STUDY TITLE:
DIAGNOSTIC TOOLS FOR THE DIRECT DETECTION OF *ORIENTIA TSUTSUGAMUSHI*

Short title: *Quick and Easy Scrub Typhus diagnostic tools*
Acronym: QuEST
Protocol number: TYP25001

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Confidentiality Statement:
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1 KEY ROLES

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Sponsoring Institution	University of Oxford UK	Oxford, UK
Funder	Wellcome trust University of Oxford	Mahidol Oxford Translational Innovations Partnership award

PROTOCOL SIGNATURE PAGE

Investigator Agreement and Conflict of Interest

"I have read this protocol and:

- Agree to abide by all provisions set forth therein.
- Agree to comply with the principles of the International Conference on Harmonisation Tripartite Guideline on Good Clinical Practice.
- and declare no conflict of interest, according to the current version of the Declaration of Helsinki"

Dr Carlo Perrone		
Principal Investigator	Principal investigator's signature	Date

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I. PROTOCOL SUMMARY

Full Title	DIAGNOSTIC TOOLS FOR THE DIRECT DETECTION OF *ORIENTIA TSUTSUGAMUSHI*
Short Title	Quick and Easy Scrub Typhus diagnostics (QuEST)
Study Type	Prospective, observational
Conducted By	MORU (Mahidol Oxford Tropical Medicine Research Unit)
PI	Carlo Perrone
Sample Size	All patients testing positive during the period of the study
Study Population	Suspected scrub typhus cases positive by Scrub Typhus IgM RDT or isothermal insulated PCR
Study Duration	12 months
Study Design	Prospective, observational
Primary Objective	Compare the performance of iiPCR for the rapid diagnosis of scrub typhus on clinical samples with commercially available RDTs, with PCR and the scrub typhus infection criteria
Secondary Objectives	1. Provide samples (buffy coat, whole blood, plasma, urine) for the development of an antigen-detection test for *Ot*. 2. Test the usability of the POCKIT system(s) in a real-life setting 3. Provide eschar punch biopsies and blood samples to elucidate the pathophysiology of and immune response to scrub typhus 4. Provide samples for testing the performance of an RPA-CRISPR/Cas diagnostic assay for scrub typhus (currently being developed) 5. Estimate G6PD deficiency in the study population and its correlation with scrub typhus outcomes
Outcome measures	Sensitivity and specificity analysis comparing the iiPCR assay with: 1. InBios IgM Ot and other RDTs used at study sites 2. MORU In-House PCR consisting of a real-time PCR assay targeting the 47kDa gene, followed by a confirmatory nested-PCR 3. The mSTIC criteria 4. G6PD deficiency estimates

II. LIST OF ABBREVIATIONS

CRF	Case Report Form
ELISA	Enzyme-linked immunosorbent assay
G6PD	Glucose-6-Phosphate Dehydrogenase
GCP	Good Clinical Practice
ICF	Informed Consent Form
ICH	.International Conference on Harmonization
IFA	Indirect Immunofluorescence Assay
IRB	Institutional Review Board
MTA	Material Transfer Agreement
Ot	*Orientia tsutsugamushi* (causative agent of scrub typhus)
PI	Principal Investigator
PIS	Participant Information Sheets
qPCR	Quantitative real time polymerase chain reaction
RAA	Recombinase assisted amplification
RDT	Rapid Diagnostic Test

2 INTRODUCTION

2.1 BACKGROUND

Scrub typhus is a potentially fatal bacterial disease caused by *Orientia tsutsugamushi (Ot)*.

The disease mostly affects farmers living in remote regions so the points of initial presentation are peripheral clinics and small hospitals. Because the disease is easily treatable with doxycycline, a cheap and easily available antibiotic, and because early treatment can avert the development of severe disease and death, it is essential to provide such facilities with rapid and reliable diagnostic tests.

Scrub typhus is common throughout the Asian continent. India, China, South Korea and Japan, are among the endemic countries, putting the population at risk at roughly one billion[1]. In Southeast Asia, it represents up to 22% of causes of fever and half of treatable causes [2].

Accurate diagnosis currently requires specific PCR or ELISA, which are only available in high level facilities, and not in rural settings, where the disease occurs.

The only commercially available point-of-care tests are antibody based and not sufficiently reliable during the first few days of illness when the development of severe disease can be prevented by initiating appropriate treatment [3]. Even when positive, up to half of scrub typhus RDTs prove to be false positives [4]. In addition to false positivity issues, sensitivity has been shown to vary greatly not only between tests but also using the same test in different settings, probably as a consequence of local epidemiology and diagnostic cut-offs used (sensitivity range: 23%-97%, specificity range 68-100%) meaning many patients with scrub typhus are not diagnosed[5], which translates into inappropriate treatment and inadequate estimates of disease burden. Aside from the direct negative consequences to patients, this promotes antibiotic misuse and resistance and prevents adequate resource allocation.

For these reasons it is of paramount importance to develop diagnostic tools capable of directly detecting *Orientia tsutsugamushi* which are easy to use in peripheral healthcare facilities.

Recent reports have, in addition, implicated G6PD deficiency with hamolysis and severe scrub typhus outcomes, we will estimate G6PD activity (genetically and phenotypically) in participants and examine its association with disease outcomes (fever clearance time and severe disease) [6, 7].

2.2 RATIONALE

PCR provides excellent sensitivity and specificity for early diagnosis but has cost limitations as it requires thermal cyclers. To obviate this, isothermal NAATs have been developed. Among them, loop-mediated isothermal amplification (LAMP), recombinase assisted amplification (RAA), CRISPR/ Cas, and isothermal solid phase DNA amplification (iSAD) have shown promising results in detecting *Orientia tsutsugamushi* and should be considered possible solutions to the problem of acute scrub-typhus diagnosis. However, these techniques

require DNA to be extracted as a substrate for the reactions, which is a time-consuming procedure[8, 9].

Genereach™ have developed a highly automated cartridge-based system which performs both DNA extraction and amplification without the need for a thermal cycler (POCKIT™). We have partnered with Genereach to develop a cartridge for the diagnosis of scrub typhus. This machine would be ideal for small health centres, because it is designed for small batches (one to six samples), it provides results in less than 90 minutes and requires only 20-30 minutes for sample preparation and minimal training. By combining the simplicity of an RDT with the accuracy of PCR, it would represent an extremely valuable tool for scrub typhus diagnosis. During the primary stage of this study we tested PCR primers targeting regions of the *Ot* genome that are present in multiple copies, which showed good performance in detecting *Ot* reference strain genomes. With the current proposal we plan to test the developed primers on the POCKIT platform to assess performance on clinical samples and usability of the machine in a real-life setting.

Samples collected will also be used for the development of an antigen-based scrub typhus diagnostic test, and to determine the performance of an assay combining recombinase polymerase amplification with CRISPR/Cas (currently being developed), targeting the same repeated regions of the *Ot* genome. Samples will also help to better comprehend certain aspects of *Ot* pathophysiology. In particular, it was recently shown that *Ot* exists in at least two distinct developmental forms, and these express different antigens on their surface. So far this has only been studied in cultured cells *in vitro.* Therefore, in the current work we will analyse the different forms of *Ot* in blood samples and eschar samples using immunofluorescence microscopy to analyse protein expression, and RNA FISH to study RNA transcripts. This will guide the development of diagnostics that can recognize all forms of *Ot* found during human infection. Preliminary analyses have been carried out using samples from a previous study (EXIST; Chiangrai Prachanukroh Hospital EC ref: EC CRH 038/60 Ex, OxTREC ref: 46-15) but there are not sufficient samples available to carry out full analysis. Therefore, in the current study we will ask participants presenting with an eschar (n=10) if they are willing to provide an eschar biopsy.

Finally, left-over samples will be used to measure G6PD activity in enrolled participants with the aim of verifying whether its deficiency is associated with more severe outcomes such as prolonged fever clearance time or the presence of disease conplications. G6PD deficiency has recently been described as the cause for severe haemolytic anaemia in a scrub typhus patient. Because scrub typhus can cause anemia, G6PD deficiency could play an important role in the pathogenesis of severe disease [6, 7, 10].

3 OBJECTIVES AND OUTCOME MEASURES

3.1 OBJECTIVES

Primary Objective
Compare the performance of iiPCR for the rapid diagnosis of scrub typhus on clinical samples with commercially available RDTs, current PCR diagnostics, and the scrub typhus infection criteria (STIC) [11].

Secondary Objectives

1. Provide samples (buffy coat, whole blood, plasma, urine) for the development of an antigen-detection test for *Ot*.
2. Test the usability of the POCKIT system(s) in a real-life setting.
3. Provide eschar punch biopsies and blood samples to elucidate the pathophysiology of and immune response to scrub typhus
4. Provide samples for testing the performance of an RPA-CRISPR/Cas diagnostic assay for scrub typhus (currently being developed)
5. Estimate G6PD deficiency in the study population and its correlation with scrub typhus outcomes

3.2 OUTCOME MEASURES

Sensitivity and specificity analysis comparing the iiPCR assay with:

1. InBios IgM Ot and other RDTs used at study sites (see Appendix B)
2. MORU In-House PCR consisting of a real-time PCR assay targeting the 47kDa gene, followed by a confirmatory nested-PCR [12]
3. The mSTIC criteria
4. G6PD deficiency estimates

4 STUDY DESIGN

4.1 TRIAL DESIGN

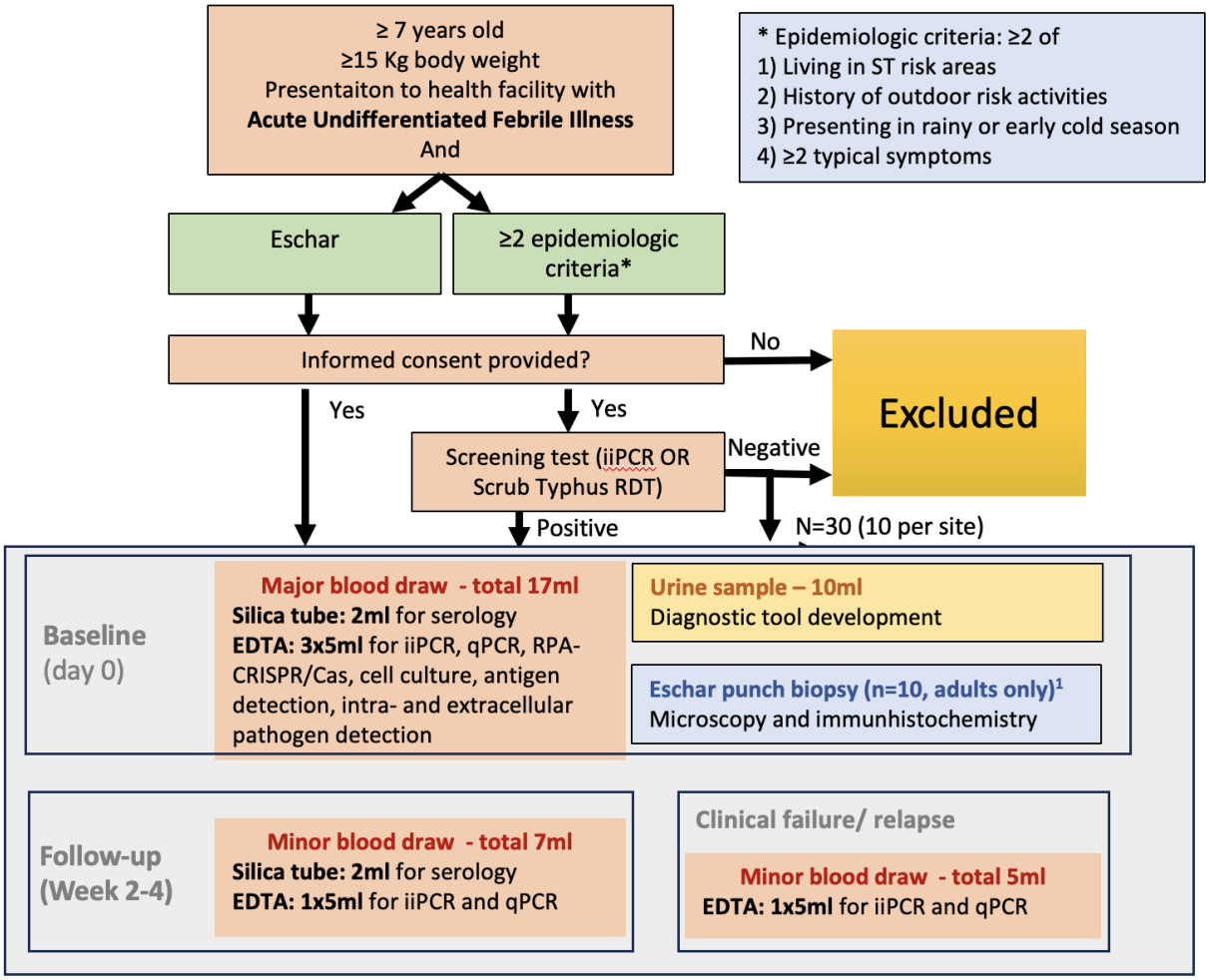
The study is a prospective, observational study in consenting out- and inpatients of all ages with acute undifferentiated fever.

4.1.1 SAMPLE SIZE

Between May 2024 and January 2025, we screened about 270 febrile patients per site, 109 of which tested positive for at least one screening test. With current positivity rates, roughly 20% of the subjects should be found to have confirmed scrub typhus. Based on subjects recruited between May 2024 and January 2025, we can estimate that 20% of those cases will have scrub typhus and therefore there should be a total of 80-100 cases of confirmed scrub typhus.

However, as we do not yet know the performance of the test, we also do not know how many true-positive cases will be detected or missed by it, which would both affect sample size estimates. To estimate test performance more accurately, 30 cases testing negative for all tests will also be included (ten per hospital).

4.1.2 STUDY FLOWCHART



[1] The eschar biopsy will be taken only if participants explicitly consent to this procedure (separate informed consent sheet)

4.2 STUDY SITES

Clinical recruitment is performed at:

1. Mae Suai District Hospital, Chiangrai, northern Thailand,
2. Mae Chan District hospital, Chiangrai, northern Thailand and
3. Mae Fa Luang District hospital, Chiangrai, northern Thailand

4.3 STUDY POPULATION

Subjects 7-years old or older and weighing at least 15 Kg presenting at the out- or inpatient departments of participating hospitals for an acute febrile illness and suspected of having scrub typhus will be screened for recruitment in the study using iiPCR and/or scrub typhus RDTs.

Suspected scrub typhus is defined as follows:

1. Eschar OR
2. Epidemiologic/ clinical suspicion, defined as:

≥ 2 of:
- Living in rural parts of Chiangrai Province or other Scrub Typhus risk areas
- History of outdoor risk activities in the 2-3 weeks before symptom onset
- Presenting during the rainy season or early cold season (June-December)
- Two or more of: headache; myalgia, cough, abdominal symptoms (abdominal pain/ nausea-vomiting); myalgia; lymphadenopathy

4.3.1 INCLUSION CRITERIA:

- ≥ 7 years old AND
- Weight>15Kg AND
- The patient and/or where relevant their parent/guardian/caretaker/LAR willing and able to give informed consent /assent for participation in the study AND
- Acute (duration ≤14 days) fever (37.5°C or higher or a history of fever in the previous 24h)

For study group
- Eschar OR Positivity to any of the screening tests (Immunochromatographic RDT or iiPCR)

For negative control
- Negativity to all screening tests (ten patients per hospital)

4.3.2 EXCLUSION CRITERIA:

- Other clear focus of infection (e.g. lobar pneumonia by chest x-ray, abscess) OR
- Other clear cause of fever (e.g. positive Dengue IgM or NS1 antigen, positive blood cultures, untreated HIV)

4.3.3 SELECTION OF NEGATIVE CONTROLS

Negative controls will be selected among patients that fulfil all inclusion criteria but test negative to all the screening tests. We will attempt to stratify controls by site, days of symptoms, and period of presentation; as follows:

Days of symptoms	Mae Suai site		Mae Chan site		Mae Fa Luang site	
	June-Aug	Sep-Dec	June-Aug	Sep-Dec	June-Aug	Sep-Dec
0-3 days	≥2	≥1	≥2	≥1	≥2	≥1
4-7 days	≥2	≥1	≥2	≥1	≥2	≥1
≥ 8 days	≥2	≥1	≥2	≥1	≥2	≥1

4.4 INFORMED CONSENT

Potential participants will be asked to provide written informed consent to carry out the screening tests for joining the study if they fulfill the other inclusion criteria, the screening tests will be carried out on leftover blood whenever possible. For participants agreeing to be tested we will collect the following information:

- Epidemiologic/ clinical criteria
- Days of symptoms at time of screening
- Age and sex

If any of the tests is positive, or if participants are enrolled as negative controls, the study will be explained in detail and written informed consent to carry out study procedures will be sought.

- To enroll febrile patients ≥ 18 years old written informed consent will be required.
- To enroll febrile patients aged ≥ 7 to <18 years old, both written parental/guardian consent and participant assent will be required.

The study will be explained to the eligible participant by trained study staff prior to obtaining informed consent. Literate participants, parents, or guardians will document their provision of informed consent/assent by signing and dating the Thai language while non-literate participants, parents, or guardians will be asked to mark their consent/assent forms with a thumbprint in the presence of a literate, third party, impartial witness who will also sign the consent form. If the patient does not speak or understand Thai, a translator will be used to relay the information about the study and assist with the informed consent procedure in a language used by the participant (e.g. hill-tribe language, Burmese, Lao, Chinese etc.). The translator will be an impartial third party (preferred) or a relative of the participant. Similarly, the witness to the informed consent procedure should be able to understand the language used to obtain consent from the participant. A video explaining the study will be prepared and used together with the PIS to explain the study to participants, it will be dubbed in Akha, Lahu, and Haw Chinese (spoken widely at the Mae Fa Luang site) to have standardized information for people who do not speak Thai.

In the event that the participant lacks capacity due to illness (e.g. acute delirium), the PIS and ICF may be presented to the parent/guardian/next of kin to consent for study participation on their behalf. If the patient regains capacity to respond during the study period, the study team will seek the direct consent of the participant.

A copy of the signed consent +/- assent forms must be provided to the participants and parents/ guardians. Signed consents +/- assents must remain in each subjects' study file.

Of adult participants presenting with an eschar (see below), we will seek to recruit ten to perform a biopsy of the lesion. They will be asked for additional, specific, written consent to the procedure with a separate participant information sheet and informed consent form.

5 STUDY CONDUCT

5.1 SCREENING AND ENROLMENT

The study team will screen patients presenting with fever at the out- and inpatient departments of study sites. A scrub typhus RDT will be performed at all sites (e.g. Scrub Typhus IgM RDT, InBios International; Scrub Typhus IgM/IgA/IgG RDT, SDBioline; STANDARD Q Tsutsugamushi IgM/IgG, SDBiosensor). Blood samples will be sent for screening at our central

laboratory in Chiangrai city, where iiPCR will also be performed. Before being screened with any of the above tests for study purposes, potential participants will be asked to provide written informed consent. Age, date of symptom onset, and history of previous antibiotic therapy will be collected for screened participants.

If participants have a positive screening test following an order form the treating physician as part of standard management (not for study purposes), this will be considered equivalent to a positive screening test; those subjects will be asked then to provide informed consent to participate in the study.

Eligible patients who agree to participate and/or their legal guardians will take part in the informed consent procedure (section 4.4).

Every screened patient will receive a screening number and details are recorded into a screening log. Screening rejection is defined as patients who have been screened but are not enrolled into the study. Reasons for rejection may include any exclusion criteria, non-conformity with the inclusion criteria, or failure to obtain informed consent. Reasons will be recorded in the screening log. Patients who do not progress from screening to trial recruitment will not be assigned with a study number or have clinical data recorded in the screening log.

5.2 INPATIENT STAY

Clinical review: Study patients will have their vital signs measured and recorded every 6 hours during hospitalisation. Details of antibiotics administered will be collected. Additional diagnostic test results (e.g. laboratory or imaging) requested by the medical team will also be collected and recorded on the CRF.

Patients admitted with scrub typhus to hospital for treatment will be discharged by the treating physician according to clinical improvement and resolution of fever.

5.3 FOLLOW-UP

Scheduled follow-up: A follow-up visits will occur at 2 weeks (+ 2 weeks). Examinations and samples are collected as outlined in the study flowchart (section 4.1.2).

If the patient cannot be followed-up, then the reason for loss-to-follow-up will be recorded on the CRF.

Study participants are asked to contact the study team if fever and/or symptoms and signs compatible with scrub typhus appear after hospital discharge until the patient exits the study (after the 2 week follow-up appointment). Clinical findings and samples are collected as outlined in the study flowchart.

If the patient was discharged or leaves the hospital prior to cessation of fever, the patient will be given a tympanic membrane monitor and asked to measure their temperature 4 times a day (flexible/pragmatic timings) and record the results in a diary until their fever has cleared.

The diary and the thermometer will be collected by the study team at follow-up and the temperature readings used to calculate the Fever clearance time (FCT).

5.4 STUDY COMPLETION:

Enrolment is planned to end on December 31st 2025, so follow-up visits should be completed by January 2026. Should the assumptions for sample size [13] calculation in section 4.1.1 not be met and/or additional funding be secured, recruitment may be extended until sufficient scrub typhus cases will have been identified to accurately estimate iiPCR and RAA-CRISPR/Cas sensitivity and specificity.

6 STUDY PROCEDURES

6.1 SAMPLES AND SPECIMENS

Specimen labeling: The prefix QES, followed by a 2-digit site identifier and a 3-digit study number i.e. QES-61-001, QES-60-001 etc. serve as patient study identifier, followed by a suffix for time of blood draw. An additional suffix for whole blood, serum, plasma (heparinized blood), plasma (citrated blood), plasma (EDTA blood), buffy coat, and eschar biopsy, will be added - e.g. QES-61-001-WB1, -S1, -PH1, -PC1, -EP1, -BU1, -EB1, e.g. QES-61-00-W0-WB1 for baseline and QES-61-001-W2-WB1 for week 2; TF for treatment failure; RL for relapse etc.

Whole blood (WB): WB is collected in silica tubes for serum or EDTA tubes for plasma and buffy coat (for PCR, iiPCR and pathophysiological tests), culture, and molecular studies.

Blood draw volume: The study requires approximately 17 ml of blood (slightly exceeding one tablespoon) at baseline, 7ml at the week 2 follow up and in case of therapeutic failure or relapse additional 5 ml (a maximum amount of about 29ml in total over two weeks), which remains well under the maximum recommended volume, both for a single blood draw and for a one-month period for participants >15Kg body weight, and takes into account the routine blood tests required for the care of the patient.

In the event that insufficient blood is obtained, we will prioritise samples according to the onsite sample collection and laboratory processing SOP.

Eschar biopsies (3mm punch biopsies): A total of n=10 eschar biopsies will be collected in this study, for microscopy and for cytokine and gene expression profiling. An experienced doctor will perform these biopsies after the application of 1% Lidocaine for local anesthesia. A biopsy size of 3mm is small, not prone to complications and, although it will cause bleeding, this specimen collection procedure usually does not require surgical sutures, but can be closed using steri-strips or similar.

Downstream analyses: microscopy, immunohistochemistry, qPCR, RNA gene expression profiling, G6PD deficiency

Urine: A urine sample (10 ml) is collected from all study patients (if possible) at baseline, for the development of urine-based rapid diagnostic tests for scrub typhus.

6.1.1 STUDY PROCEDURES OVERVIEW

Screening	Acute tests	iiPCR, ST-RDT
Baseline	Clinical data	Patient history Risk factors and previous treatment Vital signs and clinical outcomes Routine laboratory results Routine diagnostics
	Blood withdrawal	PCR *O. tsutsugamushi* antibody levels Cell culture
	Urine sample	Diagnostic antigen testing (preliminary experiments)
	Eschar punch biopsy	Spacial transcriptomics *(5 patients in total)*
Treatment failure or fever relapse[a]	Clinical data	Clinical outcomes
	Blood withdrawal	If clinical failure (PCR, cell culture)
Week 2	Clinical data	Clinical outcomes
	Blood withdrawal	*O. tsutsugamushi* antibody levels, G6PD deficiency testing
[a] Treatment failure: Persistent fever 72h after start of effective antibiotic therapy; relapse: new febrile episode 24h or more after fever clearance; within study follow-up period		

Samples overview

Type	Time point	Volumes (adult)
Major blood draw	Baseline	2ml in Silica tube 3x5ml in EDTA tube
Minor blood draw	Follow-up, persistent or relapsing fever on day 3	2ml Silica tube[b] 5ml in EDTA tube
Urine	Baseline	10 ml
Eschar	Admission	Biopsy
[b] *Only at follow-up, not in case of relapse or persistent fever*		

6.2 DIAGNOSTIC METHODS

iiPCR: A nucleic acid detection test for a highly automated POCKIT system (Genereach biotechnology Corp., Taiwan) using Ot specifically designed primers and probes.

Scrub typhus rapid diagnostic tests (RDTs): An IgM immunochromatographic RDT (InBios International, Seattle, WA, USA) will be performed by a trained study technician on plasma, serum, or whole blood samples as part of the screening process. This RDT is based on recombinant proteins from the 56kDa type-specific antigen of Karp, Kato, Gilliam, and TA716 strains, it is currently the best RDT for scrub typhus available with a sensitivity of 90% and specificity of 86% [14]. If the treating physicians will have requested other scrub typhus ICTs, we will record the results in the CRF.

RAA-CRISPR/Cas: The method is currently being developed, the aim is for it to target the same genome repeat regions of the iiPCR and qPCR. Once the assay has been developed and is shown to adequately identify *O.tsutsugamushi* DNA from cell-cultures, it will be tested on the clinical samples obtained from this project.

Serology: The gold standard serological diagnosis of scrub typhus uses a screening InBios IgM ELISA assay on convalescent sera, and positive samples are confirmed using IgM IFA on paired serum samples. Serological positivity is defined as a four-fold increase of IgM titers to a ≥ 1:3200 titer in the convalescent sample or a ≥1:3200 titer in a single admission sample, using pre-coated microscopic IFA slides with antigen from *O. tsutsugamushi* type-strains Karp, Kato and Gilliam [15]. Antibody testing on dry blood spots will also be performed.

Real-time PCR: In admission samples the bacterial load of bacteraemic (PCR-positive) patients is determined by qPCR, targeting the 47kDa *htra* gene of *Orientia tsutsugamushi*. DNA is extracted from whole blood, dry blood spots, buffy coat and/or eschar swab/crust collected (Qiagen DNeasy Blood and Tissue Kit; Qiagen, Germantown, MD, USA). Real-time or conventional PCR using the newly developed primers may also be carried out to test primer and probe performance, in case of discordant results between iiPCR and qPCR results.

Culture/isolation: Full blood or buffy coat (EDTA blood) is propagated onto L929 or Vero cell monolayers in the BSL3 laboratory at MORU. Regular samples from the cell culture flasks are taken to monitor for *Orientia* spp. growth by direct fluorescence detection and/or qPCR. If isolation is successful, the isolate is cultured, purified and DNA extracted for downstream genotyping and WGS.

6.3 LABORATORY INVESTIGATIONS AND PROCEDURES

Investigations on blood samples

- iiPCR, RPA-CRISPR/Cas, and Real-time PCR are performed on DNA extracted from whole-blood and buffy coat.
- RDTs and serological testing are performed on whole blood, serum, or plasma samples.
- Clinical *Orientia tsutsugamushi* isolates are cultured from whole blood and/or buffy coat
- Whole genome sequencing and genotyping uses whole blood, eschar, or cell culture DNA extracts.
- G6PD deficiency will be measured in left over week-2 whole blood samples targeting established single-nucleoutide polymporphisms
- The exact time of blood collection will be recorded. Sample processing is performed on site immediately or samples are stored at the appropriate temperature for processing later.

6.4 STUDY DEFINITIONS

Fever clearance time (FCT): time in hours from onset of *Ot*-effective antibiotic treatment, to the first tympanic temperature recording ≤ 37.5°C, which remains ≤ 37.5°C for 24 hours. Fever

is related to the diagnosis of scrub typhus and not explained by other factors (e.g. transfusions, dialysis).

Clinical treatment failure: unexplained persistence of fever ≥72 hours after initiation of antibiotic treatment.

Relapse and re-infection: the recurrence of fever, which the clinical and study teams have concluded as likely to be due to scrub typhus will be defined as “possible relapse”. This will be monitored during the study period only.

7 DATA MANAGEMENT

7.1 STORAGE OF DATA/SPECIMENS

Study data will be recorded on electronic Case Report Forms (CRF) and entered to a secure GCP-compliant data management system hosted at MORU. The database is password-protected.

Participants will be identified by a unique participant number in the database. Participant’s names and other personal identifiable information will not be stored in the database or used in any analysis.

A participant logbook with personal information and patient ICFs will be stored on at the CCRU building in locked cabinets that will only be accessible to study staff. These documents may be stored at the study sites (Mae Suai, Mae Chan, and Mae Fa Luang hospital) in locked cabinets accessible only to study personnel and other authorised personnel temporarily (maximum one or two weeks) for logistical reasons. The records will be retained three years after the primary research findings are published.

Enrolment logbooks will be destroyed three years after the primary research findings are published. After this point no link between patient identifiable details and study data will remain. The study database will be retained indefinitely.

With the participant’s consent, de-identified data and results from blood analyses stored in the database may be shared according to the terms defined in the MORU data sharing policy with other researchers to use in the future.

Specimens will be stored at -80°C or in liquid nitrogen, and are kept for at least 10 years after study closure for analyses by the investigators of this protocol. All records that contain names or other personal identifiers, such as informed consent/assent forms will be stored separately from study records. The database will be secured with password-protected access systems.

Residual specimens are saved for future. Any infectious disease could potentially be studied by the Principal Investigator with the exception of HIV (human immunodeficiency virus) or CJD (Creutzfeldt-Jakob Disease) because of the implications for the patient. Participants are asked to specifically consent for the storage of their left-over specimens for future research questions. Genetic analysis may be carried out in the future, participants will be asked to

specifically consent to this. No future analysis or study of legacy data/specimens will be done without seeking further regulatory (EC/IRB) approval. The majority of assays in this study will be performed in Thailand. However, some analyses need to be performed at other Institutions, allowing more complex techniques to be used. A Material Transfer Agreement (MTA) with University of Oxford, UK will be made before any specimens are sent to Oxford for testing. If the intended analyses cannot be performed inside Thailand or Oxford, then an appropriate MTA in place to allow specimens to be sent elsewhere.

The Principle Investigator is responsible for maintaining study records, including history and physical findings, laboratory data, and results of consultations in a secure storage facility for at least 10 years. These records are to be maintained in compliance with EC/IRB requirements, as well as local regulations, whichever is longest. Subject's study information will not be released without the written permission of the subject, except as necessary for study monitoring, regulatory authority inspection, and/or site EC/IRB's visit.

8 SAFETY AND RISK MANAGEMENT

8.1 BENEFIT

Participants in this study will directly benefit by getting quick and precise molecular diagnostics for scrub typhus, probably outperforming the current Rapid Diagnostic Tests (RDTs). This accuracy will make it easier for the treating physician to choose the appropriate treatment. The study also helps validate and improve diagnostic methods for future use in the participants' communities.

If G6PD deficiency was found to be associated with severe disease at a clinically relevant level it would provide a useful tool to triage patients by severity risk and improve patient management.

8.2 SAFETY MANAGEMENT

Participants will not receive any study drugs, and the interventions (blood withdrawal, punch biopsy in a minority of subjects) are minimally invasive, so adverse events are not to be expected.

Potential unexpected events following the interventions (eschar biopsy or blood withdrawal) include bleeding, haematomas or minimal scarring.

8.3 RISK MANAGEMENT

This is a minimal risk descriptive study without the involvement of experimental interventions or investigational new drugs. The total amount of blood collected from patients is considered safe as per recommendations of University of California San Francisco (Appendix A) and others. Eschar biopsy are minimally invasive procedures with a low-risk profile. The potential side-effects, including bleeding, infection, and scarring, will be mitigated through sterile sample collection by an experienced physician and minimising the biopsy size. Any adverse event or harm, experienced as a consequence of study participation, requiring additional

medical care, will be covered by the study sponsor. Stored samples will be used for diagnostic purposes relevant to the study aims. Accordingly, consent will be sought from patients to store all clinical specimens.

9 CLINICAL MONITORING STRUCTURE

The study will be conducted in compliance with this protocol, International Conference on Harmonization Good Clinical Practice (ICH GCP) and any applicable regulatory requirement(s). Monitoring the study is conducted by members of the Clinical Trials Support Group, MORU, to ensure protocol and GCP compliance.

10 STATISTICAL ANALYSIS

10.1 DESCRIPTION OF THE ANALYSES

The data obtained from this study will allow for high quality evaluation of rapid molecular diagnostic tests for scrub typhus. Also, the data on clinical presentation, therapeutic response and history of exposure will be collated with genotyping (together with similar results from ongoing and past studies) to potentially find genomic determinants of disease severity.

The assays used for acute diagnosis (iiPCR and immunochromatographic scrub typhus RDTs, especially the InBios IgM) will be compared to one another and to reference diagnostic tests (PCR and the STIC criteria). Sensitivity, specificity, positive- and negative-predictive value will be calculated on clinical samples; using the mSTIC as reference standard. In addition, the samples obtained will be tested on an RPA-CRISPR/Cas assay that is currently being developed.

Data will be analyzed with appropriate statistical tests using STATA, Graphpad or R. Data is summarized using descriptive statistics i.e. proportions for categorical data and means, medians, standard deviations and ranges, as appropriate. Differences in risk factors, levels of markers and clinical features between scrub typhus patients and controls (controls will be participants who are positive to screening tests but are not confirmed to have scrub typhus at a later stage) will be analysed. Predictors of disease severity are identified by correlations and linear regression including demographic data, clinical and biochemical features, disease severity scores and, at a later stage, genomic findings.

Usability testing of the POCKIT machine will be carried out on site.

11 ETHICS/PROTECTION OF HUMAN SUBJECTS

This study conducted in compliance with the conditions stipulated by sponsor and local EC/IRB, applicable regulations, and ICH GCP requirements. In addition, all local regulatory requirements will be adhered to, in particular those which afford greater protection to the safety of the subjects.

This study protocol will be submitted to the following ethics committees:

- CR-PHO-EC (Chiang Rai Public Health Office Ethics Committee)

- Oxford Tropical Research Ethics Committee (OxTREC), Oxford University

11.1 STUDY COMPLETION, AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

Study completion**:** Participants who complete all study requirements and sample collections as defined in the protocol inclusive of follow-up visits.

Participant discontinuation/withdrawal from study: Participant may voluntarily discontinue participation in this study at any time. The investigators may also, at their discretion, discontinue the participant from participating in this study at any time. Participant may be prematurely discontinued from the study for any of the following reasons:

- Subject cannot comply with the protocol
- At the request of the participant or investigator for the benefit of participant
- Development of an exclusionary criterion
- Study subject voluntarily withdrawing consent

The reason for discontinuation or withdrawal, if available, will be recorded.

Study termination: Reasons for terminating the study may include, but are not limited to, the following:

- Subject enrolment is unsatisfactory.
- Sample quality inadequate
- Sponsor, EC/IRB and/or regulatory authority terminate the study.

11.2 COMPENSATION

Subjects will receive compensation for traveling costs associated with traveling to the hospital and loss of work time during their stays in the hospital.

The amount of compensation will be 400 baht at baseline. Subject will also receive 400 baht for the follow-up visit.

If the subject is withdrawn from the study before the study ends, the subject will be compensated for the time that the subject is actually enrolled in the study.

11.3 STUDY INDEMNITY

The University of Oxford has a specialist insurance policy in place, which would operate in the event of any participant suffering harm as a result of their involvement in the research.

11.4 PUBLICATION POLICY

Following completion of the study, the investigator may publish the results of this study in a scientific journal, but subject names or identities will not be revealed.

12 LITERATURE

1. Rosenberg R. Drug-resistant scrub typhus: Paradigm and paradox. Parasitol Today. 1997;13(4):131-2. doi: 10.1016/s0169-4758(97)01020-x. PubMed PMID: 15275098.
2. Wangrangsimakul T, Althaus T, Mukaka M, Kantipong P, Wuthiekanun V, Chierakul W, et al. Causes of acute undifferentiated fever and the utility of biomarkers in Chiangrai, northern Thailand. PLoS Negl Trop Dis. 2018;12(5):e0006477. Epub 20180531. doi: 10.1371/journal.pntd.0006477. PubMed PMID: 29852003; PubMed Central PMCID: PMCPMC5978881.
3. Kannan K, John R, Kundu D, Dayanand D, Abhilash KPP, Mathuram AJ, et al. Performance of molecular and serologic tests for the diagnosis of scrub typhus. PLoS Negl Trop Dis. 2020;14(11):e0008747. Epub 20201112. doi: 10.1371/journal.pntd.0008747. PubMed PMID: 33180784; PubMed Central PMCID: PMCPMC7660479.
4. Wangrangsimakul T, Greer RC, Chanta C, Nedsuwan S, Blacksell SD, Day NPJ, et al. Clinical Characteristics and Outcome of Children Hospitalized With Scrub Typhus in an Area of Endemicity. J Pediatric Infect Dis Soc. 2020;9(2):202-9. doi: 10.1093/jpids/piz014. PubMed PMID: 30864670; PubMed Central PMCID: PMCPMC7192406.
5. Saraswati K, Day NPJ, Mukaka M, Blacksell SD. Scrub typhus point-of-care testing: A systematic review and meta-analysis. PLoS Negl Trop Dis. 2018;12(3):e0006330. Epub 20180326. doi: 10.1371/journal.pntd.0006330. PubMed PMID: 29579046; PubMed Central PMCID: PMCPMC5892940.
6. Shukla R, Shrestha M, Bhattarai CD, Lamichhane K, Yadav P, Tamatta D. Scrub Typhus Infection Precipitating Hemolysis in a Patient With G6PD Deficiency: A Case Report. Clin Case Rep. 2025;13(2):e70142. Epub 20250124. doi: 10.1002/ccr3.70142. PubMed PMID: 39868405; PubMed Central PMCID: PMCPMC11761397.
7. Ganguly D, Chandra A, Maitra S, Malakar SL. Acute intravascular haemolysis associated with scrub typhus. BMJ Case Rep. 2024;17(7). Epub 20240702. doi: 10.1136/bcr-2024-261003. PubMed PMID: 38960428.
8. Bhardwaj P, Nanaware NS, Behera SP, Kulkarni S, Deval H, Kumar R, et al. CRISPR/Cas12a-Based Detection Platform for Early and Rapid Diagnosis of Scrub Typhus. Biosensors. 2023;13(12) (no pagination). PubMed PMID: 2027337110.
9. Qi Y, Yin Q, Shao Y, Cao M, Li S, Chen H, et al. Development of a rapid and visual nucleotide detection method for a Chinese epidemic strain of Orientia tsutsugamushi based on recombinase polymerase amplification assay and lateral flow test. International Journal of Infectious Diseases. 2018;70:42-50. PubMed PMID: 2000613012.
10. Hazarika B, Khanna N, Bain BJ. Hematological features of scrub typhus. Am J Hematol. 2023;98(12):1951-2. Epub 20230830. doi: 10.1002/ajh.27078. PubMed PMID: 37646465.
11. Paris DH, Blacksell SD, Nawtaisong P, Jenjaroen K, Teeraratkul A, Chierakul W, et al. Diagnostic accuracy of a loop-mediated isothermal PCR assay for detection of Orientia tsutsugamushi during acute Scrub Typhus infection. PLoS Negl Trop Dis. 2011;5(9):e1307. Epub 20110913. doi: 10.1371/journal.pntd.0001307. PubMed PMID: 21931873; PubMed Central PMCID: PMCPMC3172190.
12. Rungrojn A, Batty EM, Perrone C, Abdad MY, Wangrangsimakul T, Brummaier T, et al. Molecular diagnosis and genotyping of Orientia tsutsugamushi in Maesot and Chiangrai, Thailand. Frontiers in Tropical Diseases. 2023;4. doi: 10.3389/fitd.2023.1146138.
13. Bujang MA, Adnan TH. Requirements for Minimum Sample Size for Sensitivity and Specificity Analysis. J Clin Diagn Res. 2016;10(10):Ye01-ye6. Epub 20161001. doi: 10.7860/jcdr/2016/18129.8744. PubMed PMID: 27891446; PubMed Central PMCID: PMCPMC5121784.
14. Silpasakorn S, Srisamut N, Ekpo P, Zhang Z, Chao C-C, Ching W-M, et al. Development of New, Broadly Reactive, Rapid IgG and IgM Lateral Flow Assays for Diagnosis of

Scrub Typhus. The American journal of tropical medicine and hygiene. 2012;87(1):148-52. doi: 10.4269/ajtmh.2012.11-0583. PubMed PMID: PMC3391040.
15. Lim C, Blacksell SD, Laongnualpanich A, Kantipong P, Day NP, Paris DH, et al. Optimal Cutoff Titers for Indirect Immunofluorescence Assay for Diagnosis of Scrub Typhus. J Clin Microbiol. 2015;53(11):3663-6. Epub 20150909. doi: 10.1128/jcm.01680-15. PubMed PMID: 26354819; PubMed Central PMCID: PMCPMC4609688.

APPENDIX A

The blood draw volumes in this study

The table below lists the pre-calculated allowed blood draw volumes per child body weight, as defined by the guidelines of the University of California San Francisco:

https://irb.ucsf.edu/sites/hrpp.ucsf.edu/files/risk%20Levels%20of%20Pediatric%20Procedures%20and%20Chart%20of%20Maximum%20Allowable%20Total%20Blood%20Draw%20Volumes%20in%20Children.pdf

Body Weight (Kg)	Maximum volume (mL) drawn in a 28-day period	Maximum volume (mL) drawn for any single draw*
1	5	2.5
2	10	5
3	12	6
4	16	8
5	20	10
6	24	12
7	28	14
8	32	16
9	36	18
10	40	20
11-15	44-60	22-30
16-20	64-80	32-40
21-25	84-100	42-50
26-30	104-120	52-60
31-35	124-140	62-70
36-40	144-160	72-80
41-45	164-180	82-90
46-50	184-200	92-100
51-55	204-220	102-110
56-60	224-240	112-120
61-65	244-260	122-130
66-70	264-275	132-138
Greater than 70	275	138

APPENDIX B SCRUB TYPHUS RDTS CURRENTLY IN USE AT THE STUDY SITES

Clungene Rickettsia IgG/IgM (test named with old nomenclature *Rickettsia tsutsugamushi*)

Scrub Typhus IgM/IgA/IgG SDBioline

STANDARD Q Tsutsugamushi IgM/IgG, SDBiosensor