('Bioline LFT') because it had good performance in previous studies (sensitivity 53.7–71.1%, specificity 96.0–99.6%). I chose the Standard Q Dengue IgM/ IgG, SD Biosensor LFT ('Standard Q LFT') because it was readily available in Timor-Leste and would therefore be easy to incorporate into any ongoing

surveillance programmes. First, LFTs were operated under 'field conditions', defined as whole blood being tested by a research nurses in the community, in the presence of the participant. This was intended to mimic usage in an ongoing surveillance system. Second, LFTs were operated under 'laboratory conditions', defined as serum samples (from the same individuals) being tested by laboratory scientists at LNS. In both test settings LFTs were operated according to manufacturers' instructions: After removal of packaging they were placed on a flat surface and inoculated with two drops of whole blood (field conditions) or serum (laboratory conditions). The IgG and IgM lines were interpreted visually at 15 minutes and reported in real-time. For experiments under laboratory conditions, the assays were also read at 60 minutes (i.e. beyond manufacturer recommendation), to assess whether this would affect test performance. Prior to study commencement I delivered a classroom teaching session for research nurses and laboratory scientists on the safe and appropriate use of these LFTs, which included practicing operating each device and being observed.

Participants were also tested using the Panbio indirect IgG ELISA (reference test). I chose this assay because it has been has been developed and validated for detecting previous (as opposed to acute) dengue infection and has acceptable performance compared to plaque reduction neutralisation testing (PRNT).(126,133) It has also been used in serological surveillance studies previously.(134,135). Whole blood was injected into a gel serum separation tube (SST), which underwent centrifugation in the field at 1,500 relative centrifugal force (RCF) for 10 minutes. The resulting serum samples were transported to the LNS within a field refrigerator and then stored at 4 degrees Celsius until analysis. Testing was performed in accordance with manufacturers' instructions. Cut-off values for runs were calculated by multiplying the manufacturer's batch calibration factor by the mean OD of calibrators. This was used to assign index values (IVs) to each sample by dividing their ODs by the cut-off value. Samples with IV > 0.9 were assigned 'dengue IgG detected'. The appropriateness of these serological cut-offs was verified visually by plotting a histogram of sample IVs and ensuring there were two normally distributed populations (the first corresponding

seronegative individuals and the second to seropositive individuals) with the chosen serological cut-off appropriately positioned between the two.

### 6.3.11 Lateral flow test study data analysis

I assigned LFTs which showed 'dengue IgG detected' as 'true positive' if the reference ELISA test was also positive or 'false positive' if the reference ELISA was negative. I assigned LFTs which showed 'dengue not detected' as 'true negative' if the reference ELISA result was also negative or 'false negative if the reference ELISA was positive. I defined the diagnostic sensitivity as the number of true positive results / (number of true positive results + number of false negative results). I defined the diagnostic specificity as the number of true negative results / (number of true negative results + number of false positive results). I calculated these values with 95% confidence intervals (CIs) for each LFT when used under field and laboratory conditions. I also calculated positive predictive values (PPVs) and negative predictive values (NPVs).(136)

To explore low LFT sensitivity further, I compared reference ELISA IVs (which relate to the concentration of target anti-dengue IgG in a sample) between individuals with true positive and false negative index tests, using unpaired Students T-tests.

### 6.3.12 Lateral flow test study ethics

Potential participants received verbal and written information about the purpose of the proposed study and its procedures (see participant information sheet and consent form, Appendix 6) and provided informed written consent. Participants were informed of their LFT results in the field. If their sample was assigned 'dengue IgG detected' by either LFT (but 'dengue IgM not detected'), participants were informed that they had previously been infected with a dengue virus. If their sample was assigned 'dengue IgM detected' they were informed that they may have active or recent dengue virus infection and should present to a medical facility if they feel unwell. I was awarded ethical approval to conduct this study from the INS Research Ethics Committee, Timor-Leste

(Reference: 875 MS-INS/DGE/IX/2021) and the Northern Territory Research Ethics Committee, Australia (Reference 2021-4064).

## 6.4 Results

### 6.4.1 Dried blood spot study: Assay verification results

When I compared ODs of DSS samples and to those of paired serum samples I observed linear relationship with low variation (coefficient of variation = 7.1%). I therefore considered performance of the Panbio Dengue IgG Capture ELISA assay to be equivalent between the two sample types and used the manufacturer's recommended serological cut-off for serum to assign DBS samples as either 'dengue IgG detected' or 'dengue IgG not detected'. Figure 25 shows my results from this process.

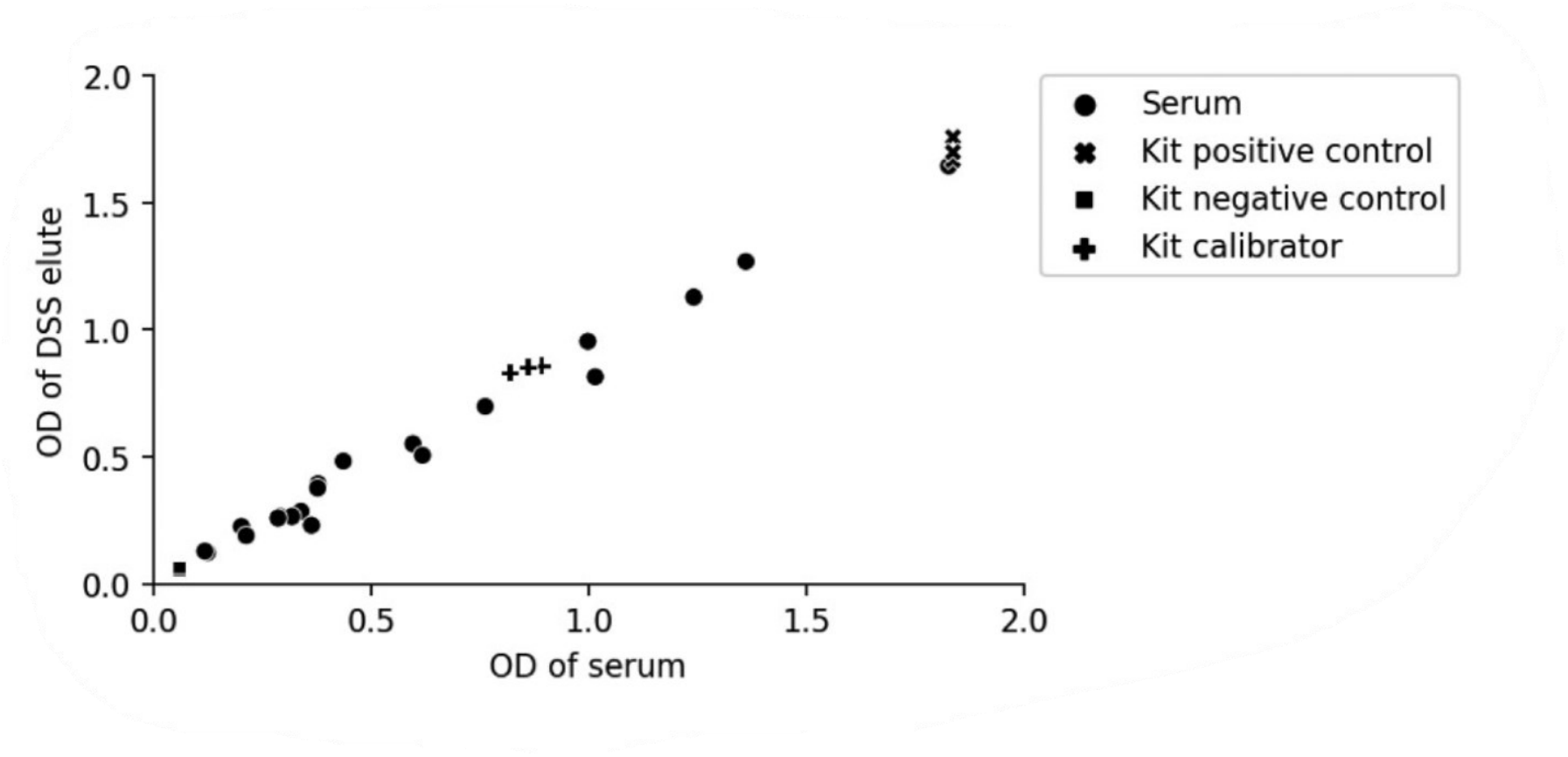



**Figure 30: Results from Panbio Dengue IgG Capture ELISA assay verification experiment.**

**Legend:** There is a linear relationship between the optical density (OD) of dried serum spot (DSS) samples and the OD of paired serum samples, with low variation (coefficient of variation = 7.1%).

### 6.4.2 Dried blood spot study: Seroprevalence results

DBS samples were successfully collected from 308 individuals. These were dried, stored and transported to LNS with ease. Thirty-six (11.7%) were rejected prior to serological analysis because visual inspection revealed that the filter paper was not fully saturated with blood. This resulted in 272 samples which underwent serological analysis, 84 (30.9%) of which were from children and 5 (1.8%) of which were from children under 5 years of age. Nineteen out of 272 (7.0%) were assigned 'dengue IgG detected'. Seroprevalence varied significantly by municipality of residence and age group but not gender (see Table 20).

**Table 20: Dengue IgG seroprevalence among individuals of different demographic groups**

| | Dengue IgG detected | Dengue IgG not detected | p = | Total |
|---|---|---|---|---|
| Gender | | | | |
| - Male | 12 (8.2%) | 135 (91.8%) | 0.332 | 147 |
| - Female | 7 (6.0%) | 110 (94.0%) | | 117 |
| Age group (years) | | | | |
| - 1-15 | 5 (6.0%) | 79 (94.0%) | **0.048** | 84 |
| - 16-30 | 11 (14.1%) | 67 (85.9%) | | 78 |
| - 31-45 | 2 (4.3%) | 45 (95.7%) | | 47 |
| - 46+ | 1 (1.8%) | 54 (98.2%) | | 55 |
| Municipality of residence | | | | |
| - Baucau | 6 (1.2%) | 44 (88.0%) | **0.014** | 50 |
| - Bobonaro | 0 (0.0%) | 29 (100.0%) | | 29 |
| - Covalima | 0 (0.0%) | 47 (100.0%) | | 47 |
| - Dili | 3 (7.9%) | 35 (92.1%) | | 38 |
| - Ermera | 2 (5.9%) | 32 (94.1%) | | 34 |
| - Liquiçá | 7 (16.7%) | 35 (83.3%) | | 42 |
| - Viqueque | 1 (3.1%) | 31 (96.9%) | | 32 |
| TOTAL | 19 (7.0%) | 253 (93.0%) | | 272 |

**Legend:** Dengue IgG seroprevalence was compared between different demographic groups using Fisher's exact and Mann–Whitney U tests for categorical and ordinal variables, respectively

Results included in this chapter have been published in Transactions of the Royal Society of Tropical Medicine and Hygiene.(137)

### 6.4.3 Lateral flow test study: Participant recruitment, seroprevalence and reference assay verification

Four-hundred-and-six individuals were included in this study and had samples taken which completed index and reference testing. This included 217 children with median age of 10 years and

189 adults with median age of 33 years. Dengue IgG seroprevalence by reference ELISA testing was 85.7% among children and 97.4% among adults (overall seroprevalence was 91.1%). Plotting a histogram of sample IVs showed that there were two populations which appeared normally distributed (the first corresponding seronegative individuals and the second to seropositive individuals), with the chosen serological cut-off appropriately positioned between the two. This is shown in Figure 26.

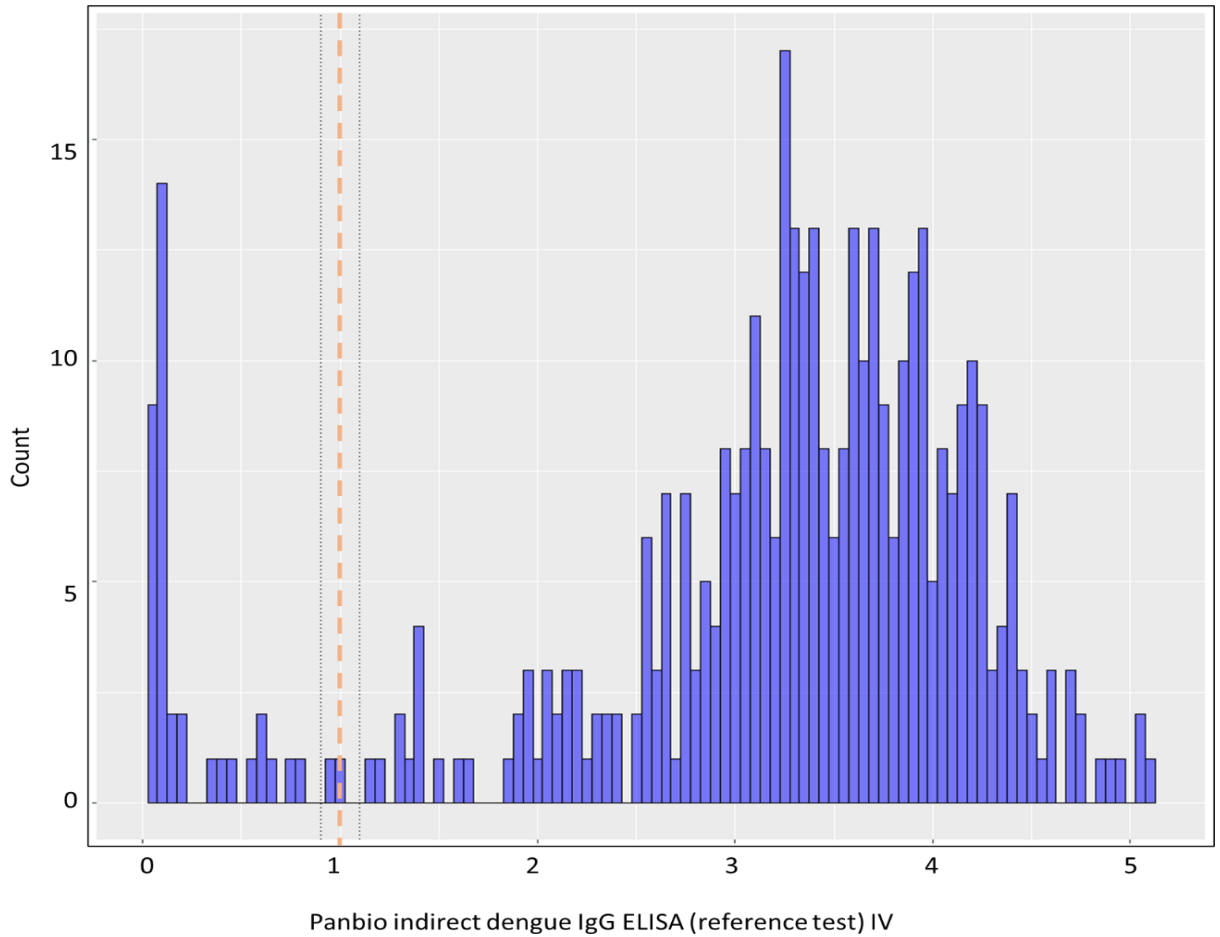



**Figure 31: Histogram of Panbio indirect dengue IgG ELISA index values**

**Legend:** Two populations are seen (blue bars), which appear normally distributed and correspond to seronegative and seropositive individuals. The chosen serological cut-off is positioned appropriately between the two (orange dotted line).

### 6.4.4 Lateral flow test study: diagnostic accuracy results

Diagnostic sensitivity and specificity of the Bioline LFT when operated under field conditions were 21.1% (95% CI = 17.2 - 25.52%) and 100.0% (95%CI = 90.4% - 100.05), respectively. Diagnostic sensitivity and specificity of the Standard Q LFT when operated under field conditions were 4.6% (95%CI = 2.9 - 7.2%) and 100% (95%CI = 81.6 - 100.0%), respectively. When LFTs were operated under laboratory conditions and when they were allowed to run for 60 minutes (i.e. beyond manufacturer recommendation) sensitivity was improved but this appeared to be at some expense to specificity. Table 21 summarises diagnostic performance characteristics of LFTs when operated under different conditions.

**Table 21: Diagnostic performance characteristics of lateral flow tests (LFTs) when repurposed to detect previous dengue virus infection.**

| | | | Panbio indirect IgG ELISA (reference method) | | Sensitivity (95%CI) | Specificity (95%CI) | Positive predictive value (95%CI) | Negative predictive value (95%CI) |
|---|---|---|---|---|---|---|---|---|
| | | | Pos | Neg | | | | |
| Bioline Dengue IgG/IgM (Abbott) | Field 15 | Pos | 78 | 0 | 21.1% (17.2-25.52) | 100.0% (90.4 - 100.0) | 100.0% 95.3-100.0) | 11.0% (8.0-14.8) |
| | | Neg | 292 | 36 | | | | |
| | Lab 15 | Pos | 158 | 1 | 42.7% (37.8-47.8) | 97.2% (85.8-99.5) | 99.4% (96.5-99.9) | 14.2% (10.4-19.1) |
| | | Neg | 212 | 35 | | | | |
| | Lab 60 | Pos | 297 | 2 | 80.3% (75.9-84.0) | 94.4% (81.9-98.5) | 99.3% (97.6 -99.8) | 31.8% (23.7-41.1) |
| | | Neg | 73 | 34 | | | | |
| Standard Q Dengue IgM/IgG (SD Biosensor) | Field 15 | Pos | 17 | 0 | 4.6% (2.9-7.2) | 100.0% (90.4-100.0) | 100.0% (81.6-100.0) | 9.3% (6.8-12.6) |
| | | Neg | 353 | 36 | | | | |
| | Lab 15 | Pos | 39 | 1 | 10.5% (7.8-14.1) | 97.2% (85.3-99.5) | 97.5% (87.12-99.7) | 9.6% (7.0-13.0) |
| | | Neg | 331 | 35 | | | | |
| | Lab 60 | Pos | 142 | 1 | 38.4% (33.6-43.4) | 97.2% (85.8-99.5) | 99.3% (96.2-99.9) | 13.3% (9.7-18.0) |
| | | Neg | 228 | 35 | | | | |

**Abbreviations**: Pos = dengue IgG detected, neg = dengue IgG not detected, field = field conditions, Lab = laboratory conditions, 15 = interpreted after 15 minutes, 60 = interpreted after 60 minutes, ELISA = enzyme-linked immunosorbent assay

**Legend:** Sensitivity, specificity, negative predictive value and positive predictive value of Bioline and Standard Q LFTs when operated under various field and laboratory conditions. The Panbio indirect IgG ELISA was used as the reference method.

### 6.4.5 Lateral flow test study: comparison of reference ELISA index values to explore low sensitivity

The mean (standard deviation, SD) reference ELISA IVs of samples which were 'true positive' when tested with the Bioline LFT under field conditions was 3.808 (0.535), whereas the mean (standard deviation, SD) reference ELISA IVs of samples which were 'false negative' was 3.300 (0.779). Similar differences were observed when considering Standard Q LFTs. Table 22 summarises findings from analysis of reference ELISA IVs.

**Table 22: Comparison of mean reference ELISA index values (IVs) between individuals who had true positive vs. false negative lateral flow test (LFT) results under differing test conditions**

| | | Reference ELISA IV | | |
|---|---|---|---|---|
| | | LFT true positive | LFT false negative | p =* |
| Bioline Dengue IgG/IgM (Abbott) | Field | 3.808 (0.535) | 3.300 (0.779) | < 0.001 |
| | Lab 15 | 3.780 (0.601) | 3.130 (0.753) | < 0.001 |
| | Lab 60 | 3.521 (0.679) | 2.944 (0.903) | < 0.001 |
| Standard Q Dengue IgM/IgG (SD Biosensor) | Field | 3.740 (0.441) | 3.392 (0.772) | 0.007 |
| | Lab 15 | 4.099 (0.517) | 3.326 (0.746) | < 0.001 |
| | Lab 60 | 3.760 (0.629) | 3.188 (0.757) | < 0.001 |

* Unpaired Student's T-tests were used to assess differences in IVs between groups

**Abbreviations:** Field = field conditions, Lab = laboratory conditions, 15 = interpreted after 15 minutes, 60 = interpreted after 60 minutes, ELISA = enzyme-linked immunosorbent assay

Results included in this chapter have been published in PLOS Neglected Tropical Diseases.(138)

## 6.5 Discussion

In this chapter I undertook two studies investigating how new applications of existing methods for participant recruitment and sample analysis may enhance serological surveillance of dengue in remote regions.

By integrating my dried blood spot study with another survey, I showed that dengue serological surveillance can be integrated with other epidemiological activities which may be occurring in remote areas. This significantly reduced the time and cost associated with fieldwork and sample collection, and provided the first ever dengue seroprevalence estimates for Timor-Leste. However, because it was tied to the LF survey design, available individual meta-data were restricted to age, gender and municipality of residence, and participants from only six out of 13 municipalities could be included. These were not representative of the underlying population. Therefore, while easier to conduct, such studies may result in more superficial and less generalisable findings than purposive serological surveillance studies. Previous reports of surveillance using blood donors or hospitalised patients as sampling frames (i.e. 'convenience samples') have reported the similar limitations.(119,139)

In this study I also demonstrated that DBS samples can be collected, transported and analysed in dengue serological surveillance programmes. Such methods could increase participation because finger-prick techniques may be more acceptable to participants than phlebotomy and reduce field consumable and cold storage requirements. However, I found that a significant number of DBSs were of insufficient quality to be analysed (assessed by visual inspection and rejected if the filter paper was not fully saturated with blood). I also found that serological analysis of DBSs was considerably more time-consuming than serum, because they must each undergo elution. Most published elution protocols involve multiple steps and take up to 24 hours.(122,140) Furthermore, many serological assays for dengue IgG have not been validated for DBS samples. While I undertook a pragmatic assay verification at LNS comparing DSSs to paired serum with good results, this was

time-consuming and other dengue IgG assays may perform less well. Therefore, when planning surveillance programmes which collect DBS samples, consideration must be given to the choice of assay, existing validation data for DBS samples (or availability of samples for performing such a study) and the laboratory infrastructure and technical expertise at the proposed location of analysis. These must be weighed against any potential advantage of collecting DBS samples over serum during fieldwork.

With these findings in mind, I proceeded to conduct a study which investigated the performance of LFTs in the diagnosis of previous dengue infection and hence whether they could be used in serological surveillance, replacing laboratory-based techniques entirely. This was among the first of such studies to be conducted under field conditions (i.e. in the intended setting), rather than under controlled laboratory conditions. LFTs were easy to transport, operate and interpret, and results were reported in real-time. I demonstrated the first use of LFTs in a community-based serological surveillance study. However, diagnostic sensitivity was low when compared to an established ELISA reference standard (21.1% for the Bioline LFT and 4.6% for the Standard Q LFT). Other studies have generally found LFTs to have higher sensitivity than this, but have been conducted under laboratory conditions. In my study, diagnostic sensitivities were improved to 80.3% and 38.4% by operating the tests under laboratory conditions and allowing them to run for 60 minutes (i.e. beyond manufacturer recommendation). However, this appeared to be at some expense to specificity, and it would largely remove the appeal of LFTs for use in serological surveillance programmes.

.

In serological surveillance studies, samples should be taken from individuals who represent the underlying population of interest and should be safely stored and transported until they undergo accurate testing. Traditional serological methods for detecting dengue IgG have most often been optimised and validated using reference- positive samples from individuals with active or recent infection, where the concentration of anti-dengue IgG is typically higher than in previous infection. Therefore, alternative methods which are intended to improve participant recruitment or reduce fieldwork logistical requirements must be verified for the purpose of serological surveillance. This includes the analysis of DBS samples instead of serum, and the use of LFTs. Ideally, this work should be undertaken in the setting or laboratory where the testing is ultimately done, within a programme of quality assurance. I carefully considered findings from this chapter and applied them when designing a nationwide population-representative serological surveillance study, which is presented in chapter 7.

## 6.6 Conclusion and key messages

New application of existing methods for participant recruitment and sample analysis may increase uptake and remove challenges associated with traditional dengue serological surveillance studies in remote areas. However, when planning studies using these methods, potential impact on the accuracy and generalisability of results must be considered.

# CHAPTER 7

## 7.0 Describing the epidemiology of dengue by determining age-stratified seroprevalence in a previously unstudied remote region

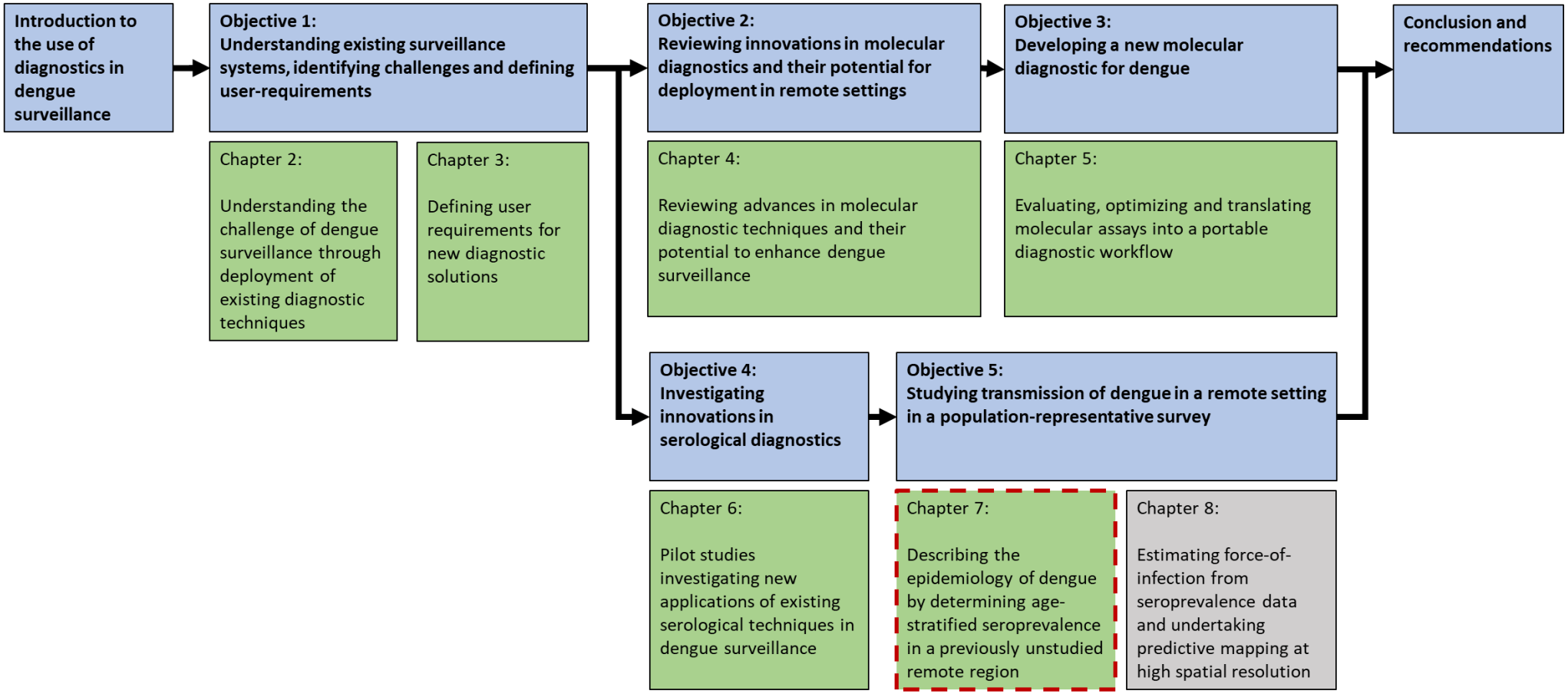



**Figure 32: Thesis overview**

## 7.1 Chapter introduction

In chapter 6 I undertook two pilot studies investigating new application of existing methods for participant recruitment and sample analysis in dengue serological surveillance. I demonstrated that the effective collection of DBS samples instead of serum samples which may reduce field consumable and cold storage requirements and potentially improve participation in surveillance activities. However, elution of DBS samples prior to their analysis is time-consuming and serological assays require verification for this new sample type. I demonstrated for the first time that available LFTs can be used in serological surveillance, removing laboratory analysis of samples entirely and producing results in real-time. However, I found that LFTs are less sensitive than traditional laboratory techniques.

Timor-Leste is a half-island nation with a population of 1.3 million people, many of whom live in remote areas and experience significant barriers to accessing healthcare.(43) . In 2005 a severe dengue outbreak in Dili, the capital city, was investigated. The majority of patients were children, and a small number of samples underwent molecular analysis to determine the infecting serotype.(45,141) More recently, an analysis of reported dengue cases across Timor-Leste between 2005 and 2013 also found children to be at higher risk than adults. Dengue incidence was highly seasonal, with outbreaks in December-January, and was related to higher mean temperature and higher precipitation.(44) These studies relied on passive surveillance systems which existed at the time of each study and findings therefore based on only the subset of individuals with acute infection who accessed healthcare and received a diagnosis of dengue which was reported. In chapter 3 I found that many such systems in other dengue endemic countries have low sensitivity, and that sensitivity may vary between regions. Since 2013, cases of acute dengue have continued to be reported through the national passive surveillance system. Overall, the annual number of notifications per-capita has been low compared to other countries with similar populations and there is anecdotal evidence of underdiagnosis and underreporting. However, incidence appears to be highest in Dili and Baucau municipalities, which include the two biggest urban centres in Timor-Leste.

Apart from my two studies presented in chapter 6, which gave preliminary seroprevalence estimates for six municipalities, there has been no serological surveillance of dengue in Timor-Leste. Accurate data relating to dengue transmission in each of Timor-Leste's municipalities are lacking. Studies to understand baseline dengue epidemiology are therefore needed. These will enable the subsequent targeting of surveillance systems, and the use of contextually appropriate tests and innovation.

In this chapter I designed and implemented a nationwide population-representative serological survey to investigate the seroprevalence of dengue in Timor-Leste. This was directly informed by my findings from chapter 6.

## 7.2 Chapter objectives

The primary aim of this chapter was to describe the epidemiology of dengue by determining age-stratified seroprevalence across a previously unstudied remote region. The secondary aim was to assess available individual and population variables for association with seropositivity.

## 7.3 Methods

### 7.3.1 Study design

National, population-representative serological survey

### 7.3.2 Setting

Recruitment for this study took place at participants' households across all 13 municipalities in Timor-Leste. Laboratory analysis occurred at LNS, Dili, which is the national diagnostic and surveillance laboratory.

### 7.3.3 Sampling methods

Timor-Leste is divided into 13 municipalities, 65 sub-municipalities ('Posto Administravos'), and 424 villages ('Sucos'). In 2015 a national census occurred which enumerated all 191,865 households in the country and geolocated them within 2320 'Enumeration Areas' (EAs), which is now the smallest administrative unit. For this study, I used 2015 census data as my sampling frame and designed a three-stage cluster sampling strategy to select individuals. First, a pre-specified number of EAs were randomly selected from each municipality, with probability proportional to municipality population. I achieved this by assigning random numbers to all EAs using the *runif()* function in R. For each municipality I selected EAs consecutively based on their random number allocation (starting with the lowest number and finishing after the required number had been selected). Second, a pre-specified number of households were randomly selected from each EA. This was achieved by Ms Vanessa Solano who is a Geospatial and Mapping Scientist at Charles Darwin University, Darwin, using the *Random Points in Layer Bounds* tool in *QGIS 3.1 (QGIS Development Team, 2020).* Positions of households were then verified based on *Sentinel-2 Satellite Imagery (European Space Agency, 2021).* Other researchers (including all individuals involved in fieldwork and data collection) were blinded to EA and household selection. Third, all available occupants who resided at each participating household were assessed for inclusion.

### 7.3.4 Sample size

I determined the sample size for this study with reference to the WHO Reference Manual for Vaccination Coverage Cluster Surveys.(142) First, I considered which age groups would be most important in which to estimate dengue IgG seroprevalence. In previous studies conducted in the region, seroprevalence rises sharply during early childhood, with most individuals being seropositive before they are 15 years of age. Additionally seroprevalence at the hypothetical vaccine-eligible age group of 5-14 years is likely to be key in deciding whether the Dengvaxia vaccine should be rolled out

in Timor-Leste.(33) Therefore, I considered it most important to estimate national seroprevalence in age groups of 1-4 and 5-14. Additionally I considered individuals in age groups 15-24 and 25-40 to be different in terms of their physiology (post-pubescent and of child-bearing age) and likely occupation (finished formal education, of working age), compared to younger groups, so considered it of additional importance to estimate seroprevalence in these two groups, as well as the oldest group (40+ years). These age groups are also reflected in the 2015 National Census report. Then, using the WHO Reference Manual, I determined the sample size which would be needed if undertaking a simple random sample from the population ('effective sample size') to be 280 for each age group. This was based on an expected seroprevalence of 50%, to give the most conservative estimate, and a precision of +/-6% for the 95% CI. I then multiplied the effective sample size by four. I used a pragmatic estimate of overall study design effect of 4 because local data relating to clustering of dengue IgG seroprevalence at each level of the sampling strategy (household, EA, municipality) were not available to calculate the expected study design effect more accurately. The required sample size for each age group and for the whole study were therefore 1120 and 5600, respectively.

The 2015 national census found that the average household occupancy in Timor-Leste was 5.7 individuals. The projected proportion of the Timorese population within each of my chosen age groups was 9.6%, 24.0%, 21.9%, 20.2% and 21.5%, respectively. Therefore I determined the number of households which would need to be visited based on the age group that is the smallest proportion of the general population (1-4 years) as:

$1120 / (0.096 \times 5.7) = 2047$

I assumed that household response rate would be 85% based on data from previous serological surveys in the region, and on local insights gained during chapter 6 of this thesis. Therefore, the I determined the number of households which would need to be targeted as:

$2047 / 0.85 = 2408$

Therefore, I decided to select 23 households from each of 113 EAs. Because four of the EAs which were ultimately selected contained fewer than 23 households, the total number of targeted households was 2558.

### 7.3.5 Fieldwork procedures

Fieldwork was undertaken by study teams which each consisted of three research nurses and a research coordinator. Study teams were also sometimes joined by local stakeholders including the following individuals who were contacted and invited to attend:

1. Municipality Administrator (one per municipality).

2. Director of Municipality Health Service (one per municipality).

3. Sub-Municipality Administrator (one for each sub-municipality).

4, Head of Community Health Centre (CHC, one for each sub-municipality).

5. Chief of Suco (one for every EA).

6. Commander of Police in Suco (one per EA being visited, at the discretion of the Chief of Suco).

Municipalities and EAs were visited sequentially, with the order being determined by up-to-date knowledge of road conditions, availability of vehicles and any local COVID-19 regulations which may have restricted movement or interaction with participants at the time. Once an EA was reached, field teams travelled by foot, using electronic tablets with the Google Earth application installed to locate households. Global Positioning System (GPS) coordinates for each household location were loaded in advance. Additionally, I collaborated with Ms Vanessa Solano, a researcher at Charles Darwin University, Australia, to produce bespoke paper maps for each EA which had households, roads, paths and landmarks marked using ArcMap V.10.4.1 with ESRI imagery base map. They were printed in colour, laminated and used during fieldwork if households could not be located using

electronic tablets. One of the 113 maps produced is shown in Appendix 6 as an example. I also developed a standard operating procedure (SOP) for locating households which is shown in Appendix 6.

### 7.3.6 Participant recruitment

Study teams approached household occupants, introduced themselves and asked to speak with the head-of-household. If the usual head-of-household was not present, they asked to speak with someone who could act as the head-of-household for the time being. If nobody was present, the study team returned to the household on two further occasions, at least one time on a different day. Study rationale and procedures were explained to the (acting) head-of-household and any other occupants who were present in their preferred language (Tetun, English or Portuguese). Each occupant was also given written information about the study in a participant information sheet (shown in Appendix 6). Household occupants were eligible to participate if they fulfilled the following criteria:

Inclusion criteria:

1. Over 1 year of age

2. Provided written informed consent (or, in the case of children, provided verbal assent and their parent or guardian provided informed written consent).

Exclusion criteria:

1. Under 1 year of age. This was because maternal transfer of antibodies can complicate the interpretation of serology results and because results from my dried blood spot study suggested this group may have been difficult to sample.

2. Needle phobia.

2. Anaemia.

5. Skin condition affecting phlebotomy or finger-prick sites.

4. Bleeding disorder.

5. Unable to communicate in Tetun (the most commonly spoken local language), English or Portuguese.

6. Reported current illness which was compatible with COVID-19, according to the national suspected case definition. This was for fieldworker safety, and to comply with local social distancing measures as the study took place during the first and second waves of the COVID-19 pandemic in Timor-Leste.

### 7.3.7 Data collection

Data were collected from the head-of-household and consenting participants using structured interview questionnaires ('household questionnaire' and 'individual participant questionnaire', respectively. I developed these based on my literature reviews and insights gained while conducting other studies presented in earlier chapters. They included questions about individuals' previous episodes of AFI, diagnoses with suspected and confirmed dengue, and efforts to protect against mosquito bites. Interview questionnaires are shown in Appendix 6. These were coded into REDCap software, such that responses could be entered directly into electronic tablets and intermittently uploaded to cloud server for storage.(143) Study teams also recorded data into an 'unable to complete' questionnaire when no household-level data could be collected after at least three visits.

### 7.3.8 Sample collection

Research nurses collected blood from participants. Venepuncture was used as the preferred technique, because it typically results in a blood draw of larger volume. However, children under 5

years of age and those who refused venepuncture were offered blood collection by finger-prick technique. I decided to include this option based on findings from studies presented in chapter 6, in an attempt to maximise participation. Venous blood was injected directly into pre-labelled SST tubes. Capillary blood was dropped directly into pre-labelled paediatric SST tubes. Table 23 shows blood collection techniques and equipment used for each participant age group. Blood samples were allowed to clot at ambient temperature out of direct sunlight, then underwent centrifugation in the field at 1500 RCF for 10 minutes, to separate serum from the cellular component. Serum was then stored at 2-8 degrees Celsius in a field refrigerator and transported to LNS. Blood from each participant was also used to create a DBS sample: Five to ten drops of blood were applied to Whatman 903 filter paper (until a 12mm circle was saturated). This was dried at ambient temperature out of direct sunlight and stored in a zip-lock bag alongside a desiccant sachet. I included the collection of a paired DBS sample as a back-up (in case the serum samples could not be analysed), and so that my samples could be used to validate serological assays for this new sample type, which was a specific need identified in chapter 6. The SOPs used for sample collection and creation of DBS samples are shown in Appendix 6, respectively.

**Table 23: Blood collection techniques and equipment used for participants in a national population-representative serological survey**

| Age group (years) | Method of blood collection | Equipment | Collection container | Target sample volume (ml) |
|---|---|---|---|---|
| 1-5 | Venepuncture<br>or<br>Finger-prick | 23G butterfly needle<br>or<br>Finger lancet | 5ml SST tube<br>or<br>2ml paediatric SST tube | 5<br><br>2 |
| 6-15 | Venepuncture | 21-23G straight needle or butterfly needle | 5ml SST tube | 5 |
| 16+ | Venepuncture | 21-23G straight needle | 5ml SST tube x2 | 10 |

### 7.3.9 Ethics

Potential participants received verbal and written information about the purpose of the proposed study and its procedures (see participant information sheet and consent form, Appendix 6) and provided informed written consent. I was awarded ethical approval to conduct this study from the INS Research Ethics Committee, Timor-Leste (Reference: 875 MS-INS/DGE/IX/2021) and the Northern Territory Research Ethics Committee, Australia (Reference: 2021-4064).

### 7.3.10 Sample analysis

Samples were analysed using the Panbio indirect dengue IgG ELISA, which contains combined NS1 antigens from all four dengue virus serotypes. It detects human IgG antibodies against any serotype but does not differentiate. I chose this assay because it has been has been developed and validated for detecting previous (as opposed to acute) dengue infection and has acceptable performance compared to PRNT.(126,133) It has also been used in serological surveillance studies previously.(134,135). Testing was performed in accordance with manufacturers' instructions. Cut-off values for runs were calculated by multiplying the manufacturer's batch calibration factor by the mean OD of calibrators. This was used to assign IVs to each sample by dividing their ODs by the cut-off value. Samples with IV > 0.9 were assigned 'dengue IgG detected'. The appropriateness of these serological cut-offs was verified visually by plotting a histogram of sample IVs and ensuring there were two normally distributed populations (the first corresponding seronegative individuals and the second to seropositive individuals) with the chosen serological cut-off appropriately positioned between the two. Samples were stored at -80 degrees Celsius in case they needed to undergo further analyses.

### 7.3.11 Fieldworker and laboratory scientist training

Before this study began I organised and delivered one week of training for fieldworkers. This included classroom sessions on 'the study protocol', 'field team composition' (structure, members, responsibilities), 'logistics, technology and map reading', 'recruitment and consent' and 'collection of data using interview questionnaires'. It then included practical sessions on 'adult and paediatric phlebotomy', 'finger-prick techniques', 'infection prevention control procedures', and the 'use of personal protective equipment' (see Appendix 6). I undertook a formative assessment of each team member throughout the training and a summative assessment using pre- and post-session written quiz.

Together with Ms Tessa Oakley who is a Researcher and Laboratory Scientist Mentor at Menzies School of Health Research, Dili, Timor-Leste, I also delivered Laboratory Scientist training. This included sessions on 'assay verification and quality' and 'procedures for processing samples and undertaking analysis using chemiluminescent and ELISA assays'.

### 7.3.12 Data analysis: Assigning remoteness indices

I used household GPS locations to determine the distance by road or path to the nearest CHC ('remoteness index'), using Google Earth. I used the following R code which automated the collection of data from Google Earth and assignment of remoteness indices:

```
install.packages(gmapsdistance)
library(gmapsdistance)
x <- read.csv("destination.csv")
y <- read.csv("destination.csv")
results <- as.data.frame(matrix(NA, (2566*55), 3))
for (i in 1:nrow(x)) {
```

```
set.api.key("AIzaSyBEzHO5Y7RfIUy…")

x <- dest [i,2]  #extract CHC name

dist$destination<- dest[i,1]

destination <- dist$destination #somehow this is how I made it a vector

s1 <- 1+(2566*(x-1))

e1 <- s1 + 2565

 results <- as.data.frame (gmapsdistance(origin, destination, "driving", shape = "long",
combination="pairwise"))

results [c(s1:e1),1] <- x

results [c(s1:e1),2] <- (results$Time.Time)/60 #Time in seconds

results [c(s1:e1),3] <- (results$Distance.Distance)/1000 #Distance in meters

print (x)

}

str(results)

write.csv(results, "Results.csv")
```

Where households were not linked to the path network, their straight-line distance to the nearest path was added to the distance from that point by road or path to the nearest CHC was used. This was done manually using Google Earth in a web browser.

### 7.3.13 Data analysis: Describing participation and assessing for sampling bias

Individuals from whom a serum sample was successfully collected and analysed for dengue IgG were included in analysis. I reviewed fieldwork and laboratory data to understand household and individual participation at each stage of the study. I represented these findings in a study flow diagram.

To assess for the presence of sampling bias at the household level, I compared households where one or more individual participated ('participating households') to those where none participated ('non-participating households') in terms of their municipality and remoteness indices. To assess for sampling bias at the individual level I compared participating individuals to those who were listed as occupants in households but did not participate in terms of their age, age-stratum, and gender. Significance of association was assessed using Fisher's exact tests and unpaired Student's t-tests for categorical and continuous variables, respectively.

### 7.3.14 Data analysis: Calculating national and municipality seroprevalence estimates

I performed analysis of dengue IgG seroprevalence in three stages. First I calculated 'crude seroprevalence' estimates for different age groups and municipalities by dividing the number of individuals with samples assigned 'dengue IgG detected' by the total number whose sample was successfully tested. Age groups included the five age groups which were determined *a priori* (1-4, 5-14, 15-24, 25-40, and 41+ years) as well as age-strata at 5-year intervals (1-4, 5-9, 10-14, 15-19, 20-24, 25-29, 30-34, 35-39, 40-44, 45-49, 50-54, 55-59, 60-64, 65-69, and 70+ years).

Second, to account for sampling bias and to compare municipalities, I calculated 'age-standardised seroprevalence' for each municipality using the 2022 projection of the 2015 national census as the reference age distribution. I used Dobson's method to calculate 95% CIs around these estimates.(144) I undertook these calculations using the dsrTest package in R Studio (R).

Third, I performed an analysis which would additionally account for various design effects in the study relating to the sampling strategy. To adjust for unequal probabilities of both selection and response at the EA and household level, I assigned sampling weights to each individual which were a factor of 'selection probability' and 'response probability' at each level. These weighted observations were then used to fit a mixed effects multivariable logistic regression model, which accounted for potential clustering of seropositivity at both the EA and household level by including

both EA and household fitted with a random intercept. Participant age-stata (5-year interval), gender and municipality were fitted with fixed effects. I then used this model to compute national and municipal 'model predicted' seroprevalence estimates with 95% CIs. These analyses were conducted using the *survey, WeMix* and *lme4* packages in *R.*

### 7.3.15 Data analysis: Assessing association of household and population variables with seropositivity

To explore associations of household and population variables with dengue IgG seropositivity I fitted a series of mixed-effects multivariable logistic regression models. These examined the effect of each variable (fitted with fixed effect) while accounting for clustering in the study design by including EA and household (which were fitted with random effect).

1. Household assigned urban vs rural at 2015 national census
2. log(Household remoteness index)
3. Number of household occupants
4. log(EA population density at 2015 national census)

These particular variables were chosen because an association was observed in previous similar studies, there were logical potential causative relationships and/or there was particular importance to assess relationships for disease control programmatic reasons.(8)

Continuous variables ('EA population density' and 'household remoteness index') were log-transformed prior to their inclusion. This transformation was decided by assessing the linearity of their univariable relationship with the log(odds) of seropositivity using a BoxCox analysis.

## 7.4 Results

### 7.4.1 Participation and sampling bias

Of the 113 EAs which were targeted, 102 (90.3%) were successfully visited. Eight were not visited due to 'poor weather and associated road damage', two were not visited because of 'inadequate time', and one was not visited because of 'field staff illness'. EA locations are shown in Figure 28.

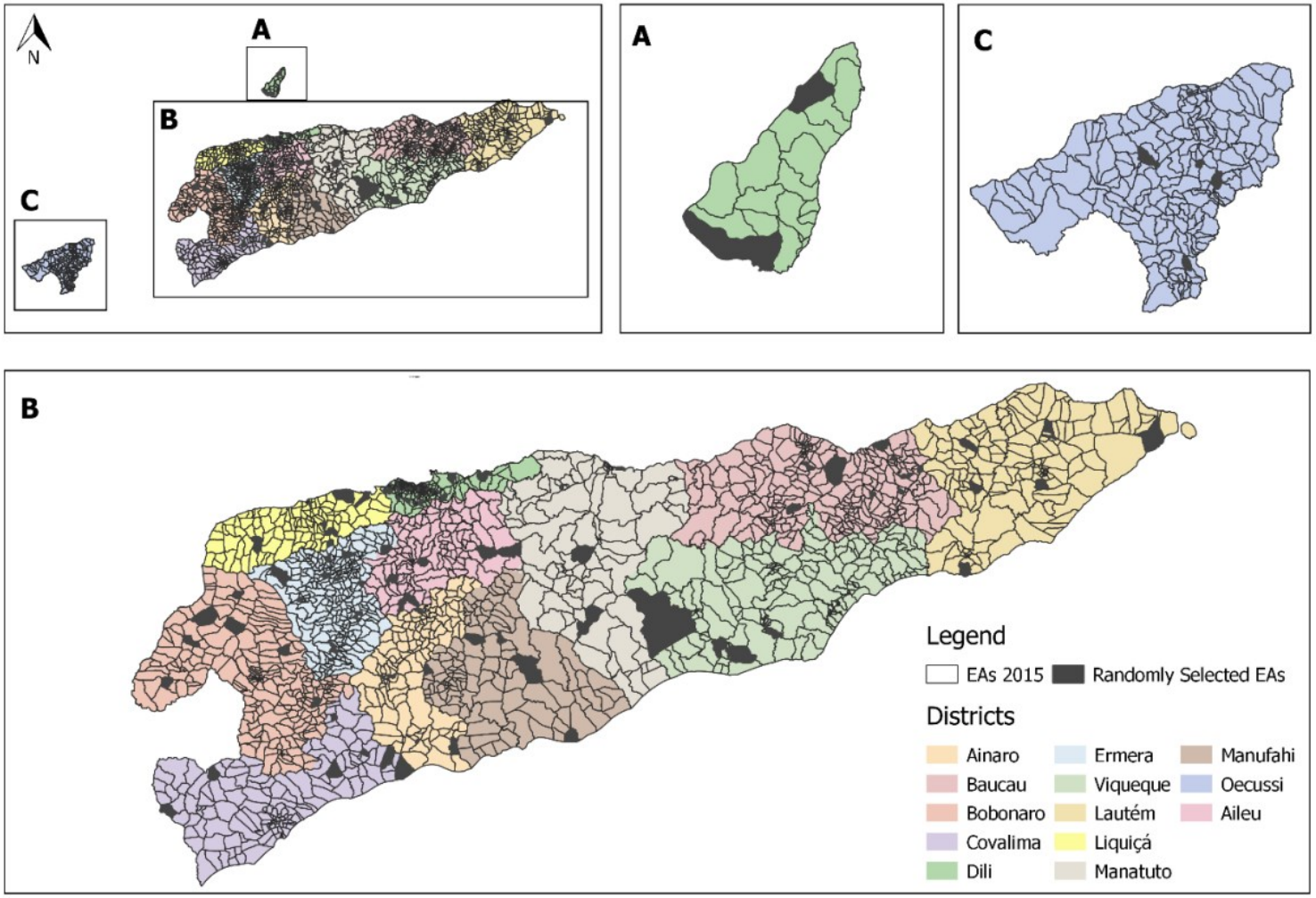



**Figure 33: Map of Timor-Leste showing selected Enumeration Areas (EAs) within 13 municipalities**

Out of the 2558 targeted households, 1853 (72.4%) provided household level data. Sixty (3.4%) of these were adjacent ‘replacement houses’, chosen because the original building was derelict, destroyed or no longer a household. Fifty-five additional households were recruited because more than one mutually exclusive group of individuals were living in separate parts of the same building. Therefore, a total of 1908 households participated and 705 did not. Table 24 shows details of participating households and compares them to non-participating households.

Household participation rate was significantly different between municipalities with lowest participation in in Ermera (42.9%), Liquica (51.9%) and Manufahi (52.6%) and highest participation in Covalima (89.9%), Manatuto (88.5%) and Baucau (87.4%, $p < 0.001$). Participation was also higher in areas which were assigned as urban at the 2015 national census (81.2%) compared to those assigned as rural (69.9%, $p < 0.001$). Participating households were also on average 1.6km closer to the nearest CHC than non-participating households (‘remoteness index', $p < 0.001$).

**Table 24: Summary of participating households and comparison to non-participating households**

| | Participating households | Non-participating households | p= | TOTAL |
|---|---|---|---|---|
| Municipality | | | | |
| - Aileu | 75 (65.2%) | 40 (34.8%) | | 115 |
| - Ainaro | 105 (75.0%) | 35 (25.0%) | | 140 |
| - Baucau | 222 (87.4%) | 32 (12.6%) | | 254 |
| - Bobonaro | 141 (76.2%) | 44 (23.8%) | | 185 |
| - Covalima | 124 (89.9%) | 14 (10.1%) | | 138 |
| - Dili | 543 (81.0%) | 127 (19.0%) | | 670 |
| - Ermera | 120 (42.9%) | 160 (57.1%) | | 280 |
| - Lautem | 102 (72.3%) | 39 (27.7%) | | 141 |
| - Liquiçá | 84 (51.9%) | 78 (48.1%) | | 162 |
| - Manatuto | 85 (88.5%) | 11 (11.5%) | | 96 |
| - Manufahi | 60 (52.6%) | 54 (47.4%) | | 114 |
| - Oecusse | 128 (83.1%) | 26 (16.9%) | | 154 |
| - Viqueque | 119 (72.6%) | 45 (27.4%) | **<0.001** | 164 |
| Mean (SD) remoteness index (distance by road/path to CHC) | 7.6 (7.9) | 9.3 (9.9) | **<0.001** | 8.1 (8.5) |
| Assignment during 2015 National Census | | | | |
| - Urban | 590 (81.2%) | 137 (18.8%) | | 727 |
| - Rural | 1318 (69.9%) | 568 (30.1%) | **<0.001** | 1886 |
| TOTAL | 1908 | 705 | | 2613 |

**Legend: Fisher's exact tests and unpaired Student's t-tests were used to test association of household participation with categorical and continuous variables, respectively.**

The total number of occupants within participating households (reported by the Head-of-Household) was 8427. Of these, 4730 (56.1%) were available during at least one visit and provided a serum sample which underwent serological analysis for dengue IgG ('participants'). Figure 29 is a flow diagram showing details of individual and household participation. Table 25 shows demographic details of participants and compares them to non-participating household occupants. Individual participation rate was significantly higher among females ($p < 0.005$) and lower among children under 5 years of age ($p < 0.005$).

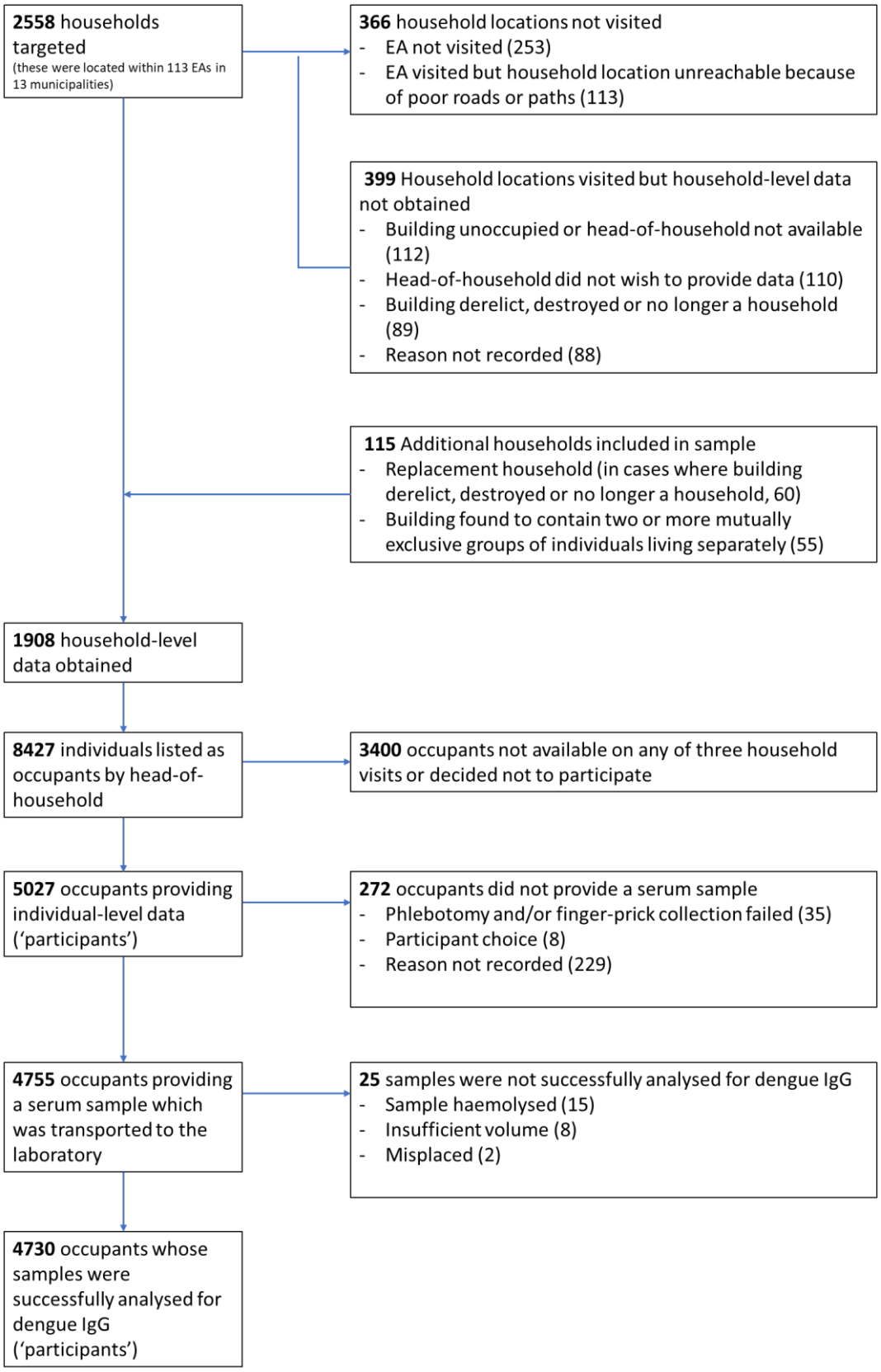



**Figure 34: Flow diagram showing household-level and individual participation -**

**Table 25: Participant characteristics and comparison of participants to non-participating household occupants**

| Individual-level characteristic | Participants | Non-participating household occupants | p= | TOTAL |
|---|---|---|---|---|
| Mean age in years (SD) | 31.0 (20.8) | 27.3 (20.2) | **<0.001** | 29.6 (20.7) |
| Age group | | | | |
| - 1-4 years | 270 (44.2%) | 341 (55.8%) | | 611 |
| - 5-14 years | 1037 (61.9%) | 639 (38.1%) | | 1676 |
| - 15-24 years | 806 (59.1%) | 558 (40.9%) | | 1364 |
| - 25-40 years | 1166 (63.2%) | 679 (36.8%) | | 1845 |
| - 41+ years | 1451 (66.2%) | 742 (33.8%) | **<0.001** | 2193 |
| - Not recorded* | 0 (0.0%) | 738 (100.0%) | | 738 |
| Adult or child | | | | |
| - Child | 1307 (51.3%) | 1241 (48.7%) | | 2548 |
| - Adult | 3423 (59.2%) | 2355 (40.8%) | **<0.001** | 5778 |
| - Not recorded* | 0 (0.0%) | 101 (100.0%) | | 101 |
| Gender | | | | |
| - Male | 1930 (50.3%) | 1908 (49.7%) | | 3838 |
| - Female | 2799 (62.4%) | 1685 (37.6%) | **<0.001** | 4484 |
| - Other/unknown/not recorded* | 1 (1.0%) | 104 (99.0%) | | 105 |
| Mean number of household occupants (SD) | 5.4 (2.4) | 5.4 (2.3) | >0.999 | 5.4 (2.4) |
| TOTAL | 4730 | 3697 | | 8427 |

*Differences in proportions of individuals with demographic details which were 'not recorded' are likely due to differences in data collection methods (i.e. because data for non-participating occupants was collected from the head-of-household rather than from the individual themselves). Therefore, these categories were omitted from hypothesis testing.

**Legend: Fisher's exact tests and unpaired Student's t-tests were used to test association of participation with categorical and continuous variables, respectively.**

### 7.4.2 Crude national and municipal seroprevalence estimates

Crude dengue IgG seroprevalence by age group and municipality are represented in Figure 30. In all municipalities seroprevalence increased with age during childhood. However, the rate of increase and age at which seroprevalence appears to plateau varies considerably between municipalities.

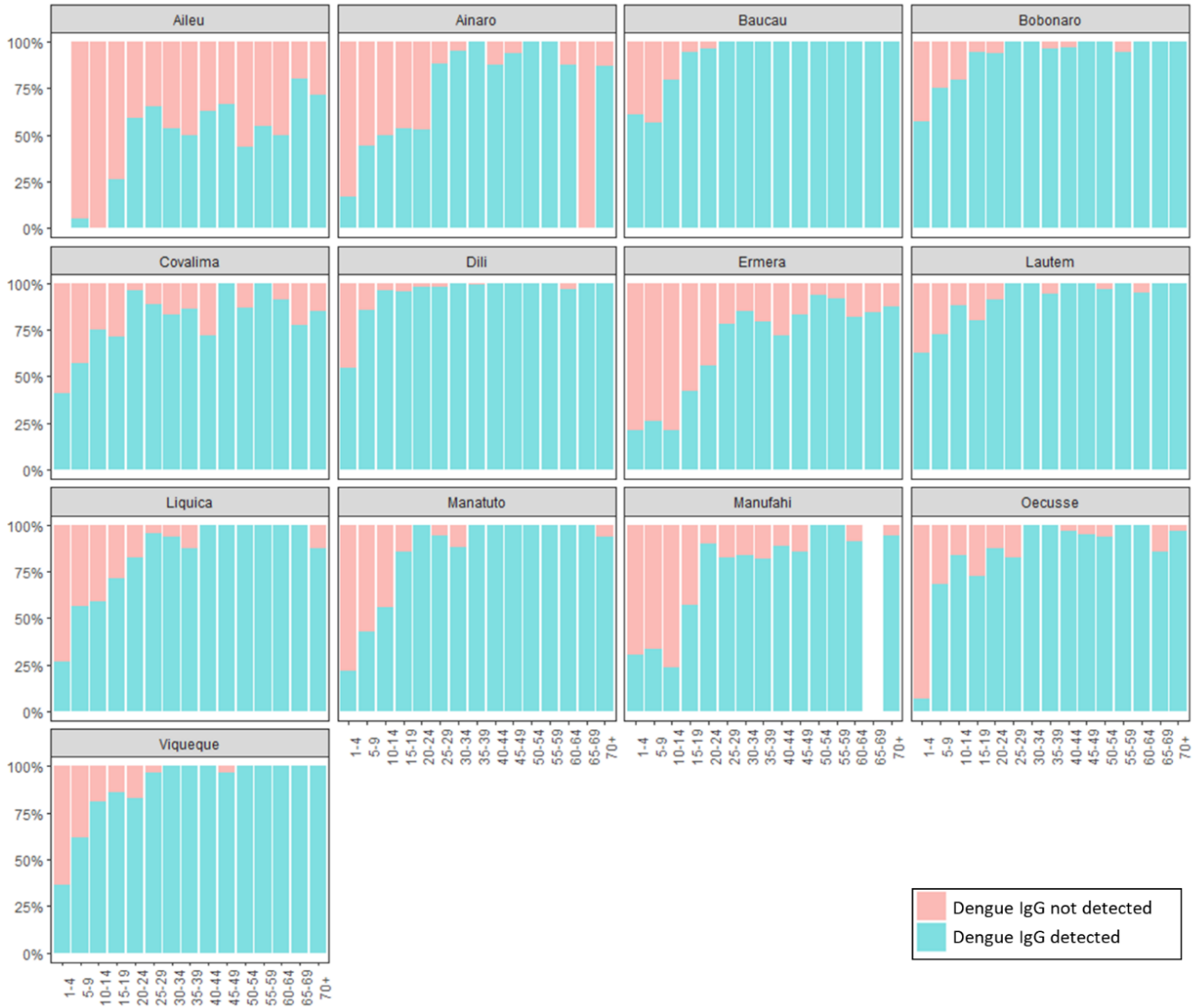



**Figure 35: Crude dengue IgG seroprevalence by age group and municipality**

### 7.4.3 Age-standardised national and municipal seroprevalence estimates

Age standardised dengue IgG seroprevalence estimates for the whole population and a hypothetical vaccine-eligible population in each municipality are shown in Table 26.

**Table 26: National and municipal crude and directly standardised dengue IgG seroprevalence estimates**

| | Dengue IgG seroprevalence | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Whole population (>1 year) | | | | Hypothetical vaccine-eligible population (5-14 years) | | | |
| | Crude rate | Directly standardised rate | 95% CI (Dobson's) | | Crude rate | Directly standardised rate | 95% CI (Dobson's) | |
| | | | Upper | Lower | | | Upper | Lower |
| Aileu | 40.3% | 36.0% | 27.4% | 46.2% | 3.0% | 2.7% | 0.1% | 14.9% |
| Ainaro | 64.9% | 60.1% | 51.2% | 70.1% | 45.6% | 45.7% | 32.0% | 63.2% |
| Baucau | 90.5% | 85.3% | 76.2% | 95.1% | 65.4% | 65.2% | 48.6% | 85.7% |
| Bobonaro | 91.3% | 86.1% | 75.7% | 97.3% | 75.9% | 75.9% | 58.0% | 97.8% |
| Covalima | 77.4% | 74.1% | 64.5% | 84.5% | 63.8% | 64.6% | 48.0% | 85.0% |
| Dili | 94.0% | 90.6% | 85.0% | 96.5% | 90.1% | 90.2% | 79.0% | 101.8% |
| Ermera | 58.5% | 49.5% | 42.3% | 57.6% | 18.3% | 18.4% | 9.1% | 32.9% |
| Lautem | 88.9% | 85.5% | 73.4% | 98.8% | 77.7% | 78.3% | 61.3% | 98.5% |
| Liquiçá | 81.8% | 73.2% | 61.8% | 85.8% | 57.6% | 57.5% | 34.6% | 89.9% |
| Manatuto | 76.8% | 74.3% | 63.1% | 86.8% | 48.4% | 47.8% | 32.1% | 68.4% |
| Manufahi | 64.2% | 60.2% | 48.7% | 73.5% | 28.9% | 28.5% | 14.2% | 51.1% |
| Oecusse | 84.2% | 75.4% | 65.3% | 86.5% | 72.6% | 74.4% | 53.8% | 100.1% |
| Viqueque | 85.8% | 79.3% | 68.7% | 90.9% | 61.5% | 63.9% | 45.3% | 87.3% |
| All municipalities | 81.9% | 76.9% | 74.4% | 79.5% | 65.0% | 65.2% | 60.3% | 70.3% |

**Legend:** Estimates for the whole population (>1 year) and for a hypothetical vaccine-eligible population (5-14 years) are shown. The 2022 projection of the 2015 National Census was used as the reference population.

### 7.4.4 Model-predicted national and municipal seroprevalence estimates

Table 27 shows the output from my mixed-effects multivariable logistic regression analysis which fitted participant ‘age-strata (5-year interval)’, ‘gender’ and 'municipality’ with fixed effects while fitting ‘EA’ and ‘household’ with random intercept.

**Table 27: Output from mixed-effects multivariable logistic regression used to compute 'model predicted' seroprevalence estimates**

| | Adj. odds ratio | Lower | Upper | p = |
|---|---|---|---|---|
| Female gender | Reference category | | | |
| Male gender | 1.257491 | 0.832829 | 1.898688 | 0.272 |
| Age 1-4 | Reference category | | | |
| Age 5-9 | 7.808 | 2.488 | 24.5 | 0.001 |
| Age 10-14 | 19.91 | 5.830 | 68.00 | <0.001 |
| Age 15-19 | 35.08 | 10.66 | 115.5 | <0.001 |
| Age 20-24 | 97.39 | 20.28 | 467.8 | <0.001 |
| Age 25-29 | 545.8 | 121.1 | 2460 | <0.001 |
| Age 30-34 | 640.1 | 112.9 | 3629 | <0.001 |
| Age 35-39 | 616.7 | 113.0 | 3367 | <0.001 |
| Age 40-44 | 580.4 | 97.92 | 3440 | <0.001 |
| Age 45-49 | 690.1 | 60.52 | 7868 | <0.001 |
| Age 50-54 | 989.3 | 129.2 | 7575 | <0.001 |
| Age 55-59 | 1090 | 143.4 | 8292 | <0.001 |
| Age 60-64 | 412.0 | 59.09 | 2872 | <0.001 |
| Age 65-69 | 857.8 | 75.39 | 9760 | <0.001 |
| Age 70+ | 648.0 | 59.11 | 7103 | <0.001 |
| Aileu | Reference category | | | |
| Ainaro | 15.75 | 2.268 | 109.3 | 0.006 |
| Baucau | 890.9 | 119.6 | 6635 | <0.001 |
| Bobonaro | 251.3 | 42.89 | 1472 | <0.001 |
| Covalima | 87.25 | 5.408 | 1408 | 0.002 |
| Dili | 1265 | 262.7 | 6097 | <0.001 |
| Ermera | 488.2 | 38.71 | 6155 | <0.001 |
| Lautem | 103.4 | 15.55 | 686.9 | <0.001 |
| Liquiçá | 69.37 | 7.515 | 640.4 | <0.001 |
| Manatuto | 12.26 | 1.494 | 100.5 | 0.020 |
| Manufahi | 69.46 | 10.76 | 448.2 | <0.001 |
| Oecusse | 105.6 | 25.06 | 445.2 | <0.001 |
| Viqueque | 15.75 | 2.268 | 109.3 | 0.006 |
| (Intercept) | 0.002742 | 0.000513 | 0.01465 | <0.001 |

| | | | | |
|---|---|---|---|---|
| EA | 3.289 | 2.128 | 5.083 | - |
| Household | 3.628 | 2.111 | 6.237 | - |

Model-predicted national and municipal seroprevalence estimates for age strata at 5-year intervals and a hypothetical vaccine-eligible subgroup are shown in Table 28.

**Table 28: National and municipal model-predicted dengue IgG seroprevalence estimates among individuals age-strata at 5-year intervals and a hypothetical vaccine-eligible subgroup**

| | 1-4 years | | | 5-9 years | | | 10-14 years | | | 15-19 years | | | 20-24 years | | | 25-29 years | | | 30-34 years | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR |
| **Aileu** | 0 | - | - | 19 | 0.526 | 0.12 (0.051-0.189) | 14 | 0.000 | 0.188 (0.105-0.271) | 23 | 0.261 | 0.235 (0.152-0.318) | 17 | 0.588 | 0.348 (0.221-0.474) | 20 | 0.600 | 0.563 (0.414-0.711) | 15 | 0.467 | 0.585 (0.402-0.767) |
| **Ainaro** | 18 | 0.111 | 0.166 (0.02-0.312) | 41 | 0.415 | 0.378 (0.16-0.596) | 38 | 0.500 | 0.498 (0.277-0.718) | 30 | 0.533 | 0.559 (0.334-0.783) | 19 | 0.526 | 0.683 (0.487-0.88) | 25 | 0.840 | 0.846 (0.722-0.97) | 20 | 0.950 | 0.859 (0.726-0.993) |
| **Baucau** | 28 | 0.607 | 0.624 (0.427-0.82) | 39 | 0.564 | 0.833 (0.703-0.962) | 39 | 0.744 | 0.896 (0.81-0.981) | 34 | 0.941 | 0.923 (0.854-0.992) | 27 | 0.926 | 0.959 (0.915-1.003) | 25 | 1.000 | 0.988 (0.973-1.003) | 50 | 1.000 | 0.989 (0.975-1.003) |
| **Bobonaro** | 14 | 0.571 | 0.469 (0.265-0.673) | 40 | 0.750 | 0.71 (0.551-0.869) | 39 | 0.769 | 0.808 (0.682-0.933) | 17 | 0.882 | 0.847 (0.737-0.956) | 33 | 0.909 | 0.912 (0.838-0.985) | 33 | 1.000 | 0.97 (0.939-1.002) | 42 | 1.000 | 0.972 (0.942-1.003) |
| **Covalima** | 27 | 0.333 | 0.34 (0.045-0.636) | 44 | 0.546 | 0.593 (0.282-0.903) | 36 | 0.750 | 0.7 (0.42-0.98) | 14 | 0.714 | 0.763 (0.512-1.013) | 27 | 0.963 | 0.843 (0.652-1.034) | 27 | 0.889 | 0.939 (0.843-1.035) | 30 | 0.833 | 0.944 (0.851-1.037) |
| **Dili** | 64 | 0.508 | 0.667 (0.549-0.785) | 141 | 0.846 | 0.861 (0.8-0.922) | 133 | 0.960 | 0.913 (0.876-0.951) | 134 | 0.947 | 0.938 (0.903-0.972) | 165 | 0.981 | 0.968 (0.947-0.988) | 108 | 0.981 | 0.991 (0.983-0.998) | 99 | 1.000 | 0.992 (0.984-0.999) |
| **Ermera** | 19 | 0.158 | 0.122 (0.064-0.181) | 27 | 0.185 | 0.297 (0.188-0.407) | 33 | 0.182 | 0.406 (0.291-0.521) | 33 | 0.394 | 0.477 (0.359-0.596) | 25 | 0.560 | 0.609 (0.478-0.741) | 23 | 0.739 | 0.79 (0.694-0.886) | 40 | 0.775 | 0.803 (0.69-0.917) |
| **Lautem** | 16 | 0.563 | 0.546 (0.27-0.822) | 51 | 0.686 | 0.781 (0.624-0.938) | 43 | 0.884 | 0.858 (0.73-0.986) | 15 | 0.800 | 0.891 (0.764-1.018) | 11 | 0.909 | 0.939 (0.856-1.022) | 11 | 1.000 | 0.98 (0.95-1.011) | 12 | 1.000 | 0.983 (0.957-1.01) |
| **Liquiçá** | 15 | 0.267 | 0.352 (0.136-0.568) | 16 | 0.563 | 0.616 (0.396-0.836) | 17 | 0.588 | 0.718 (0.535-0.902) | 14 | 0.714 | 0.777 (0.618-0.936) | 23 | 0.826 | 0.858 (0.744-0.972) | 23 | 0.957 | 0.945 (0.89-1.001) | 15 | 0.867 | 0.953 (0.892-1.013) |
| **Manatuto** | 23 | 0.217 | 0.312 (0.077-0.547) | 28 | 0.429 | 0.566 (0.309-0.824) | 34 | 0.529 | 0.678 (0.454-0.903) | 14 | 0.857 | 0.73 (0.519-0.941) | 14 | 1.000 | 0.829 (0.667-0.99) | 18 | 0.944 | 0.93 (0.842-1.017) | 17 | 0.882 | 0.936 (0.852-1.02) |
| **Manufahi** | 20 | 0.300 | 0.15 (0.004-0.296) | 21 | 0.333 | 0.35 (0.125-0.575) | 17 | 0.235 | 0.464 (0.22-0.708) | 14 | 0.571 | 0.53 (0.306-0.754) | 10 | 0.800 | 0.665 (0.454-0.876) | 17 | 0.823 | 0.824 (0.663-0.984) | 12 | 0.833 | 0.837 (0.679-0.995) |
| **Oecussi** | 15 | 0.667 | 0.313 (0.117-0.508) | 38 | 0.658 | 0.568 (0.343-0.794) | 24 | 0.833 | 0.671 (0.475-0.868) | 18 | 0.722 | 0.736 (0.55-0.923) | 24 | 0.875 | 0.829 (0.689-0.97) | 17 | 0.824 | 0.93 (0.867-0.994) | 19 | 1.000 | 0.937 (0.866-1.007) |
| **Viqueque** | 11 | 0.364 | 0.363 (0.219-0.506) | 39 | 0.513 | 0.612 (0.476-0.749) | 26 | 0.769 | 0.727 (0.627-0.828) | 28 | 0.857 | 0.778 (0.671-0.885) | 23 | 0.826 | 0.857 (0.77-0.945) | 29 | 0.931 | 0.946 (0.902-0.991) | 21 | 1.000 | 0.951 (0.911-0.992) |
| **Timor-Leste** | 270 | 0.371 | 0.373 (0.277-0.468) | 544 | 0.597 | 0.611 (0.538-0.685) | 493 | 0.704 | 0.701 (0.646-0.757) | 388 | 0.766 | 0.728 (0.675-0.781) | 418 | 0.878 | 0.837 (0.79-0.883) | 376 | 0.911 | 0.906 (0.874-0.938) | 392 | 0.925 | 0.916 (0.872-0.959) |

| | 35-39 years | | | 40-44 years | | | 45-49 years | | | 50-54 years | | | 55-59 years | | | 60-64 years | | | 65-69 years | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR | n | CPR | MPR |
| **Aileu** | 10 | 0.500 | 0.574 (0.413-0.735) | 8 | 0.625 | 0.57 (0.381-0.76) | 3 | 0.667 | 0.6 (0.418-0.782) | 16 | 0.438 | 0.638 (0.48-0.796) | 11 | 0.455 | 0.648 (0.485-0.812) | 8 | 0.500 | 0.522 (0.281-0.762) | 10 | 0.700 | 0.623 (0.481-0.764) |
| **Ainaro** | 8 | 1.000 | 0.852 (0.716-0.989) | 16 | 0.813 | 0.849 (0.706-0.992) | 16 | 0.875 | 0.866 (0.734-0.997) | 12 | 1.000 | 0.885 (0.773-0.997) | 7 | 1.000 | 0.891 (0.781-1.002) | 8 | 0.875 | 0.833 (0.649-1.016) | 4 | 0.000 | 0.883 (0.734-1.033) |
| **Baucau** | 31 | 1.000 | 0.989 (0.974-1.004) | 18 | 1.000 | 0.989 (0.974-1.004) | 36 | 1.000 | 0.99 (0.974-1.006) | 33 | 1.000 | 0.993 (0.982-1.003) | 15 | 1.000 | 0.993 (0.982-1.004) | 26 | 1.000 | 0.985 (0.962-1.009) | 15 | 1.000 | 0.991 (0.977-1.006) |
| **Bobonaro** | 26 | 0.962 | 0.972 (0.941-1.002) | 30 | 0.967 | 0.97 (0.939-1.001) | 25 | 1.000 | 0.974 (0.941-1.007) | 16 | 1.000 | 0.981 (0.959-1.003) | 17 | 0.941 | 0.982 (0.959-1.005) | 15 | 1.000 | 0.963 (0.91-1.016) | 7 | 1.000 | 0.978 (0.947-1.008) |
| **Covalima** | 29 | 0.862 | 0.944 (0.85-1.037) | 18 | 0.722 | 0.944 (0.849-1.04) | 17 | 1.000 | 0.947 (0.855-1.039) | 15 | 0.867 | 0.958 (0.884-1.031) | 10 | 1.000 | 0.961 (0.891-1.032) | 11 | 0.909 | 0.934 (0.817-1.05) | 9 | 0.778 | 0.955 (0.87-1.041) |
| **Dili** | 98 | 0.989 | 0.991 (0.984-0.999) | 55 | 1.000 | 0.991 (0.983-0.999) | 68 | 1.000 | 0.993 (0.984-1.001) | 47 | 1.000 | 0.994 (0.989-1) | 24 | 1.000 | 0.995 (0.989-1.001) | 29 | 0.960 | 0.989 (0.972-1.005) | 19 | 1.000 | 0.994 (0.987-1.002) |
| **Ermera** | 24 | 0.792 | 0.802 (0.701-0.903) | 18 | 0.667 | 0.803 (0.705-0.901) | 18 | 0.778 | 0.816 (0.669-0.962) | 16 | 0.813 | 0.845 (0.758-0.931) | 12 | 0.917 | 0.851 (0.751-0.951) | 11 | 0.819 | 0.765 (0.603-0.927) | 13 | 0.846 | 0.832 (0.7-0.964) |
| **Lautem** | 17 | 0.941 | 0.983 (0.95-1.015) | 16 | 1.000 | 0.982 (0.956-1.009) | 25 | 1.000 | 0.984 (0.955-1.012) | 31 | 0.968 | 0.988 (0.969-1.007) | 20 | 1.000 | 0.989 (0.97-1.008) | 20 | 0.950 | 0.978 (0.925-1.03) | 7 | 1.000 | 0.987 (0.964-1.011) |
| **Liquiçá** | 24 | 0.875 | 0.949 (0.892-1.005) | 8 | 1.000 | 0.95 (0.889-1.01) | 11 | 1.000 | 0.956 (0.903-1.009) | 12 | 1.000 | 0.963 (0.922-1.003) | 11 | 1.000 | 0.967 (0.922-1.011) | 13 | 1.000 | 0.934 (0.849-1.019) | 7 | 1.000 | 0.96 (0.903-1.017) |
| **Manatuto** | 18 | 1.000 | 0.934 (0.852-1.017) | 11 | 1.000 | 0.936 (0.852-1.02) | 19 | 1.000 | 0.94 (0.857-1.022) | 9 | 1.000 | 0.952 (0.887-1.016) | 11 | 0.909 | 0.956 (0.891-1.021) | 12 | 1.000 | 0.918 (0.801-1.036) | 3 | 1.000 | 0.949 (0.874-1.025) |
| **Manufahi** | 11 | 0.636 | 0.832 (0.681-0.984) | 9 | 0.889 | 0.83 (0.665-0.994) | 7 | 0.857 | 0.842 (0.681-1.003) | 7 | 1.000 | 0.872 (0.744-1) | 3 | 1.000 | 0.872 (0.729-1.015) | 11 | 0.909 | 0.804 (0.592-1.016) | 0 | - | - |
| **Oecussi** | 19 | 1.000 | 0.935 (0.863-1.006) | 30 | 0.967 | 0.933 (0.859-1.008) | 20 | 0.950 | 0.943 (0.87-1.016) | 16 | 0.938 | 0.954 (0.9-1.008) | 10 | 1.000 | 0.957 (0.902-1.012) | 10 | 1.000 | 0.92 (0.813-1.027) | 7 | 0.857 | 0.948 (0.875-1.022) |
| **Viqueque** | 21 | 1.000 | 0.951 (0.912-0.991) | 15 | 1.000 | 0.95 (0.907-0.992) | 27 | 0.963 | 0.955 (0.907-1.002) | 15 | 1.000 | 0.966 (0.937-0.995) | 3 | 1.000 | 0.968 (0.934-1.002) | 5 | 1.000 | 0.935 (0.857-1.013) | 11 | 1.000 | 0.961 (0.918-1.004) |
| **Timor-Leste** | 336 | 0.927 | 0.924 (0.888-0.96) | 252 | 0.920 | 0.912 (0.87-0.955) | 292 | 0.965 | 0.94 (0.896-0.983) | 245 | 0.933 | 0.934 (0.905-0.963) | 154 | 0.940 | 0.93 (0.891-0.969) | 179 | 0.937 | 0.907 (0.834-0.98) | 112 | 0.893 | 0.922 (0.871-0.974) |

| | 70+ years | | | Hypothetical vaccine-eligible group (5-14 years) | | |
|---|---|---|---|---|---|---|
| | n | CPR | MPR | n | CPR | MPR |
| **Aileu** | 7 | 0.286 | 0.586 (0.395-0.778) | 33 | 0.030 | 0.155 (0.081-0.229) |
| **Ainaro** | 23 | 0.870 | 0.86 (0.72-1) | 79 | 0.456 | 0.431 (0.214-0.648) |
| **Baucau** | 25 | 1.000 | 0.99 (0.974-1.005) | 78 | 0.654 | 0.863 (0.757-0.970) |
| **Bobonaro** | 25 | 1.000 | 0.974 (0.942-1.005) | 79 | 0.759 | 0.769 (0.632-0.905) |
| **Covalima** | 40 | 0.850 | 0.946 (0.85-1.042) | 80 | 0.638 | 0.643 (0.348-0.938) |
| **Dili** | 15 | 1.000 | 0.992 (0.983-1.001) | 274 | 0.898 | 0.889 (0.841-0.935) |
| **Ermera** | 16 | 0.875 | 0.805 (0.67-0.94) | 60 | 0.183 | 0.360 (0.252-0.469) |
| **Lautem** | 21 | 1.000 | 0.983 (0.955-1.012) | 94 | 0.777 | 0.818 (0.677-0.960) |
| **Liquiçá** | 16 | 0.875 | 0.951 (0.889-1.013) | 33 | 0.576 | 0.665 (0.465-0.865 |
| **Manatuto** | 15 | 0.933 | 0.937 (0.852-1.022) | 62 | 0.484 | 0.629 (0.392-0.866) |
| **Manufahi** | 17 | 0.882 | 0.843 (0.691-0.995) | 38 | 0.289 | 0.399 (0.168-0.629) |
| **Oecussi** | 31 | 0.968 | 0.939 (0.866-1.012) | 62 | 0.726 | 0.610 (0.398-0.821) |
| **Viqueque** | 28 | 1.000 | 0.953 (0.91-0.995) | 65 | 0.615 | 0.664 (0.546-0.781) |
| **Timor-Leste** | 279 | 0.920 | 0.923 (0.872-0.973) | 1037 | 0.650 | 0.655 (0.596-0.715) |

**Abbreviations:** n = number of participants, CPR = crude positive rate, MPR = model-predicted rate

### 7.4.5 Assessing linearity of potential explanatory variables' relationship with the log(odds) of seropositivity using a BoxCox analysis

Figure 31 A and B show the relationship of continuous potential explanatory variables ('EA population density' and 'household remoteness index') with the log(odds) of seropositivity. In both cases, a non-linear relationship was observed. Output from BoxCox analyses are shown in Figure 31 C and D, with the central Lambda estimate closest to zero. This indicated a log transformation on each independent variable to be most appropriate to achieve linearity. Figure 31 E and F show the relationship between the log-transformed variables and the log(odds) of seropositivity, which is more linear.

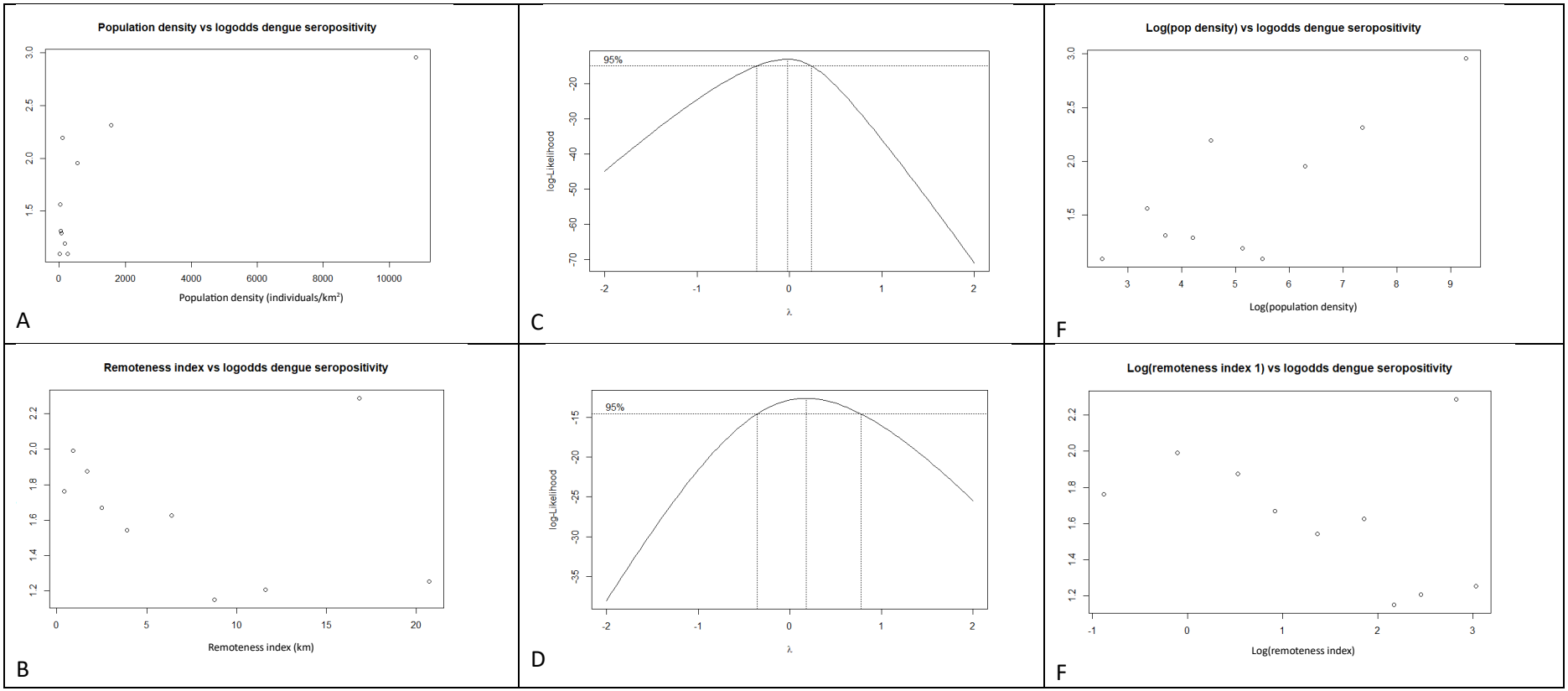



**Figure 36: Assessing linearity and transforming continuous potential explanatory variables**

**Legend:** A-B show the relationship of 'EA population density' and 'household remoteness index' with the log(odds) of dengue IgG seropositivity. C-D show outputs from BoxCox analyses, indicated log-transformation of these independent variables to be most appropriate. E-F show the relationship of 'log(EA population density)' and 'log(household remoteness index)' with the log(odds of dengue IgG seropositivity, which is approximately linear.

### 7.4.6 Association of individual and population variables with dengue IgG seropositivity

Tables 29 – 34 show the association of household and population variables with dengue IgG seropositivity when each were added on their own to a mixed-effects multivariable logistic regression model alongside EA and household (fitted with random effect to account for clustering in the study design at these levels). Where household-level ('urban household location', 'log(household remoteness index)', and 'number of household occupants') or EA-level ('log(EA population density)') fixed-effect variables are assessed, these analyses delineate the variation in seropositivity which is attributable to the fixed effect. The random term then represents and accounts for other, un-measured variation at that level.

**Table 2930: Association of 'urban household location' with dengue IgG seropositivity while accounting for clustering at the EA- and household-levels.**

| | Adjusted OR | Lower | Upper | p = |
|---|---|---|---|---|
| **Fixed effect** | | | | |
| Urban household location | 15.12 | 0.328 | 696.2 | 0.162 |
| **Random effects** | | | | |
| EA | 18.49 | 9.967 | 34.31 | - |
| Household | 5.541 | 2.890 | 10.63 | - |

**Table 3132: Association of 'log(household remoteness index)' with dengue IgG seropositivity while accounting for clustering at the EA- and household-levels.**

| | Adjusted OR | Lower | Upper | p = |
|---|---|---|---|---|
| **Fixed effect** | | | | |
| Log(household remoteness index) | 1.132 | 0.945 | 1.355 | 0.176 |
| **Random effects** | | | | |
| EA | 23.70 | 13.41 | 41.89 | - |
| Household | 5.456 | 2.856 | 10.42 | - |

**Table 33: Association of 'number of household occupants' with dengue IgG seropositivity while accounting for clustering at the EA- and household-levels**

| | Adjusted OR | Lower | Upper | p = |
|---|---|---|---|---|
| **Fixed effect** | | | | |
| Number of household occupants | 1.012 | 0.874 | 1.168 | 0.885 |
| **Random effects** | | | | |
| EA | 23.39 | 13.19 | 41.48 | - |
| Household | 5.430 | 2.811 | 10.49 | - |

**Table 3435: Association of 'log(EA population density)' with dengue IgG seropositivity while accounting for clustering at the EA- and household-levels.**

| | Adjusted OR | Lower | Upper | p = |
|---|---|---|---|---|
| **Fixed effect** | | | | |
| Log(EA population density) | 1.422 | 0.941 | 2.149 | 0.094 |
| **Random effects** | | | | |
| EA | 20.79 | 11.75 | 36.77 | - |
| Household | 5.504 | 2.874 | 10.54 | - |

None of the assessed fixed-effect variables were significantly associated dengue IgG seropositivity. Therefore, variation in dengue seropositivity at the household level was not attributable to 'urban household location', 'log(household remoteness index)' or 'number of household occupants', and variation in dengue seropositivity at the EA level was not attributable to 'log(EA population density)'.

## 7.5 Discussion

In this chapter I designed and implemented a nationwide population-representative survey which used serological techniques to investigate the proportion of individuals who had previously been infected with dengue in Timor-Leste ('seroprevalence'). Similar to other countries in the region, there was a significant positive relationship between age and seropositivity with most individuals being seropositive by adolescence.(145) While accounting for participant age and various potential study design effects relating to the sampling strategy, individuals in Dili and Baucau, which are the include the two largest urban centres, had significantly higher odds of being seropositive than those in other municipalities. These observations are consistent with other studies which have also observed dengue transmission to be higher in low-lying urban areas.(8,146,147) Seroprevalence in a hypothetical vaccine-eligible subgroup (5-14 years of age) was also significantly different between municipalities, ranging from 15.5% (8.1-22.9%) in Aileu municipality to 88.9% (84.1-93.5%) in Dili municipality. These findings will be key in deciding whether Dengvaxia should be rolled out in Timor-Leste, which is the only licenced vaccine against dengue but is contraindicated in individuals who are seronegative.

When available household-level ('urban household location', 'log(household remoteness index)' and 'number of household occupants') and EA-level ('log(EA population density)' variables were assessed in mixed-effects logistic regression analyses to delineate whether variation in dengue IgG seropositivity at each level could be attributable to those particular characteristics, none were significantly associated. This indicates that other (unmeasured) factors may determine an

individuals' risk of dengue infection and hence variation in seropositivity between households and EAs. Previous studies in other countries have indicated strong associations of climate and environmental variables with dengue transmission, which I went on to investigate in chapter 8.

This study represents the first systematic assessment of dengue transmission in Timor-Leste. It included a large number of individuals who were sampled at random from the general population based on their household enumeration in the recent National Census. It therefore took place in a diverse range of populated environments, including very remote rural areas where there has been limited dengue surveillance previously. Differences in baseline variables between participating units and non-participating units at both the household and individual levels were observed but were accounted for by assigning sampling weights to each individual which were a factor of 'selection probability' and 'response probability' at each level. Furthermore, clustering was accounted for by using a multivariable mixed-effects logistic regression model to compute 'model predicted' seroprevalence in municipalities and age-strata. Procedures for sample collection, transport, storage and analysis were carefully considered and were guided by findings from previous chapters. An ELISA assay which was optimised for the purpose of diagnosing previous dengue infection (as opposed to acute dengue) was operated by trained scientists within a programme of quality assurance.

Limitations of this study include lower participation rate among young children compared to older children and adults, which resulted in the target sample size not being achieved and lower precision estimates of seroprevalence for this group. They also include the possibility that serological testing was not 100% accurate and the fact that adjustment for imperfect assay diagnostic sensitivity or specificity was not undertaken. The potential effect of antibody waning, where antibody concentrations reduce gradually over time following an infection and without intercurrent antigenic stimulus through re-infection is not accounted for.(148) Furthermore, individuals who reported a current febrile illness (compatible with COVID-19) were not included in the study, which may have

led to a small underestimation of dengue IgG seroprevalence through their exclusion. However, since acute dengue is usually a short lived illness when compared with the duration of IgG seropositivity, this effect is likely to have been low.

## 7.6 Conclusion and key messages

Serological techniques were used to investigate dengue transmission in a large, population-representative survey in Timor-Leste. After accounting for participant age and various potential study design effects relating to the sampling strategy, there was significant variation in seroprevalence between municipalities. Findings may be used to guide disease control activities at the national and municipality level.

# CHAPTER 8

## 8.0 Estimating force-of-infection from seroprevalence data and undertaking predictive mapping at high spatial resolution

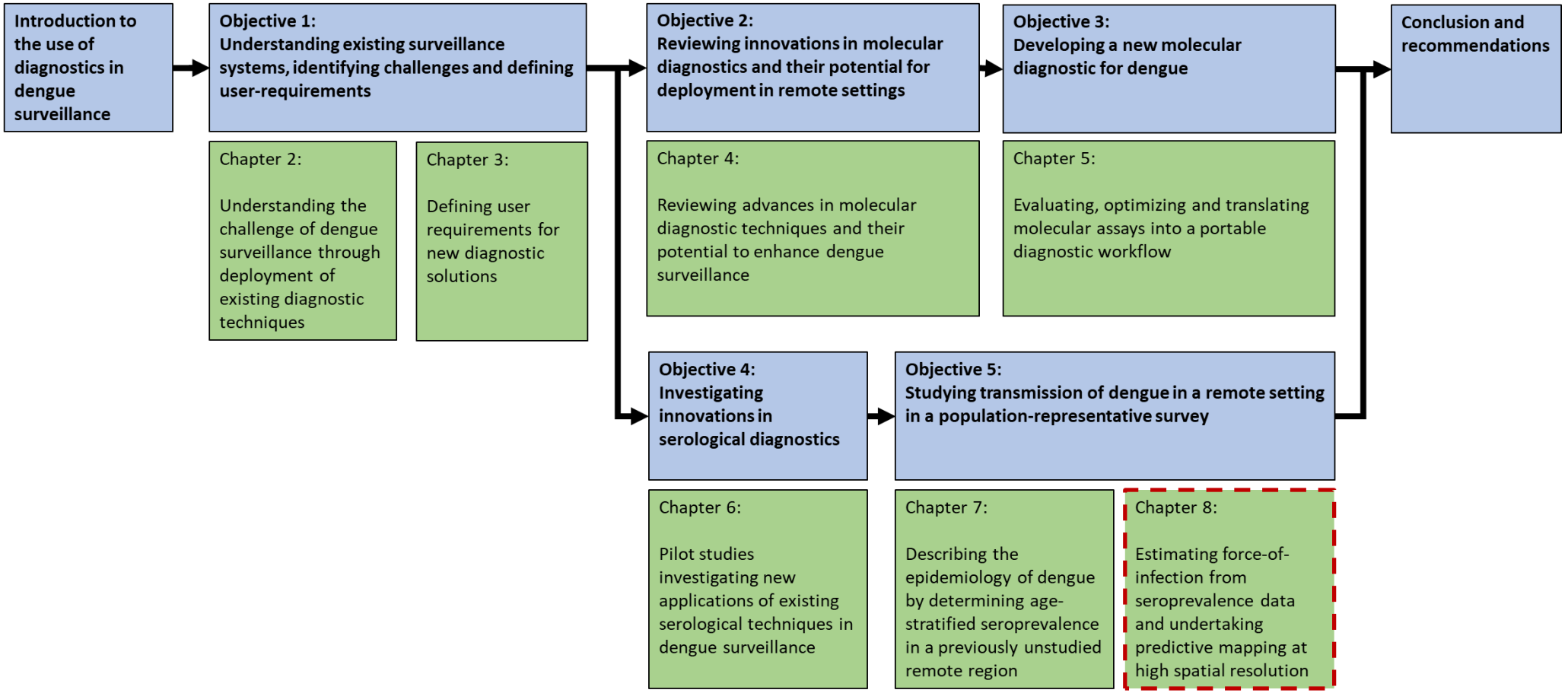



**Figure 37: Thesis overview**

## 8.1 Introduction

In chapter seven I designed and implemented a population-representative serosurvey in Timor-Leste. I found high dengue IgG seropositivity which increased with age and varied significantly between municipalities. I assessed other available individual (participant gender), and population (household remoteness index) variables for association with dengue seropositivity but found no significant relationships. Findings will guide disease control activities at the national and municipal level. However, further subgroups in the population who may be at higher risk of dengue infection were not identified, and transmission was not described at high spatial resolution.

Force-of-infection (FOI) is the rate at which susceptible individuals acquire infection (i.e. the per-capita probability of infection for a susceptible person). It is a key measure of infectious disease transmission and provides a unifying framework to assess variations in the intensity of dengue transmission across locations.(149,150). Previous studies have estimated dengue FOI from age-stratified seroprevalence data using catalytic models, an approach which allows transmission to be compared between geographic areas. In these models, large rates of increase in seroprevalence as individuals get older are explained by high FOI. Catalytic models have been used extensively for measles, rubella, Hepatitis A, Chagas disease, and dengue.(29,151,152)

Under the hypothesis that environmental conditions drive the carrying capacity and competence of Aedes species mosquitos, climate variables can also be assessed as potential predictors of dengue transmission. The largest study to date included 33 serology-based and 233 case notification-based estimates of dengue force-of-infection (FOI) which had been estimated in previous studies across Asia, Africa and Latin America. In a random-forest model (machine-learning algorithm), mean nocturnal and diurnal temperatures (and their annual seasonal variations) were strongly associated with dengue FOI. The study went on to predict dengue risk across all tropical regions, estimate the number of individuals at risk globally, and project the impact of various disease control interventions.(150) This study and others like it illustrate how climate and environmental data can

be used to understand dengue transmission and predict its intensity in areas with otherwise limited surveillance. However, they have not so far accounted for the potential effect of household clustering in their estimation of dengue FOI. Additionally, they have not assessed dengue FOI in multiple, nearby, geographic regions of small area. Therefore, data from my serosurvey provided a unique opportunity to examine dengue FOI at high spatial resolution and assess climate variables as potential predictors of dengue transmission across a previously unstudied remote area.

## 8.2 Chapter objectives

The aims of this chapter were to (a) estimate dengue FOI at the EA level from age-stratified seroprevalence data ('FOI model'), (b) model EA FOI using climate data available from satellite imagery ('climate model'), and (c) use this to predict FOI at high spatial resolution across the country.

## 8.3 Methods

### 8.3.1 Study design

Four-stage modelling study investigating dengue FOI.

Figure 38 outlines the four stages which I undertook within this chapter.

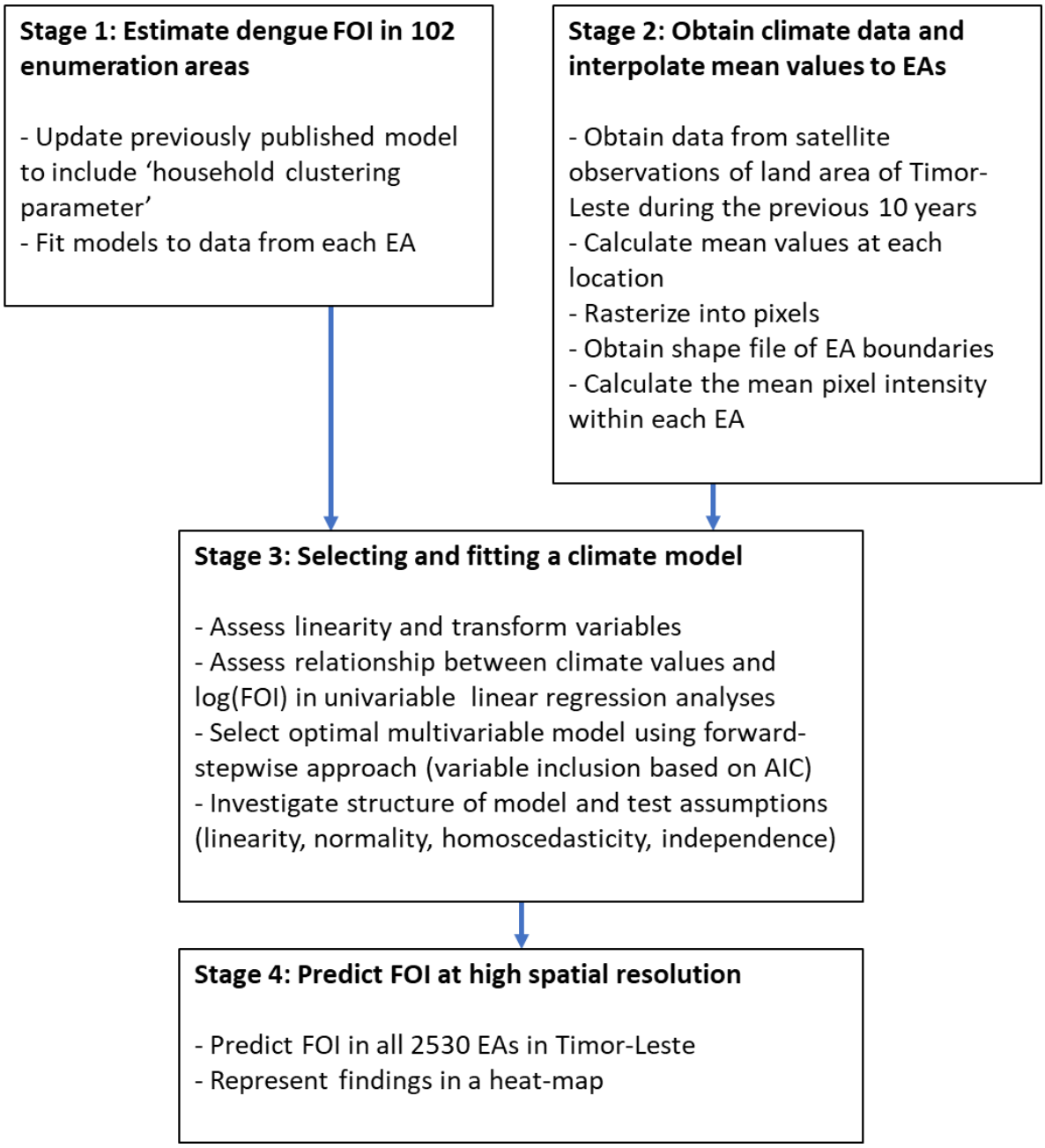



**Figure 38: Summary of the four stages undertaken in this chapter.**

**Abbreviations:** FOI = force-of-infection, EA = Enumeration Area, AIC = Akaike information criterion

### 8.3.2 Stage 1: Estimating dengue FOI in 102 Enumeration Areas

I used previously published methods for estimating the dengue FOI from age-stratified seroprevalence data. A catalytic model was fitted which assumed that upon first infection, individuals move from being seronegative to seropositive and that individuals are susceptible to

subsequent heterotypic infection. Force-of-infection was assumed to be time-constant.(153) Due to the complex sampling strategy used in my survey, this model required an update to include an additional 'household clustering parameter' which was undertaken by Anna Vicco, a researcher and PhD candidate supervised by Dr Ilaria Dorigatti, Senior Lecturer at the Faculty of Medicine, School of Public Health, Imperial College London. I used serology data from each EA to estimate FOIs with 95% CIs using *cmdstan* in R. I visualised these in a municipality-stratified forest plot.

### 8.3.3 Stage 2: Obtaining climate data and interpolating mean values to EAs

With assistance from Dr Wes Hinsley who is a Geographic information System and Database Analyst at the School of Public Health, Imperial College, London, I obtained satellite data relating to the land area of Timor-Leste from various sources, for the last 10 years. A summary of the data available to me is shown in Table 30.

**Table 36: Climate variables obtained from satellite data from various sources**

| Variable | Source | Approximate spatial resolution | Approximate temporal resolution | Number of datapoints |
|---|---|---|---|---|
| MIR | Modis | 1km | 8 days | 14057311 |
| EVI | Modis | 1km | 8 days | 14057311 |
| Precipitation | TerraClimate | 5km | 1 month | 166752 |
| Tmax | TerraClimate | 5km | 1 month | 166752 |
| Tmin | TerraClimate | 5km | 1 month | 166752 |
| RH at Tmax* | TerraClimate | 5km | 1 month | 166752 |
| RH at Tmin* | TerraClimate | 5km | 1 month | 166752 |
| Elevation | Worldclim | 1km | n/a | 25157 |
| Population** | Landscan | 1 km | 1 year | 234546 |

* Relative humidity (RH) observations were calculated from temperature and vapor pressure measurements using the equation rh = 100 * (ea / es), where rh is relative humidity, ea is air vapor pressure in mb and es is saturated vapor pressure at air temperature.

** Landscan caveat that data are not designed to be used longitudinally because the model used to make population estimations is updated annually and therefore annual predictions may not be strictly comparable. Population data were available 2011 to 2021 (inclusive), where as other variables were available 2011 to 2022 (inclusive).

**Abbreviations:** MIR = middle infrared reflectance, EVI = enhanced vegetation index, Tmax = maximum air temperature at ground level during 24-hour period, Tmin = minimum air temperature at ground level during 24-hour period, RH = relative humidity

I then calculated mean values for each point using the *aggregate* function in R as follows (MIR variable is shown as an example):

```
#Read in 2 datasets

df_mir <-read.csv('mir_2011_2022.csv.xz')

#Remove time variables

df_mir_no_time <- df_mir[,c(1,2,6)]

#Make longitude and latitude into a single variable

df_mir_no_time$coordinates_combined <-
paste(df_mir_no_time$lon,",",df_mir_no_time$lat)

#Calculate mean mir at each point

Mean_mir <- aggregate(mir ~ coordinates_combined, data = df_mir_no_time, mean)
```

Having calculated mean values for climate variables at each available point location, I proceeded to assign these to EAs in Timor-Leste. First, I rasterised point values into a regular grid of pixels, using the *rasterize* package in R as follows (precipitation is shown as an example):

```
Mean_pre_ras <-rasterFromXYZ(Mean_pre, res=c(NA,NA), crs="", digits=5)
```

Second, I obtained a kml file from the Statistics Department at the Timor-Leste Ministry of Infrastructure which included all 2350 EAs as shapefiles. I loaded this into R using the *sf* package, then determined the mean pixel intensity within each EA using the *exactextract* package, as follows:

```
Pre_estimates_ <- exact_extract(Mean_pre_ras, eas, 'mean', progress = FALSE)
```

Figure 33 illustrates how climate data available at point-locations needed to be interpolated onto EAs.

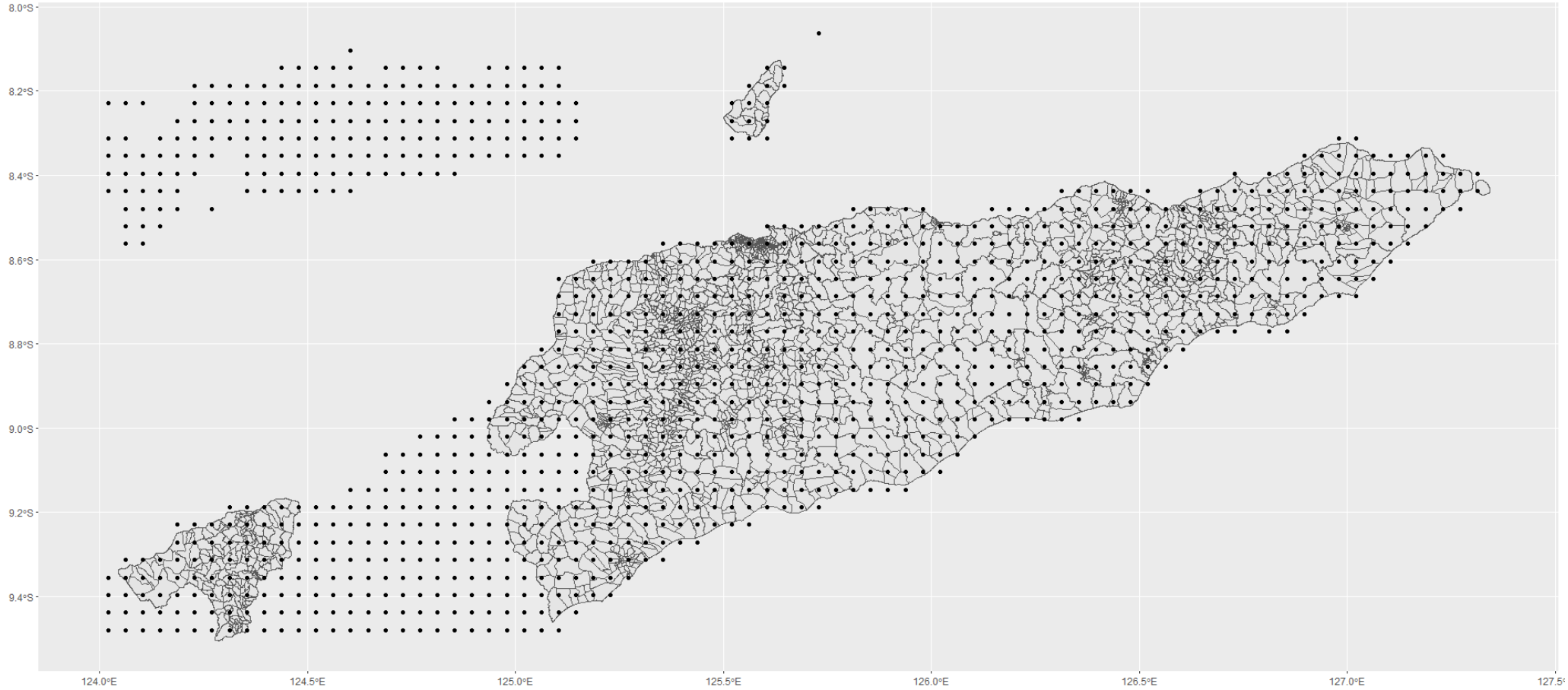



*Figure 39*: **Map of Timor-Leste illustrating challenge of data interpolation.**

**Legend:** Climate data were available at point locations (black circles). These needed to be interpolated onto 2350 Enumeration Areas (bordered by black lines).

### 8.3.4 Stage 3: Selecting and fitting a climate model

I assessed the distribution of dengue FOI across EAs to be right-skewed and therefore used log-transformed FOI values, which were approximately normally distributed, as the dependant variable in this climate model. Population density was also right-skewed and therefore I used the log-transformed population density values for this independent variable.

I assessed each climate variable for association with log(FOI) in a univariable analysis, using the *lm* function in R as follows (MIR is used as an example):

```
#Read in climate variables and merge dataframe with seroprevalence estimates
EA_climate <- read.csv('All_climate_data_nearest_points_only.csv')
EA_climate_with_estimates <- full_join(EA_climate, EA_estimates, by = 'ea_codes')
#Fit univariable linear regression model
m_mir <- lm(FOI ~ mir, data = EA_climate_with_estimates)
summary (m_mir)
```

I then used a forwards stepwise approach to build a multivariable linear regression model. Potential variables included each of the aforementioned climate variables as well as 'municipality'. They also included 'municipality percentage of population in the lowest two wealth quintiles', which was obtained from the recent Demographic and Health Survey report (termed 'low wealth' hereafter). I started with an intercept-only model, to which variables were sequentially added or removed based on assessing the potential reduction in Akaike information criterion (AIC) of each change and choosing that which resulted in the greatest reduction. Once an 'optimal' multivariable model was identified when no addition or removal of variables would result in a reduced AIC. I used the *stepAIC* package in R as follows:

```
forward_step_model_mun <- stepAIC(intercept_model_mun, direction = "both",
scope=list(upper=full_model_mun, lower=intercept_model_mun))
```

```
summary(forward_step_model_mun)
```

I then assessed model fit by calculating residuals and creating a series of plots. I used a residual vs. fitted value plot to assess whether there was constant error variance through the model range. I used a normal probability plot and histogram to assess whether residuals were normally distributed.

I received significant support in developing and implementing methods in this section from Ms Victoria Cox, Dr Ilaria Dorigatti and Prof Marta Blangiardo at the Faculty of Medicine, School of Public Health, Imperial College London

### 8.3.5 Stage 4: Predicting FOI at high spatial resolution across Timor-Leste

I used my optimal multivariable climate model to predict dengue IgG seroprevalence for all 2350 EAs across Timor-Leste. I used the *predict* function in R, as follows:

```
Predicted_df <- data.frame(predict(forward_step_model_mun, newdata =
climate_FOI_clean_mun, interval = 'confidence'))
```

I exponentiated all predicted values, including upper and lower bounds of the 95% CIs. This was necessary because I used the log-transformed value of FOI as the dependant variable in my modelling.

I represented central FOI estimates in a heatmap showing all EAs in Timor-Leste, using the *st* and *ggplot* packages in R. I coloured EAs with higher predicted dengue IgG seroprevalence in a darker shade of red:

```
EAs_sf <- st_read('EAs_2015.kml')

EAs_sf_predicted <- cbind(EAs_sf, predicted_df)

ggplot(data = EAs_sf_predicted) +

geom_sf(aes(fill = FOI)) +
```

```
scale_fill_gradient2(low = "white", high = "red")
```

## 8.4 Results from Stage 1: Estimating dengue FOI in 102 Enumeration Areas

Dengue FOI was successfully estimated for 102 EAs in Timor-Leste, using an updated catalytic model which accounted for household clustering. These are shown in Figure 34. There was uncertainty in estimation of FOI indicated by broad 95% CIs. This particularly affected high estimates, likely because these rely on a small number of datapoints from young children. Despite this caveat, significant variation in FOI was observed between EAs, emphasising the need for enhanced surveillance systems in remote areas which are sensitive to geographic variation in transmission.

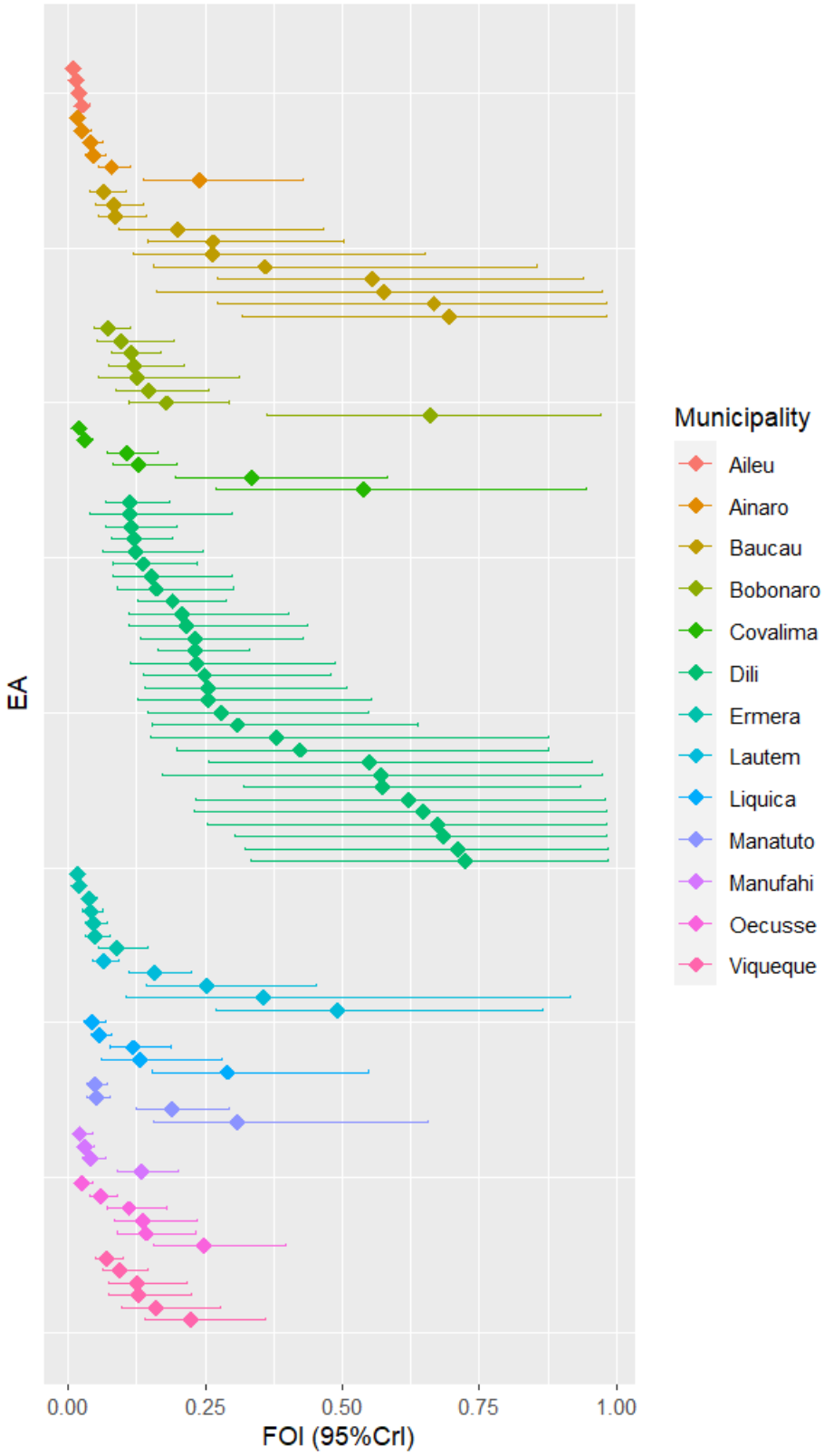



**Figure 40: Dengue force-of-infection (FOI) estimated from age-stratified seroprevalence data in 102 Enumeration Areas (EAs) within 13 municipalities in Timor-Leste**

**Legend:** Error bars represent 95% credibility intervals

## 8.5 Results from Stage 2: Obtaining climate data and interpolating mean values to EAs

I obtained climate and environmental observations from satellite data which covered the land area of Timor-Leste. I calculated mean values for each climate/environmental variable at each geolocation. I successfully interpolated these onto EAs and obtained mean values.

## 8.6 Results from Stage 3: Selecting and fitting a climate model

Results from Stage 3: Selecting and fitting a climate model

In univariable analyses, ‘MIR’, ‘Tmax’, ‘Tmin’, and ‘population density’ were positively associated with log(FOI), while 'EVI’, ‘rh_Tmax’, ‘rh_Tmin’, ‘precipitation’ and ‘log(population density)’ were negatively associated. These findings are summarised in Figure 35.

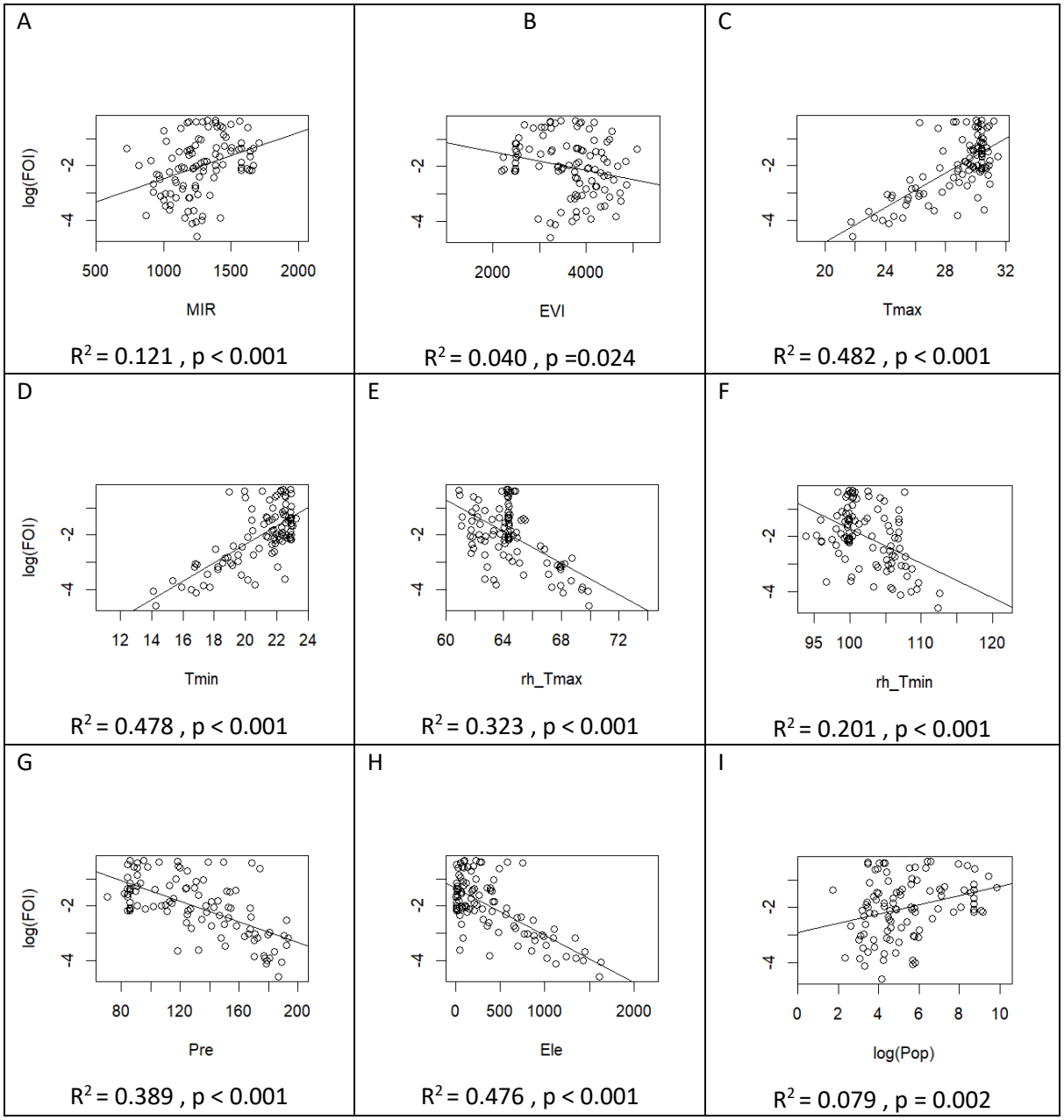



**Figure 41: Scatter plots showing the univariable relationships of climate variables with log(dengue force-of-infection)**

**Legend:** Relationships of Middle Infrared Reflectance (A), Enhanced Vegetation Index (B), maximum daily temperature (C), minimum daily temperature (D), relative humidity at Tmax (E), relative humidity at Tmin (F), precipitation (G), elevation (H), and log(population) (I) with log(dengue force-of-infection) are shown

A stepwise approach was used to build the multivariable linear regression climate model which optimally fitted the data. This is summarised in Table 31.

**Table 37: Summary of the optimal multivariable linear regression climate model**

| Variable | Coefficient | 95% CI | | p = |
|---|---|---|---|---|
| | | Lower | Upper | |
| (intercept) | 12.49 | 5.019 | 19.96 | 0.001 |
| Ainaro | 0.4802 | -0.4416 | 1.402 | 0.303 |
| Baucau | 2.135 | 1.296 | 2.974 | < 0.001 |
| Bobonaro | 0.5904 | -0.5047 | 1.685 | 0.287 |
| Covalima | 0.6502 | -0.3832 | 1.684 | 0.215 |
| Dili | 1.494 | 0.5564 | 2.431 | 0.002 |
| Ermera | 0.4199 | -0.4519 | 1.292 | 0.341 |
| Lautem | 2.176 | 1.248 | 3.104 | < 0.001 |
| Liquica | 0.6775 | -0.3681 | 1.723 | 0.201 |
| Manatuto | 0.936 | -0.1143 | 1.986 | 0.080 |
| Manufahi | 0.5198 | -0.4641 | 1.504 | 0.297 |
| Oecusse | -0.1621 | -1.392 | 1.068 | 0.794 |
| Viqueque | 1.623 | 0.7281 | 2.519 | 0.001 |
| Relative humidity at Tmin | -0.1524 | -0.2208 | -0.084 | < 0.001 |

| | |
|---|---|
| Adjusted R-squared | 0.5816 |
| p | <0.001 |

The $R^2$ for this model was 0.5816, indicating that it explained approximately 58% of the variability in log(FOI). I created a series of residual plots to assess this model. First, I plotted standardised

residuals against predicted values (see Figure 36). While residuals were roughly symmetrically distributed, there was more variability at higher predicted FOI (heteroscedasticity). This indicates that predictions for EAs with high FOI may have lower precision. Second, I plotted standardised residuals against their theoretical quantiles (see Figure 37) and a histogram of residuals. These showed that residuals are roughly normally distributed, and indicated that further transformation of variables was not likely to improve model fit further. I judged my model fit to be acceptable for predicting FOI, when the purpose is to visualise regions of relatively high or low FOI (but not for direct comparison of individual EAs).

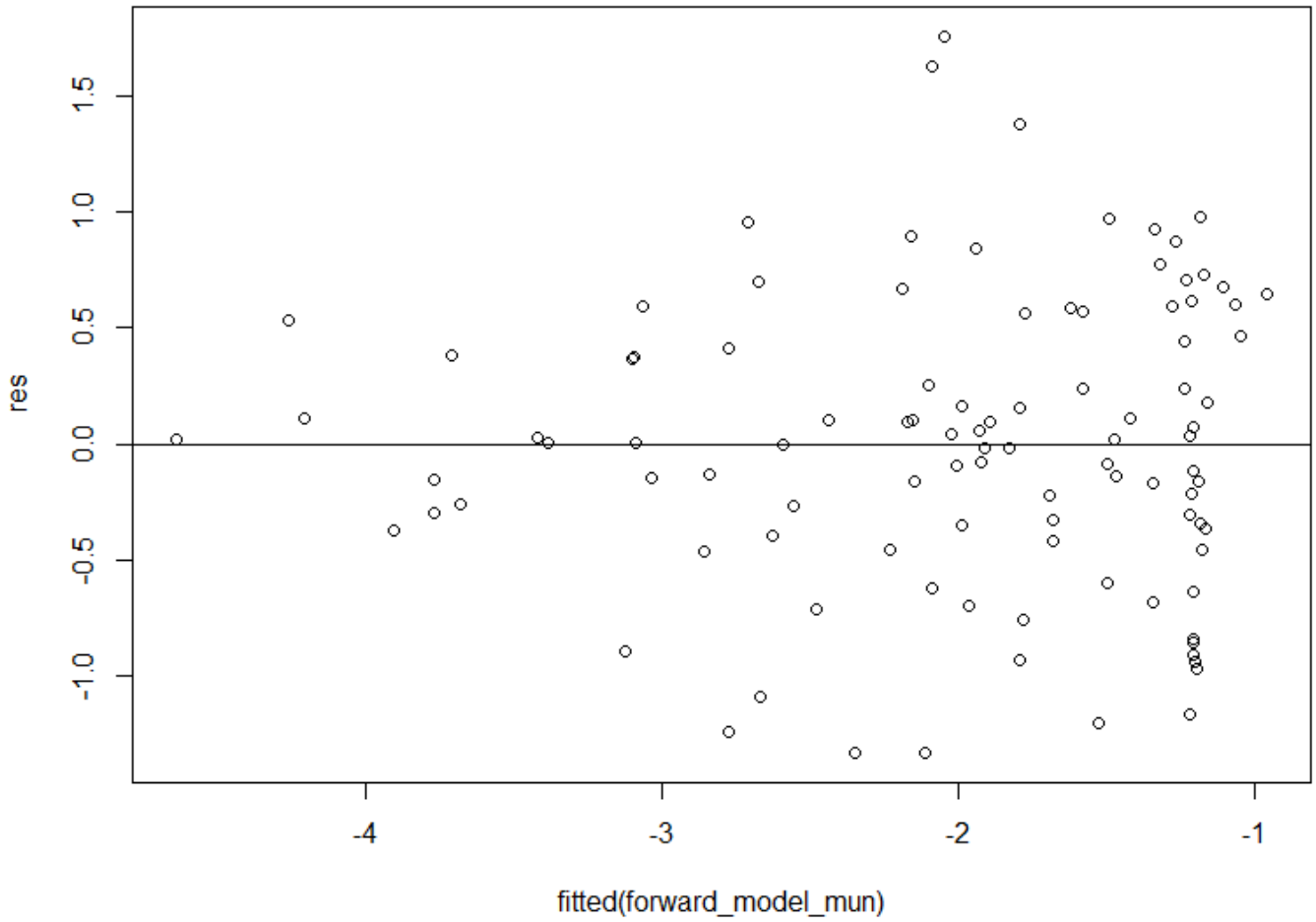



**Figure 42: Fitted vs. residual fit for optimal climate model**

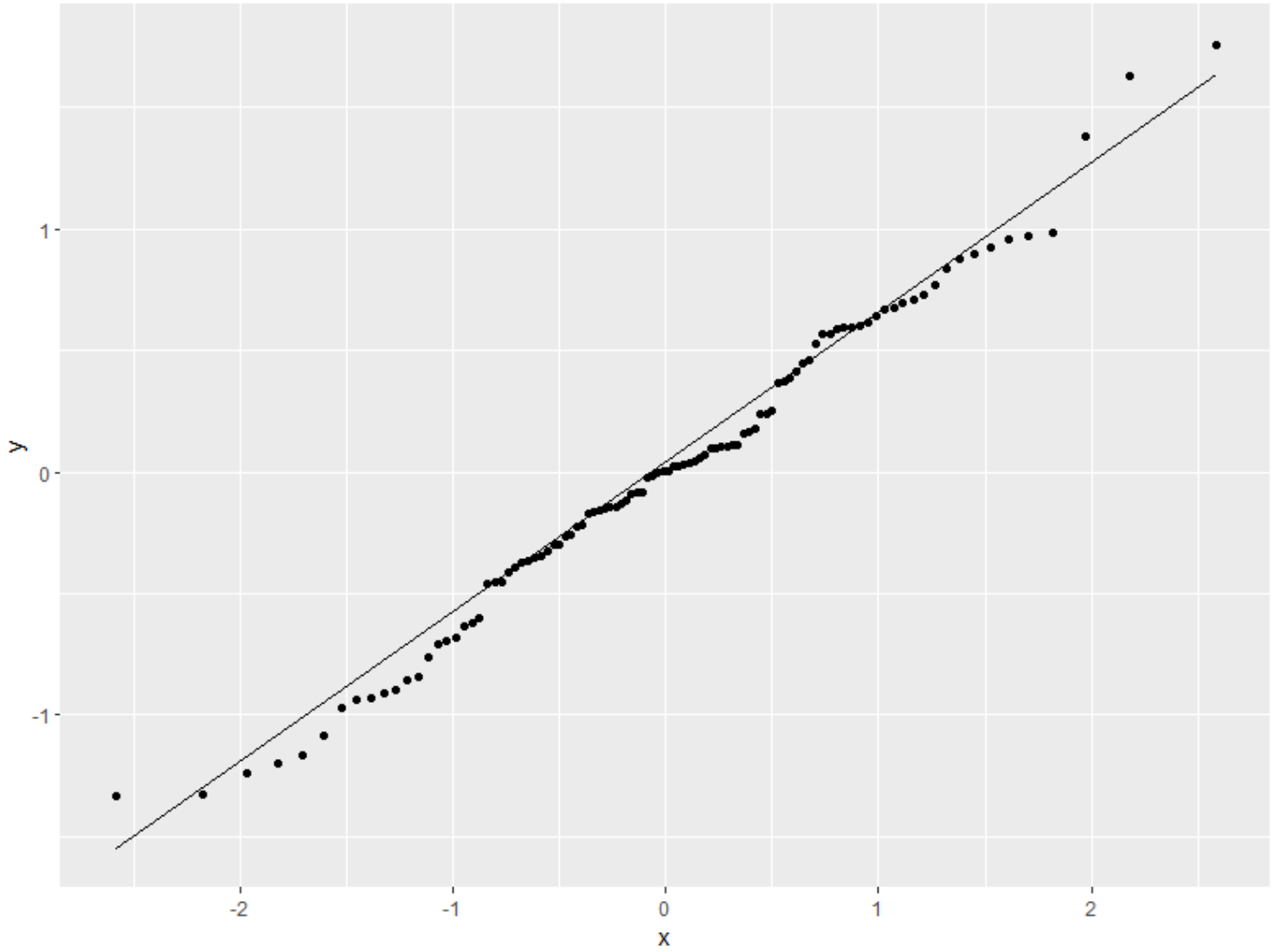



**Figure 43 Normal probability plot for optimal climate model**

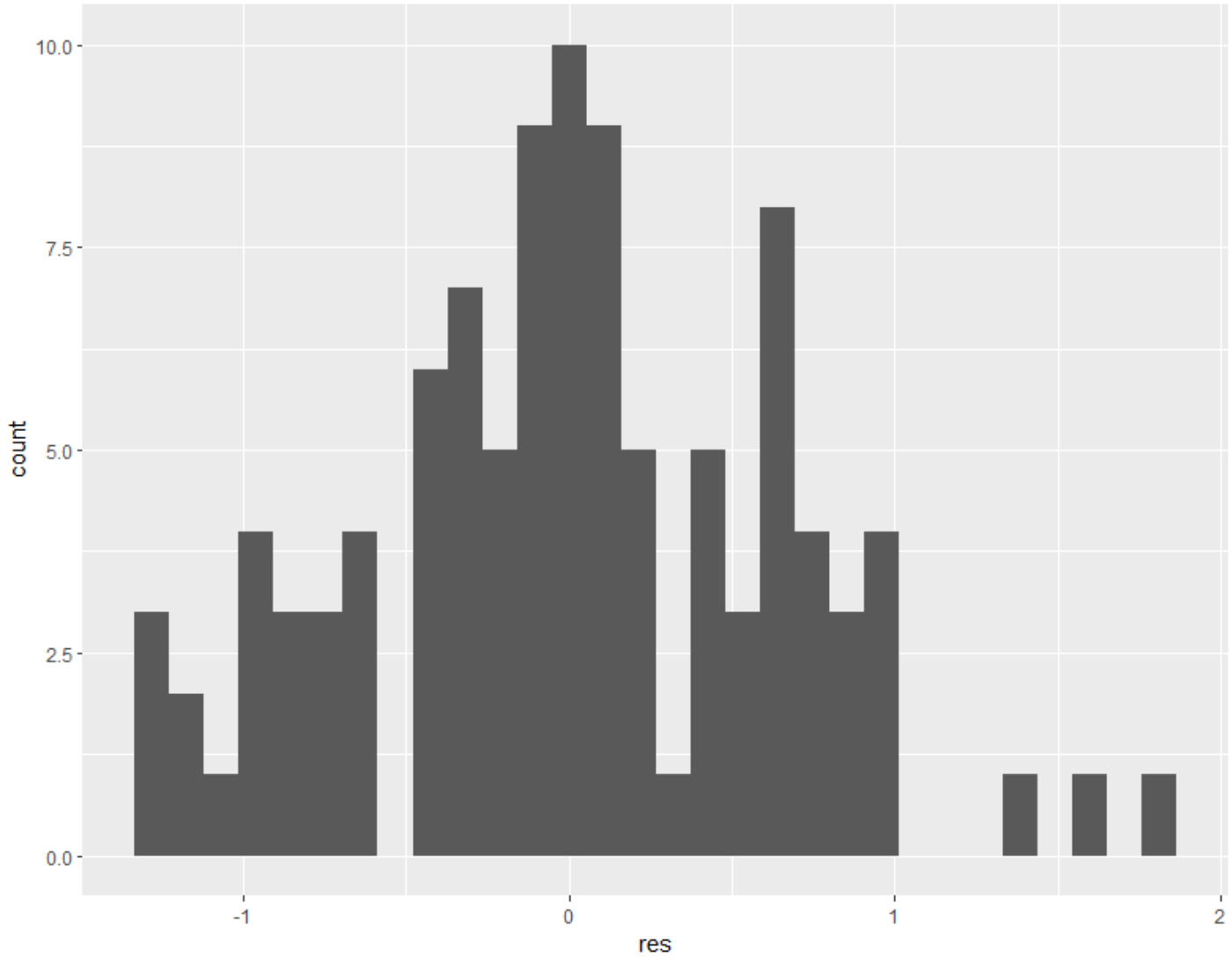



**Figure 44: Histogram of residuals plot for optimal climate model**

## 8.7 Results from stage 4: Predicting FOI at high spatial resolution across Timor-Leste

I successfully used my climate model to predict dengue FOI in all 2350 EAs across Timor-Leste. Figure 39 is a heatmap showing these findings. EAs in 3 urban centres (Dili, Baucau and Manatuto cities) as well as on Atauro Island appear to have high predicted FOI compared to other areas.

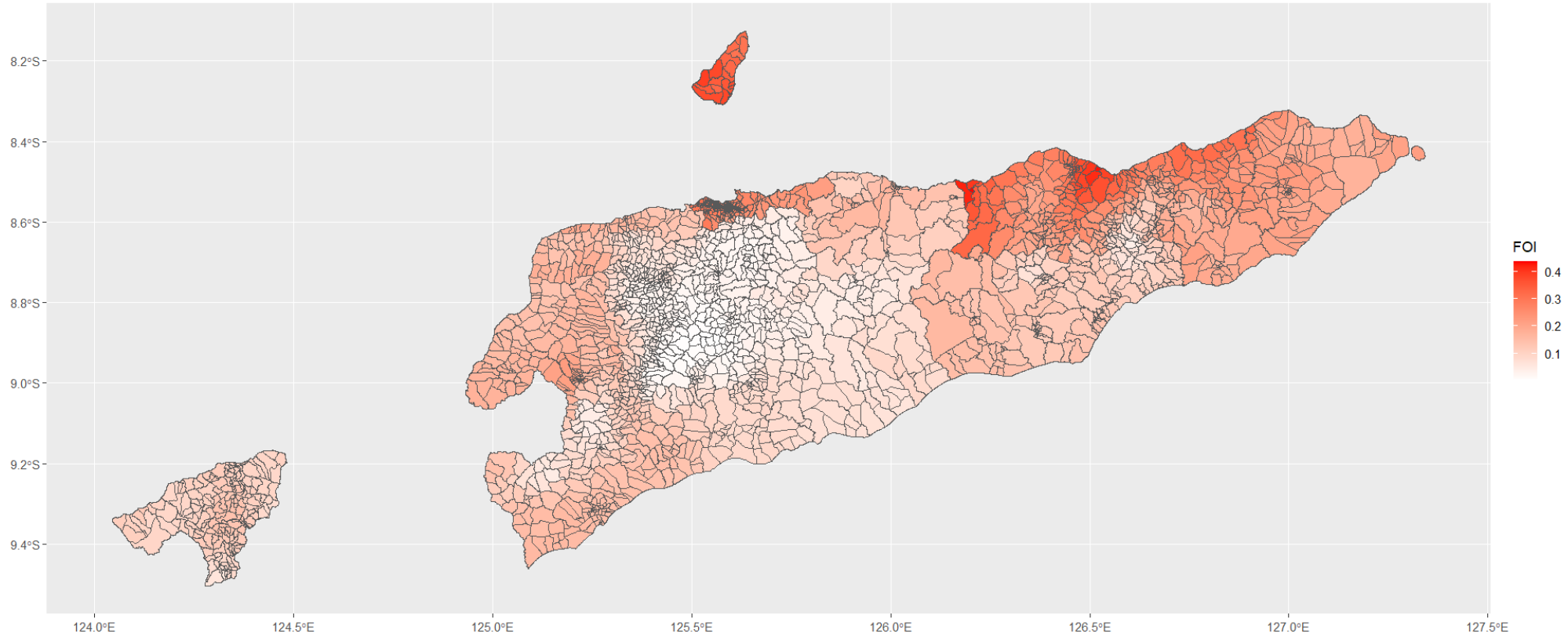



**Figure 45: Heatmap showing model-predicted dengue force-of-infection (FOI) in each of the 2350 EAs in Timor-Leste**

## 8.8 Discussion

In this chapter I estimated dengue FOI in 102 EAs in Timor-Leste from age-stratified seroprevalence data using a new catalytic which included household clustering parameters. A high degree of variation in FOI was observed, indicating that risk of dengue acquisition in Timor-Leste is highly dependant on household location. Other studies have reported significant heterogenicity in dengue FOI but not at such high spatial resolution.(29)

I obtained various climate data and calculated mean values for the past 10 years at geolocations which were in a regular grid across the land area of Timor-Leste. I interpolated these onto 2350 EAs across the country. I then assessed climate variables as predictors of dengue FOI. In univariable analyses, all were significantly associated with log(FOI). A forward stepwise variable selection process was used to identify the best-fitting ('optimal') multivariable model which included 'municipality' and 'relative humidity at minimum temperature'. $R^2$ for this model was 0.5816, indicating it explained 58.2% of the variability in log(FOI). I then predicted FOI in all 2350 EAs and represented central estimates in a heatmap. Previous studies have identified mean nocturnal and diurnal temperatures (and their annual seasonal variations), precipitation, elevation and population density to be strongly associated with dengue FOI, but have not assessed humidity. This has recently been highlighted as the 'overlooked variable in thermal biology of mosquito-borne disease'.(150,154,155).

There are several limitations to my study. First, the number of participants in each EA was small (IQR = 34-57, range = 7-100) and they were clustered within households. Despite using an updated catalytic model account for these study design effects (stage 1), FOI was only estimated with moderate precision in this disease model. Second, the catalytic model I used made several assumptions about antibody dynamics which may not always be true. These include an assumption that 100% of individuals seroconvert (i.e. transition from seronegative to seropositive) after their first dengue infection, that they remain seropositive for the rest of their lives. They also assume that

serological assays are 100% accurate in detecting previous dengue infection (and that accuracy does not vary by age, number of previous infections, or time since most recent infection). My work in previous chapters of this thesis has demonstrated that these assumptions do not always hold true. Mixture models, which are fitted to serological titre (continuous variable) rather than serological results (binary variable) and can include terms for multiple groups (i.e. 'infection naïve', 'monotypic response following one infection', 'multitypic response following more than one infection') are an alternative to catalytic models which can be used to estimate FOI while accounting for some of these factors.(156) Third, FOI estimates were simply plugged-in to my climate model (stage 2) without accounting for any uncertainty of their measurement. A 'feed-forward' approach may have been more appropriate in this study where two models were used.(157) Fourth, my analysis did not take into account any effect of spatial correlation. Using an R-INLA approach with bmy covariance structure to describe the neighbouring EAs may have been preferable. (157–159) This work is currently underway but has not yet been completed. Fifth, the serological methods used in this study are sensitive to antibody responses to all dengue viruses, but cannot differentiate the infecting serotype(s). Therefore, assessing serotype-specific transmission intensity was not possible. Samples are currently being transported to a collaborating laboratory where they will undergo an additional analysis using multiplexed microsphere immunoassay. This will indicate whether antibody responses are monotypic or multitypic, and may allow serotype-specific estimates to be made. These analyses have not been undertaken in time to be included in this thesis. Sixth, I aggregated climate and environmental data from the previous 10 years by simply taking the mean values of each parameter at each available geolocation. Therefore, my analysis did not assess for any effect of their annual seasonal variation on dengue IgG seroprevalence. Previous studies have used a fast Fornier transformations (FFT) to estimate the amount of variation in each parameter across the year, and have found this to be a predictor of dengue transmission intensity in some cases.(150) I did not do this because it would likely have resulted in me trying to fit models with too many independent variables and problems of overfitting and collinearity. Sixth, despite using climate data at highest

available resolution, many EAs were of small area in comparison to the area of pixels once climate data was rasterised. Therefore, highly localised climate patterns may not be captured, which may affect this study in Timor-Leste, which has particularly dramatic topography. Seventh, representing central predicted EA FOI in a heatmap does not capture the uncertainty of these estimations nor the assumptions made in fitting both the FOI and climate models. Eighth, as yet I have not been able to undertake validation of my FOI predictions. One option for doing this would have been to split my data into a training set (used to fit the climate model) and a testing set (used to assess how good the model is at predicting new data points). However, since I only started with a relatively small number of data points (102 FOI estimates), this would likely have adversely affected model precision. Another option would be to compare model-predicted FOI to other indications of dengue transmission intensity from other (non-serological surveillance ) methods. While a dataset including cases of dengue notified to the Timor-Leste Ministry-of-Health Surveillance Department could be available to me for this purpose, I am aware that this dataset is incomplete, does not cover many years and includes cases reported at the municipality level only (rather than EA level). Despite this, I intend to perform this external validation as part of my post-doctoral research, in part because there are very few locations in dengue endemic areas where the two approaches have been compared. This will inform global estimates of dengue incidence which are largely extrapolated or modelled from case-notification data.

## 8.9 Conclusion and key messages

In this chapter I reanalysed my age-stratified dengue IgG data together with climate data obtained from satellites to predict dengue FOI at high spatial resolution across the whole of Timor-Leste. Together with findings from chapters 6-7, this gives a detailed understanding of dengue virus transmission intensity across a remote region where other forms of surveillance have been limited. Findings will be useful to national and regional disease control activities and will guide further dengue surveillance efforts, including the potential implementation of a network of sentinel sites in areas with high predicted FOI including Dili and Baucau cities.

# CHAPTER 9

## 9.0 Integrating diagnostics with surveillance for dengue: Conclusions and recommendation

## 9.1 Conclusion

In this thesis I aimed to address the hypothesis:

> *Innovation in molecular and serological diagnostic techniques and their integration with active and passive surveillance systems can enhance understanding of dengue transmission in remote settings.*

To fulfil this aim I conducted a series of studies which investigated the challenge of dengue surveillance in remote settings, defined the need for new diagnostics, innovated molecular and serological techniques, and implemented these in surveillance systems where they have previously not been used.

In chapter 2 I undertook a pilot observational study of patients with AFI in Timor-Leste which identified a high rate of dengue infection and reported serotype-specific results to the local surveillance authority for the first time. The potential for molecular diagnostic techniques to enhance passive dengue surveillance was highlighted, alongside significant challenges relating to the deployment of assays in remote settings.

In chapter 3 I undertook a qualitative, focus group study of prospective diagnostic technology users who work in the Thai dengue surveillance system and found evidence that existing systems of passive dengue surveillance in remote settings are limited by a lack of access to diagnostic testing and a lack of effective, timely case-notification. I defined a need for new diagnostic technologies which are accurate, serotype-specific, easy to use outside laboratory settings and remote-connected such that results can be communicated to surveillance authorities and epidemiological information can be communicated to users in real-time. Devices with these design features would likely result in high user uptake and would optimally support systems of outbreak risk-assessment and responsive, targeted disease-control efforts. As such they were used as a 'target product profile' in my efforts to develop a new diagnostic workflow in chapters 4 and 5.

In chapter 4 I systematically reviewed the literature on RT-LAMP assays for dengue and assessed their potential as a solution to diagnosis and surveillance in remote areas. I found that eight sets of assays have previously been developed with published data suggesting they have high analytical and diagnostic performance. However, study reporting was not sufficiently standardised to compare performance between assays. Furthermore, none of them have so far been deployed or thoroughly evaluated outside the laboratory setting or in a system of surveillance, likely due to an ongoing requirement for sample extraction and signal detection which occurred separately using laboratory equipment in most published workflows. I concluded that there was a need for studies which objectively assess and compare performance of available RT-LAMP assays as well as those which develop equipment-free methods for nucleic acid extraction and signal detection, which I aimed to fulfil in chapter 5.

In chapter 5 I undertook a series of in-silico and in-vitro experiments to evaluate and optimise performance of serotype-specific dengue RT-LAMP assays. I found that the Hu (2015) dengue virus 1 and 2 and Parida (2005) dengue virus 3 and 4 assays had the best in-silico and in-vitro analytical performance and that an incubation duration of 25-minutes gave optimal combined assay sensitivity and specificity. I then demonstrated translation of these assays into a lyophilised, colorimetric format which allows portable amplification and signal detection (LyoLAMP strips), fulfilling previously identified user requirements for assay performance and diagnostic targets.

In chapter 6 I undertook pilot studies of various innovations in sample collection and serological diagnostics to assess whether they could be used to undertake serological surveillance of dengue in remote areas. First I showed that dengue surveillance can be integrated with other epidemiological surveys which may be occurring in remote areas through opportunistic collection of DBSs. This was achieved rapidly at low cost and provided the first ever dengue seroprevalence estimates for Timor-Leste. Second, I investigated the performance of LFTs in the diagnosis of previous dengue infection and hence whether they could be used in serological surveillance, replacing laboratory-based

techniques entirely. This was the first study which used LFTs in a community-based serological surveillance study. I used these findings to guide the design and implementation of a national population-representative dengue serological survey in Timor-Leste, presented in chapter 7.

In chapter 7 I used serological techniques in a large population-representative survey in Timor-Leste. Dengue IgG seroprevalence was high and strongly associated with participant age, with most adolescents and adults in Timor-Leste having evidence of at least one previous dengue infection. Seropositivity was associated with municipality of residence, with Dili having significantly higher seroprevalence than most other municipalities. This study represents the first systematic assessment of dengue transmission in Timor-Leste, where other forms of disease surveillance have been lacking. It is among only a small number of large population-representative dengue serological surveys which have been conducted globally.

In chapter 8 I reanalysed my seroprevalence data together with climate data obtained from satellites to predicted dengue FOI at high spatial resolution across the whole of Timor-Leste. This study demonstrates how the deployment of serological techniques within a serological surveillance and analysis of results alongside satellite data can give a detailed understanding of dengue virus transmission intensity at high resolution across remote regions.

## 9.2 Identified challenges for the future

Despite the successes outlined above, a number of research gaps remain in development and integration of dengue diagnostics in the pursuit of improved surveillance and addressing issues of access and diagnostic equity:

First, notwithstanding advances presented in this thesis, I was not able to develop and evaluate a truly portable high-performing remote-connected molecular diagnostic workflow for passive dengue surveillance. This is primarily due to the lack of a blood RNA extraction technique suitable for use

outside the laboratory setting, and systems for remote-connection and automated reporting. However, efforts to innovate and integrate these technologies are ongoing, and I aim to conduct initial field-evaluation studies of a prototype portable workflow before the end of 2023.

Second, notwithstanding significant advances towards optimal serological surveillance of dengue presented in this thesis, laboratory-based serological diagnostic techniques were used. This was because they were immediately available and my pilot studies evaluating DBSs and LFTs indicated a more complicated diagnostic workflow, and lower sensitivity, respectively. It is also notable that all these serological techniques are currently not serotype-specific. Furthermore, my studies took a cross-sectional designs, which were not responsive to temporal changes in dengue transmission. Further innovation in serological diagnostics could better support longitudinal programmes of serological surveillance. These could be integrated with passive disease surveillance systems and support the dynamic assessment of outbreak risk.

## 9.3 Recommendations

Given these identified challenges, the following recommendations for future research are made:

1. Efforts to research and develop blood RNA extraction methods which can be integrated with serotype-specific RT-LAMP assays should be maintained.

2. Systems for remote connection of dengue diagnostic workflows should be researched and developed, with a view to achieving automated case notification (upwards data transfer) and provision of real-time information about local dengue incidence to the user (downwards data transfer).

3. Serological methods for serotype-specific antibody detection should be investigated, to understand whether circulating dengue serotypes can be determined from antibody profiles.

4. An integrated approach to surveillance where molecular and serological methods are deployed longitudinally within the same geographical region should be considered. Temporal changes in serotype-specific case-detection and seropositivity should be assessed as predictors of outbreaks.

5. These technologies and study designs should continue to be researched, developed and implemented in collaboration with prospective users across surveillance systems in remote areas, to optimise solutions and maximise the chance of uptake.

## 9.4 Planned future work

This thesis represents three years of research into how innovative and integrated diagnostic and surveillance systems can be used to understand dengue transmission in remote settings. To follow this I have planned and achieved additional funding to undertake the following additional post-doctoral studies and analyses:

First, I will continue to collaborate with molecular scientists and bioengineers at Imperial College and Mahidol University in efforts to develop RNA extraction methods and systems of remote-connection which can be integrated with my LyoLAMP strips (developed in chapter 5). I hope this will ultimately result in a portable dengue diagnostic workflow for enhanced passive surveillance. We have recently been awarded additional funding by the Wellcome Trust (£50k) for this and plan to undertake initial validation studies in Thailand in October 2023.

Second, together with colleagues at the Timor-Leste Ministry of Health, I aim to set-up additional sentinel sites for passive dengue surveillance and other studies investigating aetiology and management of community acquired AFI in Timor-Leste, using the model implemented in chapter 2. These will serve as an ideal platform to validate new diagnostic workflows and assess their impact on surveillance. I aim to leverage funding which has recently been awarded to Menzies School of Health Research and Universidade de Paz in Timor-Leste by the Wellcome Trust (£80k) as part of an international collaboration on antimicrobial decision-making and optimisation for this purpose.

Third, I have achieved funding from the Department of Foreign Affairs and Trade, Australian Government (£17k) to undertake additional analysis of all samples from my serological survey (chapter 7) using a newly developed multiplexed microsphere immunoassay. This will detect antibodies against NS1 from each of the four dengue virus serotypes, determine whether responses in positive samples are monotypic or multitypic, and explore whether the predominant circulating serotype(s) can be determined from these antibody profiles.

Fourth, depending on outcomes from these projects, I will apply for an intermediate career research fellowship investigating how an integrated serotype-specific molecular and serological approach to dengue surveillance at sentinel sites may be used to assess risk and predict risk of outbreaks in real time.

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# APPENDICES

# Appendix 1: Publications arising from this thesis

**Chapter 4:**

**Paul Arkell**, Dumrong Mairiang, Adisak Songjaeng et al. Analytical and diagnostic performance characteristics of reverse-transcriptase loop-mediated isothermal amplification assays for dengue virus serotypes 1-4: A scoping review to inform potential use in portable molecular diagnostic devices. *PLoS Global Public Health.* 2023 Aug 8;3(8):e0002169. doi: 10.1371/journal.pgph.0002169. eCollection 2023.

**Chapter 6:**

**Paul Arkell**, Julia Angelina, Alberina do Carmo Vieira et al. Integrated serological surveillance of acute febrile illness in the context of a lymphatic filariasis survey in Timor-Leste: a pilot study using dried blood spots. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 2022 Jun 1;116(6):531-537. doi: 10.1093/trstmh/trab164.

**Paul Arkell**, Maria Tanesi, Nelia Gomes et al. Field evaluation of rapid diagnostic tests to determine dengue serostatus in Timor-Leste. *PLoS Neglected Tropical Diseases.* 2022 Nov 7;16(11):e0010877. doi: 10.1371/journal.pntd.0010877. eCollection 2022 Nov.

**Chapter 7:**

**Paul Arkell**, Sarah L Sheridan, Nelson Martins et al. Vaccine Preventable Disease Seroprevalence in a Nationwide Assessment of Timor-Leste (VASINA-TL): study protocol for a population-representative cross-sectional serosurvey. *BMJ Open.* 2023 May 18;13(5):e071381. doi: 10.1136/bmjopen-2022-071381.

# Appendix 2: Research Ethics Committee approvals, participant information sheets, consent forms and data collection tools for study deploying existing molecular diagnostic technology (Chapter 2)

NORTHERN TERRITORY GOVERNMENT | NT HEALTH

menzies school of health research

27 January 2023

**Ethics Administration Office**
**File Reference Number: HREC-2023-4505**
**Phone:** (08) 8946 8687 or (08) 8946 8692
**Email:** ethics@menzies.edu.au

Dr Gustodio de Jesus
Hospital Nacional Guido Valadares Emergency Department
todiva3d2021@gmail.com
CC: josh.francis@nt.gov.au; josh.francis@menzies.edu.au
*Via Email*

Dear Dr de Jesus,

**NT HREC Reference Number: 2023-4505**
**Project Title:** ***Investigating Sepsis through Implementation of a Novel Microbiological and Nucleic Acid amplification testing Strategy in Timor-Leste***

Thank you for your letter dated 16/01/2023 and taking the time to respond to the issues of concern identified by the Fast Track sub-committee of the Human Research Ethics Committee of Northern Territory Health and Menzies School of Health Research (NT HREC). This project was considered by members of the NT HREC and assessed against guidelines for human research including the NHMRC *National Statement on Ethical Conduct in Human Research 2007*.

I am pleased to advise that **full ethical approval** of this research project has been granted following assessment by representatives of the HREC. Please note that approval applies only to research conducted after the date of this letter and continued approval is dependent on annual reporting.

**Approval Date:** ***27/01/2023***

Approval is granted for the above research project until the next report due date.

**Annual progress report due:** ***31/12/2023***

**Approved timeframe (subject to compliance and annual reporting):** ***27/01/2023 to 31/12/2023***

The nominated sites participating in this project that have been approved by this HREC are:

- **Hospital Nacional Guido Valadares, Timor-Leste (primary site)**
- **Potential to expand to other TL MOH hospital sites which receive patients with AFI (secondary sites) Please ensure that this HREC is notified of any changes to sites.**

**Please note:**

- Researchers must comply with site specific governance regulations, data custodian and other stakeholder requirements.
- For site-specific authorisation for NT Health sites including access to NT Health data, please liaise with the NT Health Research Governance office, nthealth.rgo@nt.gov.au.

**The documents listed below are approved:**

| Document | Date |
|---|---|
| HREC application | 15/12/2022 |
| Response to Conditional Approval | 16/01/2023 |

**APPROVAL IS SUBJECT TO** the following conditions being met:

1. The Coordinating Principal Investigator will **immediately report anything that might warrant review** of ethical approval of the project.

2. The Coordinating Principal Investigator will notify the NT HREC of any event that requires a **modification or amendment to the protocol or other project documents** and submit any required amendments in accordance with the instructions provided by the HREC. These instructions can be found on the Menzies' website.
3. The Coordinating Principal Investigator will submit any necessary reports related to the **safety of research participants (e.g. protocol deviations, protocol violations)** in accordance with the NT HREC's policy and procedures. These guidelines can be found on the Menzies' website.
4. The Coordinating Principal Investigator will **report** to the HREC **annually** and notify the NT HREC when the project is completed at all sites using the specified forms. Forms and instructions may be found on the Menzies' website.
5. The Coordinating Principal Investigator will notify the NT HREC if the project is **discontinued at a participating site before the expected completion date** and provide the reason/s for discontinuance.
6. The Coordinating Principal Investigator will notify the NT HREC of any plan to **extend the duration of the project past the approval period listed above** and will submit any associated required documentation. The preferred time and method of requesting an extension of ethical approval is during the **annual progress report**. However, an extension may be requested at any time.
7. The Coordinating Principal Investigator will notify the NT HREC of his or her **inability to continue as Coordinating Principal Investigator**, including the name of and contact information for a replacement.
8. The safe and ethical conduct of this project is entirely the responsibility of the investigators and their institution(s).
9. Researchers should immediately report anything which might affect continuing ethical acceptance of the project, including:
    - Adverse effects of the project on participants and the steps taken to deal with these;
    - Other unforeseen events;
    - New information that may invalidate the ethical integrity of the study; and
    - Proposed changes in the project.
10. Approval for a further twelve months, within the original proposed timeframe, will be granted upon receipt of an annual progress report if the NT HREC is satisfied that the conduct of the project has been consistent with the approved protocol. Report templates are available on the Menzies ethics webpage.
11. Confidentiality of research participants should be maintained at all times as required by law.
12. The Patient Information Sheet and the Consent Form shall be printed on the relevant site letterhead with full contact details.
13. The Patient Information Sheet must provide a brief outline of the research activity including: risks and benefits, withdrawal options, contact details of the researchers and must also state that the Human Research Ethics Administrators can be contacted (telephone and email) for information concerning policies, rights of participants, concerns or complaints regarding the ethical conduct of the study.
14. You must forward a copy of this letter to all Investigators and to your institution (if applicable).

**This letter constitutes ethical approval only**.

This project, including amendments to the research protocol or conduct of the research which may affect the site acceptability of the project, cannot proceed at any site until separate research governance authorisation has been obtained from the CEO or Delegate of the institution under whose auspices the research will be conducted at that site, if not already obtained.

Any transfer of data is subject to institutional research governance arrangements for data ownership, data custodianship, and data transfer agreements.

Please forward this approval letter to the relevant research governance office.

Should you wish to discuss the above research project further, please contact the Ethics Administrators via email: NTHREC@menzies.edu.au or telephone: (08) 8946 8687 or (08) 8946 8686.

NORTHERN TERRITORY GOVERNMENT | NT HEALTH

menzies school of health research

Yours sincerely,

Dr Mary Morris
**Chair**
**Human Research Ethics Committee**
**of NT Health and Menzies School of Health Research**
http://www.menzies.edu.au/ethics

**NT HREC is registered and certified for multi-site review with the Australian National Health and Medical Research Council (NHMRC) and operates in accordance with the NHMRC *National Statement on Ethical Conduct in Human Research (2007)*. NHMRC Reg no. EC00153**

MINISTÉRIO DA
SAÚDE

INSTITUTO NACIONAL
DE SAUDE
Gabinete Diretora Ezecutiva

No. Ref.: 157 MS-INS/GDE/I/2023
Dili, 20/ 01 /2023

**APPROVAL LETTER**

**Gustodio de Jesus**
Hospital Nacional Guido Valadares

Dear. Jesus

**Project Title: Invetigating Sepsis Through Implementation Of a Novel Microbiological and Nucleic Acid Amplification Testing Strategy in Timor-Leste**

Thank you for submitting the above research project for ethical review. This project was considered by the Institute National of Health-Research Technical Committee at its meeting held on 17 January 2023, and based on your request letter that submitted on 12 October 2022 for research.

I am pleased to advise you that the Institute National of Health-Research Ethics& Technical Committee (INS-RETC) has granted **Ethics & Technical approval** of this research project, due to some technical issues and pandemic situation in Timor-Leste that affect to this research project.

Please note that if additional sites are engaged prior to the commencement of, or during the research project, the coordinating Principal Investigator is required to notify the Institute National of Health-Research Ethics & Technical Committee (INS-RETC). Notification of withdrawn sites should also be provided to the TL-HREC in timely fashion

The Approved documents include:
1. INS-RETC Application form
2. Proposal
3. Inform of Consent
4. Questioner

This approval is for period of Twelve (12) months. An ANNUAL /FINAL Project progress report is required on or before 31 January 2024

# Information sheet for adult participant

## Folha infromasaun partisipante nian

Principle Investigator: Dr Gustodio de Jesus, todiva3d2021@gmail.com, +670 7754 9505

Local Study Coordinator: Salvador Amaral, Salvador.amaral@menzies.edu.au, +670 7708 7665

INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE

(ISIN-MANAS STUDY)

INVESTIGASAUN BA SEPSE LIU HUSI IMPLEMENTASAUN BA ESTRATEJIA FOUN BA TESTE AMPLIFIKASAUN MIKROBIOLOJIKA NO BA ASIDU NUKLEIKU IHA TIMOR-LESTE

(ISIN-MANAS STUDY)

## This is for you to keep / Ida ne'e fó ba ita bo'ot atu rai

You are being invited to take part in a study about sepsis / Ita hetan konvite atu hola parte iha estudu konaba sepse

Participation: Taking part in this project is entirely voluntary. You can withdraw from the study at any time. You do not have to explain why you want to withdraw from the study, and there are no negative consequences if you withdraw. If you have any questions, please discuss them with members of our team.

Partisipasaun: Partisipa iha projetu ida ne'e kompletamente voluntariu. Ita bo'ot bele sai husi estudu iha kualker tempu. Ita bo'ot la presiza atu esplika razaun tamba saida, no sei la iha konsekuensia negativu karik ita bo'ot sai. Karik ita bo'ot iha kualker pergunta, favor bele hato'o ba membru husi ami nia ekipa.

What is sepsis? Sepsis is the body's extreme response to an infection. It is a common illness around the world and can be life-threatening. There are many different infections which can cause sepsis and these vary from place-to-place.

Saida mak sepse? Sepse mak hanesan resposta extreme husi isin ba infesaun. Ida ne'e hanesan moras komum ida iha mundu tomak no bele fo ameasa ba ema nia vida. Eziste infesaun oi-oin ne'ebé bele kauza sepse no ida ne'e la hanesan husi fatin ida ba fatin seluk.

Who is organising the project? The study is a collaboration between:

Se mak organiza projetu ida ne'e? Estudu ida ne'e kolaborasaun ida entre:

Ministry of Health, Timor-Leste / Ministeriu da Saúde, Timor-Leste

Hospital Nacional Guido Valadares

National Health Laboratory, Dili / Laboratóriu Nasional Saúde, Dili

Menzies School of Health Research, Australia

Approval for this project. This study has been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia.

Aprovasaun ba projetu ida ne'e. Estudu ida ne'e hetan ona aprovasaun husi Institutu Nasional da Saúde (INS), Timor-Leste no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research iha Australia.

Informed Consent Information / Informasaun Konsentimentu ne'ebé mak Informadu

Section 1: Purpose of the study. / Seksaun 1: Objetivu husi estudu.

The main purpose of this study is to determine which infections most commonly cause people to be unwell with sepsis in Timor-Leste. It will also describe the common symptoms and signs of sepsis in Timor-Leste, its treatment, and clinical outcomes.

Objetivu prinsipal husi estudu ida ne'e mak atu determina infesaun sira ne'ebé maka komum liu hodi kauza ema la saudavel ho sepse iha Timor-Leste. Ida ne'e mos deskreve sintoma no sinais komum sira husi sepse, ninia tratamentu, no rezultadu kliniku sira iha Timor-Leste.

Section 2: Procedures. For each person, we will:

Seksaun 2: Prosedimentu. Ba kada partisipante, ami sei:

Take an extra BLOOD SAMPLE (in addition to the samples which are being taken for your other routine tests). This will be 4mls if you are an adult, or 1ml if you are a child. / Foti amostra raan extra (alem amostra ne'ebé foti hodi halo ita nia teste rutina seluk). 4mls ba ema bo'ot no 1mls ba labarik kiik.

Test the blood sample for bacteria, viruses, parasites and/or fungi which may be causing your illness. This will occur at the National Health Laboratory (NHL) in Dili. / Halo teste amostra raan ba bakteria, virus, parasite no/ka fungu ne'ebé bele kauza moras. Ida ne'e sei hala'o iha Laboratóriu Nasional Saúde (LNS) iha Díli.

Contact you every day for 10 days and ask you about your symptoms. This will be in person if you are in hospital, or by telephone if you have gone home. / Kontaktu ita lorloron durante loron 10 nia laran no husu konaba ita nia sintoma sira. Karik ita iha hospital ida ne'e sei halo direitamente, no karik ita iha uma ida ne'e sei halo kontaktu liu husi telemovel.

If a cause of your illness is identified through this study, then you will be informed about this and will be offered treatment (if applicable). / Karik kauza husi ita nia moras identifika liu husi estudu ida ne'e, ita sei hetan informasaun konaba nia rezultadu no sei oferese tratamentu (karik iha).

Section 3: Duration of Procedures. Collection of the blood sample will take less than 5 minutes. This will be done alongside your other blood tests (using the same needle, without any extra needle sticking)

Seksaun 3: Durasaun ba Prosedimentu. Koleksaun ba amostra ran sei uza tempu pelu menus minutu 10. Prosesu ida ne'e sei hala'o hamutuk ho ita bo'ot nia teste raan sira seluk (uza daun ne'ebé hanesan, sein uza daun extra hodi hasai raan)

Section 4: Potential benefit to you and others. / Seksaun 4: Benefisiu potensial ba ita bo'ot no ema seluk.

If you participate, your blood will be tested for additional bacteria, viruses, parasites and/or fungi which might be causing your illness. If one is identified, you will be told about this and offered treatment (if applicable).

Se ita partisipa, sei halo teste ba ita nia raan hodi hetan baktéria, virus, parazita no/ka fungi adisionál sira ne'ebé bele kauza ba ita nia moras. Karik identifika ida ona, ita sei hetan informasaun konaba ida ne'e no sei oferese tratamentu (karik iha).

The results of the study will provide important and useful information about sepsis Timor-Leste. / Rezultadu sira husi estudu ida ne'e sei fornese informasaun ne'ebé importante no útil konaba sepse nian iha Timor-Leste.

Section 5: Discomforts and Risks. The potential risks of collecting a blood sample include temporary discomfort from the blood collection, bruising, bleeding, and very rarely, infection. These are unlikely to occur because experienced healthcare professionals will take samples.

Seksaun 5: Dezkomfortu no Risku sira. Risku potensiál sira husi rekolla amostra raan nian mak inklui dezkomfortu temporáriu husi kolesaun raan nian, mean, ran sai, no infesaun ne'ebé la komum. Ida ne'e dalabarak la akontese tanba profisionál saúde sira ne'ebé iha esperiénsia mak sei ba foti amostra.

In the unlikely event of any significant injury because of the blood collection procedure, the study team and/or the Ministry of Health will arrange necessary medical treatment without financial cost. However, no financial compensation will be given in case of any study-related injury.

Hare ba kualker kanek/prejuizu signifikante ne'ebé improvavel atu akontese husi prosedimentu koleksaun raan nian, ekipa estudu no/ka Ministeriu Saúde sei organiza tratamentu médiku ne'ebé presiza sein kustu finanseiru. Maske nune'e, sei la iha kompensasaun finanseiru ba kualkér kanek/prejuizu ne'ebé relasiona ho estudu ida ne'e.

Section 6: Costs for participation. Taking part in the project will cost nothing apart from your time.

Seksaun 6: Kustu ba partisipasaun. Partisipasaun iha projetu ida ne'e, sei la kobre kustu ruma exeptu ita bo'ot nia tempu.

Section 7: Compensation for participation. You will not receive any payments for being part of this project.

Seksaun 7: Kompensasaun ba Partisipasaun. Ita bo'ot sei la simu kualker pagamentu ba partisipasaun iha projetu ida ne'e.

Section 8: Study funding. This study is funded by the Australian Government Department of Foreign Affairs and Trading (DFAT). The Ministry of Health, Timor-Leste has been closely involved in the planning and development of this study and is involved in undertaking this study. The collaborators do not have any conflict of interests to declare.

Seksaun 8: Finansiamentu ba estudu. Estudu ida ne'e hetan finansiamentu husi Departamentu Asuntu Estranjeiru no Komersiu, Guverno Australia (DFAT). Ministeriu Saúde Timor-Leste, proximamente involve ona iha planeamentu no dezenvolvimentu ba estudu no envolve ba implementasaun estudu ida ne'e. Kolaboradores sira la iha kualker konflitu interese atu deklara.

Section 9: Voluntary participation. Taking part in this project is your choice, and you will have the right to stop at any time. If individuals or communities decide not to participate or decide to stop taking part at any time, there will be no penalties.

Seksaun 9: Partisipasaun Voluntariu. Partisipa iha projetu ida ne'e ita bo'ot mak hili, no ita bo'ot sei iha direitu atu hapara/sai iha kualker tempu. Karik individual sira ou komunidade deside atu la partisipa ou deside atu sai husi partisipasaun iha kualker tempu, ida ne'e sei la iha penalidade ruma.

Section 10: Delivery of results. If your blood test shows that you have a virus or parasite, you will be contacted and offered the appropriate treatment (if applicable).

Seksaun 10: Fornese rezultadu sira. Se Ita nia teste raan hatudu katak Ita iha virus ka parazita ruma, ita sei hetan kontaktu no sei oferese tratamentu ne'ebé apropriadu (karik aplika).

You will not be contacted if the test is negative. / Ita sei la hetan kontaktu ruma karik teste ne'e negativu.

Results for the whole study will be written-up and may be published. It may be used by the Ministry of Health in Timor-Leste to guide future management of sepsis in the country. / Rezultadu ba estudu tomak sei hakerek no bele publika ona. Ministério Saúde iha Timor-Leste bele uza hodi orienta jestaun sepse nian iha rai-laran iha futuru.

Section 11: Privacy and confidentiality. During this study, your personal information will be kept strictly confidential. Blood samples will be labelled using your initials, date-of-birth and patient number only. These will be transported directly to NHL where they will be stored securely before and after analysis.

Seksaun 11: Privasidade no konfidensialidade. Durante estudu ne'e, ita-boot sira nia informasaun pesoál sei nafatin asegura metin ninia konfidensialidade. Amostra raan sira sei marka deit uza ita nia inisiál naran nia, data moris no númeru pasiente. Amostra sira ne'e sei lori diretamente ba LNS, iha ne'ebé sei rai ho seguru antes no depois halo analiza.

No personally identifiable information will be shared in any project reports, publication, or presentations. We will keep each individual's participation in this project confidential to the extent permitted by law. However, it is possible that other people may become aware of their participation in this study, e.g. the following people/groups may inspect project records:

Sei la fahe informasaun personal ne'ebé mak identifikavel iha kualkér relatóriu sira projetu nian, publikasaun, ka aprezentasaun sira. Ami sei mantein ema ida-idak nia partisipasaun iha projetu ne'e ho konfidensiál to'o iha medida ne'ebé lei permite. Maske nune'e, sei iha ema balun bele hatene konaba partisipante sira iha estudu ne'e, hanesan ema/grupu sira tuir mai ne'ebé mak sei bele halo inspeksaun ba projetu nia rejistu:

The Instituto Nacional da Saúde; INS and the Ministry of Health, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia

Instituto Nacional da Saúde (INS) no Ministeriu Saude, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia

Section 12: Contact information for questions or concerns. You have the right to ask any questions you may have about this study. If you have questions, complaints or concerns or believe you may have developed an injury related to this study, please contact Salvador Amaral on email: Salvador.amaral@menzies.edu.au or mobile: +670 7708 7665. If you have questions regarding your rights as a study participant or concerns regarding the ethical conduct of the project, please contact Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au). If you have any medical problems associated with this study, such as a pain from where your blood was collected or feeling unwell, please contact the above phone numbers or attend your local hospital or clinic if urgent.

Seksaun 12: Informasaun kontaktu karik iha pergunta ou preokupasaun ruma. Ita bo'ot iha direitu hodi husu kualker pergunta ne'ebé mak relasiona ho estudu ida ne'e. Karik ita bo'ot iha pergunta, reklamasaun ou preokupasaun ou karik ita bo'ot hetan injuria relasiona ho estudu ida ne'e, favor kontaktu ba Salvador Amaral on email: Salvador.amaral@menzies.edu.au or mobile: +670 7708 7665. Karik ita bo'ot iha pergunta relasiona ho ita bo'ot nia direitu nudar partisipante ou preokupasaun relasiona ho projetu nia étika konduta, favor kontaktu Sekretariu étiku, komisaun etika peskija komite tekniku INS (Ph +670 7708 7665) ou Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (telemovel +618 8946 8600; Email ethics@menzies.edu.au). Karik ita bo'ot iha kualker problema mediku ne'ebé mak asosiadu ho estudu ida ne'e, por ezemplu hanesan moras iha fatin hasai ran ou sente la saudavel, favor kontaktu ba numeru ne'ebé mensiona iha leten ou konsulta iha hospital lokal ou klinika sira karik urgente.

Section 13: Consent to participate in project. Before making the decision about participating in this study you should have:

Discussed this project with a member of the study team

Read and understood the information in this form

Had the opportunity to ask any questions that you may have

Had time to consider whether to take part

Information about who to contact in case you have any further questions or problems

Seksaun 13: konsentimentu atu partisipa iha projetu. Antes foti desizaun atu partisipa iha estudu ida ne'e ita bo'ot tenke:

Diskute uluk konaba projetu ida ne'e ho membru husi ekipa estudu nian

Le'e no komprende informasaun iha formulariu ida ne'e

Iha oportunidade hodi husu kualker pergunta ne'ebé mak ita bo'ot iha

Iha tempu hodi hanoin antes deside atu partisipa

Infomasaun konaba se mak ita bo'ot atu kontaktu, kuandu ita bo'ot iha tan pergunta ou problema ruma.

Section 14: Ethics Committee Clearance. The ethical aspects of this research have been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia (Protocol HREC-2021-4064). If you have any concerns about this research, please contact: Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au).

Seksaun 14: Lisensa Komite Étika. Aspektu etikal husi peskiza ida ne'e hetan ona aprovasaun husi Institutu Nasional Saude Timor-Leste (INS) no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia (Protokolu HREC-2021-4064). Karik ita bo'ot iha kualker preokupasaun konaba peskiza ida ne'e, favor kontaktu: Sekretariu Étiku, Komisaun Étiku Peskija Komite Tekniku INS (Telemovel +670 7708 7665) ou the Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (Telemovel +618 8946 8600; Email: ethics@menzies.edu.au).

## Consent form for adult participant

## Formulariu Konsentimentu nian

Principle Investigator: Dr Gustodio de Jesus, todiva3d2021@gmail.com, +670 7754 9505

Local Study Coordinator: Salvador Amaral, Salvador.amaral@menzies.edu.au, +670 7708 7665

# INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE (ISIN-MANAS STUDY)

# INVESTIGASAUN BA SEPSE LIU HUSI IMPLEMENTASAUN BA ESTRATEJIA FOUN BA TESTE AMPLIFIKASAUN MIKROBIOLOJIKA NO BA ASIDU NUKLEIKU IHA TIMOR-LESTE (ISIN-MANAS STUDY)

## Informed Consent Form / Formuláriu Konsentimentu Informadu

(For adults who have capacity to make decisions about participation in this research study) / (Ba ema-boot sira ne'ebé iha kapasidade atu foti desizaun konaba partisipasaun iha estudu peskiza ida nee)

This means you can say NO / Ida ne'e signifika ita bele dehan LAE

| |
|---|
| PRINCIPAL INVESTIGATOR / INVESTIGADOR PRINSIPAL: Dr Gustodio de Jesus |
| STUDY COLLABORATORS / KOLABORADORES BA ESTUDU: |
| Hospital Nacional Guido Valadares |
| Ministry of Health (MOH), Timor-Leste / Ministeriu da Saúde, Timor-Leste |
| National Health Laboratory, Dili, Timor-Leste / Laboratoriu Nasional Saude, Dili, Timor-Leste |
| Menzies School of Health Research, Australia |

I understand the aim of this research study is to improve understanding of sepsis in Timor-Leste. Details of the study have been explained to me, and I have been provided with a written information sheet and given the opportunity to ask questions.

Hau komprende objetivu husi peskiza ne'e atu hadi'a kompriensaun konaba total ema ne'ebé hetan ona espozisaun no/ka vasinasaun kontra moras infesiozu sira iha Timor-Leste. Detallu konaba estudu ne'e esplika ona mai ha'u, no ha'u hetan ona informasaun eskrita ida no hetan oportunidade atu husu pergunta.

I acknowledge that: / Hau rekonhese katak:

Any risks and possible effects of having a venous blood test have been explained to my satisfaction;

Kualker risku no efeitu ne'ebé mak posivel atu hetan tamba teste ran venozu nian, esplika ona mai hau nia satisfasaun;

Taking part in this study is voluntary and I am aware that I can stop taking part at any time without explanation or prejudice and to withdraw any unprocessed data I have provided;

Partisipasaun iha estudu ida ne'e voluntariu no hau hatene katak hau bele hapara hau nia partisipasaun iha kualker tempu sem explikasaun ou prekonseitu, no dada fila fali kualker dadus ne'ebé seidauk prosesa, ne'ebé mak hau fornese.

Any information I give will be kept strictly confidential and that no names will be used to identify me in this study without my approval.

Kualker informasaun ne'ebé mak hau fó, sei rai estreitamente konfidensial no sei la uza kualker naran hodi identifika hau iha estudu ida ne'e sem hau nia aprovasaun

By providing my name and signature, I agree to:

Liu husi fó hau nia naran no asinatura, hau konkorda atu:

| | | | | |
|---|---|---|---|---|
| Additional blood sample being taken (at the same time as my other blood tests) / Hasai amostra raan adisional (iha tempu hnesan ho mós hau nia teste raan sira seluk) | ☐ | Yes<br>Sim | ☐ | No<br>Lae |
| My blood samples being tested for bacteria, viruses, parasites and fungi which may be causing my illness, including in partner laboratories outside Timor-Leste if tests aren't available locally/ Hau nia amostra raan teste ba bakteria, virus, parasite no fungu sira ne'ebé bele kauza hau nia moras, inklui iha laboratoriu parseria sira iha rai-liur karik teste la disponivel iha laboratoriu lokal | ☐ | Yes<br>Sim | ☐ | No<br>Lae |
| Being contacted each day for 10 days. This would be in person if I am in hospital, or by telephone if I have gone home / Hetan kontaktu lor-loron durante loron 10. Ida ne'e sei direitamente karik hau baixa iha hospital, ka husi telemovel karik hau iha ona uma | ☐ | Yes<br>Sim | ☐ | No<br>Lae |
| The use of my data for the purposes of this study (all data will be de-identified before use) / Uza hau nia dadus ba objetivu estudu ida ne'e nian (dadus hotu sei la identifikavel molok uza) | ☐ | Yes<br>Sim | ☐ | No<br>Lae |

| | |
|---|---|
| Name of participant / Naran partisipante: *(printed)* | Unique identifier: / Identifikador uniku: |
| Signature of participant: / Asinatura husi partisipante: | Date / Data: |
| Name of witness/interpreter: / Naran testemunha/tradutor: *(printed)* | Signature of witness/interpreter: / Asinatura husi testemunha/tradutor: |
| Name of researcher / Naran peskizador: *(printed)* | Signature of researcher / Asinatura husi peskizador: |

# Information sheet for parent/guardian

## Folha infromasaun ba inan-aman/kuidador

Principle Investigator: Dr Gustodio de Jesus, todiva3d2021@gmail.com, +670 7754 9505

Local Study Coordinator: Salvador Amaral, Salvador.amaral@menzies.edu.au, +670 7708 7665

INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE

(ISIN-MANAS STUDY)

INVESTIGASAUN BA SEPSE LIU HUSI IMPLEMENTASAUN BA ESTRATEJIA FOUN BA TESTE AMPLIFIKASAUN MIKROBIOLOJIKA NO BA ASIDU NUKLEIKU IHA TIMOR-LESTE

(ISIN-MANAS STUDY)

## This is for you to keep / Ida ne'e fó ba ita bo'ot atu rai

Your child is being invited to take part in a study about sepsis / Ita nia oan hetan konvite atu hola parte iha estudu konaba sepse

Participation: Taking part in this project is entirely voluntary. You can withdraw from the study at any time. You do not have to explain why you want to withdraw from the study, and there are no negative consequences if you withdraw. If you have any questions, please discuss them with members of our team.

Partisipasaun: Partisipa iha projetu ida ne'e kompletamente voluntariu. Ita bo'ot bele sai husi estudu iha kualker tempu. Ita bo'ot la presiza atu esplika razaun tamba saida, no sei la iha konsekuensia negativu karik ita bo'ot sai. Karik ita bo'ot iha kualker pergunta, favor bele hato'o ba membru husi ami nia ekipa.

What is sepsis? Sepsis is the body's extreme response to an infection. It is a common illness around the world and can be life-threatening. There are many different infections which can cause sepsis and these vary from place-to-place.

Saida mak sepse? Sepse mak hanesan resposta extreme husi isin ba infesaun. Ida ne'e hanesan moras komum ida iha mundu tomak no bele fo ameasa ba ema nia vida. Eziste infesaun oi-oin ne'ebé bele kauza sepse no ida ne'e la hanesan husi fatin ida ba fatin seluk.

Who is organising the project? The study is a collaboration between:

Se mak organiza projetu ida ne'e? Estudu ida ne'e kolaborasaun ida entre:

Ministry of Health, Timor-Leste / Ministeriu da Saúde, Timor-Leste

Hospital Nacional Guido Valadares

National Health Laboratory, Dili / Laboratóriu Nasional Saúde, Dili

Menzies School of Health Research, Australia

Approval for this project. This study has been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia.

Aprovasaun ba projetu ida ne'e. Estudu ida ne'e hetan ona aprovasaun husi Institutu Nasional da Saúde (INS), Timor-Leste no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research iha Australia.

Informed Consent Information / Informasaun Konsentimentu ne'ebé mak Informadu

Section 1: Purpose of the study. / Seksaun 1: Objetivu husi estudu.

The main purpose of this study is to determine which infections most commonly cause people to be unwell with sepsis in Timor-Leste. It will also describe the common symptoms and signs of sepsis in Timor-Leste, its treatment, and clinical outcomes.

Objetivu prinsipal husi estudu ida ne'e mak atu determina infesaun sira ne'ebé maka komum liu hodi kauza ema la saudavel ho sepse iha Timor-Leste. Ida ne'e mos deskreve sintoma no sinais komum sira husi sepse, ninia tratamentu, no rezultadu kliniku sira iha Timor-Leste.

Section 2: Procedures. For each person, we will:

Seksaun 2: Prosedimentu. Ba kada partisipante, ami sei:

Take an extra BLOOD SAMPLE (in addition to the samples which are being taken for your other routine tests). This will be 4mls if you are an adult, or 1ml if you are a child. / Foti amostra raan extra (alem amostra ne'ebé foti hodi halo ita nia teste rutina seluk). 4mls ba ema bo'ot no 1mls ba labarik kiik.

Test the blood sample for bacteria, viruses, parasites and/or fungi which may be causing your illness. This will occur at the National Health Laboratory (NHL) in Dili. / Halo teste amostra raan ba bakteria, virus, parasite no/ka fungu ne'ebé bele kauza moras. Ida ne'e sei hala'o iha Laboratóriu Nasional Saúde (LNS) iha Díli.

Contact you every day for 10 days and ask you about your child's symptoms. This will be in person if you are at the hospital, or by telephone if you are at home. / Kontaktu ita lorloron durante loron 10 nia laran no husu konaba ita nia oan nian sintoma sira. Karik ita iha hospital ida ne'e sei halo direitamente, no karik ita iha uma ida ne'e sei halo kontaktu liu husi telemovel.

If a cause of your child's illness is identified through this study, then you will be informed about this and your child will be offered treatment (if applicable). / Karik kauza husi ita nia oan nian moras identifika liu husi estudu ida ne'e, ita sei hetan informasaun konaba nia rezultadu no sei oferese tratamentu (karik iha).

Section 3: Duration of Procedures. Collection of the blood sample will take less than 5 minutes. This will be done alongside your other blood tests (using the same needle, without any extra needle sticking)

Seksaun 3: Durasaun ba Prosedimentu. Koleksaun ba amostra ran sei uza tempu pelu menus minutu 10. Prosesu ida ne'e sei hala'o hamutuk ho ita bo'ot nia teste raan sira seluk (uza daun ne'ebé hanesan, sein uza daun extra hodi hasai raan)

Section 4: Potential benefit to your child and others. / Seksaun 4: Benefisiu potensial ba ita nia oan no ema seluk.

If your child participates, their blood will be tested for additional bacteria, viruses, parasites and/or fungi which might be causing your illness. If one is identified, you will be told about this and your child will be offered treatment (if applicable).

Se ita nia oan partisipa, sei halo teste ba ita nia raan hodi hetan baktéria, virus, parazita no/ka fungi adisionál sira ne'ebé bele kauza ba ita nia moras. Karik identifika ida ona, ita sei hetan informasaun konaba ida ne'e no sei oferese tratamentu (karik iha).

The results of the study will provide important and useful information about sepsis Timor-Leste. / Rezultadu sira husi estudu ida ne'e sei fornese informasaun ne'ebé importante no útil konaba sepse nian iha Timor-Leste.

Section 5: Discomforts and Risks. The potential risks of collecting a blood sample include temporary discomfort from the blood collection, bruising, bleeding, and very rarely, infection. These are unlikely to occur because experienced healthcare professionals will take samples.

Seksaun 5: Dezkomfortu no Risku sira. Risku potensiál sira husi rekolla amostra raan nian mak inklui dezkomfortu temporáriu husi kolesaun raan nian, mean, ran sai, no infesaun ne'ebé la komum. Ida ne'e dalabarak la akontese tanba profisionál saúde sira ne'ebé iha esperiénsia mak sei ba foti amostra.

In the unlikely event of any significant injury because of the blood collection procedure, the study team and/or the Ministry of Health will arrange necessary medical treatment without financial cost. However, no financial compensation will be given in case of any study-related injury.

Hare ba kualker kanek/prejuizu signifikante ne'ebé improvavel atu akontese husi prosedimentu koleksaun raan nian, ekipa estudu no/ka Ministeriu Saúde sei organiza tratamentu médiku ne'ebé presiza sein kustu finanseiru. Maske nune'e, sei la iha kompensasaun finanseiru ba kualkér kanek/prejuizu ne'ebé relasiona ho estudu ida ne'e.

Section 6: Costs for participation. Taking part in the project will cost nothing apart from you and your child's time.

Seksaun 6: Kustu ba partisipasaun. Partisipasaun iha projetu ida ne'e, sei la kobre kustu ruma exeptu ita no ita nia oan nian tempu.

Section 7: Compensation for participation. Your family will not receive any payments for being part of this project.

Seksaun 7: Kompensasaun ba Partisipasaun. Ita nia familia sei la simu kualker pagamentu ba partisipasaun iha projetu ida ne'e.

Section 8: Study funding. This study is funded by the Australian Government Department of Foreign Affairs and Trading (DFAT). The Ministry of Health, Timor-Leste has been closely involved in the planning and development of this study and is involved in undertaking this study. The collaborators do not have any conflict of interests to declare.

Seksaun 8: Finansiamentu ba estudu. Estudu ida ne'e hetan finansiamentu husi Departamentu Asuntu Estranjeiru no Komersiu, Guverno Australia (DFAT). Ministeriu Saúde Timor-Leste, proximamente involve ona iha planeamentu no dezenvolvimentu ba estudu no envolve ba implementasaun estudu ida ne'e. Kolaboradores sira la iha kualker konflitu interese atu deklara.

Section 9: Voluntary participation. Taking part in this project is your choice, and you will have the right to stop at any time. If individuals or communities decide not to participate or decide to stop taking part at any time, there will be no penalties.

Seksaun 9: Partisipasaun Voluntariu. Partisipa iha projetu ida ne'e ita bo'ot mak hili, no ita bo'ot sei iha direitu atu hapara/sai iha kualker tempu. Karik individual sira ou komunidade deside atu la partisipa ou deside atu sai husi partisipasaun iha kualker tempu, ida ne'e sei la iha penalidade ruma.

Section 10: Delivery of results. If your child's blood test shows that they have a virus or parasite, you will be contacted and they will be offered the appropriate treatment (if applicable).

Seksaun 10: Fornese rezultadu sira. Se Ita nia oan nian teste raan hatudu katak Ita iha virus ka parazita ruma, ita sei hetan kontaktu no sei oferese tratamentu ne'ebé apropriadu (karik aplika).

You will not be contacted if the test is negative. / Ita sei la hetan kontaktu ruma karik teste ne'e negativu.

Results for the whole study will be written-up and may be published. It may be used by the Ministry of Health in Timor-Leste to guide future management of sepsis in the country. / Rezultadu estudu tomak sei hakerek no bele publikada. Ministériu Saúde iha Timor-Leste bele uza hodi orienta jestaun ba sepse iha rai-laran ba iha futuru.

Section 11: Privacy and confidentiality. During this study, your child's personal information will be kept strictly confidential. Blood samples will be labelled using their initials, date-of-birth and patient number only. These will be transported directly to NHL where they will be stored securely before and after analysis.

Seksaun 11: Privasidade no konfidensialidade. Durante estudu ne'e, ita-boot nia oan nia informasaun pesoál sei nafatin asegura metin ninia konfidensialidade. Amostra raan sira sei marka deit uza ninia inisiál naran nia, data moris no númeru pasiente. Amostra sira ne'e sei lori diretamente ba LNS, iha ne'ebé sei rai ho seguru antes no depois halo analiza.

No personally identifiable information will be shared in any project reports, publication, or presentations. We will keep each individual's participation in this project confidential to the extent permitted by law. However, it is possible that other people may become aware of their participation in this study, e.g. the following people/groups may inspect project records:

Sei la fahe informasaun personal ne'ebé mak identifikavel iha kualkér relatóriu sira projetu nian, publikasaun, ka aprezentasaun sira. Ami sei mantein ema ida-idak nia partisipasaun iha projetu ne'e ho konfidensiál to'o iha medida ne'ebé lei permite. Maske nune'e, sei iha ema balun bele hatene konaba partisipante sira iha estudu ne'e, hanesan ema/grupu sira tuir mai ne'ebé mak sei bele halo inspeksaun ba projetu nia rejistu:

The Instituto Nacional da Saúde; INS and the Ministry of Health, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia

Instituto Nacional da Saúde (INS) no Ministeriu Saude, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia

Section 12: Contact information for questions or concerns. You have the right to ask any questions you may have about this study. If you have questions, complaints or concerns or believe your child may have developed an injury related to this study, please contact Salvador Amaral on email: Salvador.amaral@menzies.edu.au or mobile: +670 7708 7665. If you have questions regarding your rights as a study participant or concerns regarding the ethical conduct of the project, please contact Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au). If your child has any medical problems associated with this study, such as a pain from where your blood was collected or feeling unwell, please contact the above phone numbers or attend your local hospital or clinic if urgent.

Seksaun 12: Informasaun kontaktu karik iha pergunta ou preokupasaun ruma. Ita bo'ot iha direitu hodi husu kualker pergunta ne'ebé mak relasiona ho estudu ida ne'e. Karik ita bo'ot iha pergunta, reklamasaun ou preokupasaun ou karik ita bo'ot nia oan hetan injuria relasiona ho estudu ida ne'e, favor kontaktu ba Salvador Amaral on email: Salvador.amaral@menzies.edu.au or mobile: +670 7708 7665. Karik ita bo'ot iha pergunta relasiona ho ita bo'ot nia direitu nudar partisipante ou preokupasaun relasiona ho projetu nia étika konduta, favor kontaktu Sekretariu étiku, komisaun etika peskija komite tekniku INS (Ph +670 7708 7665) ou Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (telemovel +618 8946 8600; Email ethics@menzies.edu.au). Karik ita bo'ot iha kualker problema mediku ne'ebé mak asosiadu ho estudu ida ne'e, por ezemplu hanesan moras iha fatin hasai ran ou sente la saudavel, favor kontaktu ba numeru ne'ebé mensiona iha leten ou konsulta iha hospital lokal ou klinika sira karik urgente.

Section 13: Consent to participate in project. Before making the decision about your child participating in this study you should have:

Discussed this project with a member of the study team

Read and understood the information in this form

Had the opportunity to ask any questions that you may have

Had time to consider whether your child should take part

Information about who to contact in case you have any further questions or problems

Seksaun 13: konsentimentu atu partisipa iha projetu. Antes foti desizaun atu partisipa iha estudu ida ne'e ita bo'ot tenke:

Diskute uluk konaba projetu ida ne'e ho membru husi ekipa estudu nian

Le'e no komprende informasaun iha formulariu ida ne'e

Iha oportunidade hodi husu kualker pergunta ne'ebé mak ita bo'ot iha

Iha tempu hodi hanoin antes deside ita nia oan atu partisipa

Infomasaun konaba se mak ita bo'ot atu kontaktu, kuandu ita bo'ot iha tan pergunta ou problema ruma.

Section 14: Ethics Committee Clearance. The ethical aspects of this research have been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia (Protocol HREC-2021-4064). If you have any concerns about this research, please contact: Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au).

Seksaun 14: Lisensa Komite Étika. Aspektu etikal husi peskiza ida ne'e hetan ona aprovasaun husi Institutu Nasional Saude Timor-Leste (INS) no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia (Protokolu HREC-2021-4064). Karik ita bo'ot iha kualker preokupasaun konaba peskiza ida ne'e, favor kontaktu: Sekretariu Étiku, Komisaun Étiku Peskija Komite Tekniku INS (Telemovel +670 7708 7665) ou the Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (Telemovel +618 8946 8600; Email: ethics@menzies.edu.au).

## Consent form for parent/guardian

## Formulariu Konsentimentu Inan-aman/kuidador

Principle Investigator: Dr Gustodio de Jesus, todiva3d2021@gmail.com, +670 7754 9505

Local Study Coordinator: Salvador Amaral, Salvador.amaral@menzies.edu.au, +670 7708 7665

# INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE (ISIN-MANAS STUDY)

# INVESTIGASAUN BA SEPSE LIU HUSI IMPLEMENTASAUN BA ESTRATEJIA FOUN BA TESTE AMPLIFIKASAUN MIKROBIOLOJIKA NO BA ASIDU NUKLEIKU IHA TIMOR-LESTE (ISIN-MANAS STUDY)

## Informed Consent Form / Formulário Konsentimentu Informadu

(For adults who self-identify as the parent/guardian of a potential participant who is a child) / (Ba adultu sira ne'e identifika an nu'udar inan-aman/kuidador ba partisipante potensial ne'ebé labarik)

This means you can say NO / Ida ne'e signifika ita bele dehan LAE

| |
|---|
| PRINCIPAL INVESTIGATOR / INVESTIGADOR PRINSIPAL: Dr Gustodio de Jesus |
| STUDY COLLABORATORS / KOLABORADORES BA ESTUDU: |
| Hospital Nacional Guido Valadares |
| Ministry of Health (MOH), Timor-Leste / Ministeriu da Saúde, Timor-Leste |
| National Health Laboratory, Dili, Timor-Leste / Laboratoriu Nasional Saude, Dili, Timor-Leste |
| Menzies School of Health Research, Australia |

I understand the aim of this research study is to improve understanding of sepsis in Timor-Leste. Details of the study have been explained to me, and I have been provided with a written information sheet and given the opportunity to ask questions.

Hau komprende objetivu husi peskiza ne'e atu hadi'a kompriensaun konaba total ema ne'ebé hetan ona espozisaun no/ka vasinasaun kontra moras infesiozu sira iha Timor-Leste. Detallu konaba estudu ne'e esplika ona mai ha'u, no ha'u hetan ona informasaun eskrita ida no hetan oportunidade atu husu pergunta.

I acknowledge that: / Hau rekonhese katak:

Any risks and possible effects of having a venous blood test have been explained to my satisfaction;

Kualker risku no efeitu ne'ebé mak posivel atu hetan tamba teste ran venozu nian, esplika ona mai hau nia satisfasaun;

Taking part in this study is voluntary and I am aware that my child can stop taking part at any time without explanation or prejudice and to withdraw any unprocessed data I have provided;

Hola parte iha estudu ida ne'e voluntariu no hau hatene katak hau nia oan nia bele para partisipa iha kualker tempu sem explikasaun ou prekonseitu, no dada fila fali kualker dadus ne'ebé seidauk prosesa, ne'ebé mak hau fornese.

Any information my child gives will be kept strictly confidential and that no names will be used to identify my child in this study without my approval.

Kualker informasaun ne'ebé mak hau nia oan fó, sei rai estreitamente konfidensial no sei la uza kualker naran hodi identifika hau iha estudu ida ne'e sem hau nia aprovasaun

By providing my name and signature, I agree to:

Liu husi fó hau nia naran no asinatura, hau konkorda atu:

| | | | | |
|---|---|---|---|---|
| Additional blood sample being taken (at the same time as my child's other blood tests) / Hasai amostra raan adisional (iha tempu hnesan ho mós hau nia oan nian teste raan sira seluk) | | Yes<br>Sim | | No<br>Lae |
| My child's blood samples being tested for bacteria, viruses, parasites and fungi which may be causing their illness, including in partner laboratories outside Timor-Leste if tests aren't available locally/ Hau nia oan nian amostra raan teste ba bakteria, virus, parasite no fungu sira ne'ebé bele kauza hau nia moras, inklui iha laboratoriu parseria seluk iha rai-liur karik teste la disponivel iha laboratoriu lokal | | Yes<br>Sim | | No<br>Lae |
| Being contacted each day for 10 days. This would be in person if I am at the hospital, or by telephone if I am at home home / Hetan kontaktu lor-loron durante loron 10. Ida ne'e sei direitamente karik hau baixa iha hospital, ka husi telemovel karik hau iha ona uma | | Yes<br>Sim | | No<br>Lae |
| The use of my child's data for the purposes of this study (all data will be de-identified before use) / Uza hau nia oan nian dadus ba objetivu estudu ida ne'e nian (dadus hotu sei la identifikavel molok uza) | | Yes<br>Sim | | No<br>Lae |

| | |
|---|---|
| Name of participant / Naran partisipante: *(printed)* | Unique identifier: / Identifikador uniku: |
| Signature of parent/guardian : / Asinatura husi inan-aman/kuidador: | Date / Data: |
| Name of witness/interpreter: / Naran testemunha/tradutor: *(printed)* | Signature of witness/interpreter: / Asinatura husi testemunha/tradutor: |
| Name of researcher / Naran peskizador: *(printed)* | Signature of researcher / Asinatura husi peskizador: |

# Information sheet for personal consultee

## Folha infromasaun ba konsultor pessoal nian

Principle Investigator / Investigador Prinsipal: Dr Gustodio de Jesus, todiva3d2021@gmail.com, +670 7754 9505

Local Study Coordinator / Kordenador Estudu Lokal : Salvador Amaral, Salvador.amaral@menzies.edu.au, +670 7708 7665

INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE

(ISIN-MANAS STUDY)

INVESTIGASAUN BA SEPSE LIU HUSI IMPLEMENTASAUN BA ESTRATEJIA FOUN BA TESTE AMPLIFIKASAUN MIKROBIOLOJIKA NO BA ASIDU NUKLEIKU IHA TIMOR-LESTE

(ISIN-MANAS STUDY)

## This is for you to keep / Ida ne'e fó ba ita bo'ot atu rai

Your friend/spouse/relative is being invited to take part in a study about sepsis / Ita nian kolega/kaben/relativu hetan konvite atu hola parte iha estudu konaba sepse nian

Participation: Taking part in this project is entirely voluntary. You can withdraw from the study at any time. You do not have to explain why you want to withdraw from the study, and there are no negative consequences if you withdraw. If you have any questions, please discuss them with members of our team.

Partisipasaun: Partisipa iha projetu ida ne'e kompletamente voluntariu. Ita bo'ot bele sai husi estudu iha kualker tempu. Ita bo'ot la presiza atu esplika razaun tamba saida, no sei la iha konsekuensia negativu karik ita bo'ot sai. Karik ita bo'ot iha kualker pergunta, favor bele hato'o ba membru husi ami nia ekipa.

What is sepsis? Sepsis is the body's extreme response to an infection. It is a common illness around the world and can be life-threatening. There are many different infections which can cause sepsis and these vary from place-to-place.

Saida mak sepse? Sepse mak hanesan resposta extreme husi isin ba infesaun. Ida ne'e hanesan moras komum ida iha mundu tomak no bele fo ameasa ba ema nia vida. Eziste infesaun oi-oin ne'ebé bele kauza sepse no ida ne'e la hanesan husi fatin ida ba fatin seluk.

Who is organising the project? The study is a collaboration between:

Se mak organiza projetu ida ne'e? Estudu ida ne'e kolaborasaun ida entre:

Ministry of Health, Timor-Leste / Ministeriu da Saúde, Timor-Leste

Hospital Nacional Guido Valadares

National Health Laboratory, Dili / Laboratóriu Nasional Saúde, Dili

Menzies School of Health Research, Australia

Approval for this project. This study has been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia.

Aprovasaun ba projetu ida ne'e. Estudu ida ne'e hetan ona aprovasaun husi Institutu Nasional da Saúde (INS), Timor-Leste no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research iha Australia.

Informed Consent Information / Informasaun Konsentimentu ne'ebé mak Informadu

Section 1: Purpose of the study. / Seksaun 1: Objetivu husi estudu.

The main purpose of this study is to determine which infections most commonly cause people to be unwell with sepsis in Timor-Leste. It will also describe the common symptoms and signs of sepsis in Timor-Leste, its treatment, and clinical outcomes.

Objetivu prinsipal husi estudu ida ne'e mak atu determina infesaun sira ne'ebé maka komum liu hodi kauza ema la saudavel ho sepse iha Timor-Leste. Ida ne'e mos deskreve sintoma no sinais komum sira husi sepse, ninia tratamentu, no rezultadu kliniku sira iha Timor-Leste.

Section 2: Procedures. For each person, we will:

Seksaun 2: Prosedimentu. Ba kada partisipante, ami sei:

Take an extra BLOOD SAMPLE (in addition to the samples which are being taken for your other routine tests). This will be 4mls if you are an adult, or 1ml if you are a child. / Foti amostra raan extra (alem amostra ne'ebé foti hodi halo ita nia teste rutina seluk). 4mls ba ema bo'ot no 1mls ba labarik kiik.

Test the blood sample for bacteria, viruses, parasites and/or fungi which may be causing your illness. This will occur at the National Health Laboratory (NHL) in Dili. / Halo teste amostra raan ba bakteria, virus, parasite no/ka fungu ne'ebé bele kauza moras. Ida ne'e sei hala'o iha Laboratóriu Nasional Saúde (LNS) iha Díli.

Contact you every day for 10 days and ask you about your friend/spouse/relative's symptoms. This will be in person if you are at the hospital, or by telephone if you are at home. / Kontaktu ita lor-loron durante loron 10 nia laran no husu konaba ita nia kolega/kaben/relativu nian sintoma sira. Karik ita iha hospital ida ne'e sei halo direitamente, no karik ita iha uma ida ne'e sei halo kontaktu liu husi telemovel.

If a cause of your friend/spouse/relative's illness is identified through this study, then you will be informed about this and your friend/spouse/relative will be offered treatment (if applicable). / Karik kauza husi ita nia kolega/kaben/relativu nian moras identifika liu husi estudu ida ne'e, ita sei hetan informasaun konaba ida ne'e no ita nia kolega/kaben/relativu sei hetan tratamentu (karik iha).

Section 3: Duration of Procedures. Collection of the blood sample will take less than 5 minutes. This will be done alongside your other blood tests (using the same needle, without any extra needle sticking)

Seksaun 3: Durasaun ba Prosedimentu. Koleksaun ba amostra ran sei uza tempu pelu menus minutu 10. Prosesu ida ne'e sei hala'o hamutuk ho ita bo'ot nia teste raan sira seluk (uza daun ne'ebé hanesan, sein uza daun extra hodi hasai raan)

Section 4: Potential benefit to your friend/spouse/relative and others. / Seksaun 4: Benefisiu potensial ba ita nia kolega/kaben/familia no ema seluk.

If your friend/spouse/relative participates, their blood will be tested for additional bacteria, viruses, parasites and/or fungi which might be causing their illness. If one is identified, you will be told about this and your friend/spouse/relative will be offered treatment (if applicable).

Se ita nia kolega/kaben/familia partisipa, sei halo teste ba ita nia raan hodi hetan baktéria, virus, parazita no/ka fungi adisionál sira ne'ebé bele kauza ba sira nian moras. Karik identifika ida ona, ita sei hetan informasaun ida ne'e no ita nia kolega/kaben/relativu sei hetan tratamentu (karik iha).

The results of the study will provide important and useful information about sepsis Timor-Leste. / Rezultadu sira husi estudu ida ne'e sei fornese informasaun ne'ebé importante no útil konaba sepse nian iha Timor-Leste.

Section 5: Discomforts and Risks. The potential risks of collecting a blood sample include temporary discomfort from the blood collection, bruising, bleeding, and very rarely, infection. These are unlikely to occur because experienced healthcare professionals will take samples.

Seksaun 5: Dezkomfortu no Risku sira. Risku potensiál sira husi rekolla amostra raan nian mak inklui dezkomfortu temporáriu husi kolesaun raan nian, mean, ran sai, no infesaun ne'ebé la komum. Ida ne'e dalabarak la akontese tanba profisionál saúde sira ne'ebé iha esperiénsia mak sei ba foti amostra.

In the unlikely event of any significant injury because of the blood collection procedure, the study team and/or the Ministry of Health will arrange necessary medical treatment without financial cost. However, no financial compensation will be given in case of any study-related injury.

Hare ba kualker kanek/prejuizu signifikante ne'ebé improvavel atu akontese husi prosedimentu koleksaun raan nian, ekipa estudu no/ka Ministeriu Saúde sei organiza tratamentu médiku ne'ebé presiza sein kustu finanseiru. Maske nune'e, sei la iha kompensasaun finanseiru ba kualkér kanek/prejuizu ne'ebé relasiona ho estudu ida ne'e.

Section 6: Costs for participation. Taking part in the project will cost nothing apart from you and your friend/spouse/relative's time.

Seksaun 6: Kustu ba partisipasaun. Partisipasaun iha projetu ida ne'e, sei la kobre kustu ruma exeptu ita bo'ot no ita nia kolega/kaben/relativu nia tempu.

Section 7: Compensation for participation. You nor your friend/spous/relative will not receive any payments for being part of this project.

Seksaun 7: Kompensasaun ba Partisipasaun. Ita bo'ot ka ita nia kolega/kaben/relativu sei la simu kualker pagamentu ba partisipasaun iha projetu ida ne'e.

Section 8: Study funding. This study is funded by the Australian Government Department of Foreign Affairs and Trading (DFAT). The Ministry of Health, Timor-Leste has been closely involved in the planning and development of this study and is involved in undertaking this study. The collaborators do not have any conflict of interests to declare.

Seksaun 8: Finansiamentu ba estudu. Estudu ida ne'e hetan finansiamentu husi Departamentu Asuntu Estranjeiru no Komersiu, Guverno Australia (DFAT). Ministeriu Saúde Timor-Leste, proximamente involve ona iha planeamentu no dezenvolvimentu ba estudu no envolve ba implementasaun estudu ida ne'e. Kolaboradores sira la iha kualker konflitu interese atu deklara.

Section 9: Voluntary participation. Taking part in this project is your choice, and you will have the right to stop at any time. If individuals or communities decide not to participate or decide to stop taking part at any time, there will be no penalties.

Seksaun 9: Partisipasaun Voluntariu. Partisipa iha projetu ida ne'e ita bo'ot mak hili, no ita bo'ot sei iha direitu atu hapara/sai iha kualker tempu. Karik individual sira ou komunidade deside atu la partisipa ou deside atu sai husi partisipasaun iha kualker tempu, ida ne'e sei la iha penalidade ruma.

Section 10: Delivery of results. If your friend/spouse/relative's blood test shows that they have a virus or parasite, you will be contacted and they will be offered the appropriate treatment (if applicable).

Seksaun 10: Fornese rezultadu sira. Se Ita nia kolega/kaben/relativu nian teste raan hatudu katak Ita iha virus ka parazita ruma, ita sei hetan kontaktu no sei hetan tratamentu ne'ebé apropriadu (karik aplika).

You will not be contacted if the test is negative. / Ita sei la hetan kontaktu ruma karik teste ne'e negativu.

Results for the whole study will be written-up and may be published. It may be used by the Ministry of Health in Timor-Leste to guide future management of sepsis in the country. / Rezultadu ba estudu tomak sei hakerek no bele publika. Ministériu Saúde iha Timor-Leste bele uza hodi orienta jestaun ba sepese nian iha rai-laran ba iha futuru.

Section 11: Privacy and confidentiality. During this study, your friend/spouse/relative's personal information will be kept strictly confidential. Blood samples will be labelled using their initials, date-of-birth and patient number only. These will be transported directly to NHL where they will be stored securely before and after analysis.

Seksaun 11: Privasidade no konfidensialidade. Durante estudu ne'e, ita nia kolega/kaben/relativu nian informasaun pesoál sei asegura metin ninia konfidensialidade. Amostra raan sira sei marka deit uza sira nian nia inisiál naran nian, data moris no númeru pasiente de'it. Amostra sira ne'e sei lori diretamente ba LNS, iha ne'ebé sei rai ho seguru antes no depoisde halo analiza.

No personally identifiable information will be shared in any project reports, publication, or presentations. We will keep each individual's participation in this project confidential to the extent permitted by law. However, it is possible that other people may become aware of their participation in this study, e.g. the following people/groups may inspect project records:

Sei la fahe informasaun personal ne'ebé mak identifikavel iha kualkér relatóriu sira projetu nian, publikasaun, ka aprezentasaun sira. Ami sei mantein ema ida-idak nia partisipasaun iha projetu ne'e ho konfidensiál to'o iha medida ne'ebé lei permite. Maske nune'e, sei iha ema balun bele hatene konaba partisipante sira iha estudu ne'e, hanesan ema/grupu sira tuir mai ne'ebé mak sei bele halo inspeksaun ba projetu nia rejistu:

The Instituto Nacional da Saúde; INS and the Ministry of Health, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia

Instituto Nacional da Saúde (INS) no Ministeriu Saude, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia

Section 12: Contact information for questions or concerns. You have the right to ask any questions you may have about this study. If you have questions, complaints or concerns or believe your friend/spouse/relative may have developed an injury related to this study, please contact Salvador Amaral on email: Salvador.amaral@menzies.edu.au or mobile: +670 7708 7665. If you have questions regarding your rights as a study participant or concerns regarding the ethical conduct of the project, please contact Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au). If your friend/spouse/relative has any medical problems associated with this study, such as a pain from where your blood was collected or feeling unwell, please contact the above phone numbers or attend your local hospital or clinic if urgent.

Seksaun 12: Informasaun kontaktu karik iha pergunta ou preokupasaun ruma. Ita bo'ot iha direitu hodi husu kualker pergunta ne'ebé mak relasiona ho estudu ida ne'e. Karik ita bo'ot iha pergunta, reklamasaun ou preokupasaun ou karik ita nia kolega/kaben/relativu hetan injuria relasiona ho estudu ida ne'e, favor kontaktu ba Salvador Amaral on email: Salvador.amaral@menzies.edu.au or mobile: +670 7708 7665. Karik ita bo'ot iha pergunta relasiona ho ita bo'ot nia direitu nudar partisipante ou preokupasaun relasiona ho projetu nia étika konduta, favor kontaktu Sekretariu étiku, komisaun etika peskija komite tekniku INS (Ph +670 7708 7665) ou Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (telemovel +618 8946 8600; Email ethics@menzies.edu.au). Karik ita nia kolega/kaben/relativu hetan kualker problema mediku ne'ebé mak asosiadu ho estudu ida ne'e, por ezemplu hanesan moras iha fatin hasai ran ou sente la saudavel, favor kontaktu ba numeru ne'ebé mensiona iha leten ou konsulta iha hospital lokal ou klinika sira karik urgente.

Section 13: Consent to participate in project. Before making the decision about your friend/spouse/relative participating in this study you should have:

Discussed this project with a member of the study team

Read and understood the information in this form

Had the opportunity to ask any questions that you may have

Had time to consider whether your friend/spouse/relative should take part

Information about who to contact in case you have any further questions or problems

Seksaun 13: konsentimentu atu partisipa iha projetu. Antes foti desizaun atu partisipa iha estudu ida ne'e ita nia kolega/kaben/relativu tenke:

Diskute uluk konaba projetu ida ne'e ho membru husi ekipa estudu nian

Le'e no komprende informasaun iha formulariu ida ne'e

Iha oportunidade hodi husu kualker pergunta ne'ebé mak ita bo'ot iha

Iha tempu hodi hanoin antes ita nia kolega/kaben/relativu deside atu partisipa

Infomasaun konaba se mak ita bo'ot atu kontaktu, kuandu ita bo'ot iha tan pergunta ou problema ruma.

Section 14: Ethics Committee Clearance. The ethical aspects of this research have been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia (Protocol HREC-2021-4064). If you have any concerns about this research, please contact: Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au).

Seksaun 14: Lisensa Komite Étika. Aspektu etikal husi peskiza ida ne'e hetan ona aprovasaun husi Institutu Nasional Saude Timor-Leste (INS) no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia (Protokolu HREC-2021-4064). Karik ita bo'ot iha kualker preokupasaun konaba peskiza ida ne'e, favor kontaktu: Sekretariu Étiku, Komisaun Étiku Peskija Komite Tekniku INS (Telemovel +670 7708 7665) ou the Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (Telemovel +618 8946 8600; Email: ethics@menzies.edu.au).

## Declaration form for personal consultee

## Formulariu Konsentimentu ba konsultor pessoal nian

Principle Investigator: Dr Gustodio de Jesus, todiva3d2021@gmail.com, +670 7754 9505

Local Study Coordinator: Salvador Amaral, Salvador.amaral@menzies.edu.au, +670 7708 7665

# INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE (ISIN-MANAS STUDY)

# INVESTIGASAUN BA SEPSE LIU HUSI IMPLEMENTASAUN BA ESTRATEJIA FOUN BA TESTE AMPLIFIKASAUN MIKROBIOLOJIKA NO BA ASIDU NUKLEIKU IHA TIMOR-LESTE (ISIN-MANAS STUDY)

## Informed Declaration Form / Formulário Deklarasaun Informadu

(For those who self-identify as the friend/spouse/relative of a potential participant who lacks capacity to make decisions about participation themselves) / (Ba sira ne'ebé identifika an nu'udar kolega/kaben/relativu ba partisipante potensiál ne'ebé laiha kapasidade atu foti desizaun kona-ba partisipasaun ba nia an rasik)

This means you can say NO / Ida ne'e signifika ita bele dehan LAE

| |
|---|
| PRINCIPAL INVESTIGATOR / INVESTIGADOR PRINSIPAL: Dr Gustodio de Jesus |
| STUDY COLLABORATORS / KOLABORADORES BA ESTUDU: |
| Hospital Nacional Guido Valadares |
| Ministry of Health (MOH), Timor-Leste / Ministeriu da Saúde, Timor-Leste |
| National Health Laboratory, Dili, Timor-Leste / Laboratoriu Nasional Saude, Dili, Timor-Leste |
| Menzies School of Health Research, Australia |

I understand the aim of this research study is to improve understanding of sepsis in Timor-Leste. Details of the study have been explained to me, and I have been provided with a written information sheet and given the opportunity to ask questions.

Hau komprende objetivu husi peskiza ne'e atu hadi'a kompriensaun konaba total ema ne'ebé hetan ona espozisaun no/ka vasinasaun kontra moras infesiozu sira iha Timor-Leste. Detallu konaba estudu ne'e esplika ona mai ha'u, no ha'u hetan ona informasaun eskrita ida no hetan oportunidade atu husu pergunta.

I acknowledge that: / Hau rekonhese katak:

Any risks and possible effects of having a venous blood test have been explained to my satisfaction;

Kualker risku no efeitu ne'ebé mak posivel atu hetan tamba teste ran venozu nian, esplika ona mai hau nia satisfasaun;

Taking part in this study is voluntary and I believe that my friend/spouse/relative would agree to participate if they were able to make decisions for themselves.

Hola parte iha estudu ida ne'e voluntariu no hau fiar katak hau nia kolega/kaben/relative sei aseita hodi partisipa karik sira bele foti desizaun rasik ba sira nian an.

Any information my friend/spouse/relative gives will be kept strictly confidential and that no names will be used to identify my friend/spouse/relative in this study without my approval.

Kualker informasaun ne'ebé mak hau nia kolega/kaben/relative fó sei rai estreitamente konfidensial no sei la uza kualker naran hodi identifika hau nia kolega/kaben/relativu iha estudu ida ne'e sem hau nia aprovasaun

By providing my name and signature, I understand that:

Liu husi fó hau nia naran no asinatura, hau konkorda atu:

| | | | | |
|---|---|---|---|---|
| An additional blood sample will be taken (at the same time as my friend/spouse/relative's other blood tests) / Hasai amostra raan adisional (iha tempu hnesan ho mós hau nia kolega/kaben/relative nain teste raan sira seluk) | | Yes<br>Sim | | No<br>Lae |
| My friend/spouse/relative's blood samples will be tested for bacteria, viruses, parasites and fungi which may be causing their illness, including in partner laboratories outside Timor-Leste if tests aren't available locally/ Hau nia kolega/kaben/relative nian amostra raan teste ba bakteria, virus, parasite no fungu sira ne'ebé bele kauza hau nia moras, inklui iha laboratoriu parseria sira iha rai-liur karik teste la disponivel iha laboratoriu lokal | | Yes<br>Sim | | No<br>Lae |
| I may be contacted each day for 10 days. This would be in person if I am at the hospital, or by telephone if I am at home home / Hetan kontaktu lor-loron durante loron 10. Ida ne'e sei direitamente karik hau baixa iha hospital, ka husi telemovel karik hau iha ona uma | | Yes<br>Sim | | No<br>Lae |
| My friend/spouse/relative's data will be used for the purposes of this study (all data will be de-identified before use) / Uza hau nia kolega/kaben/relativu dadus ba objetivu estudu ida ne'e nian (dadus hotu sei la identifikavel molok uza) | | Yes<br>Sim | | No<br>Lae |

| | |
|---|---|
| Name of participant / Naran partisipante: *(printed)* | Unique identifier: / Identifikador uniku: |
| Signature of personal consultee: / Asinatura husi konsultor pessoal: | Date / Data: |
| Name of witness/interpreter: / Naran testemunha/tradutor: *(printed)* | Signature of witness/interpreter: / Asinatura husi testemunha/tradutor: |
| Name of researcher / Naran peskizador: *(printed)* | Signature of researcher / Asinatura husi peskizador: |

INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE (ISIN-MANAS STUDY)

BASELINE DATA COLLECTON FORM

FORMULARIU BA KOLESAUN DADUS IHA BAZE

(To be filled by attending clinical team and sent to lab with samples)

(prienxe husi ekipa klinikal ne'ebé partisipa no haruka ba laboratóriu hamutuk ho amostra sira)

## DEMOGRAPHIC INFORMATION / INFORMASAUN DEMOGRAFIA

| | | |
|---|---|---|
| 1 | Unique identifier / Identifikador Úniku | <Attach sticker here><br>< Anexa sticker iha ne'e> |
| 3 | Date of hospital attendance / Data atendimentu husi hospital | ___/___/___ |
| 4 | First name / Naran prinsipal | |
| 5 | Family name / Naran Familia (Apelidu) | |
| 6 | Gender / Jeneru | □ Female/Feto □ Male/Mane |
| 7 | Age in years / Idade iha tinan ida ne'e | |
| 8 | Date of birth / Data Moris | ___/___/___ □ Unknown / La hatene |
| 9 | Primary telephone number / Numeru telemovel primariu | |

| | | |
|---|---|---|
| 10 | Secondary telephone number / Numeru telemovel sekundariu | |
| 11 | Other telephone number / Numeru telemovel seluk<br>(If applicable) (karik iha) | |
| 12 | Address / Hela fatin | Municipality / Munisipiu:<br>Suco / Suku:<br>Aldeia: |

## CLINICAL ASSESSMENT / AVALIASAUN KLINIKAL

| | | |
|---|---|---|
| 13 | Duration of illness (days) / Durasaun husi moras nian (loron) | |
| 14 | Duration of fever symptoms (days) / Durasaun husi sintoma isin manas nian (loron) | |
| 15 | Has the participant been an inpatient at any healthcare facility during the last 7 days? / Pasiente baixa ona iha sentru saude balun durante loron 7 ikus ne'e? | □ Yes / Sim □ No / Lae |
| 16 | If yes, which facility? / Karik sim, fasilidade ida ne'ebé? | |
| 17 | Did the participant receive any antimicrobial treatment during the last 7 days? / Pasiente simu tratamentu antimikribiu ruma durante loron 7 ikus ne'e?<br>(This might be antimicrobials which were bought over the counter from a pharmacy or shop, or those prescribed by a healthcare worker) / (Ida ne'e bele inklui mos antimikrobiu ne'ebe hola sem reseita husi apotik ka loja, ka reseita husi professional saude) | □ Yes / Sim □ No / Lae |
| 18 | If yes, provide details<br>Karik sim, favor fornese nia detallu sira | Drug 1: Dose: Route:<br>Ai-moruk 1: Doze: Rota:<br>Drug 2: Dose: Route:<br>Ai-moruk 2: Doze: Rota:<br>Drug 3: Dose: Route:<br>Ai-moruk 3: Doze: Rota:<br>Drug 4: Dose: Route:<br>Ai-moruk 4: Doze: Rota: |
| 19 | Are there any clinical features which localise an infection to any of the following sites?<br>(Leave all boxes unticked if the febrile illness is truly 'undifferentiated') / Karik iha karateristika klinikal ruma ne'ebé bele lokaliza infesaun ba fatin sira tuir mai? (Lalika tau vistu karik isin- | □ Lower respiratory tract / Vias respiratoriu inferior<br>□ Gastro-intestinal tract / intra-abdominal infection / Tratu-gastrointestinal / infesaun intra-abdominal<br>□ Urinary tract / Tratu urinariu<br>□ Skin / soft tissue / Kulit/tesidu mole<br>□ Central nervous system / Sistema nervozu sentral<br>□ Other (please specify): / Seluk (favor espesifika): |

| | | |
|---|---|---|
| | manas la bele halo diferensia ka 'indiferensiada') | |
| 20 | Vital signs at presentation<br>(Must be ≥37.5°C for inclusion in study)<br>Sinál vital sira iha aprezentasaun<br>(Tenke ≥37.5°C hodi bele inklui iha estudu) | Temperature (°C): / Temperatura (°C):<br>Pulse rate (bpm): / Frekuensia pulsu nian (bpm):<br>Blood pressure (mmHg): / Presaun sanguínea/ran (mmHg):<br>Sp02 / oxygen saturation (%): / Sp02 / saturasaun ba oxijêniu (%):<br>Respiratory rate (bpm): / Frekuensia respiratóriu (bpm): |

## MANAGEMENT / JESTAUN

| | | |
|---|---|---|
| 21 | Admission diagnosis / Admisaun Diagnóstiku | |
| 22 | Details of antimicrobial treatments given on admission<br>Detallu konaba tratamentu antikimrobial ne'ebé fó momentu admisaun/konsulta/baixa. | Drug 1: Dose: Route:<br>Ai-moruk 1: Doze: Rota:<br>Drug 2: Dose: Route:<br>Ai-moruk 2: Doze: Rota:<br>Drug 3: Dose: Route:<br>Ai-moruk 3: Doze: Rota:<br>Drug 4: Dose: Route:<br>Ai-moruk 4: Doze: Rota: |
| 23 | Planned discharge destination<br>(Leave blank if this is not decided yet)<br>Planu fatin deskarga<br>(Husik mamuk karik ida ne'e seidauk deside) | □ Admit to this hospital / Tama iha hospital ida ne'e<br>□ Transfer to another hospital / Muda ba hospital seluk<br>□ Stay in emergency department (until the next day) / Baixa iha departamentu emerjensia (to'o loron tuir mai)<br>□ Discharge home / Fila ba uma<br>□ Died / Obitu |
| 24 | If admitted, which ward did the participant go to?<br>Karik baixa, entaun pasiente baixa iha enfermaria ida ne'ebé? | |

FOR LAB TEAM ONLY:

| | | |
|---|---|---|
| 25 | Write the blood culture accession number here (once blood culture is booked-in):<br><br>Hakerek numero accession cultivo ran nian iha ne'e (wainhira simu ona cultivo ran) | |

INVESTIGATION OF SEPSIS THROUGH THE IMPLEMENTATION OF A NOVEL MICROBIOLOGICAL AND NUCLEIC ACID AMPLIFICATION TESTING STRATEGY IN TIMOR-LESTE (ISIN-MANAS STUDY)

FOLLOW-UP DATA COLLECTON FORM
FORMULÁRIU KOLLETA DADUS AKOMPANHAMENTU NIAN

(To be filled by research nurses every day for 10 days following study enrolment, and on day 30) / (Preenxhe husi emfremeiru peskiza sira lor-loron durante loron 10 nia laran depoisde halo inskrisaun ba estudu, no iha loron 30)

## DEMOGRAPHIC INFORMATION / INFORMASAUN DEMOGRAFIKU

| | | |
|---|---|---|
| 1 | Unique identifier / Identifikador Úniku | |
| 2 | Date of follow-up / Loron akompanhamentu | ___/___/___ |
| 3 | Confirmation of identification of participant by checking full name, age, and address / Komfirmasaun identifikasaun husi partisipante, verifika naran kompletu, tinan no hela fatin | |

## FOLLOW-UP / AKOMPANHAMENTU

| | | |
|---|---|---|
| 13 | Where is the participant currently? / Agora dauduan partisipante iha ne'ebé? | □ Outpatient / Pasiente ne'ebé la baixa ka ambulatoria<br>□ Inpatient (please specify which healthcare facility): / Pasiente ne'ebé baixa (favor espesifika fasilidade saúde ida ne'ebé): |
| 13 | Has there been ongoing symptoms of fever since the last follow-up? / Karik iha sintoma ne'ebé kontinuada desde akompanhamentu ikus?<br>(Ask: "have you experienced fever symptoms since you were enrolled into the study / we last spoke") / (Husu: "ita iha sintoma isin-manas desde kedas administra iha estudu na'a / desde tempu ne'ebé ita kulia'') | □ Yes/Sim □ No/Lae |
| 14 | Has there been any fever ≥37.5°C recorded in the medical notes since the last follow-up? / Karik iha isin-manas ho temperatura ≥37.5°C rejistu ona iha nota medical nian desde akomapnhamentu ikus?<br>(Only answer if participant is currently an inpatient) / (So bele hatan karik pasiente agora dadaun baixa) | □ Yes/Sim □ No/Lae |

| | | |
|---|---|---|
| 18 | Did the participant take any antimicrobial treatment since the last follow-up? / Partisipante simu tratamentu antimikrobiu ruma desde akompanhamentu ikus?<br>(This might be antimicrobials which were bought over the counter from a pharmacy or shop, those which were prescribed by a healthcare worker, or those given while in hospital. Ask specifically whether each medication listed at last follow-up has been continued/stopped, and ask specifically if any new medications have been started) / (Ida ne'e bele hanesan antomikrobiu sira ne'ebé hola husi farmasia ka loja sira ne'ebé preskreve husi professional kuidadu saude, ka ne'ebé hetan iha hospital Husu espesífiku karik ai-moruk ida-idak ne'ebé lista ona iha akompanhamentu ikus kontinua ka lae, no husu espesífiku se ai-moruk foun ruma hahú ona) | Drug 1: Dose: Route:<br>Ai-moruk 1: Doze: Rota:<br>Drug 2: Dose: Route:<br>Ai-moruk 2: Doze: Rota:<br>Drug 3: Dose: Route:<br>Ai-moruk 3: Doze: Rota:<br>Drug 4: Dose: Route:<br>Ai-moruk 4: Doze: Rota:<br>Drug 5: Dose: Route:<br>Ai-moruk 5: Doze: Rota: |

## OUTCOME / REZULTADU

| | | |
|---|---|---|
| 18 | Was the participant discharged from hospital since the last follow-up? / Partisipante alta desde akompanhamentu ikus? | □ Yes/Sim □ No/Lae<br>If yes, date of discharge: ___/___/___<br>Karik sim, data alta nian: ___/___/___<br>If yes, discharge diagnosis / Karik sim, alta ho diagnostiku: |
| 18 | Is the participant known to have died since the last follow-up? / Partisipente obitu desde akompanhamentu ikus? | □ Yes/Sim □ No/Lae<br>If yes, date of death: ___/___/___<br>Karik sim, data obitu: ___/___/___<br>If yes, cause of death? / Karik sim, kauza husi obitu? |

# Appendix 3: Research Ethics Committee approval, participant information sheet and consent form for study defining user requirements for new molecular diagnostic workflows (Chapter 3)

สำนักงานคณะกรรมการจริยธรรมการวิจัยในคน
สำนักงานบริการการวิจัย คณะเวชศาสตร์เขตร้อน
โทรศัพท์ 1349 ต่อ 16/11%08 2306 9126

ท อว78.1116fiÜ.395 duu17 nqvnonu 2565

อนุมัติโครงการและส่งเอกสารรับรองโครงการพร้อมแนวทางปฏิบัติสำหรับโครงการวิจัยที่ได้รับการรับรอง

(โครงการหมายเลข TMEC 22-007)

L%ou รองศาสตราจารย์ ffi.uyquduni<aofiunn iou

ตามที่ท่านส่งโครงการวิจัยฉบับแก้ไขตามมติของคณะกรรมการฯ ครั้งที่ 1 เรื่อง " '(na
นำมาใช้ของเครื่องมือใหม่สำหรับการวินิจฉัยทางโมเลกุล ณ จุดบริการของโรคไข้เดงกี่ในประเทศไทย:
มีส่วนร่วมของผู้ใช้ (Development and implementation of a novel platform for near-pati
diagnosis of dengue in Thailand: a user-engagement study)" (โครงการหมายเลข TM
เพื่อให้คณะกรรมการจริยธรรมการวิจัยในคน คณะเวชศาสตร์เขตร้อน พิจารณาอีกครั้งหนึ่ง นั้น

คณะกรรมการฯ ได้พิจารณาแล้วเห็นชอบและอนุมัติให้รับรองเอกสารโครงการวิจัย ดังนี้

gnivuunuag mfnunnonu for near-patient molecular

TMEC 22-007)

Research proposal (FTM ECF-034-01): version 2 date 12 April 2022

Participant Information Sheet (FTM ECF-020-06(EN)) for Phase 1: English version 2.0 date 20 April 2022

Participant Information Sheet (FTM ECF-020-06(EN)) for Phase 2 : English version 2.0 date 20 April 2022

Participant Information Sheet (FTM ECF-020-06(EN)) for Phase 3 : English version 2.0 date 20 April 2022

Participant Information Sheet (FTM ECF-020-06(TH)) for Phase 1: Thai version 2 date

May 2022

Participant Information Sheet (FTM ECF-020-06(TH)) for Phase 2: Thai version 2 date

May 2022

Participant Information Sheet (FTM ECF-020-06(TH)) for Phase 3: Thai version 2 date

May 2022

Informed Consent Form (FTM ECF-021-08(TH)): Thai version 1.0 date 3 November

2021

Topic Guideline for Focus group for Phase 1; Thai version (date not affixed)

Topic Guideline for Focus group for Phase 1; English version (date not affixed)

Interview Guideline for User Experience Evaluation for Phase 2 ; Thai version (date not affixed)

Interview Guideline for User Experience Evaluation for Phase 2 ; English version

(date not affixed)

Questionnaire for User Acceptance for Phase 3: Thai version (date not affixed)

Questionnaire for User Acceptance for Phase 3: English version (date not affixed)

tan 1 1 d) Progress Report Form/ Certificate of Ethical Approval Extension Request Form (FTM ECF-008-RR) 2 L%udøuäuuun uoninfinnäiöupimüunoauäua%qäu FTM ECF-

010-RR (Notification of Study Closure) lufiguuooulau[f] q;tJüüUmfiÖtJtUflU [6]tu 4 60 TJ

จึงเรียนมาเพื่อโปรดทราบ

2

MUTM 2022-031-01

CERTIFICATE OF ETHICAL APPROVAL

Ethics Committee of the Faculty of Tropical Medicine, Mahidol University

420/6 Ratchawithi Rd., Ratchathewi, Bangkok 10400, Thailand

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This Certificate of Ethical Approval (MUTM 2022-031-01) applies to the

Project entitled: Development and implementation of a novel platform for near-patient molecular diagnosis of dengue in Thailand: a user-engagement study

EC Submission No.: TMEC 22-007 with the following relevant documents:

Research proposal (FTM ECF-034-Ol): version 2 date 12 April 2022

Participant Information Sheet (FTM ECF-020-06(EN)) for Phase 1: English version 2.0 date 20 April 2022

Participant Information Sheet (FTM ECF-020-06(EN)) for Phase 2 : English version 2.0 date 20 April 2022

Participant Information Sheet (FTM ECF-020-06(EN)) for Phase 3 : English version 2.0 date 20 April 2022

Participant Information Sheet (FTM ECF-020-06(TH)) for Phase 1: Thai version 2 date I May

2022

Participant Information Sheet (FTM ECF-020-06(TH)) for Phase 2: Thai version 2 date 1 May

2022

Participant Information Sheet (FTM ECF-020-06(TH)) for Phase 3: Thai version 2 date I May 2022

Informed Consent Form (FTM ECF-021-08(TH)): Thai version 1.0 date 3 November 2021

Topic Guideline for Focus group for Phase l ; Thai version (date not affixed)

Topic Guideline for Focus group for Phase 1; English version (date not affixed)

I l . Interview Guideline for User Experience Evaluation for Phase 2 ; Thai version (date not affixed)

Interview Guideline for User Experience Evaluation for Phase 2 ; English version (date not affixed)

Questionnaire for User Acceptance for Phase 3: Thai version (date not affixed)

Questionnaire for User Acceptance for Phase 3: English version (date not affixed)

I FTM ECF-013-06

| | |
|---|---|
| Principal Investigator: | Assoc. Prof. Dr.Saranath Lawpoolsri Niyom/ Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University |
| Co-investigator(s): | 1. Mr.Sanhapon Ketklao, M.D./ Division of Dengue Hemorrhagic Fever Research, Faculty of Medicine Siriraj Hospital, Mahidol University<br>Assoc. Prof. Dr.Panisadee Avirutnan/ Department of Research and<br>Development, Faculty of Medicine Siriraj Hospital, Mahidol University<br>Dr.Paul Arkell/ Menzies School of Health Research, Timor-Leste Office +<br>Clinical Research Fellow, Imperial College, London<br>Prof.Alison Holmes/ Department of Infectious Diseases, Imperial College,<br>London<br>Dr.Kanokwan Sriruksa, M.D./ Department of Pediatric, Khon Kaen Hospital<br>Dr.Sopon lamsirithaworn, M.D./ Department of Disease Control,<br>The Ministry of Public Health, Thailand<br>This project has been approved for the period from 13 May 2022 to 12 May 2023 |

The Ethics Committee of Faculty of Tropical Medicine certify that we are in compliance with Declaration of Helsinki, ICH Guidelines for Good Clinical Practice and other International Guidelines for Human Research Protection

Signature Signature

(Assoc. Prof. Dr. Jaranit Kaewkungwal) (Mrs. Pornpimon Adams)

Chairperson Member Secretary

Ethics Committee of the Ethics Committee of the

Faculty of Tropical Medicine Faculty of Tropical Medicine i MAY 2022

Date..... Date 17 MAY 2022

2 FTM ECF-013-06

Post-approval Requirements

Ethics Committee, Faculty of Tropical Medicine, Mahidol University

The Certificate of Ethical Approval (CEA) for each research study is valid for 1 year only, from the date of approval.

PI must extend the certificate using Progress Report Form/ Certificate of Ethical Approval Extension Request Form (FTM ECF-008-RR) 2 months prior to the expiry date.

If extension without modifying the project details, PI are requested to send copy ofthe stamped approval research documents (Only documents that wish to be extended this time)

PI must submit the signed letter of permission from authorized person of the implementing institution when the PI received.

PI must submit progress reports after approval according to the stipulated EC requirements related to the identified risk that specified in the cover letter, using Progress Report Form/ Certificate of Ethical Approval Extension Request Form (FTM ECF-008-RR).

Reporting Serious Adverse Event

For reporting Local SAE

- Local serious adverse events which are fatal or life threatening: PI must report to EC immediately, no later than 24 hours after the PI becomes aware of the event.

- Local serious adverse events which is non-fatal or non life threatening: PI must report to EC immediately, no later than 7 calendar days after the PI becomes aware of the event.

For reporting any Non-Local Serious Adverse Reactions

- Sponsor must report non-local serious adverse reaction including SUSARs to EC at least every 6 months accompanied by a brief report highlighting the main point of concern.

-Other adverse reactions that may increase risks to subjects, the sponsor must report to EC as soon as possible but no later than 15 calendar days.

-Other type of reports, the sponsor must report to EC at least every year or periodically or on request.

The document format is a photocopy of completed SAE report form according to provision of the sponsor.

Reporting Suspected Unexpected Serious Adverse Reactions

For reporting Local SUSARs which are fatal or life threatening:

Sponsor must report to EC as soon as possible using CIOMS form, no later than 7 calendar days after the sponsor becomes aware of the event.

If the initial report is incomplete, the sponsor must report to EC relevant follow-up information and complete report as soon as possible, within additional 8 calendar days.

- Sponsor must report any significant new information as a follow up report within 15 calendar days 5.2 Local SUSARs which are non-fatal or non life-threatening:

- Sponsor must repolt to EC as soon as possible using CIOMS form, no later than 15 calendar days after the sponsor becomes aware of the event.

- Further relevant follow-up information should be given as soon as possible.

All Adverse Events (AE) related and not related to the study are required to notify the EC in 1 year of the event notification to PI using PI form or the sponsor-required documentation.

For the unanticipated problems and unscheduled mandatory (medication errors, unexpected complications, protocol violations) must be reported in written to EC within 5 working days of the event notification to PI form or the sponsor-required documentation. For protocol deviation must be reported in 1 month.

If the PI wishes to amend any research document (e.g. Research Proposal, Participant Information Sheet, Informed Consent Form/ Informed Assent Form, Leaflet or Questionnaire), the Request for Protocol Amendment Form (FTM ECF-023-RR) should be filed, with the amended documents attached, and with the specified version and date shown in the footer of each document. The signatures of the PI (and any CO-PI) must be inscribed in the research proposal in ink.

If the PI wishes to notify the Ethics Committee of study closure, the Notification of Study Closure form (FTM ECF-OI O-RR), and final report should be filed.

Remark: 3 copies of the documentation sent to the EC. If a full board review is necessary, an EC officer will advise the PI of any additional documentation requirements.

Development and implementation of a novel platform for near-patient molecular diagnosis of dengue in Thailand: a user-engagement study

**Participant information sheet and consent form**

*This document may contain some statements that you do not understand. Please ask the Principal Investigator or his/her representative to give you explanations until everything is clearly understood. To assist in your decision-making process for participation in this research, you may take this document home to read and consult your relatives, intimates, personal doctor, or other doctors.*

**Title of Research Project** Development and implementation of a novel platform for near-patient molecular diagnosis of dengue in Thailand: a user-engagement study

**Name of Principal Investigator** Assoc. Prof. Saranath Lawpoolsri Niyom

**Research Site(s)**

Khon Kaen Hospital

The Provincial Health Control Office, Khon Kaen Province

**Source(s) of Funding** Wellcome Trust – Innovator Award (2018)

**Objective(s) of the Research Project:**

Our research group is currently developing a new diagnostic test for dengue virus. In this project, we aim to understand and describe requirements for dengue tests, engage users in design, assess their experience of prototypes, and evaluate user acceptance during implementation of the finished device. By doing this study, we hope to produce an optimized solution for the diagnosis and surveillance of dengue virus in Thailand.

You are invited to participate in this research project because you have been identified by our research team as someone who is a 'potential user' of diagnostic tests for dengue virus. During the various phases of this study, we aim to include clinicians, other healthcare professionals, laboratory scientists, and public health practitioners.

There will be a total of 80-100 participants, and the research study will last for 3 years. The study will be conducted in 3 phases.

Phase 1: Formative evaluation and co-design of device (6 participants).

Phase 2: Evaluation of user experience (6-12 participants).

Phase 3: Evaluation of user acceptance and cloud connectivity (70 participants).

You are being asked to participate in the **Phase 1** study.

What you will be asked to do, if you agree to participate in the study?

If you agree to participate in this study, and sign the Informed Consent Form, the researcher will ask you to attend a series of four focus group discussions, each lasting up to 2 hours. During the first discussion you will be asked about your experience of how dengue virus is generally diagnosed and surveyed in Thailand. The following three discussions will be focused around each of the following specific design features of the device: '*The diagnostic workflow*', '*the molecular assay*' and '*remote connectivity*'.

During all phases of this study, you may choose not to answer any questions which make you feel uncomfortable. You may also withdraw from the study at any time, without needing to give any specific reason. If, at the time you withdraw, data has already been collected, you will be asked if you wish also to withdraw your data from the study, in which case it will be destroyed and will not be analysed.

Data/ Specimen sharing with other researcher(s)

If you are participating in a focus group and/or in-depth interview, these will be recorded and transcribed into a MS Word Document. If you are participating in a self-administered questionnaire, your responses will be input into a MS Access Database. Data will be anonymized (it will not contain any personal identifying information, for example names, addresses or telephone numbers). Electronic data will be stored on a secure password-protected computer at the Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, and will be shared with researchers who work at Imperial College, London using a secure cloud server. Paper questionnaires will be stored in a locked filing cabinet at the Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University.

The data you provide during this study will be used by researchers in this study. Analysis will occur at Mahidol University (Bangkok) and Imperial College (London). It will only be used for this project. We aim to publish the findings of this research in a scientific journal. However, the information shared will not be personally identifiable.

Risk and/or potential discomfort

The main risk of participating in this study is that you could become distressed during data collection. If this occurs, you should inform the researcher(s) and the focus group or interview will be paused. You will be given time to decide whether you wish to continue with the session or withdraw.

Participation in this research project is voluntary:

If you decide not to participate in this research project, there will be no impact on you in the present or the future. There will be no impact on your employment or medical treatment.

Name of the researcher who can be contacted:

If any concern arises and you wish to inquire about the research or if you are injured/ill due to the research study, you can contact

Assoc. Prof. Saranath Lawpoolsri Niyom

Department of Tropical Hygiene, Faculty of Tropical Medicine,

Mahidol University

420/6 Ratchawithee Road, Ratchathewee, Bangkok 10400

Mobile phone: 081-9188688

Compensation:

Participation in this research is entirely voluntary. However, you will be given 500 THB for compensation for your time spent in each focus group discussion. The participants can participate up to 4 focus groups.

Your own expense(s) (if any):

It is anticipated that this research study will take place virtually. Therefore, the only associated expenses will be using a personal mobile device or computer with an internet connection

Benefit(s) to the research participant and others:

Participation in this research study will not directly benefit you. However, the data you provide will help us to develop a new diagnostic test for dengue which is optimized for use in Thailand. Therefore, participation may indirectly benefit people in Thailand and other parts of the world where dengue is a problem.

If relevant information arises about benefits and risks related to this research project, the researchers will inform you immediately and without concealing any details.

**Assuring confidentiality**

During data collection we will not ask you to provide any personal identifying information. We may use your name during focus group discussions and interviews, but these will be removed at the time of transcription. We will keep a single record of your name and contact details, on a secure password-protected computer at the Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University. This will only be used when contacting potential participants and organizing times and locations for data collection (i.e. when/where it is best to meet you.)

The anonymized data you provide will be kept confidential. It will not be disclosed individually, but will be disseminated as part of the overall results. Individual information may be examined by groups of persons, e.g. the Ethics Committee, funding organizations.

You have the right to withdraw from the project at any time without prior notice, and refusal to participate or withdrawal from the research project will result in no consequences.

If you are not treated as indicated in this information sheet, you can inform the Ethics Committee at the Ethics Committee, Office of Research Services, 4th Floor, The 60th Anniversary of His Majesty the King's Accession to the Throne Building, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Ratchathewi, Bangkok 10400. Tel 66-2-306-9126.

eเอกสารชี้แจงผู้เข้าร่วมการวิจัย

**(Participant Information Sheet)**

**การวิจัยส่วนที่ 1 การศึกษาข้อมูลเพื่อนำไปร่วมในการออกแบบเครื่องมือ**

*ในเอกสารนี้อาจมีข้อความที่ท่านอ่านแล้วยังไม่เข้าใจ โปรดสอบถามหัวหน้าโครงการวิจัย หรือผู้แทนให้ช่วยอธิบายจนกว่าจะเข้าใจดี*
*ท่านอาจจะขอเอกสารนี้กลับ ไปอ่านที่บ้านเพื่อปรึกษาหารือกับญาติพี่น้อง เพื่อนสนิท แพทย์ประจำตัวของท่าน หรือแพทย์ท่านอื่น เพื่อช่วยในการตัดสินใจเข้าร่วมการวิจัย*

**ชื่อโครงการ (ภาษาไทย)** การพัฒนาและนำมาใช้ของเครื่องมือใหม่สำหรับการวินิจฉัยทางโมเลกุล ณ จุดบริการผู้ป่วยของโรคไข้เลือดออกเด็งกี่ในประเทศไทย: การศึกษาความมีส่วนร่วมของผู้ใช้

**ชื่อผู้วิจัย** รศ.ดร.พญ. สารนาถ ล้อพูลศรี นิยม

**สถานที่วิจัย**

โรงพยาบาลขอนแก่น

สำนักงานสาธารณสุข จังหวัดขอนแก่น

**ผู้ให้ทุน** Wellcome Trust – Innovator Award (2018)

**วัตถุประสงค์ของการวิจัย**
โครงการวิจัยนี้จัดทำขึ้นเพื่อศึกษาความต้องการของผู้ใช้เครื่องมือในการตรวจโรคไข้เดงกี่ เพื่อนำข้อมูลในส่วนของผู้ใช้งานเข้าไปมีส่วนร่วมในการออกแบบเครื่องมือในการวินิจฉัยการติดเชื้อเดงกี่ โดยใช้วิธีการทางโมเลกุล ณ จุดบริการ รวมถึงการประเมินประสบการณ์การใช้อุปกรณ์ต้นแบบ และประเมินความยอมรับของผู้ใช้เมื่อมีการนำเครื่องมือมาใช้จริง
ซึ่งจะมีประโยชน์ที่คาดว่าจะได้รับคือจะใช้พัฒนาเครื่องมือการตรวจวินิจฉัยโรคไข้เดงกี่แบบใหม่ที่มีความแม่นยำสูง และเหมาะสมกับผู้ใช้งานจริงในประเทศไทย

ท่านได้รับเชิญให้เข้าร่วมการวิจัยนี้เนื่องจากท่านเป็นผู้ที่ทำงานในส่วนที่เกี่ยวข้องกับการใช้เครื่องมือหรือข้อมูลในการวินิจฉัยการติดเชื้อไวรัสเดงกี่ โดยในช่วงต่าง ๆ ของการวิจัยจะมีการเชิญแพทย์ บุคลากรทางสาธารณสุข นักวิทยาศาสตร์ในห้องปฏิบัติการ และนักวิชาการสาธารณสุข มาเข้าร่วมการวิจัย

จะมีผู้เข้าร่วมการวิจัยนี้ทั้งสิ้นประมาณ 80-100 คน ระยะเวลาที่จะทำการวิจัยทั้งสิ้น 3 ปี
โครงการวิจัยนี้แบ่งเป็น 3 ส่วน ส่วนที่ 1 เป็นการศึกษาข้อมูลเพื่อนำไปร่วมในการออกแบบเครื่องมือ (ผู้เข้าร่วมวิจัยประมาณ 6 ท่าน) ส่วนที่ 2 เป็นการประเมินประสบการณ์ของผู้ใช้งาน (ผู้เข้าร่วมวิจัยประมาณ 6-12 ท่าน) ส่วนที่ 3 เป็นการประเมินการยอมรับของผู้ใช้และการเชื่อมต่อคลาวด์ (ผู้เข้าร่วมวิจัย 70 ท่าน)

ซึ่งในเอกสารนี้ จะเป็นการเข้าร่วมโครงการวิจัยในส่วนที่ 1

ขั้นตอนการปฏิบัติตัวหากท่านเข้าร่วมโครงการวิจัย

หากท่านสมัครใจเข้าร่วมโครงการและลงนามในเอกสารยินยอมโดยได้รับการบอกกล่าวแล้ว ผู้วิจัยจะขอให้ท่านเข้าร่วมการอภิปรายกลุ่มสนใจ 4 กลุ่ม ใช้เวลาครั้งละ 2 ชั่วโมง
การอภิปรายครั้งแรกท่านจะถูกถามเกี่ยวกับประสบการณ์ของท่านต่อการวินิจฉัยและสำรวจการติดเชื้อไวรัสเด็งกี่ในประเทศไทย การอภิปรายครั้งถัดไปอีก 3
ครั้งจะมุ่งไปที่การออกแบบคุณลักษณะเฉพาะของเครื่องมือดังต่อไปนี้: *‘ขั้นตอนการวินิจฉัย’*, *‘การทดสอบทางโมเลกุล’* และ *‘การเชื่อมต่อระยะไกล’*

ตลอดทุกช่วงการศึกษา ท่านสามารถเลือกไม่ตอบคำถามใดก็ตามที่ทำให้ท่านไม่สบายใจ
ท่านสามารถถอนตัวจากการวิจัยได้ทุกเมื่อโดยไม่ต้องให้เหตุผลเป็นการเฉพาะ
หากทีการเก็บข้อมูลไปแล้วในช่วงที่ท่านถอนตัว
ท่านจะถูกถามว่าต้องการให้ถอนข้อมูลของท่านจากการศึกษาด้วยหรือไม่
ในกรณีนั้นข้อมูลของท่านจะถูกทำลายและไม่ถูกนำมาวิเคราะห์

การแบ่งปันข้อมูล/สิ่งส่งตรวจของท่านกับผู้วิจัยอื่น

หากท่านเข้าร่วมในกลุ่มสนใจ และ/หรือการสัมภาษณ์เชิงลึก
ข้อมูลเหล่านี้จะถูกบันทึกและแปลงลงในเอกสาร
หากท่านเข้าร่วมการตอบแบบสอบถามแบบตอบด้วยตนเอง ข้อมูลการตอบจะถูกใส่ลงในฐานข้อมูล
ข้อมูลจะถูกทำให้ระบุตัวตนไม่ได้ (จะไม่มีข้อมูลส่วนตัว เช่น ชื่อ ที่อยู่ หรือเบอร์โทรศัพท์)
ข้อมูลอิเล็กทรอนิกส์จะถูกเก็บในคอมพิวเตอร์ที่เข้ารหัสที่ภาควิชาสุขวิทยาเขตร้อน คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล กรุงเทพฯ และจะมีการแบ่งปันข้อมูลกับนักวิจัยที่วิทยาลัยอิมพีเรียลลอนดอน (Imperial College London) โดยใช้คลาวด์เซิร์ฟเวอร์ (cloud server) ที่ปลอดภัย
แบบสอบถามที่เป็นกระดาษจะถูกเก็บในตู้เอกสารที่ถูกล็อคที่หน่วยวิจัยโรคไข้เลือดออก

ข้อมูลที่ท่านให้ในช่วงการศึกษาจะถูกใช้โดยนักวิจัยในการศึกษานี้ การวิเคราะห์จะทำที่มหาวิทยาลัยมหิดล (กรุงเทพฯ) และวิทยาลัยอิมพีเรียลลอนดอน (ลอนดอน) ข้อมูลจะถูกใช้สำหรับการวิจัยนี้เท่านั้น
อย่างไรก็ตามข้อมูลที่แบ่งปันจะไม่ถูกระบุเป็นรายบุคคล
การรายงานผลจะเป็นข้อมูลแบบรวมไม่ระบุตัวบุคคล

ความเสี่ยงและ/หรือความไม่สบายที่อาจเกิดขึ้น

ความเสี่ยงที่อาจจะเกิดขึ้นเมื่อเข้าร่วมการวิจัยคือท่านอาจเกิดความทุกข์ใจในช่วงการเก็บข้อมูล หากสิ่งนี้เกิดขึ้น ท่านสามารถบอกผู้วิจัยได้ตลอดเวลา การอภิปรายกลุ่มจะถูกหยุดชั่วคราว
และจะให้เวลาท่านในการตัดสินใจว่าต้องการร่วมการวิจัยต่อไปหรือต้องการถอนตัว

การเข้าร่วมโครงการวิจัยของท่านต้องเป็นไปด้วยความสมัครใจ

หากท่านไม่เข้าร่วมในโครงการวิจัยนี้จะไม่มีผลกระทบใด ๆ
ทั้งในปัจจุบันและอนาคตด้านการจ้างงานและการรักษาทางการแพทย์

**ระบุชื่อผู้วิจัยที่จะสามารถติดต่อได้**
หากมีข้อข้องใจที่จะสอบถามเกี่ยวข้องกับการวิจัย หรือเมื่อบาดเจ็บ/ เจ็บป่วยจากการวิจัย ท่านสามารถติดต่อ
รศ.ดร.พญ. สารนาถ ล้อพูลศรี นิยม

ภาควิชาสุขวิทยาเขตร้อน คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

420/6 ถ.ราชวิถี เขตราชเทวี กทม. 10400

โทรศัพท์มือถือ 081-9188688

ค่าตอบแทนที่จะได้รับ การเข้าร่วมงานวิจัยนี้เป็นไปด้วยความสมัครใจ
อย่างไรก็ตามท่านจะได้รับเงินจำนวน 500 บาท
เพื่อเป็นการชดเชยการเสียเวลาของท่านในการเข้าร่วมการอภิปรายกลุ่มในแต่ละครั้ง

ค่าใช้จ่ายที่ผู้เข้าร่วมการวิจัยจะต้องรับผิดชอบเองมีอะไรบ้าง การดำเนินการวิจัยนี้ จะเป็นการคุยทางไกล ดังนั้นท่านอาจมีค่าใช้จ่ายที่เกี่ยวข้องกับการเชื่อมต่ออินเทอร์เน็ตในระหว่างการอภิปราย

ประโยชน์ที่จะเกิดแก่ตัวท่านและผู้อื่น
ท่านจะไม่ได้รับประโยชน์โดยตรงจากการเข้าร่วมการวิจัยนี้ อย่างไรก็ตาม

ข้อมูลที่ท่านให้จะช่วยเราในการพัฒนาการตรวจวินิจฉัยใหม่สำหรับโรคไข้เลือดออกเด็งกี่ที่พัฒนาสำหรับประเทศไทย ดังนั้นการเข้าร่วมการวิจัยของท่านจะเป็นประโยชน์ต่อประชาชนคนไทยในทางอ้อม รวมถึงส่วนอื่นของโลกที่โรคไข้เดงกี่กำลังเป็นปัญหา

หากมีข้อมูลเพิ่มเติมทั้งด้านประโยชน์และโทษที่เกี่ยวข้องกับการวิจัยนี้ ผู้วิจัยจะแจ้งให้ทราบโดยรวดเร็ว ไม่ปิดบัง

การรักษาความลับ

ระหว่างการเก็บข้อมูล คณะผู้วิจัยจะไม่มีการให้ข้อมูลส่วนตัว เราอาจใช้ชื่อของท่านในช่วงการอภิปรายกลุ่มสนใจหรือการสัมภาษณ์ แต่จะถูกนำออกเมื่อถอดความ คณะผู้วิจัยจะเก็บชื่อและข้อมูลการติดต่อของท่านครั้งเดียวในคอมพิวเตอร์ที่เข้ารหัสที่ภาควิชาสุขวิทยาเขตร้อน คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล ข้อมูลจะใช้เมื่อติดต่อผู้เข้าร่วมวิจัยที่สนใจ และใช้ในการนัดหมายเวลาและสถานที่สำหรับเก็บข้อมูลเท่านั้น

ข้อมูลส่วนตัวของท่านจะถูกเก็บรักษาไว้ ไม่เปิดเผยต่อสาธารณะเป็นรายบุคคล แต่จะรายงานผลการวิจัยเป็นข้อมูลส่วนรวม ข้อมูลของผู้ร่วมการวิจัยเป็นรายบุคคลอาจมีคณะบุคลบางกลุ่มเข้ามาตรวจสอบได้ เช่น คณะกรรมการจริยธรรมฯ ผู้ให้ทุนวิจัย เป็นต้น

ท่านมีสิทธิ์ถอนตัวออกจากโครงการวิจัยเมื่อใดก็ได้ โดยไม่ต้องแจ้งให้ทราบล่วงหน้า และการไม่เข้าร่วมการวิจัยหรือถอนตัวออกจากโครงการวิจัยนี้จะไม่ส่งผลกระทบใด ๆ

หากท่านได้รับการปฏิบัติที่ไม่ตรงตามที่ได้ระบุไว้ในเอกสารชี้แจงนี้ ท่านจะสามารถแจ้งให้คณะกรรมการจริยธรรมฯ ทราบได้ที่
ฝ่ายเลขานุการคณะกรรมการจริยธรรมการวิจัยในคน สำนักงานบริการการวิจัย ชั้น 4 อาคารเฉลิมพระเกียรติฉลองสิริราชสมบัติครบ 60 ปี คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล 420/6 ถนนราชวิถี เขตราชเทวี กรุงเทพฯ 10400 โทร 02-306-9126

หมายเหตุ :

หากผู้เข้าร่วมวิจัยเป็นผู้เยาว์ (อายุต่ำกว่า 18 ปีบริบูรณ์)
และเอกสารชี้แจงนี้ทำให้ผู้ปกครอง/ผู้แทนโดยชอบธรรมเป็นผู้อ่าน ให้เปลี่ยนสรรพนาม “ท่าน” เป็น “เด็กในปกครองของท่าน” ตามตำแหน่งที่เหมาะสม

ผู้วิจัยควรมอบสำเนาแบบยินยอมอาสาสมัครพร้อมแนบคำชี้แจงอาสาสมัครอย่างละ 1 ชุด
ให้อาสาสมัครหรือผู้ปกครองด้วย

Appendix 4: Material transfer agreements for samples used in studies of portable diagnostic workflows (Chapter 5)

# Material Transfer Request - Implementing Letter

PROVIDER: Organization providing the ORIGINAL MATERIAL

Organization: Mahidol University by Faculty of Medicine Siriraj Hospital

Address: 2, Wanglang Road, Siriraj, Bangkoknoi, Bangkok, Thailand 10700

PROVIDER SCIENTIST:

Name: Assoc.Prof.Dr. Panisadee Avirutnan

Address: 2, Wanglang Road, Siriraj, Bangkoknoi, Bangkok, Thailand 10700

RECIPIENT: Organization receiving the ORIGINAL MATERIAL

Organization: Imperial College London

Address: Research Office, Level 5 Sherfield Building, Exhibition Road, South Kensington Campus, London, SW7 2AZ

RECIPIENT SCIENTIST:

Name: Dr Paul Arkell

Address: Centre for Antimicrobial Optimisation, Imperial College London, Commonwealth Building, Hammersmith

Hospital Campus, Du Cane Road, London, W 12 ONN

ORIGINAL MATERIAL

Name of the ORIGINAL MATERIAL: Extracted total RNA prepared from spiked-dengue virus culture in human blood specimen of one healthy donor

Date of request: dd/mm/yyyy: 26 August 2021

Purpose of use: For research

Title of Project: Multi-dimensional Diagnostics for Rapid detection of Infectious Diseases in Developing Countries

Quantity:

| TYPE | Infected or Not Infected | Specify and Quantity ( . . ml/tube) |
|---|---|---|

| | | | |
|---|---|---|---|
| | Human | Yes | ☐ Blood.....<br>☐ Plasma.....<br>☐ Serum.....<br>☐ Tissue.....<br>Uri e.....<br>Other..... |
| | Animal | Yes | .....<br>..... |
| | Microorganism | Yes | .....<br>..... |
| | other | Yes | Specify: Extracted total RNA from spikeddengue virus cultured in one healthy blood donor<br>Quantity: 372 tubes (10 microliters/tube) |

Transmittal Fee: The ORIGINAL MATERIAL is provided at no cost. A reasonable transmittal fee for preparation, handling

and distribution is optional requested.

The PROVIDER and PROVIDER SCIENTIST have agreed to distribute the ORIGINAL MATERIAL under the

Material Transfer Agreements (identified below).

MATERIAL TRANSFER AGREEMENT: the following agreements are between RECIPIENT and PROVIDER. See enclosed

exhibit

Using a separate form not part of this implementing letter, RECIPIENT SCIENTIST has acknowledged to having read and understood the Material Transfer Agreements identified above.

By executing this implementing letter, RECIPIENT agrees to the terms ofthe Material Transfer Agreements identified above.

RECIPIENT ORGANIZATION CERTIFICATION:

You, the person signing this form, certify that

l) you are the Authorized Representative whose name appears below, or you have been given authority by the

Authorized Representative whose name appears below to complete this form,

the Authorized Representative has the authority to sign Material Transfer Agreements on behalf of RECIPIENT,

RECIPIENT is a non-profit research organization (qualified under a government or state non-profit statute), or a university or other institution of higher education, or a government agency conducting research, and

RECIPIENT agrees to the transfer of the ORIGINAL MATERIAL as described in this letter.

Authorized Representative:

| | |
| --- | --- |
| Provider Scientist | |
| | Recipient Scientist |
| Signature..... ..,,,. | |
| Name: Assoc.Prof.Dr. Panisadee Avirutnan | Signature |
| Authorized person | Name Dr Paul Arkell |
| Provider Organization | Authorized person |
| | Recipient Organization |
| Signature..... ..... | Signature. |
| Printed Name: Prof.Prasert Auewarakul, M.D.Ph.D. | Name. Sharna Crosdale |
| Job PositionDeputy Dean for Research, Faculty of Medicine Sirirai Hosoital, MAHIDOL UNIVERSITY | Job Position. Contracts Officer MTA's (RO) |
| | Date 6th October 2021 |

# MU Material Transfer Agreement

## Article 1 Definitions

PROVIDER: Organization providing the ORIGINAL MATERIAL. The name and address of this party will be specified in an implementing letter.

PROVIDER SCIENTIST: The name and address of this party will be specified in an implementing letter.

3. RECIPIENT: Organization receiving the ORIGINAL MATERIAL. The name and address of this party will be specified in an implementing letter.

RECIPIENT SCIENTIST: The name and address of this party will be specified in an implementing letter.

ORIGINAL MATERIAL: The description of the material being transferred will be specified in an implementing letter.

MATERIAL: ORIGINAL MATERIAL, PROGENY, and UNMODIFIED DERIVATIVES.

The MATERIAL shall not include: (a) MODIFICATIONS, or (b) other substances created by the RECIPIENT through the use of the MATERIAL which are not MODIFICATIONS, PROGENY, or UNMODIFIED DERIVATIVES.

PROGENY: Unmodified descendant from the MATERIAL, such as virus from virus, cell from cell, or organism from organism.

UNMODIFIED DERIVATIVES: Substances created by the RECIPIENT which constitute an unmodified functional subunit or product expressed by the ORIGINAL MATERIAL. Some examples include: subclones of unmodified cell lines, purified or fractionated subsets of the ORIGINAL MATERIAL, proteins expressed by DNA/RNA supplied by the

PROVIDER, or monoclonal antibodies secreted by a hybridoma cell line.

MODIFICATIONS: Substances created by the RECIPIENT which contain/incorporate the MATERIAL.

Article 2 Ownership and Intellectual Properties of Materials

l. Provider retains ownership of the material including any materials contained or incotporate in the Modification

The Recipient and/or the RECIPIENT SCIENTIST retains ownership of:

the Modifications,

those substances create through the use of material or modifications but which are not PROGENY,

UNMODIFIED DERIVATIVES or MODIFICATIONS (i.e., do not contain the ORIGINAL MATERIAL,

PROGENY, UNMODIFIED DERIVATIVES).

If either 2(a) or 2 (b) result from collaboration efforts of the Provider and the Recipient, joint ownership may be negotiated.

The RECIPIENT may distribute MODIFICATIONS to NONPROFIT ORGANIZATION(S) for research and teaching purposes only.

Without written consent from the PROVIDER, the RECIPIENT and/or the RECIPIENT SCIENTIST may NOT provide MODIFICATIONS for COMMERCIAL PURPOSES. It is recognized by the RECIPIENT that such COMMERCIAL PURPOSES may require a commercial license from the PROVIDER and the PROVIDER has no obligation to grant a commercial license to its ownership interest in the MATERIAL incorporated in the MODIFICATIONS. Nothing in this paragraph, however, shall prevent the RECIPIENT from granting commercial licenses under the RECIPIENT's intellectual property rights claiming such MODIFICATIONS, or methods of their manufacture or their use.

The RECIPIENT acknowledges that the MATERIAL is or may be the subject of a patent application. Except as provided in this Agreement, no express or implied licenses or other rights are provided to the RECIPIENT under any patents, patent applications, trade secrets or other proprietary rights of the PROVIDER, including any altered fonns of the MATERIAL made by the PROVIDER. In particular, no express or implied licenses or other rights are provided to use the MATERIAL, MODIFICATIONS, or any related patents of the PROVIDER for COMMERCIAL PURPOSES.

If the RECIPIENT desires to use or license the MATERIAL or MODIFICATIONS for COMMERCIAL PURPOSES, the RECIPIENT agrees, in advance of such use, to negotiate in good faith with the PROVIDER to establish the terms of a commercial license. It is understood by the RECIPIENT that the PROVIDER shall have no obligation to grant such a license to the RECIPIENT, and may grant exclusive or non-exclusive commercial licenses to others, or sell or assign all or part of the rights in the MATERIAL to any third party(ies), subject to any pre-existing rights held by others and obligations to the Federal Government

The RECIPIENT is free to file patent application(s) claiming inventions made by the RECIPIENT through the use of the

MATERIAL but agrees to notify the PROVIDER upon filing a patent application claiming MODIFICATIONS or method(s) of manufacture or use(s) of the MATERIAL.

Article 3 Obligations of Provider and Recipient

Recipient shall use the Materials:

for the Purpose of Use specify in the implementing letter and not for any other purpose.

is to be used solely for teaching and academic research purposes;

will not be used in human subjects, in clinical trials, or for diagnostic purposes involving human subjects without the written consent of the PROVIDER;

is to be used only at the RECIPIENT organization and only in the RECIPIENT SCIENTIST's laboratory under the direction of the RECIPIENT SCIENTIST or others working under his/her direct supervision; and

will not be transferred to anyone else within the RECIPIENT organization without the prior written consent of the PROVIDER

Recipient shall use the Materials in compliance with all applicable (national and international) laws and regulations, including without limitation, Public Health Service and those relating to research involving the Use of Animals,

Pathogen and Animal Toxin Act, Stem cell or recombinant DNA, Convention on Biological Diversity.

Recipient shall obtain the prior approval of Provider, if it desires to alter, modify or otherwise change the condition of the Materials from their state at the date of transfer to establish the Modifications.

The PROVIDER and the PROVIDER SCIENTIST represent and warrant to the RECIPIENT that:

all MATERIALS supplied under this Agreement are or have been procured and supplied to RECIPIENT ethically in full compliance with any and all applicable nation laws, regulations, code of practices relating to the use of the

MATERIAL providing protection for subjects in Thailand;

all necessary consents, licenses, permissions, authorizations required (either from human subjects, Thai government agencies or otherwise) have been obtained in connection with the supply of the MATERIALS to the

RECIPIENT and for taking any other actions they are required under this Agreement;

the donor of the MATERIALS (the "DONOR") has given informed consent to use the MATERIALS; and

all MATERIALS supplied to RECIPIENT may be used to provide data in support of commercial product development and were procured without inappropriate financial benefit to the DONOR.

The MATERIALS shall be de-identified or 'coded' to protect the identity and confidentiality of the DONOR and shall be supplied to the RECIPIENT without any information or data that could allow the RECIPIENT to personally identify the DONOR.

MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND. THERE ARE NO IMPLIED

WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE ORIGINAL MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS.

Limitation of Liability: to the extent permitted by law, RECIPIENT assumes all liability for damages that may arise from RECIPIENT's use, storage or disposal of the ORIGINAL MATERIAL. PROVIDER, its agents and its successors and their respective directors, officers, members, employees, and agents will not be liable to RECIPIENT for any loss, claim or demand made by the RECIPIENT, or made against RECIPIENT by any other party, due to or arising from the use of the ORIGINAL MATERIAL by RECIPIENT, except to the extent permitted by law when caused by the gross negligence or willful misconduct of PROVIDER. IN NO EVENT SHALL PROVIDER 'S CUMULATIVE LIABILITY EXCEED THE FEES PAID BY RECIPIENT TO MU FOR ORIGINAL MATERIAL FOR THE TWELVE (12) MONTH PERIOD PRECEDING THE DATE OF THE EVENT GIVING RISE TO THE CLAIM.

Indemnification: to the extent permitted by law, RECIPIENT shall indemnify and hold harmless PROVIDER, its agents and its successors and their respective directors, officers, members, employees, and agents, from and against any and all losses, claims, damages, expenses and liabilities arising at any time as a result of RECIPIENT's use and disposal of the ORIGINAL MATERIAL, RECIPIENT's breach ofthese Additional Terms, and RECIPIENT's breach of the applicable Material Transfer Agreements, except when caused by the gross negligence or willful misconduct of PROVIDER.

Conflicts: in the event of a conflict between these Additional Terms and the applicable Material Transfer Agreements that govern

RECIPIENT's use of the ORIGINAL MATERIAL the applicable Material Transfer Agreements shall prevail.

MATERIAL TRANSFER AGREEMENT

As principal investigator I agree on behalf of all those involved in the project to accept the following conditions relating to the use of material and information from the Imperial College Healthcare NHS Trust Tissue Bank (ICHTB).

| | |
|---|---|
| **Title of Project** | Lamp Assays For Dengue Detection And Serotyping |
| **Start Date** | 30th Jan 2023 |
| **End Date** | 30th Apr 2023 |
| **Principal Investigator** | Professor Alison Holmes |
| **Address** | |
| **Tel** | 07788491441 |
| **Email** | p.arkell@imperial.ac.uk |

The recipient/investigator agrees that the materials provided by the ICHTB will be used only for the purposes specified in this application.

The recipient agrees not to attempt to obtain information identifying the individuals providing materials to the ICHTB.

The recipient agrees that they shall not sell any portion of the materials provided by the ICHTB, or products directly extracted from these materials (e.g. protein, mRNA or DNA).

The recipient also agrees that they shall not transfer tissue (or any portion thereof) supplied by the ICHTB to third parties without the prior written permission of the ICHTB. Any subsequent transfer that may be made to other parties, with prior agreement from ICHTB, will require signature of this agreement between the final recipients of the material and the ICHTB.

Any publication relating from the use of samples from the ICHTB must state the following 'Tissue samples were provided by the Imperial College Healthcare NHS Trust Tissue Bank funded by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health'. This is essential for continued financial support of the tissue bank.

The following statement should be used in publications requiring a statement of ethical approval "Human samples used in this research project were obtained from the Imperial College Healthcare Tissue Bank (ICHTB). ICHTB is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. ICHTB is approved by Wales REC3 to release human material for research (22/WA/0214 ), and the samples for this project (give approved project number e.g. R120004) were issued from sub-collection reference number xxx." The project number can be found on the letter or approval issued for access to samples, and the sub-collection reference number can be found on the original

application to access. If you require further information please contact the Tissue Bank (tissuebank@imperial.ac.uk)

The recipient understands that while the ICHTB attempts to avoid providing materials that are contaminated with highly infectious agents such as hepatitis and HIV, all materials should be handled as if potentially infectious. The individuals who have supplied tissue to the ICHTB have not agreed to have clinical tests performed on this tissue (e.g. for the presence of infective agents such as hepatitis).

The recipient acknowledges that the institution where the tissue will be used follows the appropriate local regulations for handling human specimens and will instruct their staff to abide by those rules. The recipient further agrees to assume all responsibility for informing and training personnel in the dangers and procedures for safe handling of human materials.

Materials are provided as a service to the research community without warranty of merchantability or fitness for a particular purpose or any other warranty, express or implied. The ICHTB accepts no responsibility for any injury (including death), damages or loss that may arise either directly or indirectly from their use.

All results should be documented using the ICHTB code number. This is particularly important as material from the same patient may be made available to several researchers.

A brief report on the work of the project will be made within 3 months of the end of the study. This shall include the results of all investigations carried out on each sample. The report should be sent to the ICHTB Secretariat (tissuebank@imperial.ac.uk) for use within the ICHTB. It will not be otherwise disseminated without the agreement of the Principal Investigator.

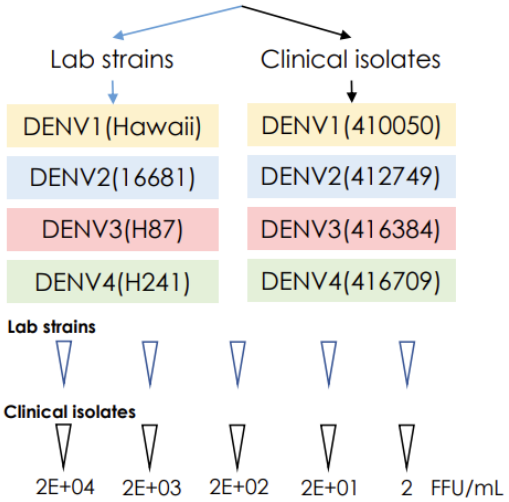
The Principal Investigator's institution agrees to assume all risks and responsibility in connection with the receipt, handling, storage and use of materials from the ICHTB. It further agrees to indemnify and hold harmless the ICHTB and the funders of the ICHTB from any claims costs, damages or expenses resulting from the use of the materials provided by the ICHTB. The undersigned warrant that they have authority to execute this agreement on behalf of the recipient institution.

**BY MY SIGNATURE I AGREE TO THE TERMS SET FORTH IN THE ABOVE AGREEMENT**

| | | | |
|---|---|---|---|
| Principal Investigator | **Professor Alison Holmes** | Institution | Imperial College London |
| Signature of Principal Investigator | | Date | 30.01.2023 |

# List samples of extracted RNA spiked DENV1-4 in three materials.

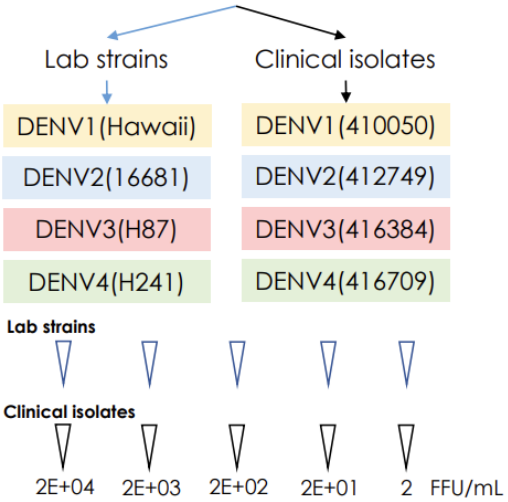
## Medium




Extracted viral RNA = 10ul x 3 tubes in each concentration.

- **Box No.1:**
  DENV1-4 Lab strains = 60 tubes
  Negative control materials = 2 tubes.
- **Box No.2:**
  DENV1-4 clinical isolates = 60 tubes
  Negative control materials = 2 tubes.

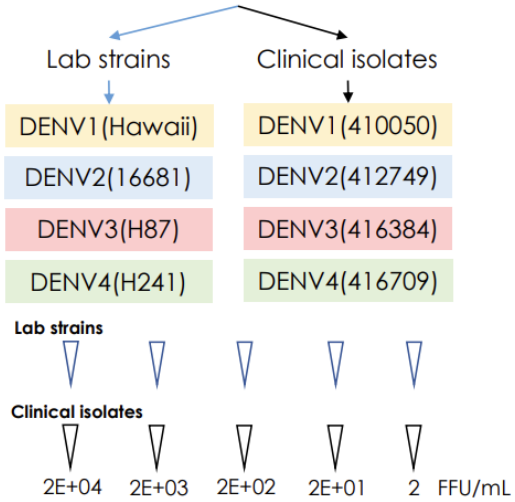
## Plasma




Extracted viral RNA = 10ul x 3 tubes in each concentration.

- **Box No.3:**
  DENV1-4 Lab strains = 60 tubes
  Negative control materials = 2 tubes.
- **Box No.4:**
  DENV1-4 clinical isolates = 60 tubes
  Negative control materials = 2 tubes.

## Whole blood




Extracted viral RNA = 10ul x 3 tubes in each concentration.

- **Box No.5:**
  DENV1-4 Lab strains = 60 tubes
  Negative control materials = 2 tubes.
- **Box No.6:**
  DENV1-4 clinical isolates = 60 tubes
  Negative control materials = 2 tubes.

Note : Clinical isolates mean Thai strains.

"Division of Dengue Hemorrhagic Fever Research", Faculty of Medicine Siriraj Hospital, Mahidol University
Adulyadej vikrom Building 12 th Floor, 2 Wang Lang Road, Siriraj Subdistrict, Bangkok Noi, Bangkok 10700, Thailand. Tel: +662-4197000 ext. 6667-6668

*TB-DOC-App1 v7 13/9/18*

# MATERIAL TRANSFER AGREEMENT

As principal investigator I agree on behalf of all those involved in the project to accept the following conditions relating to the use of material and information from the Imperial College Healthcare NHS Trust Tissue Bank (ICHTB).

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| **Title of Project** | Lamp Assays For Dengue Detection And Serotyping |
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| **Principal Investigator** | Professor Alison Holmes |
| **Address** | |
| **Tel** | 07788491441 |
| **Email** | p.arkell@imperial.ac.uk |

1. The recipient/investigator agrees that the materials provided by the ICHTB will be used only for the purposes specified in this application.
2. The recipient agrees not to attempt to obtain information identifying the individuals providing materials to the ICHTB.
3. The recipient agrees that they shall not sell any portion of the materials provided by the ICHTB, or products directly extracted from these materials (e.g. protein, mRNA or DNA).
4. The recipient also agrees that they shall not transfer tissue (or any portion thereof) supplied by the ICHTB to third parties without the prior written permission of the ICHTB. Any subsequent transfer that may be made to other parties, with prior agreement from ICHTB, will require signature of this agreement between the final recipients of the material and the ICHTB.
5. Any publication relating from the use of samples from the ICHTB must state the following 'Tissue samples were provided by the Imperial College Healthcare NHS Trust Tissue Bank funded by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health'. This is essential for continued financial support of the tissue bank.
6. The following statement should be used in publications requiring a statement of ethical approval "Human samples used in this research project were obtained from the Imperial College Healthcare Tissue Bank (ICHTB). ICHTB is supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. ICHTB is approved by Wales REC3 to release human material for research (22/WA/0214 ), and the samples for this project (give approved project number e.g. R120004) were issued from sub-collection reference number xxx." The project number can be found on the letter or approval issued for access to samples, and the sub-collection reference number can be found on the original application to access. If you require further information please contact the Tissue Bank (tissuebank@imperial.ac.uk)
7. The recipient understands that while the ICHTB attempts to avoid providing materials that are contaminated with highly infectious agents such as hepatitis and HIV, all materials should be

handled as if potentially infectious. The individuals who have supplied tissue to the ICHTB have not agreed to have clinical tests performed on this tissue (e.g. for the presence of infective agents such as hepatitis).

8. The recipient acknowledges that the institution where the tissue will be used follows the appropriate local regulations for handling human specimens and will instruct their staff to abide by those rules. The recipient further agrees to assume all responsibility for informing and training personnel in the dangers and procedures for safe handling of human materials.
9. Materials are provided as a service to the research community without warranty of merchantability or fitness for a particular purpose or any other warranty, express or implied. The ICHTB accepts no responsibility for any injury (including death), damages or loss that may arise either directly or indirectly from their use.
10. All results should be documented using the ICHTB code number. This is particularly important as material from the same patient may be made available to several researchers.
11. A brief report on the work of the project will be made within 3 months of the end of the study. This shall include the results of all investigations carried out on each sample. The report should be sent to the ICHTB Secretariat (tissuebank@imperial.ac.uk) for use within the ICHTB. It will not be otherwise disseminated without the agreement of the Principal Investigator.
12. The Principal Investigator's institution agrees to assume all risks and responsibility in connection with the receipt, handling, storage and use of materials from the ICHTB. It further agrees to indemnify and hold harmless the ICHTB and the funders of the ICHTB from any claims costs, damages or expenses resulting from the use of the materials provided by the ICHTB. The undersigned warrant that they have authority to execute this agreement on behalf of the recipient institution.

**BY MY SIGNATURE I AGREE TO THE TERMS SET FORTH IN THE ABOVE AGREEMENT**

| | | | |
|---|---|---|---|
| Principal Investigator | **Professor Alison Holmes** | Institution | Imperial College London |

# Appendix 5: Research Ethics Committee approvals, participant information sheet and consent form for dried blood spot study (Chapter 6)

VIII GOVERNO CONSTITUCIONAL

MINISTÉRIO DA SAÜDE

Gabinete Garantia Kualidade ba Saude

Dili, 07 Marsu 2019

No. Ref : MS-GGKS/11V2019/.ï.P

KARTA APROVASAUN ETIKA / LETTER OF ETHICS APPROVAL

Data 07 Marsu 2019

Sr : Dr. Joshua Francis

Darwin (Royal Darwin Hospital Campus), John Mathews Building (JMB) Building 58,

TELP : +61423528381

GGKS Reference Number: GGKS/EP/2019/1. 001.0001

Titulu Peskiza: Serological investigation into Potential arthropod-borne and Zoonotic Causes of fever in Timor- Leste

Respeita ba Sr. Joshua Francis

Agradese tanba submete proposta peskiza ho topiku ne'ebe mensiona iha leten atu hetan revizaun etika.

Ho laran klosok hau informa ba ita bo'ot katak Gabinete de Garantia de Qualidade na Saude (GGQS) fé tiha ona aprovasaun etika ba proposta ne'e. Fatin ne'ebe rekomenda ba projetu ida ne'e maka:

Iha Munisipiu: Baucau, Covalima, Dili, Ermera, Liquica no Viqueque

Favor anota katak karik presiza atumenta lokalizasaun ne'ebe partisipa iha projetu ne'e, antes atu hahu ou durante ezekusaun projetu ne'e, Peskizador Prinsipal tenki informa GGQS atempadamente .

Dokumentu aprovasaun inklui:

Proposta peskiza INS

Deklarasaun Peskijador

• Karta deklarasun Instituisaun

Formulariu/Template Peskija INS

Formulariu konsentimentu

Formulariu Informasaun ba Autorizasaun no Deklarasaun ba Partisipantes

Formulariu Apresiasaun Partisipantes

Karta Deklarasaun husi Laboratorium Nasional

Karta Deklarasaun husi Departemento CDC -DNSP

Kalendariu ne'ebe aprova ba Projetu: 07 2019 to'0 30 2019

KONDISOENS BA VALIDADE APROVASAUN mak sira tui mai ne'e:

Peskizador Prinsipal tenki relata situasaun hotu ne'ebe presiza revizaun ba aprovasaun etika ba projetu

Peskizador Prinsipal tenki notifika GGQS konaba eventu sira hotu ne'ebe presiza modifikasaun protokolu ou dokumentu seluk projetu nian no submete amandamentu hotu mai GGQS

Peskizador Prinsipal tenki submete relatoriu sira hotu relasiona ho seguransa peskiza nia partisipante sira

Peskizador Prinsipal tenki halo relatoriu ba GGQS wainhira projetu iha lokalizasaun hotu-hotu remata ona

Peskizador Prinsipal tenki notifika GGQS wainhira deskotinua ou termina projetu iha naran lokalizasaun ida antes husi tempu projetu remata, no aprezenta rasaun sira ba terminasaun

Peskizador Prinsipal tenki notifika GGQS wainhira iha planu atu extende durasaun projetu liu husi tempu determinadu iha leten • hamutuk ho dokumentasaun neebe relasiona ho extensaun

Peskizador Prinsipal etenki notifika GGQS wainhira nia labele ona kontinua nudar Peskizador Prinsipal inklui mos ho naran no detalles kontaktu ema ne'ebe atu troka nia

Peskizador sira no sira nia instituisaun sira tenki assume responsabilidade tomak ba seguransa no kunduta etika projetu ida ne'e

Peskizador sira tenki relata imediatamente situasaun hotu ne'ebe bele afeta kontuinuasaun aprovasaun etika projetu ida ne'e, inklui:

Efeitu adversu sira husi projetu ba sujeitu sira no prosedimentu sira ne'ebe halo ona atu resolve situasaun sira ne'e;

Eventu sira ne'ebe la preve antes hahu projetu;

Informasun foun ruma ne'ebe bele invalida integridade etika husi projetu ida ne'e, no

Proposta ba mudansa ruma iha projetu

Tenki mantein konfidensialidade projetu nia partisipante sira durante tempu tomak tuir lei ne'ebe Vigora

Folletu Informasaun ba Pasiente no Formulariu Konsentimentu tenki imprime ho kesallu no detalle kontaktu sira kompletu tuir lokalizasaun projetu

Folletu Informasaun ba Pasiente tenki fo deskrisaun breve konaba atividade peskiza ne'e, inklui risku no benefisiu sira, opsaun atu sai husi peskiza, detalle kontaktu peskizador sira no tenki mensiona mos katak bele kontaktu GGQS liu husi numeru 3331308/ +670 78256372 atu hetan informasaun konaba politika, direitu partisipante sira, prekausaun no keixa sira konaba konduta etika peskiza ne'e

18-Mar-2019

Dear Associate Professor Susana Vaz Nery,

| | |
|---|---|
| **Project Title** | Serological investigation into potential arthropod-borne and zoonotic causes of fever in Timor-Leste |
| **HC No** | HC190056 |
| **Re** | HC190056 Notification of Ethics Approval |
| **Approval Period** | 18-Mar-2019 - 17-Mar-2024 |

Thank you for submitting the above research project to the **HREC Executive** for ethical review. This project was considered by the **HREC Executive** at its meeting on **14-Mar-2019**.

I am pleased to advise you that the **HREC Executive** has granted ethical approval of this research project. The following condition(s) must be met before data collection commences:

**Conditions of Approval:** N/A

**Conditions of Approval - All Projects:**

- The Chief Investigator will immediately report anything that might warrant review of ethical approval of the project.
- The Chief Investigator will seek approval from the **HREC Executive** for any modifications to the protocol or other project documents.
- The Chief Investigator will notify the **HREC Executive** immediately of any protocol deviation or adverse events or safety events related to the project.
- The Chief Investigator will report to the **HREC Executive** annually in the specified format and notify the **HREC Executive** when the project is completed at all sites.

The Chief Investigator will notify the **HREC Executive** if the project is discontinued before the expected completion date, with reasons provided.

The Chief Investigator will notify the **HREC Executive** of his or her inability to continue as Coordinating Chief Investigator including the name of and contact information for a replacement.

The **HREC Executive** Terms of Reference, Standard Operating Procedures, membership and standard forms are available from https://research.unsw.edu.au/research-ethics-and-compliance-support-recs.

If you would like any assistance, or further information, please contact the ethics office on:

P: +61 2 9385 6222, + 61 2 9385 7257 or + 61 2 9385 7007

E: humanethics@unsw.edu.au

Kind Regards,

HREC Presiding Chairperson

This HREC is constituted and operates in accordance with the National Health and Medical

Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007)*. The processes used by this HREC to review multi-centre research proposals have been certified by the National Health and Medical Research Council.

**PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM**

Serological survey of potential causes of fever in Timor-Leste

**What is the research study about?**

This is an invitation for you and your family to take part in this research project which studies causes of fever. Fever is common in Timor-Leste, but little is known about which type of infections cause it. By testing blood samples, this study aims to measure how many people have had previous exposure to infectious which can cause fever. These include dengue virus, malaria, Chikungunya virus, Japanese encephalitis virus, zika virus, leptospirosis, rickettsia, and others. The study is important in assisting public health officials and health workers to improve diagnosis and treatment of fever, and plan preventive measures.

**Who is conducting this research?**

The study is being carried out by the following researchers:

Dr Joshua Francis (Menzies School of Health Research, Charles Darwin University, Phone +61 423 528 381)

Dr Susana Vaz Nery (Australian National University, College of Health and Medicine, Phone +61 (2) 6125 0155)

Dr Paul Arkell (Menzies School of Health Research, Charles Darwin University, Phone +44 7788 491441)

Dr Merita Monteiro (Communicable Diseases Control Programme, Ministry of Health, Timor-Leste, +67078258709

**Who is being invited to take part in this research?**

Adults and children who are already taking part in the MOH lymphatic filariasis survey (which has a separate consent form) will be invited to take part in this study.

**Do I have to take part in this research study?**

Participation in this research study is voluntary. If you or your child does not want to take part, you do not have to. If you decide to take part and later change your mind, you are free to withdraw from the study at any stage.

If you decide you want to take part in the research study, you should:

Read or listen to the information carefully (ask questions if necessary);

Sign and return the consent form if you decide to participate in the study;

Take a copy of this form with you to keep.

**What does participation in this research require, and are there any risks involved?**

If you consent to take part in the research study, three drops of blood will be taken from your finger. The first drop of blood is already being taken for the lymphatic filariasis survey (which has a separate consent form). The second and third drops of blood are for this study, and will be put onto paper, dried, stored, and later tested for previous exposure to dengue virus, malaria, Chikungunya virus, Japanese encephalitis virus, zika virus, leptospirosis, rickettsia, strongyloides and others.

We do not expect this study to cause any harm. However, you may experience a small amount of discomfort, which is normal during finger-prick blood sample collection. Only a single finger-prick is needed to get all three drops of blood, therefore participating in this study should not cause additional pain.

**What are the possible benefits of participation?**

We hope to use information we get from this research to improve the prevention, diagnosis and treatment of fever in Timor-Leste. Your participation in this study is a free gift in the spirit of human kindness to help others fight illness and remain healthy. Taking part in the study will not incur any cost to you.

**What will happen to information about me?**
By signing the consent form you consent to the research team collecting and using information about you for the research study. All information that you provide will be considered confidential. No names will be attached to your dried blood sample. Rather, it will be assigned a study number that cannot be used by anyone outside the study to identify it. All information collected will only be linked to the study number. Only researchers who were directly involved in this research will have access to information collected in this study. The information you provide will be used in presentations and published articles, but no mention of your name will appear in any of these.

Hard data containing laboratory results will be kept at each laboratory that will analyse the samples. A soft copy of the record will be kept in computer files at the National Health Laboratory in Dili, Menzies School of Health Research in Darwin, and the Kirby Institute, University of New South Wales in Sydney. The computers will be password protected and will only be accessed by the research team.

With your permission your dried blood sample will be stored in a locked cabinet in the National Health Laboratory in Dili for up to 5 years from date of collection. None of your personal information will be linked to this stored specimen. The stored blood sample may be used in the future in related research projects that investigate infectious pathogens causing illness. If you decide that you do not want your samples and/or other gathered data to be used for future research, you can tell us at any time and we will destroy them.

**How and when will I find out what the results of the research study are?**

The research team intend to publish and report the results of the research study in a variety of ways. All information published will be done in a way that will not identify you or your child.

If you would like to receive a copy of the results you can let the research team know by adding your email address or telephone number (for text messages) within the consent form. You can also send a request to 'Dr Paul Arkell, c/o Dr Joshua Francis, Darwin (Royal Darwin Hospital Campus),

John Mathews Building (JMB), Building 58, Royal Darwin Hospital Campus, e-mail paularkell@doctors.org.uk'. We will only use these details to send you the results of the research.

**What if I want to withdraw from the research study?**

If you do consent to participate, you may withdraw at any time. You can do so by completing the 'Withdrawal of Consent Form' which is provided at the end of this document. Alternatively, you can ring the research team and tell them you no longer want your child to participate. Your decision not to participate or to withdraw from the study will not affect your relationship with Timor-Leste Ministry of Health, UNSW Sydney, Menzies School of Health Research or any other institutions which are involved in this study. If you decide to leave the research study, the researchers will not collect additional information from you. Any identifiable information about you will be withdrawn from the research project. The research team will destroy any information about you that was collected during your participation in the study.

**What should I do if I have further questions about my involvement in the research study?**

The person you may need to contact will depend on the nature of your query. If you want any further information concerning this project or if you have any problems which may be related to your involvement in the project, you can contact the following member/s of the research team:

**Research Team Contact Details**

| | |
|---|---|
| **Name** | Dr Paul Arkell |
| **Position** | Co-investigator |
| **Telephone** | +670 77311945 (or +447788 491441) |
| **Email** | paularkell@doctors.org.uk |

**What if I have a complaint or any concerns about the research study?**

If you have any complaints about any aspect of the project, the way it is being conducted, then you may contact:

**Complaints Contact**

| | |
|---|---|
| **Position** | Human Research Ethics Coordinator |
| **Telephone** | + 61 2 9385 6222 |
| **Email** | humanethics@unsw.edu.au |

Or you can also contact Dr Paul Arkell, Co-investigator, phone: +670 77311945 (or +447788 491441), e-mail: paularkell@doctors.org.uk.

# Consent Form – Parent/Guardian Consent

**Declaration by the participant**

I understand I am being asked to provide consent to participate (and/or allow my child to participate) in this research project;

I have read the Participant Information Sheet or someone has read it to me in a language that I understand;

I understand the aim of this research and have been given a description of the study, and I understand what I/we will be asked to do;

I provide my consent for the information collected about me (and/or my child) to be used for the purpose of this research study only.

I have had an opportunity to ask questions and I am satisfied with the answers I have received;

I freely agree to participate in this research study as described and understand that I am free to withdraw at any time during the project and withdrawal will not affect my relationship with any of the named organisations and/or research team members;

I understand that I will be given a signed copy of this document to keep;

I would like to receive a copy of the study results via email or post, I have provided my details below and ask that they be used for this purpose only;

**Name: _____Address: _____Email Address: _____**

**Participant or parent/guardian signature**

| | |
|---|---|
| Name of Participant(s) (please print) | Participant:……<br>Child 1: …… Child 2: ……<br>Child 3: …… Child 4: …… |
| Signature of Research Participant | |
| Date | |

**Declaration by Researcher***

I have given a verbal explanation of the research study, its study activities and risks and I believe that the participant has understood that explanation.

**Researcher Signature***

| | |
|---|---|
| Name of Researcher (please print) | |
| Signature of Researcher | |
| Date | |

**[+]An appropriately qualified member of the research team must provide the explanation of, and information concerning the research study.**

# Form for Withdrawal of Participation

I wish to **WITHDRAW** my consent for my child to participate in this research study described above and understand that such withdrawal **WILL NOT** affect my relationship with Timor-Leste Ministry of Health, UNSW Sydney, Menzies School of Health Research or any other institutions which are involved in this study. In withdrawing my consent I would like any information collected from me or my child that has been provided for the purpose of this research project withdrawn.

**Participant Signature**

| | |
|---|---|
| Name of Participant (s) (please print) | Participant:……<br>Child 1: …… Child 2: ……<br>Child 3: …… Child 4: …… |
| Signature of Research Participant | |
| Date | |

**The section for Withdrawal of Participation should be forwarded to:**

| | |
|---|---|
| Investigator Name: | *Dr Paul Arkell* |
| Email: | paularkell@doctors.org.uk |
| Phone: | +447788 491441 |
| Postal Address: | *c/o Dr Joshua Francis, Darwin (Royal Darwin Hospital Campus), John Mathews Building (JMB), Building 58, Royal Darwin Hospital Campus* |

**PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM**

Serological survey of potential causes of fever in Timor-Leste

**1.Saida mak Estudu ba peskiza ida ne'e?**

Ida ne'e mak konvite ida ba imi no imi-nia família sira atu bele mai partisipa iha projetu peskiza ida ne ' ebé sei hala'o kona ba estudu kauza ba moras febrile. Febrile mak moras ida ne'e komún iha Timor-Leste, maibé deskobre uitoan deit ba tipu infeksaun ne'ebe maka hetan Hosi ijame teste sample raan. estudu ne'e ho nia objetivu atu sukat ema nain hira ona mak hetan infeksaun ne ' ebé bele causa husi moras febrile iha pasadu. Ne'e inklui mos virus sira hanesan; vírus dengue, vírus chikungunya, virus zika no virus sira seluk. Estudu ne'e importante tebes katak bele fo tulun funsionáriu saúde públika no traballadór saúde sira-hodi desembolve sira nia diagnóstiku no tratamentu ba moras febrile sira, no bele halo planu ba medida preventiva.

**2.Sése mak hala'o peskiza ida ne'e ?**

Estudu ne'e hala'o hosi peskizador sira ne'ebe mak hanesan tuir mai ne'e:

Dr Joshua Francis (Menzies School of Health Research, Charles Darwin University, contaktu +61 423 528 381)

Dr Susana Vaz Nery (Australian National University, College of Health and Medicine, contaktu +61 (2) 6125 0155)

Dr Paul Arkell(Menzies School of Health Research, Charles Darwin University, contaktu +44 7788 491441)

Dr Merita Monteiro (Communicable Diseases Control Programme, Ministry of Health, Timor-Leste, contaktu +67078258709)

**3. Sé los mak hetan ona konvite hodi hola parte iha peskiza ida ne'e?**

Labarik no Ema adultu sira -ne ' ebé uluk hola parte iha dadus levantamentu lymphatic filariasis husi Ministériu De Saude (ne ' ebé iha formatu konsentimentu ketak ), sira mos sei hetan konvite atu mai partisipa iha estudu ne'e.

**4. Ha'u mos bele hola parte iha estudu peskiza ida ne'e ga lae?**

Partisipasaun ba estudu peskiza ida ne'e halao voluntáriumente. Karik ita ka ita-nia oan lakohi atu hola parte, ami sei la obriga. Se ita deside atu partisipa no ikus mai troka ita bot nia hanoin, ita mos iha liberdade atu hasees a'an husi estudu iha faze ruma.

Se ita hakarak duni atu partisipa iha estudu peskiza ida ne'e, ita tenke halo tuir kriteria sira hanesan tuir mai ne'e;

Lee didiak informasaun no husu pergunta (se karik ita la komprende);

Hafoin ita lee hotu tia asina no entrega fila fali formatu konsentimentu ida ne'e karik ita deside atu partisipa;

Kopia formatu ida no rai kopia ho ita.

**5. Partisipasaun iha peskiza ida ne'e nia rekerementu mak saida, no ida ne'e involve iha risku ruma ka lae?**

Se ita hakarak atu partisipa iha studu peskiza, ita sei hasai ita nia rann husi liman-fuan ba turuk dala tolu.. raan turuk dahuluk sei foti hodi halo ba levantamentu lymphatic filariasis (ne ' ebé iha forma konsentimentu ketak ida). raan turuk daruak no datoluk sei halo estudu, ida ne'e, sei tau ba surat tahan, hamaran , rai hela, no depois sei koko ba infeksaun uluk ho dengue, malaria, chikungunya, Japanese encephalitis, zika, leptospirosis, rickettsia, strongyloides ho seluk tan.

Ita la espera katak estudu ida ne'e atu halo estragu ou hakanek ruma. Maibé, ita mos preukupa ida ne'e sei sai esperiénsia ' ebé mak normál durante hasai ran no rekolla ran. Presiza ran Husi liman fuan ida hetan raan turuk tolu, tanba ne'e partisipa iha estudu ne'e sei la hakanek ita .

**6. Benefísiu saida mak ita sei hetan hosi partisipasaun ida ne'e?**

Ami espera katak informasaun ne ' ebé ita hetan hosi peskiza ida ne'e bele halo mudansa di'ak liután ba prevensaun, diagnóstiku no tratamentu ba moras febrile iha Timor-Leste. Ita-nia partisipasaun iha estudu ida ne'e fornese grratuita hanesan prezente ida-ne ' ebé mak mai husi ema ne'ebe iha espíritu no laran-di'ak atu ajuda ema seluk hodi kombate moras no moris ho isin-di'ak. Hola parte iha estudu ida ne'e gratuita no sei la kustu buat ruma ba imi.

**7. Oisa mak hau bele hatene hau nia informasaun husi partispasaun ida ne'e?**

Husi asinatura formatu konsentimentu ne'ebe imi autoriza rasik ba ekipa peskiza hodi rekolha no foti informasaun kona-ba ita nia an ba estudu peskiza. Informasaun hotu-hotu ne ' ebé ita fornese sei konsidera hanesan konfidensiál. No ita boot nia naran sei la temi ( atached) ba ita nia sample ran maran. ida-ne'e sei atribuí númeru estudu ne ' ebé maka la autoriza ema liur atu bele uza ba estudu hodi identifika . Informasaun hotu-hotu ne ' ebé halibur sei ligadu de'it ba númeru estudu. peskizador ne ' ebé mak involve diretamente iha peskiza sei iha asesu ba informasaun ne ' ebé halibur iha estudu ne'e. Informasaun ne ' ebé ita fornese sei uza iha aprezentasaun no publika artigu, maibé la temi kona-ba ita-nia naran no sei la publika .

Dadus (Hard data) ne ' ebé hamutuk ho rezultadu laboratóriu sei kontinua rai iha laboratóriu ida-idak ne ' ebé sei halo analiza ba sample refere. Ba kópia lista nia ga soft copy sei rai iha compute iha r arkivu Laboratóriu Nasionál de Saúde Dili, Menzies Eskola Saúde Peskiza iha Darwin, no ba Institutu Kirby, Universidade New South Wales iha Sydney.

Komputadór sira sei iha protesaun password no sei asesu deit hosi ekipa peskizador sira

Ho ita-nia autorizasaun ita nia ran maran sei rai iha gabinete Laboratóriu Nasionál de Saúde iha Dili to'o tinan 5 laran hosi loron ne'ebe iha halo koleksaun. La iha informasaun pesoál ne'ebe sei ligadu ba specimen stored ida-ne'e. raan sample sira ne'e be rai hela sei bele uza iha futuru pa projetu peskiza sira ne ' ebé relasiona ho investigasaun pathogens poténsia sira ne ' ebé causa moras. Se imi deside katak imi lakaoi imi-nia sample ran atu manda ba rai liur ka atu rai no usa iha futuru ba peskiza , imi bele fó-hatene ami iha kualkér tempu no ami sei bele halo destroi ba imi nia dadus.

**8. Oinsa no wainhira ita sei buka saida mk resultado sira ne?**

Husi grupo hare fali iha relatorio nebe mak hatama ona iha sistema sira kona ba resultado estuda nebe mak verifica tiha ona.  Informasaun sira nebe mka  hatama ona iha sistema maibe sei la identifica ita,ho ita boot nia oan .

Sekarik ita boot hakarak simu ou kopia resultado ita boot bele fo hatene ba ita nia grupo ou liu husi Email Address ka numero kontakto atu haruka mensagen husi papel,ita bot mos bele haruka karta ba DR.Paulo Arkell,C\O Dr Joshua Francis,Darwin(Royal Darwin Hospital Campus),Jhon Mathews Building (JMB) Building 58 Royal Darwin Hospital Campus,e-mail paullarkell@doctors.org.uk.Ami sei iha deit sistema sira nea tu haruka ita boot nia resultado kona ba informasaun.

**9.saida mak hau hakarak hetan husi estuda ne?**

Sekarik ita boot fokus no activo,iha loron ikus mai ita boot bele halo completo ou ita buka dalan papel seluk atu fahe ita boot nia nia dukumentasaun sira t oba iha nebe deit ou bele buka opcao seluk atu fahe informasaun kona b aita nia grupo no fohatene sira no sei lakeur maibe hakarak ita nia oan tenki participa ho ita bot nia desizaun laos partisiopa  programa sira ne hodi halo affeta ba ita nia familia.Ministerio Saude Timor leste UNSW Syoney,minzie eskola konaba saude ou iha instituisau seleuk sira nebe mak  involve iha estuda ida ne,sekarik ita boot deside atu husik formasaun estuda,ita boot nia grupo sei la hatama adicional informasaun husi ita boot,no sei la identifica konaba informasaun nebe mak ita boot halo husi grupo ga projeto.

Ita boot nia grupo sei la fo sai informasaun  ou hatama ita nia relatorio sira durante ita boot participa iha estudo.

**10.  Saida mak hau prisija no halo iha pergunta kona ba involvimento grupo estuda?**

Ema nebe mak ita boot presija atu kontakto depende ba ita,karik ita boot hakarak buka tan informasaun seluk atu halo acordó ho ita nia projeto ou sekarik ita iha problema ida nebe mak relasiona ho ita boot nia involvimento sira iha ita nia projeto.ita bele kontakto ou halo tuir membros sira iha grupo ne.

**11. Grupo nia aplikasaun:**

| | |
|---|---|
| **Naran** | Dr Paul Arkell |
| **Posisaun** | Co-investigator |
| **Numero Telefone** | +670 77311945 (or +447788 491441) |
| **Email** | paularkell@doctors.org.uk |

**12. Saida mak hau iha karik atu submete  iha papel konaba estuda?**

Karik hau iha duvida kona ba aspecto iha projeto,dalan ba hau atu confirma ou bele contakto;

| | |
|---|---|
| **Posisaun** | Coordenador |
| **Numero Telefone** | + 61 2 9385 6222 |
| **Email** | humanethics@unsw.edu.au |

Ita boot Bele kontakto Dr.Paul Arkell, Co-investigator, Numero Telefone: +670 77311945 (or +447788 491441), email: paularkell@doctors.org.uk

# Papel

**Declarasaun husi participante:**

Hau hatene hau husu no fahe papel konoba participante (no/ka hatan hau –nia oan atu participa iha projeto ida-ne'e;

Hau lee informasaun konoba participasaun ka ema lee mai hau iha lian neebe hau komprende;

Hau komprende ojectivo iha grupu no fo mai hau papel konaba estuda, no hau komprende saida mak hau/ami bele husu atu halo;

Hau fo hau-nia papel konaba informasaun hetan konoba hau (no/ka hau-nia oan) atu bele usa ba objectivo ida nee mak estuda deit.

Hau iha oportunidade atu husu pregunta no hau satisfeto ho resposta neebe hau simu;

Hau libre hatan atu participa iha programa ho estuda hanesan hakerek iha papel ida ne'e no hau komprende katak ha'u libre atu sai iha horas neebe deit durante projeto no sai sei la afecto ba hau-nia relasaun ho ema neebe iha organisasaun o membru grupu-nian;

Hau komprende katak ha'u sei fo asignatura kopi ba documentus ne'e rai;

Ha'u hakarak simu kopi husi resultado estuda o liu husi email o post. Ha'u fo hau-nia documentos sira iha kraik no husu katak sira usa objectivo ida ne'e deit;

**Naran: _____ Hela Fatin: _____ Email: _____**

**Asignatura:**

| | |
|---|---|
| Participasaun Naran<br>( favor ida hakerek) | Participasaun:……<br>Oan 1: …… Oan 2: ……<br>Oan 3: …… Oan 4: …… |
| Asignatura husi participante | |
| Data | |

**Declarasaun husi Servisu***

Ha'u explica tiha ona konoba estuda, ida ne'e actividades, no risku ho ha'u fiar katak participante komprende konoba explicasaun ida ne'e..

**Asignatura Servisu***

| | |
|---|---|
| Naran servisu ( favor ida hakerek) | |
| Asignatura servisu | |
| Data | |

***Capacidade iha cualidade membru iha grupo servisu tenke fo explicasaun konoba, informasaun konoba papel estuda.**

# Papel konoba sai participante

Ha'u hakarak sai ha'u-nia papel ba ha'u-nia oan atu participasaun iha estuda ida ne'e no ha'u komprende katak se ha'u sai sei la afeta ha'u-nia relasaun ho Ministerio Saude Timor L'este, UNSW, o institusaun ruma sire neebe mak involve iha estuda ida ne'e. Se ha'u sai ha'u-nia papel hakarak sai mos informasaun konoba hau o hau nia oan neebe hetan tiha ona fo objectivo konoba projeto ida ne'e.

**Asignatura:**

| | |
|---|---|
| Participasaun Naran ( favor ida hakerek) | Participasaun:……<br>Oan 1: …… Oan 2: ……<br>Oan 3: …… Oan 4: …… |
| Asignatura husi participante | |
| Data | |

**Secsaun ida ne'e ba participasaun neebe hakarek sai tenke haruka carta:**

| | |
|---|---|
| Investigasaun naran: | *Dr Paul Arkell* |
| Email: | paularkell@doctors.org.uk |
| Numero Telefone: | +447788 491441 |
| Hela Fatin: | *c/o Dr Joshua Francis, Darwin (Royal Darwin Hospital Campus), John Mathews Building (JMB), Building 58, Royal Darwin Hospital Campus* |

# Appendix 6: Research Ethics Committee approval, participant information sheet, consent form, and data collection tools for lateral flow study and study determining age-stratified dengue seroprevalence (Chapter 6 and 7)

MINISTÉRIO DA SAÚDE

Instituto Nacional de Safidc

Gabinete Executivo

No. Ref.: 895 MS-INS/GDE/1X/2021

Dili, Septemberl,st 2021

ETHICS APPROVAL LETTER

Professor DR. Nelson Martins, MD, IMHM PhD

Plaza Hotel, Office of Menzies School of Health Research, Dili Timor Leste

Ph: +670 74178262 (WhatsApp)

Email:nmdasilaku@gmail.com

## Project Title: National Scro-Prevalcnce Study of C 19, Measles-Rubclla, IIcp-B and Dengue inTimor-Lcste

Thank you for submitting the above research project _for technical and ethical review. This project was considered by the Institute National of Health-Research Technical Committee with Ethical Part from Cabinet of Guarantee and Health Quality in MOH for their consideration at its meeting held on June 15 th 2021 and passed long process of getting final ethical approval letter from INS.

I am pleased to advise you that the Institute National of Health-Research Ethics & Technical Committee (INS-RETC) has granted Ethics & Technical approval of this research project.

Please note that if additional sites are engaged prior to the commencement of, or during the research project, the coordinating Principal Investigator is required to notify the Institute National of Health-Research Ethics & Technical Committee (INS-RETC), Notification of withdrawn sites should also be provided to the TL-IIREC in tirnely fashion

The Approved documents include:

l. INS-RETC Application form

2. Proposal

3, Inform consent

Questionnaire

This approval is for period of twelve (12) months. An ANNUAL /FINAL Project progress report is requil'ed on or before September 30th 2022

APROVAL IS SUBJECT to the following conditions being met:

The Coordinating Principal Investigator will iminediately report anything that rnight warrant review of technical and ethical approval of the project.

The coordinating Principal Investigator will notify the Institute National of HealthResearch Ethics& Technical Committee (INS-RETC) or any event that requires a modification to the Protocol or other project document and submit any required amendments and accordance with the instructions provided by the INS-RETC,

7, The Coordinating Principal Investigator will sublilit any necessary repol't related to the safety of research participant (i.e. Protocol deviation, protocol violations) in

Edificio Instituto 'Vacional de Satide,

Rua de Cmnoro Dili Il'ebsile;www.ins-gov-tl.cmn,.

Telf; 33310099

MINISTÉRIO DA

Instituto Nacional de Safide

SAÜDEGabinete Diret6r Executivo

accordance with Institute National of Health-Research Ethics& Technical Committee (INS-RETC) policy and procedures.

The coordinating PI will report to the INS-RETC, Annually in the specified rormat and Notify the HREC when the project is completed at all sites.

The coordinating PI will notify the INS-RETC if the project is discontinued at a participating site before the expectcd completion date, with reason provided.

The coordinating PI will notify the INS-RETC of any plan to extend the duration of the project past the approval period listed above and will submit any associated required documentation.

l1 . The coordinating PI will notify the INS-RETC of his or her inability to continue as coordinating PI including the name of and contact infornyttion for a replacement.

The safe and ethical conduct of this project is entirely the responsibility of the investigators and their institution(s).

Researcher should report immediately anything which might affect continuing ethical acceptance of the project, including:

13.1 . Adverse of the project on subject and steps taken to deal with these;

13.2.Other unforeseen events;

New Inforlnation that may invalidate the ethical integrity of the study; and

Propose changes in the project.

Approval for further twelve months will be granted if the INS-RETC is satisfied that the conducted of the project has been consisted with the original protocol.

Confidentiality Research participants should be Inaintained at all times as required by law

The patient inforrnation sheet and the consent form shall be printed on the relevant site letterhead with full contact details.

This Letter Constitutes Ethical & Technical Approval Only.

Should you have any queries about the Institute National of Health-Research Ethics& Technical Committee (INS-RETC)'s Consideration of your project please contact Mr. Caetano Gusmao,MPH, with contact number +670 7723 4849, 7732 7595 and email address: wairisi@yahoo.eonl.au INS-RETC wishes you every success in your research.

Domingas

Executive Director of INS

Edificlo Instituto de Sati(le, Rua de Conujro Dili Website;vewn'.ins-gov-d.com,.

Tell: 33310099



menzies
school of health research

Postal: PO Box 41096, Casuarina NT 0811, Australia
Location: John Mathews Building (Bldg 58),
Royal Darwin Hospital Campus, Rocklands Drv, Cas
Ph: (08) 8946 8600 Fax: (08) 8946 8464
Website: menzies.edu.au
ABN: 70 413 542 847

14 September 2021

Ethics Administration Office
File Reference Number: HREC-2021-4064
Phone: (08) 8946 8687 or (08) 8946 8692
Email: ethics@menzies.edu.au

Dr Nelson Martins

Universidade da Paz

Menzies School of Health Research nelson.martins@menzies.edu.au CC: stacey.kopf@menzies.edu.au

Via Email

Dear Dr Martins,

HREC Reference Number: 2021-4064

Project Title: National Seroprevalence Study of COVID-19, measles, rubella, hepatitis B, and dengue in Timor-Leste

Thank you for email dated 09/09/2021 and taking the time to respond to the issues of concern identified by the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) at its meeting held on the 23/06/2021.

This project was considered by the HREC and assessed against guidelines for human research including the NHMRC National Statement on Ethical Conduct in Human Research 2007.

I am pleased to advise that full ethical approval of this research project has been granted following assessment by representatives of the HREC. Please note that approval applies only to research conducted after the date of this letter and continued approval is dependent on annual reporting.

Approval Date: 14/09/2021

Approval is granted for the above research project until the next report due date.

Annual progress report due: 14/09/2022

Approved timeframe (subject to compliance and annual reporting): 14/09/2021 to 14/09/2022

The nominated sites/s participating in this project that have been approved by this HREC is/are:

122 Enumeration Areas (EA) in Timor-Leste (groups of households, in the community) • Timor-Leste National Health Laboratory

Please note:

Researchers must comply with site specific governance regulations, data custodian and other stakeholder requirements.

APPROVAL IS SUBJECT TO the following conditions being met:

1 . The Coordinating Principal Investigator will immediately report anything that might warrant review of ethical approval of the project.

The Coordinating Principal Investigator will notify the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (HREC) of any event that requires a modification or amendment to the protocol or other project documents and submit any required amendments in accordance with the instructions provided by the HREC. These instructions can be found on the Menzies' website.

The Coordinating Principal Investigator will submit any necessary reports related to the safety of research participants (e.g. protocol deviations, protocol violations) in accordance with the HREC's policy and procedures. These guidelines can be found on the Menzies' website.

The Coordinating Principal Investigator will report to the HREC annually and notify the HREC when the project is completed at all sites using the specified forms. Forms and instructions may be found on the Menzies' website.

The Coordinating Principal Investigator will notify the HREC if the project is discontinued at a participating site before the expected completion date and provide the reason/s for discontinuance.

The Coordinating Principal Investigator will notify the HREC of any plan to extend the duration of the project past the approval period listed above and will submit any associated required documentation. The preferred time and method of requesting an extension of ethical approval is during the annual progress report. However, an extension may be requested at any time.

The Coordinating Principal Investigator will notify the HREC of his or her inability to continue as Coordinating Principal Investigator, including the name of and contact information for a

replacement.

The safe and ethical conduct of this project is entirely the responsibility of the investigators and their institution(s).

Researchers should immediately report anything which might affect continuing ethical acceptance of the project, including:

Adverse effects of the project on participants and the steps taken to deal with these; • Other unforeseen events;

New information that may invalidate the ethical integrity of the study; and • Proposed changes in the project.

Approval for a further twelve months, within the original proposed timeframe, will be granted upon receipt of an annual progress report if the HREC is satisfied that the conduct of the project has been consistent with the approved protocol, Report templates are available on the Menzies ethics webpage.

Confidentiality of research participants should be maintained at all times as required by law.

The Patient Information Sheet and the Consent Form shall be printed on the relevant site letterhead with full contact details.

The Patient Information Sheet must provide a brief outline of the research activity including: risks and benefits, withdrawal options, contact details of the researchers and must also state that the Human Research Ethics Administrators can be contacted (telephone and email) for information concerning policies, rights of participants, concerns or complaints regarding the ethical conduct of the study.

You must forward a copy of this letter to all Investigators and to your institution (if applicable).

This letter constitutes ethical approval only. This project, including amendments to the research protocol or conduct of the research which may affect the site acceptability of the project, cannot proceed at any site until separate site specific assessment or research governance authorisation has been obtained from the CEO or Delegate of the institution under whose auspices the research will be conducted at that site, if not already obtained. Please forward this approval letter to the relevant research governance office.

Should you wish to discuss the above research project further, please contact the Ethics Administrators via email: ethics@menzies.edu.au or telephone: (08) 8946 8687 or (08) 8946

8686.

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research wishes you every continued success in your research.

Yours sincerely,

Dr. Mary Morris

Chair

Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research http:llwww.menzies.edu.aulethics

This HREC is registered with the Australian National Health and Medical Research Council (NHMRC) and operates in accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007). NHMRC Reg no. EC00153

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**Participant information sheet**

Local Study Coordinator Name, National Health Laboratory, Dili

## SEROLOGICAL STUDY OF VACCINE-PREVENTABLE DISEASES IN TIMOR-LESTE

## This is for you to keep / Ida ne'e fó ba ita bo'ot atu rai

You are being invited to take part in a study about vaccine-preventable diseases, which include COVID-19, Hepatitis B, Measles, Rubella and Dengue.

**Participation:** Taking part in this project is entirely voluntary. You can withdraw from the study at any time. You do not have to explain why you want to withdraw from the study, and there are no negative consequences if you withdraw. If you have any questions, please discuss them with members of our team.

**Partisipasaun:** Partisipa iha projetu ida ne'e kompletamente voluntariu. Ita bo'ot bele sai husi estudu iha kualker tempu. Ita bo'ot la presiza atu esplika razaun tamba saida, no sei la iha konsekuensia negativu karik ita bo'ot sai. Karik ita bo'ot iha kualker pergunta, favor bele hato'o ba membru husi ami nia ekipa.

**What are vaccine preventable diseases?** Vaccine preventable diseases are infectious diseases which can be prevented through vaccination. These include COVID-19, Hepatitis B, Measles, Rubella and Dengue.

COVID-19 is the disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is a type of coronavirus. The virus was first identified in December 2019 and is new for humans. Coronavirus is spread from person to person mostly by droplets and contaminated surfaces. Coronavirus can cause both mild and severe disease, especially in older people and people with medical conditions. Some people with coronavirus may have no symptoms. Common symptoms of coronavirus are fever and cough. Other symptoms include headache, sore throat, tiredness, shortness of breath, sore muscles, loss of taste and smell, chills and vomiting.

COVID-19 started spreading in Timor-Leste in February 2021, and vaccines have been available since April 2021.

Hepatitis B is a virus which is spread by contact with blood. This usually occurs from mother to child (during childbirth), but can also occur by contaminated needles or blood transfusions. In some people the virus causes a short infection, the goes away. In other people, the virus causes a long-lasting infection, which can cause damage to the liver over time. Vaccines are given to new-born babies in Timor-Leste, to prevent them from getting Hepatitis B.

Measles and rubella are infections which can cause outbreaks in children. They usually cause fever and rash, and can occasionally cause serious illness. Vaccines are given to young children to prevent them from getting measles and rubella.

Dengue is a virus spread by mosquitoes which can cause illness in children and adults. Usually it causes fever with headache and rash, sometimes the illness can be serious. Currently vaccines are not used against dengue in Timor-Leste.

**Who is organising the project?** The study is a collaboration between:

**Se mak organiza projetu ida ne'e?** Estudu ida ne'e kolaborasaun entre:

Ministry of Health, Timor-Leste / Ministeriu da Saúde, Timor-Leste

Menzies School of Health Research, Australia

National Health Laboratory, Dili / Laboratóriu Nasional Saúde, Dili

National Centre for Immunisation Research and Surveillance, Australia / Sentru Nasional ba Peskiza no Vigilansia Imunizasaun, Australia

Approval for this project. This study has been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia.

Aprovasaun ba projetu ida ne'e. Estudu ida ne'e hetan ona aprovasaun husi Institutu Nasional da Saúde (INS), Timor-Leste no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research iha Australia.

Informed Consent Information / Informasaun Konsentimentu ne'ebé mak Informadu

Section 1: Purpose of the study.

The main purpose of this study is to see how many people in Timor-Leste have antibodies against different vaccine-preventable diseases. If a person has antibodies to a particular infection, it means they have either been exposed in the past, or have been vaccinated. It sometimes means that the person has protection against the infection ('immunity').

We will test the blood for antibodies against COVID-19, hepatitis B, measles, rubella and dengue. These tests will be done in the laboratory in Dili (National Health Laboratory)

Approximately 5600 people in Timor-Leste will be invited to participate in the project.

Section 2: Procedures. For each person, we will:

Seksaun 2: Prosedimentu. Ba kada partisipante, ami sei:

Ask some **QUESTIONS** including name, age, gender, contact details, occupation, vaccination details.

Take a **BLOOD SAMPLE** (5mL). The blood samples will be used to test for previous exposure or vaccination against COVID-19, hepatitis B, measles, rubella and dengue. The blood sample will also be used to test for hepatitis B infection, and to validate check the accuracy of different tests.

Take a **DRIED BLOOD SPOT** (3 drops of blood). This will be made by applying drops of blood to a small piece of filter paper, then drying.

The **blood samples may also be used for validating different tests, and for potential future use to answer medical research questions relating to communicable or non-communicable diseases in Timor-Leste**. All results will be de-identified, and only grouped results will be reported. Anonymized blood samples will be stored at the National Health Laboratory for 10 years before being disposed of.

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**Section 3: Duration of Procedures.** Collection of the blood sample will take less than 10 minutes. The questionnaire should take about 5 minutes to complete and can be answered in Tetun or English.

**Seksaun 3: Durasaun ba Prosedimentu.** Koleksaun ba amostra ran sei uza tempu pelu menus minutu 10. Kuestionariu sei uza tempu durante minutu 5 hodi kompleta, no bele resposta uza lian Tetun ou Ingles.

**Section 4: Potential benefit to you and others. / Seksaun 4: Benefisiu potensial ba ita bo'ot no ema seluk.**

The blood tests for COVID-19, measles, rubella and dengue provides evidence of past infection or vaccination, but do not test for current infection. There is no need for any treatment if these tests are positive. The blood test for hepatitis B will check for active infection. If this is positive, you will be offered assessment and management (which may include treatment) at the hepatology clinic at Hospital Nacional Guido Valadares (NHGV).

The results of the study will provide important and useful information for Timor-Leste on the success of managing patients with COVID-19 in health care facilities without the transmission of coronavirus transmission.

**Section 5: Discomforts and Risks.** The potential risks of collecting a venous blood sample include temporary discomfort from the needle, bruising, bleeding, and very rarely, infection. Experienced doctors, nurses, or laboratory technicians will take the blood samples. The risk of problems is very small. In the unlikely event of any significant injury because of the blood collection procedure, the study team and/or the Ministry of Health will arrange necessary medical treatment without financial cost. However, no financial compensation will be given in case of any study-related injury.

**Seksaun 5: Deskonfortus no Risku.** Potensia ba risku husi kolekta ran venozu sei inklui mos deskonfortu temporariu husi daun, kulit mean (bubu), ran sai, no infeksaun raramente. Doutor, enfermeiru/a, ou tekniku laboratoriu sira ne'ebé mak iha ona esperiensia mak sei foti amostra ran. Risku ba problema ne'e kiik tebes. Karik mosu kualker prejuizu signifikante relasiona ho prosedimentu ba koleksaun ran nian, ekipa estudu/Ministeriu Saude sei aranja tratamentu medikal ne'ebé mak nesesariu sem kustu finansial. No entantu, sei la iha kompensasaun finansial ba kualker injuria ne'ebé mak mosu relasiona ho prosesu estudu.

If you are found to have active hepatitis B, you will be referred to the hepatology clinic and you will be offered further tests to see if any treatment is required.

**Section 6: Costs for participation.** Taking part in the project will cost nothing apart from your time.

**Seksaun 6: Kustu ba partisipasaun.** Partisipasaun iha projetu ida ne'e, sei la kobre kustu ruma exeptu ita bo'ot nia tempu.

**Section 7: Compensation for participation.** You will not receive any payments for being part of this project.

**Seksaun 7: Kompensasaun ba Partisipasaun.** Ita bo'ot sei la simu kualker pagamentu ba partisipasaun iha projetu ida ne'e.

**Section 8: Study funding.** This study is funded by the Australian Government Department of Foreign Affairs and Trading (DFAT). The Ministry of Health, Timor-Leste has been closely involved in the planning and development of this study and is involved in undertaking this study. The collaborators do not have any conflict of interests to declare.

**Seksaun 8: Finansiamentu ba estudu.** Estudu ida ne'e hetan finansiamentu husi Departamentu Asuntu Estranjeiru no Komersiu, Guverno Australia (DFAT). Ministeriu Saude Timor-Leste, proximamente involve ona iha planeamentu no dezenvolvimentu ba estudu ida ne'e no envolve ba implementasaun estudu ida ne'e. Kolaboradores sira la iha kualker konflitu interese atu deklara.

**Section 9: Voluntary participation.** Taking part in this project is your choice, and you will have the right to stop at any time. If individuals or communities decide not to participate or decide to stop taking part at any time, there will be no penalties.

**Seksaun 9: Partisipasaun Voluntariu.** Partisipa iha projetu ida ne'e ita bo'ot mak hili, no ita bo'ot sei iha direitu atu hapara/sai iha kualker tempu. Karik individual sira ou komunidade deside atu la partisipa ou deside atu sai husi partisipasaun iha kualker tempu, ida ne'e sei la iha penalidade ruma.

**Section 10: Delivery of results.** If your blood test shows that you have active hepatitis B, you will be contacted and offered assessment and management (which may include treatment) at the hepatology clinic at Hospital Nacional Guido Valadares (NHGV).

You will not be contacted with results of the other blood tests (COVID-19, measles, rubella and dengue antibodies). This is because we are planning on testing 5600 people, the tests may take several months to complete, and it would be very difficult to contact everyone. Additionally, these tests are not for active infections, so there is no need for any treatment if they are positive.

Results for the whole study will be written-up and may be published. It may be used by the Ministry of Health to plan additional vaccines and other disease-control activities in Timor-Leste. During this whole process, individual results will remain strictly confidential.

**Section 11: Privacy and confidentiality.** A unique code will be used to identify and link the questionnaire data and blood sample results. Study records and completed questionnaires will be reviewed, stored, and analyzed on a computer at the Menzies office in Dili and will be kept in secured areas and password protected computers. Blood samples collected for laboratory testing will be labeled with the unique code and securely stored at the National Health Laboratory in Dili, Timor-Leste. The list that matches participants' names with unique codes will be kept in a password protected file on a computer in the Menzies office in Dili. No personally identifiable information will be shared in any project reports, publication, or presentations. We will keep each individual's participation in this project confidential to the extent permitted by law. However, it is possible that other people may become aware of their participation in this study, e.g. the following people/groups may inspect project records:

The Instituto Nacional da Saúde; INS and the Ministry of Health, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia

**Seksaun 6: Privasidade no Konfidensialidade.** Sei uza kodigu uniku hodi identifika no liga dadus husi kuestionariu no rezultadu amostra ran. Registrus no kuestionariu ne'ebé kompleta ona husi estudu sei reviza, armazena, no halo analizasaun uza komputador iha Menzies nia office Dili, no sei rai iha area seguru no iha komputador ne'ebé mak proteje uza password. Amostra ran ne'ebé mak koletadu hodi halo teste iha laboratoriu sei tau rotulagem/label ho kodigu uniku no seguramente sei rai iha Laboratoriu Nasional Saude, Dili Timor-Leste. Lista ne'ebé mak kombinadu ho partisipante nia naran ho kodigu uniku ne'e sei rai iha arkivu 1 protektidu ho password iha komputador iha Menzies nia Office, Dili. Kualker informasaun personal ne'ebé mak identifikavel sei la kompartilha iha kualker projetu nia relatoriu, publikasaun, ou aprezentasaun sira. Ami sei mantein kada individual nia partisipasaun iha projetu ida ne'e konfidensial tuir lei haruka. No entantu, iha posibilidade ba ema balu atu notifika konaba ita bo'ot sira nia partisipasaun iha estudu ida ne'e, por ezemplu ema/grupu hirak hanesan tuir mai ne'e sei bele halo inspeksaun ba projetu nia registrus:

Instituto Nacional da Saúde (INS) no Ministeriu Saude, Timor-Leste

Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia

Some of these records could contain information that tells them who participated in the project. However, the results of blood test and answers to the questionnaires will not be linked to this information. Your test results, including the hepatitis B results will not be shared with any person outside the study team.

Husi registrasaun sira ne'e balun sei inklui informasaun ne'ebé mak sei informa konaba partisipante sira iha projetu ida ne'e. No entantu, rezultadu teste ran nian no resposta iha kuestionariu sira, sei la inklui iha informasaun refere. Ita bo'ot nia rezultadu teste, inklui rezultadu hepatitis B sei la kompartilha ho kualker ema ne'ebé mak laos ekipa estudu.

**Section 12: Contact information for questions or concerns.** You have the right to ask any questions you may have about this study. If you have questions, complaints or concerns or believe you may have developed an injury related to this study, please contact Local Study Coordinator Name, National Health Laboratory, Dili, Phone: TBC; Email: TBC. If you have questions regarding your rights as a study participant or concerns regarding the ethical conduct of the project, please contact Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au). If you have any medical problems associated with this study, such as a pain from where your blood was collected or feeling unwell, please contact the above phone numbers or attend your local hospital or clinic if urgent.

**Seksaun 12: Informasaun kontaktu karik iha pergunta ou preokupasaun ruma.** Ita bo'ot iha direitu hodi husu kualker pergunta ne'ebé mak relasiona ho estudu ida ne'e. Karik ita bo'ot iha pergunta, reklamasaun ou preokupasaun ou karik ita bo'ot hetan injuria relasiona ho estudu ida ne'e, favor kontaktu Local Study Coordinator Name, National Health Laboratory, Dili, Phone: TBC; Email: TBC. Karik ita bo'ot iha pergunta relasiona ho ita bo'ot nia direitu nudar partisipante ou preokupasaun relasiona ho projetu nia étika konduta, favor kontaktu Sekretariu étiku, komisaun etika peskija komite tekniku INS (Ph +670 7708 7665) ou Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (telemovel +618 8946 8600; Email ethics@menzies.edu.au). Karik ita bo'ot iha kualker problema mediku ne'ebé mak asosiadu ho

estudu ida ne'e, por ezemplu hanesan moras iha fatin hasai ran ou sente la saudavel, favor kontaktu ba numeru ne'ebé mensiona iha leten ou konsulta iha hospital lokal ou klinika sira karik urgente.

**Section 13: Consent to participate in project.** Before making the decision about participating in this study you should have:

Discussed this project with a member of the study team

Read and understood the information in this form

Had the opportunity to ask any questions that you may have

Had time to consider whether to take part

Information about who to contact in case you have any further questions or problems

Seksaun 13: konsentimentu atu partisipa iha projetu. Antes foti desizaun atu partisipa iha estudu ida ne'e ita bo'ot tenke:

Diskute uluk konaba projetu ida ne'e ho membru husi ekipa estudu nian

Le'e no komprende informasaun iha formulariu ida ne'e

Iha oportunidade hodi husu kualker pergunta ne'ebé mak ita bo'ot iha

Iha tempu hodi hanoin antes deside atu partisipa

Infomasaun konaba se mak ita bo'ot atu kontaktu, kuandu ita bo'ot iha tan pergunta ou problema ruma.

Section 14: Ethics Committee Clearance. The ethical aspects of this research have been approved by the National Institute of Health, Timor-Leste (Instituto Nacional da Saúde; INS) and the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia (Protocol HREC-2021-4064). If you have any concerns about this research, please contact: Secretario ethiku, Komisaun etiku peskija komite tekniku INS (Ph +670 7708 7665) or the Northern Territory Department of Health and Menzies School of Health Research Human Research Ethics Committee (Phone +618 8946 8600; Email ethics@menzies.edu.au).

**Seksaun 14: Lisensa Komite Étika.** Aspektu etikal husi peskiza ida ne'e hetan ona aprovasaun husi Institutu Nasional Saude Timor-Leste (INS) no Human Research Ethics Committee of the Northern Territory Department of Health no Menzies School of Health Research, iha Australia (Protokolu HREC-2021-4064). Karik ita bo'ot iha kualker preokupasaun konaba peskiza ida ne'e, favor kontaktu: Sekretariu Étiku, Komisaun Étiku Peskija Komite Tekniku INS (Telemovel +670 7708 7665) ou the Northern Territory Department of Health no Menzies School of Health Research Human Research Ethics Committee (Telemovel +618 8946 8600; Email: ethics@menzies.edu.au).

## Consent form

## Formulariu Konsentimentu

Local Study Coordinator Name, National Health Laboratory, Dili

**SEROLOGICAL STUDY OF VACCINE-PREVENTABLE DISEASES IN TIMOR-LESTE**

Informed Consent /Assent Form

**This means you can say NO /**

| |
|---|
| PRINCIPAL INVESTIGATOR / INVESTIGADOR PRINSIPAL: Dr Nelson Martins |
| STUDY COLLABORATORS / KOLABORADORES BA ESTUDU: |
| Ministry of Health (MOH), Timor-Leste / Ministeriu da Saúde, Timor-Leste |
| Menzies School of Health Research, Australia |
| National Health Laboratory, Dili, Timor-Leste / Laboratoriu Nasional Saude, Dili, Timor-Leste |
| National Centre for Immunisation Research and Surveillance, Australia / Sentru Nasional ba Peskiza no Vigilansia Imunizasaun, Australia |

I understand the aim of this research study is **to improve understanding of the number of people who have been exposed and/or vaccinated against infectious diseases in Timor-Leste.** Details of the study have been explained to me, and I have been provided with a written information sheet and given the opportunity to ask questions.

I acknowledge that: / Hau rekonhese katak:

Any risks and possible effects of having a **venous blood test** have been explained to my satisfaction;

Kualker risku no efeitu ne'ebé mak posivel atu hetan tamba **teste ran venozu** nian, esplika ona mai hau nia satisfasaun;

Taking part in this study is voluntary and I am aware that I can stop taking part at any time without explanation or prejudice and to withdraw any unprocessed data I have provided;

Partisipasaun iha estudu ida ne'e voluntariu no hau hatene katak hau bele hapara hau nia partisipasaun iha kualker tempu sem explikasaun ou prekonseitu, no dada fila fali hau nia dadus ne'ebé mak seidauk prosesa

Any information I give will be kept strictly confidential and that no names will be used to identify me in this study without my approval.

Kualker informasaun ne'ebé mak hau fó, sei rai estreitamente konfidensial no sei la uza kualker naran hodi identifika hau iha estudu ida ne'e sem hau nia aprovasaun

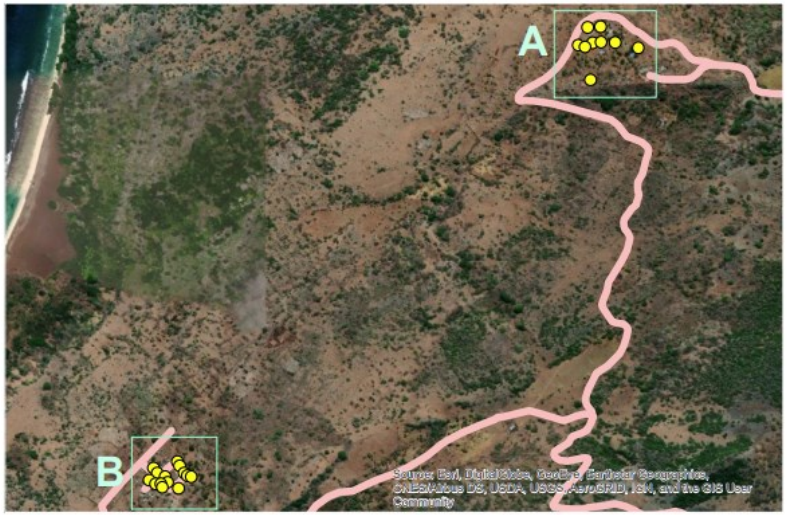
**By providing my name and signature, I agree to:**

**Liu husi fó hau nia naran no asinatura, hau konkorda atu:**

| | | | | |
|---|---|---|---|---|
| A venous blood test for past exposure or vaccination against COVID-19 | | **Yes**<br>**Sim** | | **No**<br>**Lae** |
| A venous blood test for past exposure or vaccination against measles<br>Teste ran venozu hodi hatene hau nia imunidade ba Sarampo | | **Yes**<br>**Sim** | | **No**<br>**Lae** |
| A venous blood test for past exposure or vaccination against rubella<br>Teste ran venozu hodi hatene hau nia imunidade ba rubella | | **Yes**<br>**Sim** | | **No**<br>**Lae** |
| A blood test for past exposure to dengue | | | | |
| A venous blood test for past exposure to hepatitis B and active infection with hepatitis B<br>Teste ran venozu hodi hatene hau nia imunidade ba hepatitis B no infeksaun | | **Yes**<br>**Sim** | | **No**<br>**Lae** |
| My blood being used to make a 'dried blood spot' | | | | |
| The anonymised blood sample and 'dried blood spot' being stored for 10 years and being used to validate tests for infections, and potentially tested for other communicable or non-communicable diseases | | **Yes**<br>**Sim** | | **No**<br>**Lae** |
| Completing a questionnaire with the help of a research assistant<br>Kompleta kuestinariu ho ajuda husi asistente peskiza nian | | **Yes**<br>**Sim** | | **No**<br>**Lae** |
| Being contacted again about vaccine-preventable diseases | | **Yes**<br>**Sim** | | **No**<br>**Lae** |
| Being contacted again about my hepatitis B results | | **Yes**<br>**Sim** | | **No**<br>**Lae** |

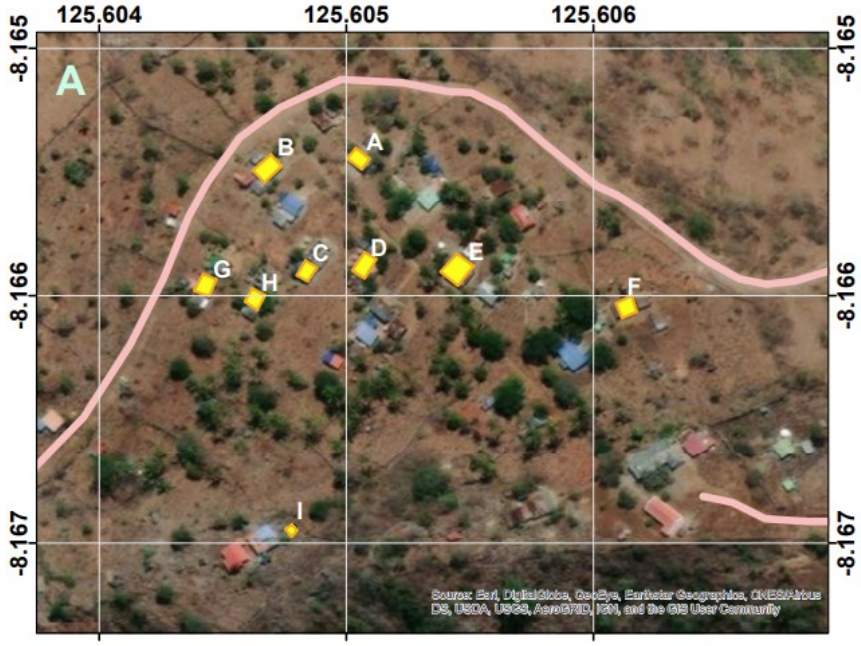
| | |
|---|---|
| **Name of participant / Naran partisipante:** *(printed)* | **Unique identifier: / Identifikador uniku:** |
| **Signature of participant or parent/guardian:** | **Date / Data:** |
| **Name of witness/interpreter: / Naran testemunha/tradutor:** *(printed)* | **Signature of witness/interpreter: / Asinatura husi testemunha/tradutor:** |
| **Name of researcher / Naran peskizador:** *(printed)* | **Signature of researcher / Asinatura husi peskizador:** |

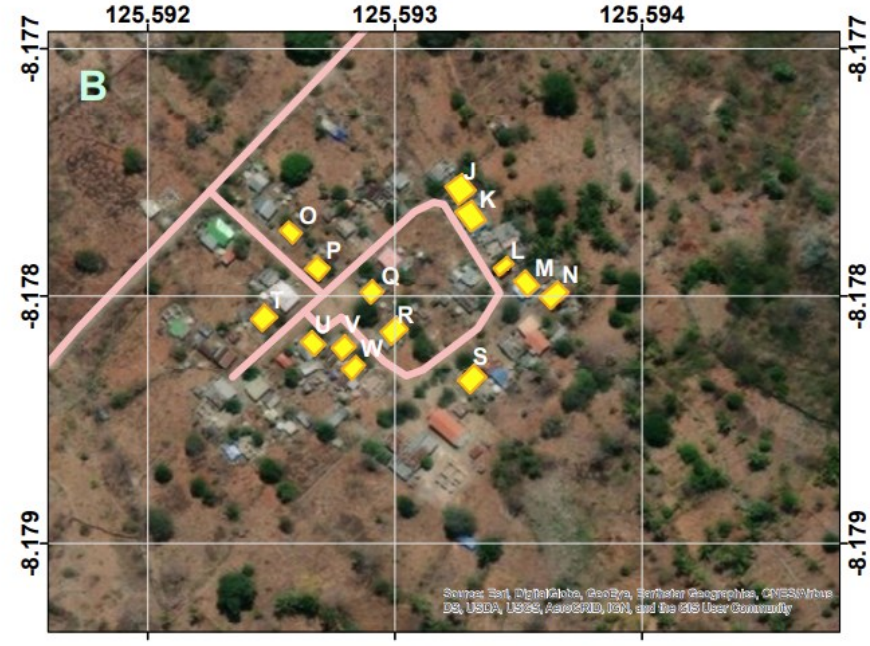
Example bespoke map




# ATAURO
# EA: 060102042

Houses
Streets
N
0 75 150
m



## Timor-Leste National Vaccine-Preventable Diseases Seroprevalence Survey

## Fieldwork SOP – Household Location

**Navigate to household using GPS device and printed maps**

Make sure you identify the exact house (don't accept another household nearby!)

**If at least one adult household member is present, move to *Data and Sample Collection SOP***

**If the household was found, but there are no household members present (or if one or more household members are missing):**

Arrange and conduct a second and third visit. Try to make this at different times of day.

If there are no household members present on all three visits, fill out the Fill out the *'Unable to Complete Questionnaire'* on RedCap

Do not find another household to replace this one

**If the household was found, but it looks derelict**

Fill out the *'Unable to Complete Questionnaire'* on RedCap

Choose the nearest occupied house and sample from here instead. If there are two equidistant houses, choose the one on the Left. When collecting data for the new household, add "B" to the end of the household number (e.g. "12345**B**")

**If the household cannot be found**

Fill out the *'Unable to Complete Questionnaire'* on RedCap

Make sure you enter the reason that the household cannot be found

Do not find another household to replace this one

Household questionnaire

| | |
|---|---|
| Record ID | _____ |
| Household number | _____ |
| How many people currently live in this household? | ○ 1<br>○ 2<br>○ 3<br>○ 4<br>○ 5<br>○ 6<br>○ 7<br>○ 8<br>○ 9<br>○ 10<br>○ 11<br>○ 12<br>○ 13<br>○ 14<br>○ 15<br>○ 16<br>○ 17<br>○ 18<br>○ 19<br>○ 20<br>(If the household has more than 20 mebers, fill out a second Household Questionnaire) |
| Gender of household member 1 | ○ Male<br>○ Female |
| Age of household member 1 | _____ |
| Study ID number of household member 1 | _____<br>( Only fill if he/she chooses to participate) |
| Gender of household member 2 | ○ Male<br>○ Female |
| Age of household member 2 | _____ |
| Study ID number of household member 2 | _____<br>( Only fill if he/she chooses to participate) |
| Gender of household member 3 | ○ Male<br>○ Female |
| Age of household member 3 | _____ |

| | |
|---|---|
| Study ID number of household member 3 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 4 | ○ Male<br>○ Female |
| Age of household member 4 | _____ |
| Study ID number of household member 4 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 5 | ○ Male<br>○ Female |
| Age of household member 5 | _____ |
| Study ID number of household member 5 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 6 | ○ Male<br>○ Female |
| Age of household member 6 | _____ |
| Study ID number of household member 6 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 7 | ○ Male<br>○ Female |
| Age of household member 7 | _____ |
| Study ID number of household member 7 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 8 | ○ Male<br>○ Female |
| Age of household member 8 | _____ |
| Study ID number of household member 8 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 9 | ○ Male<br>○ Female |

| | |
|---|---|
| Age of household member 9 | _____ |
| Study ID number of household member 9 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 10 | ◯ Male<br>◯ Female |
| Age of household member 10 | _____ |
| Study ID number of household member 10 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 11 | ◯ Male<br>◯ Female |
| Age of household member 11 | _____ |
| Study ID number of household member 11 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 12 | ◯ Male<br>◯ Female |
| Age of household member 12 | _____ |
| Study ID number of household member 12 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 13 | ◯ Male<br>◯ Female |
| Age of household member 13 | _____ |
| Study ID number of household member 13 | _____ ( Only fill if he/she chooses to participate) |
| Gender of household member 14 | ◯ Male<br>◯ Female |
| Age of household member 14 | _____ |
| Study ID number of household member 14 | _____ ( Only fill if he/she chooses to participate) |

| | |
|---|---|
| Gender of household member 15 | ○ Male<br>○ Female |
| Age of household member 15 | _____ |
| Study ID number of household member 15 | _____<br>( Only fill if he/she chooses to participate) |
| Gender of household member 16 | ○ Male<br>○ Female |
| Age of household member 16 | _____ |
| Study ID number of household member 16 | _____<br>( Only fill if he/she chooses to participate) |
| Gender of household member 17 | ○ Male<br>○ Female |
| Age of household member 17 | _____ |
| Study ID number of household member 17 | _____<br>( Only fill if he/she chooses to participate) |
| Gender of household member 18 | ○ Male<br>○ Female |
| Age of household member 18 | _____ |
| Study ID number of household member 18 | _____<br>( Only fill if he/she chooses to participate) |
| Gender of household member 19 | ○ Male<br>○ Female |
| Age of household member 19 | _____ |
| Study ID number of household member 19 | _____<br>( Only fill if he/she chooses to participate) |
| Gender of household member 20 | ○ Male<br>○ Female |
| Age of household member 20 | _____ |

| | |
|---|---|
| Study ID number of household member 20 | _____<br>( Only fill if he/she chooses to participate) |
| At any time in the past 12 months, has anyone sprayed the interior walls of your house? | ☐ Yes<br>☐ No<br>☐ Don't know |
| What do you use to stop being bitten by mosquitoes at night time? | ☐ Sprays<br>☐ Coils<br>☐ Bednets<br>☐ Other<br>☐ None |
| Please specify | _____ |

Individual participant questionnaire

| | |
|---|---|
| Record ID | _____ |
| **Demographic information** | |
| Household number | _____ |
| Study ID number | _____ (Use number on sample sticker) |
| Name of interviewer | _____ |
| Date of interview | _____ |
| First name / Naran prinsipal | _____ |
| Family name / Naran Familia | _____ |
| Gender / Jeneru | ◯ Female / Feto<br>◯ Male / Mane<br>◯ Other/Unknown / Seluk/La hatene |
| Date of birth | _____ |
| Age / Idade | _____ (Only need to fill if date-of-birth is not known) |
| Phone number(s) / Numeru Telemovel | _____ |
| Email address | _____ |
| Nationality / Nasionalidade | ◯ Timorese / Timor oan<br>◯ Other / seluk |
| Specify / espesifika | _____ |
| Have you traveled outside Timor-Leste (including West Timor, NTT) within the last 6 MONTHS? | ◯ Yes<br>◯ No<br>◯ Don't know |
| Where did you travel (in the last 6 months)? | ◯ West Timor (NTT)<br>◯ Other |

| | |
|---|---|
| Please specify the country (or countries) you went to (in the last 6 months) | _____ |
| Have you ever traveled outside Timor-Leste in your lifetime (but MORE THAN 6 MONTHS AGO)? This includes travel to West Timor (NTT). | ◯ Yes<br>◯ No<br>◯ Don't know |
| Where did you travel (MORE THAN 6 MONTHS AGO)? | ◯ West Timor (NTT)<br>◯ Other |
| Please specify the country (or countries) you went to MORE THAN 6 MONTHS AGO | _____ |

**Questions about febrile illness within the last 6 months**

| | |
|---|---|
| Have you been unwell with a FEVER within the last 6 months? | ◯ Yes<br>◯ No<br>◯ Don't know |
| When you had this illness, did you seek any medical care? | ◯ Yes<br>◯ No<br>◯ Don't know<br>(Includes medical clinic, pharmacy, hospital etc.) |
| When you sought medical care with fever in the last 6 months, did you get diagnosed by a healthcare professional with any of the following infections? | ☐ COVID-19<br>☐ Dengue<br>☐ Malaria<br>☐ Other<br>☐ No specific diagnosis was made |
| When you were diagnosed with COVID-19, how was this diagnosis made? | ◯ I was tested for COVID-19 and the test was POSITIVE<br>◯ I did NOT receive a positive test, but I was diagnosed with COVID-19 anyway<br>◯ I don't know if I was tested or not |
| When you were diagnosed with DENGUE, how was this diagnosis made? | ◯ I was tested for DENGUE and the test was POSITIVE<br>◯ I did NOT receive a positive test, but I was diagnosed with DENGUE anyway<br>◯ I don't know if I was tested or not |
| When you were diagnosed with MALARIA, how was this diagnosis made? | ◯ I was tested for MALARIA and the test was POSITIVE<br>◯ I did NOT receive a positive test, but I was diagnosed with MALARIA anyway<br>◯ I don't know if I was tested or not |
| Have you ever been diagnosed with COVID-19 by a healthcare worker in your life (even MORE THAN 6 MONTHS AGO)? | ◯ Yes<br>◯ No<br>◯ Don't know<br>(Answer 'yes', even if a COVID diagnosis within 6 months was reported in previous answers) |
| When did you get diagnosed with COVID-19? | _____<br>(Enter approximate date if unsure, leave blank if unknown) |

| | |
|---|---|
| Have you ever been diagnosed by a healthcare worker with DENGUE in your life? | ◯ Yes<br>◯ No<br>◯ Don't know<br>(Answer 'yes', even if a DENGUE diagnosis within 6 months was reported in previous answers) |
| Have you ever been diagnosed by a healthcare worker with MALARIA in your life? | ◯ Yes<br>◯ No<br>◯ Don't know<br>(Answer 'yes', even if a MALARIA diagnosis within 6 months was reported in previous answers) |
| Have you received vaccination for COVID-19? / Ita bo'ot simu ona vasinasaun ba COVID-19? | ◯ Yes / Sim<br>◯ No / Lae |
| Number of doses / Numeru dose: | ◯ 1<br>◯ 2<br>◯ 3<br>◯ More than 3 |
| When was your FIRST COVID-19 vaccine | _____<br>(Enter approximate date if unsure, leave blank if unknown) |
| Which type of COVID-19 vaccine was your FIRST dose? | ☐ AstraZenica<br>☐ Sinovac<br>☐ Pfizer<br>☐ Other<br>☐ Don't know |
| When was your SECOND COVID-19 vaccine | _____<br>(Enter approximate date if unsure, leave blank if unknown) |
| Which type of COVID-19 vaccine was your SECOND dose? | ☐ AstraZenica<br>☐ Sinovac<br>☐ Pfizer<br>☐ Other<br>☐ Don't know |
| When was your THIRD COVID-19 vaccine | _____<br>(Enter approximate date if unsure, leave blank if unknown) |
| Which type of COVID-19 vaccine was your THIRD dose? | ☐ AstraZenica<br>☐ Sinovac<br>☐ Pfizer<br>☐ Other<br>☐ Don't know |
| If you have had more than three COVID-19 vaccines, when was your MOST RECENT dose? | _____<br>(Enter approximate date if unsure, leave blank if unknown) |

| | |
|---|---|
| If you have had more than three doses of COVID vaccines, which type have you recieved for your MOST RECENT DOSE? | ☐ AstraZenica<br>☐ Sinovac<br>☐ Pfizer<br>☐ Other<br>☐ Don't know |
| Do you wish to be vaccinated for COVID-19? | ◯ Yes / Sim<br>◯ No / Lae<br>◯ Don't know / La hatene<br>◯ Not applicable - too young to answer this question<br>((Only for participants who have not received any doses of COVID-19 vaccines)) |
| Why have you not received a SECOND dose of COVID-19 vaccine? | ◯ It is less than 3 months since my first dose, therefore my second dose is not due yet<br>◯ It is more than 3 months since my first dose, but I have not been offered a second dose yet<br>◯ I got COVID-19 infection after my first dose, therefore I don't think I need another dose<br>◯ I don't want to have another dose<br>◯ Other<br>((Only asked to participants who have received just one dose of COVID-19 vaccine)) |
| Why don't you want another dose? | _____ |
| Please specify | _____ |

## VASINA Fieldwork SOP – data and sample collection

### For each household, do the following:

**Introduce research team to head-of-household and other household member(s)**

Explain study, using patient information sheet

Wear mask and visor for this

**Conduct *Household Questionnaire* on RedCap with head-of-household**

Fill this out even if no household members give consent for individual data or samples to be collected (if this is the case, head-of-household should still fill consent form)

If head-of-household does not wish to fill *Household Questionnaire* on RedCap, fill *Unable to Complete Questionnaire* on RedCap

**Each time you enrol an individual, give them a sticker with their Study ID number on it**

Children less than 1 year old should not be enrolled and do not need to be assigned a Study ID number (but their details should still be included on the *Household Questionnaire*)

For individuals who are not currently present, assign a study ID number, but keep his/her sticker until the next visit

### For each participant, do the following:

**Receive written informed consent (signed form)**

- For children, receive written informed consent from one of their parents

- Also receive 'verbal assent' from the child

**Conduct *Individual Participant Questionnaire* on RedCap**

**Don PPE**

**Phlebotomy (maximum x2 attempts)**
- Adults: use needle and syringe to collect 10ml (x2 tubes)
- Children: use needle and syringe or butterfly to collect 5ml (x1 tube)

- Also place x9-12 drops onto filter paper to make three blood spots (*see Dried Blood Spot SOP*).

- Make sure blood tubes and dried blood spots are clearly labelled with stickers

**Finger-prick blood sample (where phlebotomy failed)**

- Collect 2mls of blood drops into paediatric serum tubes

- Collect x9-12 drops onto filter paper to make three blood spots.

- Make sure blood tubes and dried blood spots are clearly labelled with stickers

**Store samples**

- Put labelled blood samples into cool box

- Allow blood spot to dry for 4 hours, put into zip-lock bag with desiccant sachet. Put this into cool box

**Equipment disposal**

- Place all sharps into sharps container

- Place all other phlebotomy equipment into clinical waste bags

- Dengue RDT can be disposed of, after good-quality picture and interpretation is done

**Thank household member(s) and make sure they have a copy of the participant information sheet**

**Make sure gloves and apron are changed and hands are washed/alcohol gelled before moving onto next participant**

**Timor-Leste National Vaccine-Preventable Diseases Seroprevalence Survey**

**Fieldwork SOP – Preparation of Blood Spots on Filter Paper**

## 1. PURPOSE AND SCOPE

To describe the procedure for preparing dried blood spots on filter paper suitable for serological analysis.

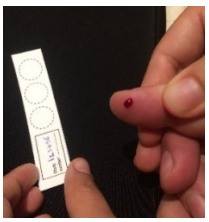
## 2. BACKGROUND

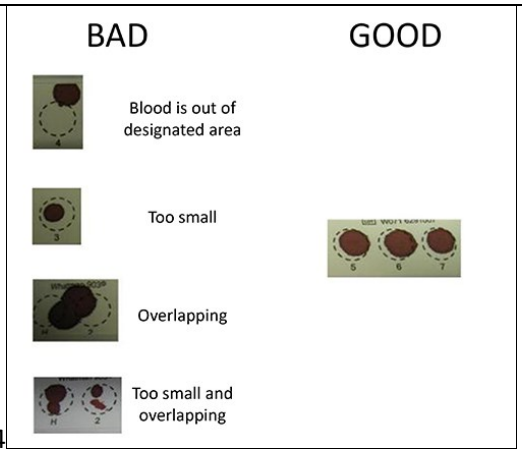
Dried blood spot samples can be tested for the presence of various antibodies and/or antigens in serological assays. In this study, samples will be tested for IgG or total antibody to various infections, looking for *exposure* or *previous infection*.

## 3. SUPPLIES, MATERIALS AND EQUIPMENT

- Filter paper cards (Whatman 903), one for each participant
- Ziplock plastic bags, one for each participant
- Desiccant sachets, one for each participant
- Fresh whole blood, obtained from participant as part of lymphatic filariasis (LF) survey
- Plastic box, for storage of dried blood spots while on site-visit
- Freezer at –20 °C, for storage of samples, at National Health Laboratory

## 4. PROCEDURE

| | |
|---|---|
| 1. Label the filter paper with the participant's Study ID number (use sticker) | |
| 2. Touch 2-3 drops of blood onto the circular area designated for the first spot. Ensure the circle is **saturated and filled** with blood<br>- If phlebotomy was successful, use the last ½ ml of blood from the syringe (after filling the serum tubes)<br>- Alternatively, if phlebotomy was not successful, use blood directly from the finger (after filling paediatric serum tubes) | 2  or  |
| 3. Preparing three spots for each participant | 3  |
| 4. Make sure blood spots are good quality | |

| | |
|---|---|
| 5. Allow to dry for at least 1 hour at room temperature (do not apply heat or place in direct sunlight to shorten drying time)<br><br>6. Place into plastic ziplock bag<br><br>7. Add a desiccant sachet inside each ziplock plastic bag containing filter paper samples<br><br>8. Place ziplock plastic bag into plastic box<br><br>8. Store and transport the dried samples at room temperature, with the desiccant, during site visits<br><br>9. After site-visit, store dried blood spot samples in freezer at –20 °C | 4 <br>6  |

**5. PROCEDURE NOTES**

- Wear gloves to prevent contamination of filter paper
- Universal precautions should be used, including relevant personal protective equipment

**Paediatric phlebotomy training materials**

**PROPÓZITU**

*To ensure the correct procedure is undertaken for paediatric venepunctures.*

**ÁMBITU**

*This Method applies to all phlebotomists, laboratory staff, nurses and doctors.*

**SAFETY**

Perform hand hygiene





Any cuts or abrasions are to be fully covered with a dressing



Don gloves, mask and apron



Discard sharps immediately after use into an approved sharps container



**(EQUIPMENT)**

Clean tray

23g or 22g Butterfly

2.5ml or 5ml Syringe

Alcohol swabs

Appropriate blood tubes

Tourniquet

Safety Box

Tape (3 pieces)

Non sterile Gloves

Request form

Pen

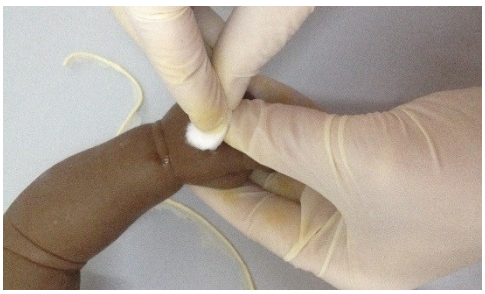
**PREPARATION**

| | | |
|---|---|---|
| | Wash hands with soap and water or Hand Alcohol |   |
| 5.2 | Clean trolley with Alcohol 70%<br><br>Clean tray with Alcohol 70%<br>Sharps container (safety box)<br>Tourniquet (soft gauze or glove)<br>22g or 23g butterfly or needle<br>2.5ml or 5ml syringe<br>Alcohol 70%<br>Sterile gauze<br>Blood tubes<br>Tape (3 pieces)<br>New non-sterile Gloves<br>Request form<br>Pen |   |
| 5.3 | Take trolley to cot, warmer, bed or treatment room. | |

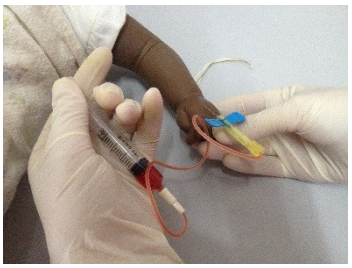
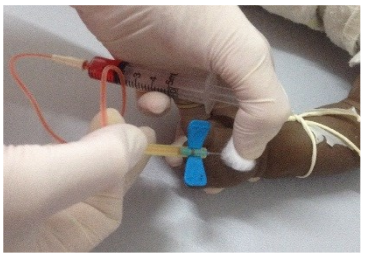
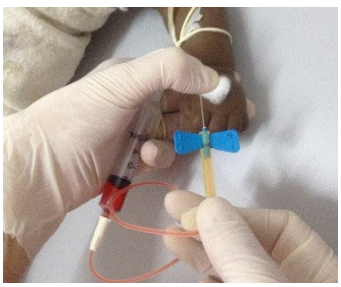
**PATIENT IDENTIFICATION**

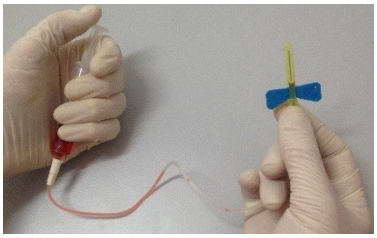
| | | |
|---|---|---|
| 6.1 | Greet the family and explain what the procedure is and why it is being done and ask for consent | |
| 6.2 | Identify patient by confirming<br><br>Full name<br>Date of birth<br>Address<br>Medical Record Number<br><br>Confirm identity with parent and patients wrist band<br>Add any missing information or correct any wrong information. |  |
| 6.3 | Wash hands with soap and water or hand alcohol |   |

**VENEPUNCTURE METHOD**

| | | |
|---|---|---|
| | Place the tourniquet on the patient's upper arm/lower leg and tighten to help locate a vein in the hand/foot.<br><br>Check both arms/legs if necessary and choose the best vein |  |
| | Loosen tourniquet | |
| 7.3 | Swab the site with an alcohol swab using one wipe straight down the vein and allow to dry |  |
| 7.4 | Ensure all equipment is within easy reach, attach syringe to butterfly needle | |
| 7.5 | **Position patient** – Child in lying position (usually young children).<br><br>Lie child on their back in bed. Or parnts lap. Have a family or staff member assist with holding.<br><br>Ensure arms and legs are still.<br><br>Instruct assistant to calm young child or baby, if needed, with positive words or other distraction stratagies (pictures ,toys ,singing) |  |

| | | |
|---|---|---|
| 7.6 | **Position patient** - Child in sitting position (usually older children).<br><br>Have family or staff member sit in chair with child on their lap.<br><br>Place child's legs between assistants' legs.<br><br>Instruct assistant to hold the child's arm **not** being used for blood test with the assistants opposite hand.<br><br>Instruct assistant to use their other hand to securely hold child's shoulder above the area to be used for blood test.<br>Instruct assistant to calm child, if needed, with positive words or other distraction stratagies. |  |
| 7.7 | Wash hands |   |
| 7.8 | Don gloves |  |
| 7.9 | Re-tighten tourniquet |  |

| | | |
|---|---|---|
| 7.10 | Anchor skin securely **below** venepuncture site |  |
| 7.11 | Insert the needle with the bevel hole facing upwards into the vein |  |
| 7.12 | Release tourniquet once the flow of blood into the butterfly tubing has commenced |  |
| 7.13 | Withdraw blood by **gently** pulling back the plunger of the syringe<br><br>5 mls is recommended amount |  |
| 7.14 | Take sterile gauze and place lightly over the venepuncture site |  |
| 7.15 | Remove the needle from the vein |  |

| | | |
|---|---|---|
| 7.16 | Pull on syringe (remove blood from syringe) |  |
| 7.17 | Discard the butterfly needle into the Safety Box | |
| 7.18 | Place blood filled syringe on tray |  |
| 7.19 | Ask family or staff member to apply firm pressure on the site, minimum of 2 minutes | |

**BLOOD SAMPLE PROCESSING**

| | | |
|---|---|---|
| 8.1 | The needle should be inserted into the rubber stopper from the top in straight postion with tube sitting in rack do not remove rubber stopper because vaccumm will be lost stopper if no rubber stopper gently syringe blood into the anticoagulant tubes first purple and the serum other colour tubes last. Replace tops securely to prevent accidental blood spillage |   |
| 8.2 | NOTE: Order of draw in peadtric phelbotomy when using a butterfly/needle and syringe to collect blood<br><br>First EDTA (CBC tube = purple)<br>Second SST (Kimia tube = Yellow) | |
| 8.3 | Gently invert x5 each blood tube after removaL |   |

**LABELING TUBES AND PATHOLOGY REQUEST**

| | | |
|---|---|---|
| 9.1 | Label the blood tubes while with the patient;<br>Full name<br>Date of birth<br>Time and date of collection | |
| 9.2 | Complete request form details while with the patient:<br>Patient details<br>Date and time of collection<br>Nurse signature | |
| 9.3 | Lift the gauze swab and check that the venepuncture site has stopped bleeding | |
| 9.4 | Tape the gauze swab to the hand/foot | |